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MOLECULAR BASES OF CIRCADIAN RHYTHMS

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■ **Abstract** Circadian rhythms are found in most eukaryotes and some prokaryotes. The mechanism by which organisms maintain these roughly 24-h rhythms in the absence of environmental stimuli has long been a mystery and has recently been the subject of intense research. In the past few years, we have seen explosive progress in the understanding of the molecular basis of circadian rhythms in model systems ranging from cyanobacteria to mammals. This review attempts to outline these primarily genetic and biochemical findings and encompasses work done in cyanobacteria, *Neurospora*, higher plants, *Drosophila*, and rodents. Although actual clock components do not seem to be conserved between kingdoms, central clock mechanisms are conserved. Somewhat paradoxically, clock components that are conserved between species can be used in diverse ways. The different uses of common components may reflect the important role that the circadian clock plays in adaptation of species to particular environmental niches.

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WHAT IS A CIRCADIAN CLOCK?

People have long been fascinated by the human relationship to time. Pericles is reported to have advised a belligerent warrior to "be ruled by time, the wisest counselor of all" (Plutarch 75 A.D.). Although the target of this advice ignored it (with fatal consequences), most organisms heed Pericles' words. Much of the behavior, physiology, and biochemistry of organisms changes rhythmically over the course of each day. Some of these changes occur only in response to environmental stimuli such as light:dark (LD) cycles, whereas other rhythms persist even in the absence of environmental changes. Rhythms that occur with a periodicity roughly matching that of the earth's rotation on its axis and that continue in the absence of external stimuli are termed circadian (Figure 1). There are many types of circadian rhythms, ranging from the subtle (such as rhythms in photosynthetic activity in cyanobacteria) to the obvious (such as activity rhythms in animals). In fact, the first account of a circadian rhythm dates to about 100 years after Pericles gave his sage advice, in a description of the rhythmic opening and closing of the leaves of the tamarind tree by the Greek Androsthenes in the 4th century B.C. (Moore-Ede et al. 1982).

Circadian rhythms are controlled by an endogenous oscillator, the circadian clock. The circadian clock allows organisms to anticipate rhythmic changes in the environment and accordingly change their physiological state, thus providing them with an adaptive advantage (Ouyang et al. 1998). In addition to persistence in the absence of external cues, circadian rhythms exhibit other hallmarks. They are temperature compensated: the period of their rhythmicity is roughly consistent even over a wide range of temperatures. Although the necessity of this trait is obvious (since a clock that runs faster on hot days than cold ones would be largely useless), its mechanistic basis is a mystery because the rate of almost all biochemical reactions is extremely temperature dependent. Although the above traits emphasize the stability of the clock and its independence from external factors, circadian clocks must respond to changes in the environment to be of use. Such resetting of the clock so that internal time matches local time (entrainment) can be accomplished by signals (called zeitgebers, meaning time-givers) such as changes in light, temperature, activity, and nutrient availability (Figure 1). In addition, circadian rhythmicity arises as a cell-autonomous trait even in multicellular organisms. A corollary is that multicellular organisms have functional clocks in many different cell types. For example, whereas the circadian behavior of vertebrates is largely controlled by a portion of the brain called the suprachiasmatic nucleus (SCN), vertebrates also

have clocks in peripheral tissues such as kidney and skeletal muscle (reviewed in Hastings & Maywood 2000).

These traits common to circadian clocks in all organisms underlie an impressive variety of clock outputs. Cyanobacteria, photosynthetic prokaryotes, exhibit circadian control of photosynthesis, nitrogen fixation, and even cell division. In mammals, processes influenced by the circadian clock include digestion, regulation of body temperature, hormone secretion, and behaviors such as time of sleep onset. In fact, a mutation in a human clock gene (*hPer2*) has recently been linked to a family with an extreme bias toward activity very early in the morning (Toh et al. 2001). This review focuses on the (somewhat paradoxical) twin themes of conservation and divergence that have emerged from the study of the molecular bases of circadian rhythms in various model organisms. Given the scope of this field, this review is not meant to be exhaustive, but rather to illustrate general concepts with specific examples. There are an abundance of recent reviews covering particular aspects of circadian biology in more detail, which are recommended to the reader throughout this review.

THE CENTRAL OSCILLATOR

The circadian system is often described as consisting of three parts: a central oscillator that generates rhythmicity; input pathways that receive and relay environmental cues that entrain the oscillator; and output components that create overt rhythms. This characterization is an oversimplification because clock input components such as photoreceptors can be clock outputs as well (Bognar et al. 1999, Emery et al. 1998). Furthermore, clock outputs can feed back on the clock itself, affecting the pace at which the central oscillator runs (Lopez-Molina et al. 1997). Therefore, a more realistic model might be one in which there is no central oscillator per se, but rather multiple overlapping feedback loops that contribute to timekeeping (Roenneberg & Merrow 1998). However, the central oscillator paradigm has proven conceptually useful and is described in more detail below.

Loops of Transcriptional Feedback Inhibition

Delayed negative feedback loops are at the heart of oscillatory processes, so it is no surprise to find them implicated in all circadian clocks investigated to date. What is perhaps surprising is that in all clocks, important negative feedback occurs at the transcriptional level. Transcription factors induce the expression of other clock genes that then act to negatively regulate their own transcription, creating oscillating patterns of gene expression. Although in principle only one component in such a loop need cycle, in all clock feedback loops described thus far all negative elements and some of the positive elements cycle at the mRNA and/or protein level. These important loops have been emphasized in previous reviews (Dunlap 1999). However, if no delays were built into such systems, they would soon dampen to some constant level of gene expression. Intriguingly, at least one negative

component in each feedback loop described below exhibits a significant delay between peak levels of message and protein, suggesting that post-transcriptional regulation is key for proper clock control. Often, these negative elements are the first to be affected by environmental signals that reset the clock, and their phase determines the overall phase of the oscillator (see below). We first describe the transcriptional feedback loops implicated in clock regulation in cyanobacteria, *Neurospora crassa*, *Drosophila melanogaster*, mammals, and higher plants and then consider various post-transcriptional mechanisms that allow circadian clocks to run with an approximately 24-h period.

CYANOBACTERIA Although it was originally assumed that prokaryotes would not have circadian rhythms, work in the late 1980s and early 1990s showed that in fact cyanobacteria do (reviewed in Golden et al. 1997) and therefore that an organism does not need a nucleus to have a functional circadian clock. Forward genetic screens in these photosynthetic bacteria led to the identification of mutants with long-period, short-period, and arrhythmic phenotypes (Kondo et al. 1994). All these mutants had changes in one of three adjacent genes: *kaiA*, *kaiB*, or *kaiC*, with mutations in *kaiC* accounting for all three possible clock phenotypes (Ishiura et al. 1998). Transcription of these genes is rhythmic, with mRNA levels peaking near the end of the subjective day at CT 9–12 (CT, or circadian time, is used to indicate the subjective time of day in constant conditions. By convention, CT 0 corresponds to subjective dawn (lights on) and CT 12 corresponds to subjective dusk (lights off). Overexpression of *kaiC* suppresses expression of both *kaiB* and *kaiC*, which share a single promoter and are co-transcribed. On the other hand, overexpression of *kaiA* enhances expression of *kaiBC*, whereas loss of *kaiA* leads to a marked decrease in *kaiBC* expression (Ishiura et al. 1998). Therefore, KaiA acts as the positive element in the *kaiBC* feedback loop, and KaiC acts as a negative element to inhibit its own transcription (Figure 2*A*). KaiA protein levels do not cycle, whereas KaiB and KaiC both peak at around CT 15 (Xu et al. 2000a), several hours after the peak of mRNA levels. Such delays between peak RNA levels and peak protein levels of clock proteins are also found in *Neurospora*, *Drosophila*, and mammals.

The biochemical roles of the Kai proteins, none of which has an obvious DNA-binding motif, are unclear. However, all three proteins can bind to each other in vitro and in yeast two-hybrid assays. These intermolecular protein:protein interactions are likely to be of physiological relevance because the interaction between KaiB and a mutant version of KaiA that confers a long-period phenotype is enhanced (Iwasaki et al. 1999). A KaiC-interacting protein, SasA, has also been identified. Disruption of the gene encoding this two-component histidine kinase protein affects the period and amplitude of *kai* expression, demonstrating that it acts close to the cyanobacterial oscillator (Iwasaki et al. 2000). A more detailed discussion of the cyanobacterial central oscillator can be found in Iwasaki & Kondo (2000).

NEUROSPORA Circadian rhythm mutants in the fungus *Neurospora crassa* were first identified in the 1970s. Mutations in three genes, *frq*, *wc-1*, and *wc-2*, cause period length defects or arrhythmicity (reviewed in Bell-Pedersen 2000). The

message levels of *frq* cycle, peaking at around CT 4 (Aronson et al. 1994), whereas FRQ protein levels peak around CT 8, again demonstrating post-transcriptional delay (Garceau et al. 1997). FRQ protein negatively regulates *frq* mRNA levels (Aronson et al. 1994), an effect assumed to occur at the transcriptional level. FRQ does not have any obvious protein motifs, although it has some sequence features suggesting it may be a transcription factor (Lewis et al. 1997). *frq* expression requires WC-1 and WC-2 (Crosthwaite et al. 1997), two proteins with PAS domains (named for PER, ARNT, and SIM, the first three proteins identified with this motif) and zinc finger DNA-binding domains (Ballario & Macino 1997). PAS domains can mediate protein dimerization, often with other PAS domain-containing proteins. These domains are found in a number of clock genes (see below) and are also found in other genes implicated in adaptation to environmental changes (Gu et al 2000).

WC-1 and WC-2 heterodimerize in vivo, are required for induction of gene expression in response to blue light, and may be involved directly in both light perception and gene activation (Talora et al. 1999). As transcription factors required for *frq* expression, WC-1 and WC-2 are assumed to bind to the *frq* promoter, although this has not been demonstrated, and are the positive elements in the *Neurospora* feedback loop (Figure 2*B*). *wc-1* and *wc-2* message levels and the WC-2 protein show no significant cycling. However, levels of the WC-1 protein cycle in a FRQ-dependent manner, creating a second feedback loop (Lee et al. 2000, Dunlap 1999). FRQ has been shown to bind to WC-2 and to co-immunoprecipitate with WC-2 and WC-1, suggesting that it may inhibit its own transcription by interfering with WC-1 and WC-2 activity (Merrow et al. 2001). See Bell-Pedersen (2000) and Dunlap (1999) for more details on the *Neurospora* central oscillator.

DROSOPHILA The molecular-genetic study of circadian rhythms was initiated in *Drosophila*, with the identification and eventual cloning of the *period* (*dper*) mutant gene based on its perturbation of fly eclosion (emergence of adults from their pupal cases) and activity rhythms (Konopka & Benzer 1971, Reddy et al. 1984). The next fly clock gene cloned was *timeless* (*dtim*), identified both by the ability of dTIM to bind to the dPER protein and by positional cloning of a mutant gene responsible for altered activity rhythms (Gekakis et al. 1995, Myers et al. 1995). Both *dper* and *dtim* mRNA levels cycle, peaking in the early night around CT 14 (Hardin et al. 1990, Sehgal et al. 1995). Protein levels also cycle, but peak with a delayed phase relative to the mRNA levels, around the middle of the night at CT 18 (Siwicki et al. 1988, Zerr et al. 1990, Hunter-Ensor et al. 1996, Myers et al. 1996, Zeng et al. 1996). dPER interacts with dTIM via a PAS domain, but there are no other obvious functional domains in dPER or dTIM. The roles of these proteins remained mysterious until two other *Drosophila* clock components were discovered.

These two genes, *dclock* (*dclk*) and *cycle* (*cyc*), encode proteins containing both basic helix-loop-helix (bHLH) DNA-binding domains and PAS domains. The steady-state mRNA levels of *dclk* cycle and peak near subjective dawn, as do dCLK protein levels (Darlington et al. 1998; Bae et al. 1998, 2000). Contrary to its name, CYC does not cycle, either at the mRNA or protein level (Rutila

et al. 1998, Bae et al. 2000). However, *dper* and *dtim* message levels are drastically reduced and do not cycle in *dclk* or *cyc* mutants, indicating that these bHLH factors regulate transcription of *dper* and *dtim* (Rutila et al. 1998, Allada et al. 1998). This is thought to be a direct effect because dCLK/CYC heterodimers bind directly to E-box elements found in the *dper* and *dtim* promoters and enhance expression of reporter genes driven by these E-box elements (Darlington et al. 1998, Hogenesch et al. 1998). This induction of gene expression is antagonized by the actions of dPER and dTIM (Darlington et al. 1998). It has recently been shown that binding of dPER and dTIM to the dCLK/CYC complex prevents their binding to DNA but does not disrupt their association (Lee et al. 1999, Lee et al. 1998, Bae et al. 2000). Therefore, dCLK and CYC form the positive elements of the transcriptional loop, whereas dPER and dTIM mediate negative feedback (Figure 2*C*).

Somewhat surprisingly, dPER and dTIM are required for high-level *dclk* expression (Bae et al. 1998), a requirement that is lost in the absence of functional dCLK or CYC (Glossop et al. 1999). This epistatic analysis indicates that dCLK and CYC somehow repress the transcription of *dclk* through an unknown mechanism and that this inhibition is antagonized by dPER and dTIM. Therefore, there is a second transcriptional feedback loop in the *Drosophila* central oscillator (Figure 2*C*). Matters are probably even more complex than this, as there is evidence that a protein called VRILLE (VRI), a transcription factor essential for development, belongs in these core feedback loops as well. *vri* message oscillates in phase with *dper* and *dtim*, and disruption of *vri* expression alters circadian rhythms and *dtim* expression (Blau & Young 1999). Further discussion of the feedback loops at the core of the *Drosophila* circadian clock can be found in Scully & Kay (2000) and Edery (2000).

MOUSE At first glance, the mammalian circadian clock seems quite similar to the fly clock because a host of mammalian genes homologous to the *Drosophila* clock genes described above have been discovered. These include three *period* homologues (*mper1*, *2*, and *3*), a *tim* homologue (*mtim*), a *clk* homologue (*mclk*), and a gene homologous to *cyc* called either *bmal1* or *mop3*. Only one of these genes, *mclk*, was discovered in a forward genetic screen; the rest were identified primarily by homology to their fly counterparts. Functional analysis of these mammalian genes has shown that there are a number of important differences between the fly and mouse clock mechanisms. A further complication is that these clock genes often behave differently in the SCN and in peripheral tissues (Damiola et al. 2000, Stokkan et al. 2001). Because the SCN is thought to coordinate and regulate peripheral clocks, discussion in this section is limited to gene function in the SCN.

The positive elements of the mouse clock, as in the fly clock, are the bHLH/PAS transcription factors mCLK and BMAL1. *mclk* was originally identified in a genetic screen for mice with aberrant locomotor activity rhythms (Vitaterna et al. 1994, King et al. 1997). BMAL1 was identified by virtue of its interaction with mCLK (Hogenesch et al. 1998, Gekakis et al. 1998). Subsequent experiments have shown that disruption of *bmal1* causes immediate loss of locomotor rhythms in mice held

in constant conditions (Bunger et al. 2000). In rodents, *bmal1* message and protein levels cycle, both peaking around the middle of the subjective night at CT 18. In contrast, *mclk* message does not cycle (Shearman et al. 2000b, Oishi et al. 2000, Tamaru et al. 2000). This situation is the reverse of that seen in flies, where *dclk* mRNA levels cycle and *cyc* mRNA levels do not. *mper* transcripts are reduced in both *mclk* and *bmal1* mutant mice (Gekakis et al. 1998, Jin et al. 1999; Bunger et al. 2000), suggesting that mCLK and BMAL1 act as positive elements in the mouse clock just as their homologues do in the fly clock.

Things become more confusing once we turn to the negative elements in the mammalian clock. The three *mper* homologues all cycle at both the mRNA and protein levels. Their steady-state message levels peak at slightly different times throughout the subjective day (Sun et al. 1997; Albrecht et al. 1997; Shearman et al. 1997; Takumi et al. 1998a,b; Zheng et al. 1999). These variant expression patterns suggest that these genes may be controlled by different mechanisms, a possibility reinforced by their variable responses to light. The three mPER proteins peak coordinately in the SCN near the end of the subjective day at CT 10–13 (Hastings et al. 1999, Kume et al. 1999). All three mPER proteins have some antagonistic effects on mCLK/BMAL1-mediated gene activation, but unlike dPER, this action is independent of TIM and is not as potent as that mediated by dPER/dTIM on dCLK/CYC (Griffin et al. 1999, Kume et al. 1999, Sangoram et al. 1998). Furthermore, it is questionable whether mTIM plays any role in the mammalian circadian system. mTIM is more similar to a second fly gene than it is to dTIM (Gotter et al. 2000). The *mtim* knockout shows no circadian phenotype (Gotter et al. 2000), and no association was found between mPER and mTIM proteins in the SCN (Field et al. 2000).

It is possible that negative feedback on the mammalian circadian system is supplied by two cryptochromes, mCRY1 and mCRY2. Cryptochromes may bind pterin and flavin chromophores and are related to the DNA repair enzyme photolyase, but have no repair activity. In plants and flies, cryptochromes act as blue-light photoreceptors and transmit light information to the circadian clock (see below, and reviewed in Sancar 2000 and Devlin & Kay 2001). A light-dependent activity has yet to be shown for the mCRYs, but they inhibit mCLK/BMAL1-mediated gene activation just as effectively as dPER/dTIM complexes inhibit dCLK/CYC (Griffin et al. 1999, Kume et al. 1999, Okamura et al. 1999, van der Horst et al. 1999, Vitaterna et al. 1999). *mcry1* message levels and mCRY1 and mCRY2 proteins cycle coordinately with the *mper* transcripts and proteins in the SCN. Also, like the *mper* transcripts, both *mcry1* and *mcry2* mRNA levels are reduced in *clk* mutant mice (even though *mcry2* message levels do not cycle) (Kume et al. 1999). Genetic evidence suggests that at least mCRY1, mCRY2, and mPER2 play significant roles in maintaining clock function in mice (Zheng et al. 1999, Thresher et al. 1998, van der Horst et al. 1999), whereas mPER3 seems to have mostly non-essential functions in maintaining rhythmicity (Shearman et al. 2000a).

The biochemical functions of mCRY and mPER proteins remain unclear. All three mPERs associate with each other and the two mCRYs in vitro and in yeast

(Zylka et al. 1998, Kume et al. 1999, Griffin et al. 1999, Yagita et al. 2000), and all these proteins co-immunoprecipitate from the SCN (Field et al. 2000). In addition, the mCRYs have been shown to bind to BMAL1 and mCLK in yeast two-hybrid assays (Griffin et al. 1999). These proteins may act alone or in higher-order complexes to inhibit mCLK/BMAL1 from activating gene expression, as suggested in Figure 2*D*. These proteins are likely to have other functions as well; for example, the mCRY proteins may affect the intracellular localization of mPER proteins (see below) and stabilize mPER2 (Shearman et al. 2000a). See Edery (2000) and King & Takahashi (2000) for further information on the mammalian central oscillator.

ARABIDOPSIS Although a number of genes implicated in circadian clock function have recently been isolated in *Arabidopsis*, there is as yet no clear model in which to place them. However, their predicted functions are suggestive. Two transcription factors, *CCA1* and *LHY*, cause arrhythmicity when overexpressed (Schaffer et al. 1998, Wang & Tobin 1998). These two genes are 39% identical overall, and both have single Myb-like DNA-binding domains that are 78% identical. The mRNA levels of both genes and the protein level of at least CCA1 cycle coordinately in constant conditions and peak near subjective dawn. Overexpression of either gene leads to drastically reduced levels of both transcripts, indicating that they may be involved in negative feedback loops. Plants lacking *CCA1* have a relatively modest short-period-length phenotype, perhaps because *LHY* and *CCA1* act redundantly (Green & Tobin 1999). The circadian phenotype of *CCA1*/*LHY* loss-of-function double mutants will be of great interest and will help to determine whether these genes represent clock components or clock outputs that feed back upon the clock. A number of genes with similar, single Myb domains that also cycle at the RNA level have recently been identified as members of the REVEILLE gene family (C.R. Andersson, S.L. Harmer & S.A. Kay, unpublished data). These *RVE* genes may control clock outputs and/or may act close to the clock itself.

Another plant gene thought to act close to or in the central clock is *TOC1*. Mutation of *TOC1* causes a short-period phenotype that is independent of light input to the clock (Somers et al. 1998b). *TOC1* encodes an atypical response regulator protein, similar to those found in bacterial two-component signal transduction systems (Strayer et al. 2000). *TOC1* mRNA levels cycle, peaking at the end of the subjective day. Circadian-regulated homologues of *TOC1* have also been recently reported (Matsushika et al. 2000). Moreover, these proteins lack some critical amino acid residues required for normal response-regulator function. These pseudo-response-regulator transcripts peak at different times throughout the day, suggesting that they may be important for the proper phasing of clock outputs through the day. Another mutant, *tej*, also causes increased period length independent of light input. TEJ regulates the poly-ADP ribosylation state of nuclear proteins (S. Panda & S.A. Kay, unpublished data). Such post-translational modification can alter activities of transcription factors by changing their affinity to target DNA sites (Desnoyers et al. 1995). A third *Arabidopsis* clock mutant, *ztl*, was also recently cloned. Unlike *toc1* or *tej*, the *ztl* long-period phenotype is altered by light

treatment, suggesting that it may play a role in light signal transduction to the clock (see below). Other mutant alleles found to influence rhythms in *Arabidopsis* include *elf3-1*, which causes arrhythmicity in plants held in the light but not in plants held in the dark (Hicks et al. 1996), and *gi-2*, which simultaneously lengthens some clock outputs while shortening others (Park et al. 1999). Although more work is required to sort out where these genes fit with regard to a central feedback loop, recent evidence suggests that TOC1, CCA1, and LHY regulate each other's transcription (D. Alabadi, T. Oyama, M.J. Yanovsky, F.G. Harmon, P. Mas & S.A. Kay, submitted). See McClung (2001) and Golden & Strayer (2001) for a more in-depth discussion of these issues.

POST-TRANSCRIPTIONAL CONTROLS IMPOSE DELAYS

Although the transcription/translation feedback loops described above allow the construction of satisfying models, these models are not sufficient to describe circadian rhythmicity. Multiple levels of post-transcriptional controls are built into these systems, presumably to delay the cycles so that they take a full 24 h, maintain robust amplitude of cycling from the transcription of clock components all the way to physiological outputs, and buffer the clock mechanism against abrupt changes. In addition, post-transcriptional control provides mechanisms by which the clock can be reset by environmental inputs.

RNA Splicing and Stabilization

As *Drosophila* clock genes are among the best characterized, examples of post-transcriptional clock control of RNA are drawn here from studies of the fly *dper* gene. A surprising finding in the fly was that a promoterless *dper* genomic fragment rescues rhythmic locomotor activity in *dper* null mutants (Hamblen et al. 1986). Although transcription from this *dper* gene is not rhythmic, steady-state levels of *dper* mRNA nonetheless cycle with a two- to threefold amplitude (Frisch et al. 1994, So & Rosbash 1997). In wild-type flies, a comparison of the rate of transcription from the *dper* promoter and the rate of *dper* mRNA accumulation has led to the suggestion that *dper* mRNA half-life is different during its accumulating and declining phases. Studies with reporter genes transcriptionally or translationally fused to *dper* have led to similar conclusions (Stanewsky et al. 1997). dTIM may play an important role in this process, as dTIM or dPER/dTIM complexes upregulate *dper* mRNA levels through a post-transcriptional mechanism (Suri et al. 1999). Splicing of *dper* RNA has also been shown to be a regulated process. There are two *dper* transcripts, generated by alternative splicing within the *dper* 3′ untranslated region (UTR). Removal of this intron is stimulated at low temperatures and correlates with an earlier rise in *dper* transcript and dPER protein, perhaps by enhancing the processing of the pre-mRNA in other ways. This cold-dependent effect may be important for the appropriate phasing of fly activity during the cold and short days of winter (Majercak et al. 1999). Similar controls of RNA stability

and splicing probably occur in other clock genes as well; for example, the mRNA cycling of a clock-controlled gene in *Arabidopsis* apparently occurs through a post-transcriptional mechanism (Pilgrim et al. 1993).

Translational Control

A number of observations suggest post-transcriptional regulation of clock component protein levels. Significantly, at least one component of the negative limb of each clock characterized thus far shows a several-hour lag between peak RNA and protein levels: *kaiB* and *kaiC* in cyanobacteria (Xu et al. 2000a); *frq* in *Neurospora* (Luo et al. 1998); *dper* and *dtim* in *Drosophila* (Sehgal et al. 1995); and *mper1* and *mper2* (Field et al. 2000) in mouse. This may be essential for clock function, as the delayed accumulation of a repressor may allow enough time for transcription of its message to increase substantially before protein levels have increased sufficiently to inhibit transcription (Zerr et al. 1990, Marrus et al. 1996, Edery 1999, Hastings et al. 1999, Scheper et al. 1999). In flies, although the delayed accumulation of dPER may be caused by its instability in the absence of dTIM (Price et al. 1998), the phase lag between peak RNA and protein levels of *dtim* in constant darkness remains unexplained. Further evidence of regulation of this process comes from two *dbt* mutants, which when held in LD cycles accumulate dPER and dTIM with wild-type kinetics even though the corresponding RNA rhythms are substantially delayed, as if the delay in protein accumulation is uncoupled from message levels (Suri et al. 2000).

Other clear examples of clock-control of translation are found in *Neurospora* and mouse. Under constant free-running conditions, levels of WC-1 and mCRY2 cycle even though their transcript levels do not exhibit any circadian rhythmicity (Lee et al. 2000, Kume et al. 1999). Because there are several possible translational control mechanisms (Gray & Wickens 1998), the exact mechanism for the post-transcriptional regulation of WC-1 and mCRY2 is not clear. There are, however, examples of translational regulation of clock outputs that may provide clues. In the marine alga *Gonyaulax polyhedra*, luciferase-binding protein (*lbp*) (Morse et al. 1989) and chloroplast glyceraldehyde-3-phosphate dehydrogenase (cpGAPDH) (Fagan et al. 1999) exhibit circadian rhythms in protein levels while their mRNA levels remain constant. The translational control of cpGAPDH is proposed to be at an elongation step, whereas synthesis of LBP is postulated to be repressed by a protein that binds to the 3′-UTR of the *lbp* transcript during the day phase (Mittag et al. 1994). In *Drosophila*, the RNA-binding protein LARK may also be translationally regulated (McNeil et al. 1998).

Nuclear Transport

In eukaryotic organisms, the transcriptional feedback loops described above are obviously dependent upon nuclear localization of the positive and negative clock components. Although all the positive elements investigated thus far seem to be constitutively nuclear, regulated entry of the negative elements plays an important

role in *Drosophila* and perhaps in mouse as well. As mentioned above, dTIM and dPER are both required for rhythmicity. The nuclear localization of dPER and dTIM requires expression of both proteins (Vosshall et al. 1994, Hunter-Ensor et al. 1996, Myers et al. 1996, Saez & Young 1996). Although these proteins both possess nuclear localization sequences, they also contain cytoplasmic localization domains (CLDs) that block nuclear entry of monomeric dPER or dTIM. CLD function is blocked by the formation of dPER/dTIM complexes, which are competent to enter the nucleus. In experiments performed in a heterologous cell culture system, high levels of dPER and dTIM accumulated in the cytoplasm several hours before these proteins could be detected in the nucleus (Saez & Young 1996). Such a delay in nuclear entry is also seen in vivo and may be an important determinant of the observed delay between dPER and dTIM accumulation and their negative effect on transcription (Curtin et al. 1995). Such regulated nuclear entry could involve the transcription factor *vri*, which may inhibit the nuclear translocation of dPER and dTIM (Blau & Young 1999). Recent data suggest that nuclear dPER inhibits dCLK/CYC-mediated transcription in the absence of dTIM, thus indicating that dTIM is only required for the translocation of dPER from the cytoplasm to the nucleus (Rothenflüh et al. 2000).

Just as the roles of the negative elements in the mammalian circadian clock are mysterious, the mechanism by which their intracellular localization is controlled is also unclear. In studies done in heterologous cell systems, mCRY1 and mCRY2 are found predominantly in the nucleus, whereas the mPER proteins are found in both the cytoplasm and nucleus. Co-expression of the mPER proteins with each other only modestly affects their intracellular localization, whereas their co-expression with the mCRYs leads to their efficient nuclear localization. However, the mPER proteins are found predominantly in the nucleus both in mCRY-deficient mice and in the nuclei of cell lines derived from these mice, indicating that they do not require mCRY for nuclear localization (Yagita et al. 2000, Kume et al. 1999, Shearman et al. 2000b). A further complication is raised by the finding that co-expression of mPER1 with casein kinase Iε (CKIε) in a cell line leads to the phosphorylation-dependent retention of mPER1 in the cytoplasm (Vielhaber et al. 2000). CKIε is a homologue of the *Drosophila* gene *double-time* (*dbt*), which plays an important role in the phosphorylation and destabilization of dPER (see below). Thus the nuclear localization of the mPER proteins in vivo may depend upon a number of factors. Regulation of nuclear localization is likely to play important roles in other clock systems as well. For example, a photoreceptor known to signal to the plant circadian clock translocates from the cytoplasm to the nucleus in a light-dependent manner (see Somers et al. 1998a and Yamamoto & Deng 1999 for further details).

Protein Stability and Degradation

For an oscillator to produce robust cycling, periodic inactivation of its components is essential. This can be achieved by degradation of at least one clock component. As described above, protein levels of many clock components do cycle, including

KaiC in cyanobacteria; FRQ and WC-1 in *Neurospora*; dPER, dTIM, and dCLK in flies; and PER1 and PER2 in mammals. The near-symmetric cycling of these proteins indicates that they have a short half-life and do not undergo sudden degradation at a specific phase of the oscillator. A common feature of all these proteins is their progressive phosphorylation prior to degradation (Xu et al. 2000b, Lee et al. 1998, Edery et al. 1994, Keesler et al. 2000, Liu et al. 2000, Sancar 2000). In a few cases, changes in phosphorylation status are accompanied by altered stability, suggesting a direct link between phosphorylation and protein degradation. Mutations that abolish a phosphorylation site in FRQ lead to delayed turnover of the protein (Liu et al. 2000), whereas a mutation that may affect the activity of the putative casein kinase DBT leads to hypo-phosphorylation and increased levels of dPER (Price et al. 1998, Kloss et al. 1998). Similarly, a mutation in a hamster CKIε homolog causes a short-period phenotype in these animals (Lowrey et al. 2000). A recently identified mutant version of human *per2*, with an alteration in a CKIε-binding region, is hypo-phosphorylated by CKIε in vitro (Toh et al. 2001). It will be interesting to see whether this mutated hPER shows any defect in its degradation.

In some cases, phosphorylation may trigger degradation by the proteosome (Deshaies et al. 1995, Lahav-Baratz et al. 1995). Light treatment causes tyrosine phosphorylation and ubiquitination of dTIM, followed by its proteosomal degradation (Naidoo et al. 1999). This degradation mechanism, however, is rapid and may not be involved in the relatively slow turnover of dTIM proteins during normal progression of the oscillator. In *Arabidopsis*, the F-box and kelch domain-containing protein ZTL may recruit specific substrates to a core ubiquitin ligase complex (SCF) for ubiquitination and subsequent proteolytic degradation (Somers et al. 2000). The *ztl* mutation has a light-dependent period-lengthening effect, suggesting that ZTL plays a role in light input to the clock. Interestingly, ZTL also contains a PAS-related LOV motif that has been shown to bind chromophore in the plant photoreceptor NPH1. Therefore, it is possible that ZTL takes part in a novel light-mediated protein degradation process involved in setting the pace of the oscillator.

There are other examples of altered phosphorylation that affects period length. The KaiC protein of cyanobacteria can autophosphorylate in vitro and has an ATP-binding site that when mutated causes arrhythmicity (Nishiwaki et al. 2000). In *Arabidopsis*, over-expression of a casein kinase that phosphorylates the clock-associated protein CCA1 in vitro alters free-running period length (Sugano et al. 1998, 1999). However, it is far from clear whether these effects are mediated through changes in protein stability. Phosphorylation may modulate clock protein function by other means, perhaps by regulating their localization, interactions, or ability to modulate transcription (Garceau et al. 1997, Liu et al. 2000).

CLOCK RESETTING

A fundamental property of a circadian oscillator is its ability to reset its phase to synchronize with the changing photoperiod in nature. Although normally both ambient light and temperature cycle on a daily basis, light is often (but not always)

the predominant zeitgeber. Many studies of light input to the clock are done by treating organisms held in free-running conditions of constant darkness or dim light with a brief pulse of light (non-parametric entrainment). The resulting change in phase of the overt output reflects a change in phase of the oscillator. A pulse of light will elicit a small change in phase when given during the subjective day, but will cause significant phase delays when given during the early evening or phase advances when given before dawn. In clever experiments using two temporally separated pulses of light (the first pulse resetting the clock and the second, later pulse determining whether phase resetting by the first pulse was completed in the time separating the two light treatments), phase resetting of the clock was achieved quite rapidly: 0.75 h in *Neurospora* (Crosthwaite et al. 1995), 2 h in mouse (Best et al. 1999), and 3 h in *Drosophila* (Pittendrigh 1979). These studies establish the temporal window within which the primary molecular mechanisms of circadian resetting must occur. As a broad generalization, it may be suggested that light either degrades a clock component that usually declines during the subjective day or induces a component that usually rises during the subjective day. Experimental evidence suggests that the mechanism of phase resetting by light may be different from the changes that occur during normal progression of the oscillator. In all clocks studied so far, light affects the phase of a negative element that then determines the phase of the oscillator. Therefore, light-triggered degradation has to overcome the mechanism that normally stabilizes a component and, conversely, induction of a component has to overcome the normal clock-mediated repression of its transcription. The clock itself regulates one or more of the components of this resetting pathway so that the clock is more prone to phase resetting at certain times than others, a phenomenon described as the gating of light input.

Cyanobacteria and *Neurospora*

A protein important for light input to the clock in cyanobacteria was recently identified. CikA is a bacteriophytochrome that possesses histidine kinase motifs and acts in the light-resetting pathway. Despite being a bacteriophytochrome, CikA may not act as a photoreceptor because it lacks a conserved residue important for chromophore binding in other phytochromes (Schmitz et al. 2000). A possible target of CikA may be KaiC. The phase of KaiC expression dictates the phase of the oscillator, since induction of *kaiC* within the physiological range resets the phase of transcription of the output gene *psbAI* (Xu et al. 2000b).

In *Neurospora*, light acts through WC-1 and WC-2 to reset the oscillator by the rapid transcriptional induction of *frq* (Crosthwaite et al. 1995, 1997). Interestingly, in addition to its DNA-binding domain, WC-1 has a LOV motif such as the one shown to bind flavin in the *Arabidopsis* blue-light photoreceptor NPH1, and many loss-of-function mutations in WC-1 map to this LOV motif (Christie et al. 1999, Crosthwaite et al. 1997). WC-1 may therefore serve both as a photoreceptor and a transcription factor mediating light response. Recent data suggest that FRQ is also essential for light responsiveness (Merrow et al. 2001). The magnitude of light-induced *frq* transcription and subsequent FRQ translation is several-fold

higher than the peak levels seen during free-running oscillations, suggesting that the acute induction of *frq* occurs by a unique mechanism. Under continuous illumination, FRQ levels remain high and constant, indicating that light induction of *frq* overcomes FRQ negative autoregulation and causes arrhythmicity. Light also triggers the rapid phosphorylation of WC-1, followed by WC-1 degradation (Talora et al. 1999). It is yet to be seen whether this phosphorylation is involved in the acute high level of *frq* transcription. Elucidation of the exact molecular events in light-induced *frq* transcription will likely also shed light on the molecular mechanism of FRQ negative autoregulation.

Temperature resetting of *Neurospora* clock appears to act instantly through translational control (Liu et al. 1998). *Neurospora* free running at different temperatures have very different levels of FRQ protein in spite of similar limits of oscillating message. FRQ amount at the lowest point in the curve (subjective dawn) at 28°C is higher than the highest point in the curve (subjective dusk) at 21°C; therefore, a given FRQ level at different temperatures reflects different circadian phases. Temperature changes, therefore, result in the interpretation of the existing FRQ level as a different phase, and FRQ oscillation is gradually adjusted to the new limits.

Drosophila

Several studies have shown that dTIM is rapidly degraded in response to light and suggest that this is the key molecular event involved in light-induced phase resetting (Lee et al. 1996, Hunter-Ensor et al. 1996, Zeng et al. 1996, Myers et al. 1996, Suri et al. 1998). An important question is whether the visual (opsin-based) photoreceptors are also the circadian photoreceptors. In flies, light acts through both ocular and extraocular pathways (Stanewsky et al. 1998, Suri et al. 1998, Yang et al. 1998). Although not much is known about ocular photoentrainment of the clock, non-ocular light entrainment is well studied. Genetically eyeless, opsin-depleted, or blind flies can be entrained to LD cycles, albeit with reduced efficiency (Helfrich-Forster & Homberg 1993, Helfrich-Forster 1997, Stanewsky et al. 1998). This entrainment is thought to be mediated by dCRY, which acts in a cell-autonomous manner (Emery et al. 2000).

dCRY interacts with dTIM in a light-dependent manner (Ceriani et al. 1999), suggesting that there is no signaling intermediate between the light receptor and its target, dTIM. This interaction correlates with dTIM degradation. Because dTIM stabilizes dPER, dPER is also degraded following light treatment. Thus in the early evening when dPER and dTIM levels are increasing, a light-induced decrease in dPER/dTIM results in a delay, and in late night when dPER/dTIM levels are normally falling, the same mechanism of degradation results in an advance to the next subjective day. Under constant light, dCRY-mediated dTIM degradation inhibits accumulation of dTIM and stops the clock, causing arrhythmic behavior.

In contrast to wild type, mutant cry^b flies show normal behavioral rhythmicity under constant light as if they are blind to light, conclusively establishing dCRY as a

unique circadian photoreceptor (Emery et al. 2000). These mutants are also deficient in short, light pulse–induced phase resetting (non-parametric entrainment), but their behavioral rhythms can be entrained to a LD cycle (parametric phase resetting) (Stanewsky et al. 1998). This suggests a role for ocular light signaling in parametric entrainment. dCRY does not play an essential role in oscillator function, as *cry*^{b} flies show normal behavioral rhythms in constant darkness. Furthermore, in the lateral neuron cells (LN_vs) that control behavioral rhythmicity in flies just as the SCN does in mammals, circadian oscillations of dPER/dTIM are intact (Emery et al. 1998, 2000). These findings indicate the mechanism of dCRY-mediated dTIM degradation is different from the mechanism that normally regulates dTIM protein oscillation in constant dark.

Mammals

Surgical experiments in rodents have established the retina as the primary anatomical site of photoreception. Light signals are transmitted from the retina to the master oscillator in the SCN, both directly via the retinohypothalamic tract (RHT) and indirectly by way of the intergeniculate leaflet of the lateral geniculate nucleus (reviewed in King & Takahashi 2000). Photoreceptors in the retina perceive light and translate it into a neuronal signal that travels through the RHT, primarily by ionotropic glutaminergic neurotransmission (Ding et al. 1994, Mintz et al. 1999). To effect a phase change, the retinoreceipient cells in the SCN must then translate this signal into a change in the level of an oscillator component. Mammalian clock resetting, unlike that seen in flies, has many intermediate steps from light perception to clock resetting. The intermediate components may integrate signals from many nonphotic entrainment stimuli. Direct photic resetting of the clock without any anatomical signaling intermediate, however, cannot be ruled out.

Very little is known about the retinal photoreceptive mechanism in this entrainment pathway. The maximal spectral sensitivity for phase shifting of locomotor activity is near 500 nm (Yoshimura & Ebihara 1996, Takahashi et al. 1984), similar to the action spectra of retinaldehyde-based visual pigments found in the retina. However, studies in mice lacking all rod cells and most cone cells suggest that the retinal visual rhodopsins and cone opsins are not required for light input to the clock. Even though these *rd* mutant mice are visually and electrophysiologically blind by three months of age, light entrainment of the circadian oscillator and light masking (inhibition of locomotor activity in presence of light) are intact in these animals (Mrosovsky et al. 1999, 2000; Selby et al. 2000). This, along with the finding that cryptochromes act as circadian photoreceptors in other systems, has renewed interest in the possibility that these proteins may act as circadian photoreceptors in mammals as well (reviewed in King & Takahashi 2000).

As mentioned above, the mCRYs are thought to play an essential role in clock function because mice deficient in both CRYs exhibit behavioral arrhythmicity in constant darkness. Since cryptochromes are essential for clock function, it is difficult to prove that they also play a role in pre-SCN photoreception (Selby et al.

2000). However, mCRY expression is seen in the inner retina as well as the SCN, suggesting a role in photoentrainment. Both *rd* mutants and *mcry* double mutants show some rhythmicity in LD, whereas mice deficient for all three genes are arrhythmic in LD. Therefore, both visual photoreceptors and the cryptochromes may contribute to circadian entrainment. Novel opsins may also contribute to light signaling to the SCN (reviewed in King & Takahashi 2000).

The target of light-mediated clock resetting in the oscillator is thought to be *mPER1*, whose transcript levels are usually high during the day. Light causes acute induction of *mper1* mRNA levels (Shigeyoshi et al. 1997), which peak an hour after the start of the light pulse and rapidly decline to the pre-pulse level within three hours. Light-induced *mper1* message transiently reaches the same level as the peak value seen in the free-running DD cycle, but an increase in protein level is seen only four hours after the light pulse (Field et al. 2000). Therefore, although light induction of *mper1* message seems to override the normal transcriptional repression mechanism of the clock, the mechanism that maintains a lag between message level and protein level still operates. In the early evening, when *mper1* levels are beginning to fall, a light pulse–induced increase in *mper1* delays that decline and produces a phase delay. In the late night, when the mPER1 protein level is beginning to rise, *mper1* induction advances the phase. In rats, light also triggers the degradation of the clock component BMAL1 (Tamaru et al. 2000), raising the possibility that light may target multiple components during phase resetting.

Many immediate changes in response to phase-resetting pulses of light have been documented in the SCN, some of which may act upstream of clock components. They include phosphorylation of cAMP (cyclic AMP) response element–binding protein (CREB) (Golombek & Ralph 1995) and induction of a set of genes including c-*fos* (Kornhauser et al. 1996, Boissin-Agasse et al. 1996). Pharmacological agents inducing some of these changes can mimic the effect of light on phase resetting, thereby placing these events upstream of clock resetting. Multiple kinases can phosphorylate CREB, including protein kinase A (PKA), Ca^{2+}/calmodulin-dependent kinase, and MAP kinase (Xing et al. 1996, Gonzalez & Montminy 1989, Sheng et al. 1991, Impey et al. 1998). Therefore, this pathway may integrate signaling from various pathways including nonphotic light signaling. Phosphorylated CREB activates transcription from genes containing CRE sites in their promoter. It is yet to be seen whether phospho-CREB directly induces transcription from the *per1* promoter, which contains multiple CRE sites (Hida et al. 2000), or whether more signaling intermediates are involved. cAMP, protein kinase C, glucocorticoid hormones, and Ca^{2+} can all trigger a transient surge of *mper1* transcription and its subsequent rhythmic expression in Rat-1 cells (Balsalobre et al. 2000), suggesting that many pathways can cause clock resetting in mammals.

Arabidopsis

Light interacts with the plant clock in a way qualitatively different from its interaction with fly, mammalian, or fungal clocks. Oscillators in these other systems

apparently cease to operate either in constant light (*Drosophila*, rodents, *Neurospora*) or under constant dark (cyanobacteria), while plant oscillators function under constant light or dark conditions (Millar & Kay 1991, Strayer et al. 2000). Furthermore, under constant light, the period length is dependent on both quality and fluence rate (intensity of light), suggesting an active role of light input pathway in setting the pace of the oscillator (Somers et al. 1998a). Circadian photoreceptors were first unequivocally identified in plants (Somers et al. 1998a). Plants contain an array of photoreceptors for optimal function in both different light intensities and qualities. In *Arabidopsis*, two classes of photoreceptors are implicated in circadian entrainment: phytochromes, which perceive red light; and cryptochromes, which perceive blue light (although there is some overlapping function between these classes) (Smith 2000, Casal 2000). PhyA, PhyB, PhyD, and PhyE act additively in red-light input to the clock. The blue-light photoreceptors aCRY1 and aCRY2 act redundantly in the blue-light input pathway. aCRY1 is also required for PhyA signaling in both red and blue light, whereas PhyB is required for aCRY2 function in white light (Mas et al. 2000; Somers et al. 1998a; Devlin & Kay 1999, 2000). PhyA has also been shown to mediate far-red-light input to the clock (Yanovsky et al. 2000a). Interestingly, *Arabidopsis cry1/cry2* double mutants still show robust rhythmicity (Devlin & Kay 2000, Yanovsky et al. 2000b), which indicates that cryptochromes do not play an oscillator role in plants as they do in mammals. The downstream signaling pathways from the Phys and aCRYs are complex (for recent reviews, see Smith 2000, Lin 2000). One interesting light-signaling component is a bHLH transcription factor, PIF3, that binds to the promoters of various light-induced genes including *CCA1* and *LHY*. PIF3 may, therefore, play a role in light-mediated resetting of the *Arabidopsis* clock (Martinez-Garcia et al. 2000).

Recent development of a new circadian marker (*ccr2::luc*) that shows robust rhythmicity in dark-adapted seedlings (Strayer et al. 2000) has facilitated the investigation of both non-parametric entrainment and light-independent circadian function in plants. It will now be possible to determine whether the photoreceptors and downstream components implicated in parametric entrainment also participate in non-parametric entrainment. A gene implicated in both kinds of entrainment has been identified and its properties studied using this marker. *elf3-1* plants have an intact clock in continuous darkness but are arrhythmic in constant light conditions. These plants also exhibit aberrant responses to light pulses. ELF3 levels cycle, and ELF3 attenuates light signaling from both blue- and red-light photoreceptors, indicating that it contributes to a gating mechanism for general light responses (Hicks et al. 1996, McWatters et al. 2000, Covington et al. 2001).

CLOCK OUTPUTS

The discussion thus far has been limited primarily to consideration of the core components of circadian oscillators. Now we consider outputs controlled by circadian clocks in the various model systems.

The simplest way to generate cycling outputs is to use the *cis*- and *trans*-acting elements of the central oscillator to generate cycling transcription of output genes. These immediate clock outputs may, in turn, regulate downstream outputs in a complex web of events, ultimately regulating various cellular processes. Most of our current knowledge of circadian outputs is based on rhythmic cellular or physiological processes, although there are several gene products known to cycle at the mRNA and/or protein levels. Systematic approaches to the identification of genes exhibiting cycling expression levels have begun to give us a genome-wide perspective on transcriptional regulation by the central oscillator (Liu et al. 1995, Harmer et al. 2000, Bell-Pedersen et al. 1996b; J.B. Hogenesch, M.F. Ceriani & S.A. Kay, unpublished data).

Given the nature of the clock outputs identified so far, a few generalized comments can be made. In all organisms, the clock coordinates aspects of energy metabolism with regard to the expected time of food availability, either via direct regulation of enzymes or via hormones known to regulate energy metabolism. The clock also plays a role in maintaining genome integrity in the presence of mutagenizing radiation from the sun. For example, cell division in the alga *Chlamydomonas* occurs during the dark phase (Nikaido & Johnson 2000, Mori & Johnson 2000), while genes encoding enzymes involved the synthesis of UV-protective compounds peak just before dawn in plants (Harmer et al. 2000). Many clock outputs also feed back on the oscillator and thereby may synchronize clocks in different cells and maintain clock homeostasis.

Neurospora

Attempts to identify clock-controlled genes (*ccgs*) in *Neurospora* have led to the estimate that fewer than 10% of its transcripts are cycling (Bell-Pedersen et al. 1996b). Most of the cycling messages identified to date peak around CT 4, similar to *frq* mRNA. It is possible that the same machinery that produces cycling transcription of *frq* message may be regulating a majority of clock-controlled genes. It is not yet known what *cis*-acting elements in the *frq* promoter confer cycling; however, progress has been made in the identification of a clock-responsive regulatory element found near the transcriptional start site of *ccg-2* (Bell-Pedersen et al. 1996a). Characterization of the nuclear factors that bind to this sequence should identify the *trans*-acting elements regulating cycling transcription. Over a dozen *ccgs* have been identified in *Neurospora*, and they contribute to rhythmic control of a variety of cellular processes (Loros 1998), including clock-regulated sugar metabolism [trehalose synthase (*ccg-1*) and glyceraldehyde 3-phosphate dehydrogenase (*ccg-7*)], stress responses [copper metallothionene (*ccg-12*)], and development [*ccg-2* (hydrophobin), *ccg-4*, *ccg-6*, *con-6* and *con-10*] (Bell-Pedersen et al. 1992, 1996b; Lee & Ebbole 1998; Shinohara et al. 1998).

Drosophila

Because the LN_vs in *Drosophila* control circadian regulation of locomotor activity in flies, genes expressed in these cells are of special interest. A few clock-regulated

transcripts have been localized to the LN_vs, and many feed back on the oscillator. In these cells, dCLK and CYC directly regulate transcription of the bZIP transcription factor VRI, which may be part of the central oscillator (Blau & Young 1999). One transcriptional target of VRI is the neuropeptide PDF, which regulates activity rhythms in flies (Renn et al. 1999). *pdf* is also regulated by the clock proteins dCLK and CYC, although its steady-state message levels do not show any rhythmicity (Park et al. 2000). dPER and dTIM act through some unknown mechanism to produce a rhythm in PDF peptide in the terminals, but not cell bodies, of axons projecting from the chronobiologically important small LN_vs. PDF has been suggested to synchronize oscillators in different cells (Park et al. 2000). Another output that feeds back to affect the oscillator is CREB. As in mammals, the phosphorylation state of dCREB cycles, and mutations in *dcreb*, affects the period of locomotor activity rhythms (Belvin et al. 1999).

There are also many outputs that do not feed back on the fly clock. The novel clock-controlled gene *takeout* (*to*), which does not seem to be regulated directly by dCLK and CYC, was isolated using a subtractive hybridization approach. TO is involved in a pathway that conveys temporal and food status information to feeding-relevant metabolisms and behaviors (So et al. 2000, Sarov-Blat et al. 2000). *to* shares sequence similarity with the fly gene *0.9kb*. Although *0.9kb* mRNA does not show circadian cycling, it is indirectly regulated by the clock as its levels peak just before eclosion (Lorenz et al. 1989). Another clock-regulated gene implicated in a circadian eclosion phenotype is *lark*. Molecular genetic analysis indicates that rhythmic changes in the abundance of the LARK RNA-binding protein are important for circadian regulation of adult eclosion (Newby & Jackson 1993). LARK protein oscillates in abundance within a defined group of neuropeptide-containing neurons of the ventral nervous system, which in many insects are thought to contain cellular elements of the clock output pathway regulating eclosion (McNeil et al. 1998). There are other clock-controlled genes, such as *dreg-5* and *crg-1*, whose functions remain unknown (Van Gelder & Krasnow 1996, Van Gelder et al. 1995, Rouyer et al. 1997). Peripheral tissues in the fly also maintain circadian rhythms independent of the LN_vs. The peripheral clock in the antennae may directly regulate a circadian rhythm in electrophysiological responses to olfactory stimuli (Krishnan et al. 1999). In addition, decapitated flies show a rhythm in dopamine receptor responsiveness that is abrogated in per^o flies lacking a functional clock. This suggests that the same clock components are important for rhythms in central and peripheral tissues (Andretic & Hirsh 2000).

Mammals

DNA microarrays now make it possible to simultaneously monitor changes in expression of thousands of genes (Lockhart & Winzeler 2000). High throughput gene expression analysis using DNA microarray technology has begun to identify transcripts with a circadian rhythm of accumulation in whole *Arabidopsis* seedlings (see below) and in a variety of mouse tissues (J.B. Hogenesch & S.A. Kay, unpublished data). Although a small percentage of gene transcripts cycle in

any given mammalian tissue, very little overlap is seen between sets of cycling mRNAs from different tissue types. Therefore, the total number of genes showing circadian regulation in at least one organ may be substantial. Particularly interesting will be the cycling of components involved in the regulation of hormones and their receptors, as they may amplify oscillator outputs and impose circadian regulation on a number of physiological processes.

In mammals, the mCLK/BMAL1/E-box system mediates cycling transcription of some clock outputs in addition to generating cycling of clock components. Transcription of DBP (an albumin-D binding protein containing a basic leucine zipper) is activated by mCLK/BMAL1 through E-boxes and inhibited by the mPER and mCRY proteins (Yamaguchi et al. 2000, Ripperger et al. 2000). The expression of the *dbp* gene oscillates with a large amplitude in liver, kidney, pancreas, heart muscle, and lungs (Fonjallaz et al. 1996, Lavery & Schibler 1993, Wuarin et al. 1992, Wuarin & Schibler 1990) and with a moderate two- to threefold amplitude in brain (Lopez-Molina et al. 1997). DBP feeds back on the central oscillator by binding a DBP consensus site in the *mper1* promoter and enhancing mCLK/BMAL1-mediated transcription of *mper1* (Yamaguchi et al. 2000). Mice lacking DBP display a shorter circadian period in locomotor activity and are less active. DBP also regulates several downstream processes such as circadian sleep consolidation and rhythmic EEG activity (Franken et al. 2000). Transcriptional targets of DBP include *cyp2a4* and *cyp2a5*, whose message levels cycle in a circadian manner in liver. Protein products of these two genes are involved in the metabolism of the sex hormones testosterone and estradiol (Lavery et al. 1999, Lavery & Schibler 1993). Two other proteins, thyroid embryonic factor (TEF) and hepatocyte leukemia factor (HLF), share extensive sequence similarity with DBP in the basic leucine zipper region and an adjacent segment rich in prolines and acidic amino acids (Drolet et al. 1991, Hunger et al. 1992, Inaba et al. 1992). *tef* and *hlf* message levels also cycle in several tissues (Falvey et al. 1995, Fonjallaz et al. 1996).

Other clock-regulated genes include the neuropeptide arginine vasopressin (AVP), the bHLH/PAS protein cycle-like factor (CLIF), and the retinoid-related orphan receptor β (RORβ). AVP is transcribed, synthesized, and released in a circadian manner from the SCN (Carter & Murphy 1992). It is involved in salt and water balance in the periphery and in some central nervous system functions. AVP transcription may be directly regulated by dCLK/BMAL1 through a putative E-box in its promoter (Jin et al. 1999). CLIF is rhythmically expressed in endothelial cells and in the SCN. CLIF can dimerize with mCLK in vitro to drive transcription from an E-box found in the promoter of the target gene plasminogen activator inhibitor-1 (PAI-I). Furthermore, mPER2 and mCRY1 inhibit PAI-1 promoter activation by dCLK/CLIF heterodimers. PAI-I may be involved in a circadian variation in fibrinolytic activity and may provide a molecular basis for the morning onset of myocardial infarction (Maemura et al. 2000). RORβ expression is rhythmic in the SCN, the retina, the pineal gland, and parts of the central nervous system involved in processing sensory information. RORβ-deficient mice exhibit a duck-like gait, transient male sterility, retinal defects, and lengthened locomotor activity rhythms

in constant darkness (Andre et al. 1998). It is interesting that the clock regulates developmentally critical genes, even though the absence of a functional clock does not affect normal development in many arrhythmic mutants.

Other clock-controlled processes include transcriptional activation of promoters containing cAMP response elements (CREs). This clock control of CRE-mediated gene expression occurs only in the SCN, and correlates with a rhythm in phosphorylation of the transcription factor CREB (Obrietan et al. 1999). CREB phosphorylation in the mouse SCN is regulated both by circadian oscillator and light (see above) (Ginty et al. 1993). Similarly, circadian rhythmicity in the phosphorylation state and, thus, activity of MAPK have been shown in *Drosophila* (Belvin et al. 1999), the chicken pineal gland (Sanada et al. 2000), the bullfrog retina (Harada et al. 2000), and the mouse SCN (Obrietan et al. 1998). A major mediator of clock-regulated physiology in animals is the hormone melatonin. It is synthesized in the pineal of all vertebrates and exhibits a daily rhythm with elevated nighttime levels. A second site of melatonin synthesis is the retina, where it may regulate some retinal physiology. The enzyme arylalkylamine *N*-acetyltransferase (AANAT) catalyzes the synthesis of melatonin from serotonin and is transcriptionally regulated via an E-box in chickens (Chong et al. 2000). In mammals, the SCN indirectly regulates pineal function via circadian regulation of norepinephrine (NE) release in the pineal glands. NE may act *trans*-synaptically through β-adrenergic receptors to induce cAMP, which may act through CREB to induce expression of a transcription factor that drives AANAT transcription (Baler & Klein 1995).

Many clock-controlled processes have been reported in peripheral tissues. Interestingly, several genes involved in feeding and digestion exhibit clock control (Scheving 2000). Intestinal expression of the high-affinity Na^+/glucose cotransporter-1 (SGLT1), which absorbs dietary glucose and galactose, displays both circadian periodicity in its activity and gated induction by dietary carbohydrates (Rhoads et al. 1998). Its peak transcription rate is phased to the morning. Two transcription factors, hepatocyte nuclear factor (HNF)1-α and HNF1-β, regulate SGLT1 transcription; HNF1-α is found bound to the promoter throughout the day while HNF1-β shows a circadian rhythm in binding to the promoter. Uroguanylyn (UGN) and guanylyn (GN) are the intestinal receptors for guanylyl cyclase. Transcription of these three genes in the intestine is under circadian control, and the protein levels of at least UGN and GN pro-hormones cycle in LD cycles with a peak in the night. They cycle with an anticipatory rise in the evening, suggesting this regulation is circadian (Scheving & Jin 1999).

Cyanobacteria and Higher Plants

Although the make-up of the central oscillator remains obscure in plants, our knowledge of plant clock outputs is considerable, both at the physiological and molecular levels. The rich history of this field began in 1727 when de Mairan noted that rhythmic leaf movements persisted in plants held in constant darkness,

the first recognition that circadian rhythms persist even in the absence of LD cycles (Sweeney 1987). Other physiological rhythms controlled by the circadian clock in plants include changes in cell elongation rates, stomatal aperture, CO_2 assimilation, Calvin cycle reactions, ethylene production, and the opening and closing of flowers in some species. See Sweeney (1987) and Somers (1999) for a more detailed discussion of these plant output pathways.

Physiological events controlled by the clock in cyanobacteria are also varied and include cell division, nitrogen fixation, and photosynthesis (Xu et al. 2000b, Golden et al. 1997). An important function of the circadian clock in these organisms is thought to be the temporal separation of nitrogen fixation, which relies on enzymes that are poisoned by oxygen, and photosynthesis, which produces free oxygen as a byproduct. Studies of circadian control of gene expression in cyanobacteria have yielded surprising findings. When a promoterless luciferase reporter gene was inserted randomly into the cyanobacterial genome, almost all of the resulting 800 bioluminescent strains showed a circadian rhythm in light production. Almost 80% of the clones exhibited peaks in light production around CT 12, with smaller groups of genes peaking at other phases. However, no colonies had a peak phase of luminescence in the first three quarters of the subjective night (CT 12–21) (Liu et al. 1995). This ubiquitous rhythmicity of gene expression suggests that the circadian clock regulates the general transcriptional machinery or the structure of the genome. However, other layers of control exist for regulation of genes that need be expressed in other phases; for example, a sigma factor that modifies circadian expression of only a subset of genes has been identified (Tsinoremas et al. 1996). It is important to note that these luciferase-based assays measure the rate of transcription but not the steady-state mRNA levels of the genes in which the reporter construct is inserted. Differences between bioluminescence rhythms and mRNA accumulation have indeed been found in some of these cyanobacterial strains (Golden et al. 1997).

In *Arabidopsis*, experiments using either oligonucleotide-based or spotted microarrays have shown that 6% or 2%, respectively, of the genes in the *Arabidopsis* genome are controlled by the circadian clock (Harmer et al. 2000, Schaffer et al. 2001). These different estimates are likely due to differences in experimental protocols, and both probably underestimate the true number of clock-controlled genes in *Arabidopsis*. Notably, peak transcript levels occur at all phases of the subjective day and night (Figure 3*A*), in contrast to what has been found in cyanobacteria and other organisms. These clock-controlled genes can be clustered into groups with similar phases of peak expression and predicted functions. These groupings have provided us with testable models for how the central clock may control physiological rhythms and have also revealed new pathways that may be influenced by the clock (Figure 3*B*) (Harmer et al. 2000, Schaffer et al. 2001). The identification of hundreds of cycling transcripts will likely facilitate the characterization of the central oscillator as well. A novel clock-regulated promoter element was identified in the promoters of genes that peaked late in the subjective day (Figure 3*C*). Mutation or deletion of the "evening element" causes a significant

decrease in the ability of a clock-regulated promoter to confer rhythmicity on reporter genes (Staiger & Apel 1999, Harmer et al. 2000). This promoter motif bears marked similarity to a CCA1-binding motif, and in fact CCA1 binds to it in vitro (D. Albadi, T. Oyama, M.J. Yanovsy, F.G. Harmon, P. Mas & S.A. Kay, submitted). It may be that CCA1 acts as a transcriptional repressor at the promoters of these evening-phased genes in contrast to its positive action at other promoters (Wang et al. 1997). Thus CCA1 and/or LHY may regulate two groups of genes whose peak phases of expression occur at very different times. Such positive and negative actions by the same transcription factor have been previously observed (Sauer & Jackle 1993, Willy et al. 2000). It seems likely that the study of clock outputs may lead us back to the central oscillator, in plants and in other systems as well.

SEASONAL RHYTHMS

Plants

Plants and animals manifest obvious rhythms matching the annual seasonal changes in the environment. In plants, these seasonal rhythms include the formation of flowers at the most appropriate times of the year to ensure reproductive success. Work in plants first revealed that seasonal rhythms are controlled by changes in day length, a phenomenon called photoperiodism. Bunning was the first to suggest that circadian timing systems also control photoperiodic timing (Figure 1). This has now been amply demonstrated both in plants and animals using classic physiological techniques (Sweeney 1987, Bunning 1973). More recent molecular genetic studies in plants have made the link between the photoperiodic control of flowering and circadian rhythms even more clear.

A number of *Arabidopsis* mutants have been described with aberrant circadian rhythms and disrupted regulation of flowering time. Normally, *Arabidopsis* plants flower much more rapidly when grown under long-day conditions mimicking summer than when grown under short-day conditions characteristic of winter. However, misregulation or mutation of genes implicated in clock function disrupts this response. For example, overexpression of either *CCA1* or *LHY* leads to late flowering in long days, while mutation of the putative light input factor *ELF3* leads to photoperiod-insensitive early flowering (see Samach & Coupland 2000 for a recent review). The short-period mutant *toc1-1* also flowers early in short days and is unresponsive to photoperiod (Somers et al. 1998b) when grown under days of the normal 24-h length. However, when *toc1-1* plants are grown under day:night cycles with a total length of 21 h, they can distinguish between short days and long days. Notably, these artificially short days approximate the period of the free-running clock in the *toc1-1* mutants, demonstrating that the *toc1-1* flowering defect can be fully explained by its circadian defect (Strayer et al. 2000).

A possible explanation for circadian regulation of photoperiodism in both plants and animals is given by the external coincidence model, in which the sensitivity of the organism to light input is posited to be dependent upon some internal

clock-controlled rhythm. Only at certain phases of that rhythm, perhaps when some gene is expressed above a threshold level, is the organism able to respond to light. In a long-day plant such as *Arabidopsis*, such a coincidence would occur only under long-day conditions and would act to promote flowering. The transcript levels of a number of genes involved in flowering time determination have recently been shown to cycle (Park et al. 1999, Fowler et al. 1999, Schaffer et al. 1998, Wang & Tobin 1998) and may constitute the internal rhythms sensitive to light proposed in the external coincidence model. The aberrant flowering of the *toc1-1* mutant may result from disruption of the phase or waveform of such clock-controlled flowering time genes when the period of the external environment does not match the plant's endogenous period (Strayer et al. 2000). These data are also consistent with an internal coincidence model in which two or more clock-controlled factors would be brought into the appropriate phase to evoke flowering only under long-day conditions. In either case, there must be a complex interplay between endogenous oscillators and light signal transduction pathways. Given the powerful genetic tools available in *Arabidopsis*, we can now test these models.

Animals

Seasonal rhythms influenced by the circadian clock in animals include changes in reproductive activity, food consumption, body mass, and hibernation. Much recent work has focused on how environmental stimuli interact with the clock to regulate these processes. One proposal is that the central oscillator responds to both the lights on (morning) and lights off (evening) signals. However, the clocks of most model organisms studied so far respond only to lights on or to lights off. A circadian system consisting of two independent oscillators might act to memorize the photoperiod, perhaps with two clusters of cells within the same animal cycling with different phases, one group tracking the morning and the other tracking the evening. Interestingly, hamster SCN sectioned in a vertical plane (instead of the usual coronal sections) exhibits two peaks of neuronal firing activity, one tied to the morning and the other tied to the evening. Recordings from hamsters entrained to different photoperiods showed that the onset of the first peak tracked the previous dawn while the offset of the second peak tracked the projected dusk (Jagota et al. 2000). Similarly, application of glutamate, which mimics a light pulse, has different phase-shifting effects on these two peaks of firing activity when administered in the early evening versus the late night.

These molecular observations complement recent observations made in house sparrow, a day-active organism, and Siberian hamster, a nocturnal organism. Under constant environmental conditions, sparrows previously exposed to long photoperiods continued to have long feeding activity times and, conversely, those previously exposed to short-day conditions maintained short feeding activity times. Cycling levels of secreted melatonin in sparrows held in constant darkness also follow the long- or short-day patterns seen in the entraining conditions. Explanted pineal glands showed the same patterns of melatonin secretion as found

in intact sparrows, demonstrating the tissue-autonomous nature of this response (Brandstatter et al. 2000). Similarly, when Siberian hamsters were subjected to different photoperiods, abundance of haPER-1 and haPER-2 proteins in the SCN closely followed the entraining photoperiod even after the animals were released to free-running conditions (Nuesslein-Hildesheim et al. 2000, Messager et al. 2000). This photoperiod-modulated *per* expression might affect clock outputs that mediate seasonal rhythms. For example, under long photoperiods, prolonged PER expression may repress arginine vasopressin and lead to a seasonal modulation of hormone levels. Thus independent oscillators may track dawn and dusk, and their combined outputs may contribute to photoperiodic time measurement and seasonal rhythms in animals.

MULTIPLE OSCILLATORS

An ongoing debate in the oscillator field is whether there is one molecular clock that affects all overt rhythms or multiple oscillators that control different sets of rhythms. In unicellular organisms, where all overt rhythms probably result from individual cells, it is relatively easy to investigate the presence of multiple oscillators by looking for overt rhythms in the absence of the already identified molecular clock. For example, although FRQ-deficient *Neurospora* show no circadian rhythm in sporulation or transcription of known clock-controlled genes, a second oscillator seems to generate some cyclic outputs. In these *frq* mutants, there is a persistent DAG rhythm regulated by what is presently known as the *Frq*-less oscillator (Ramsdale & Lakin-Thomas 2000). The molecular nature of this oscillator is still unknown. The marine alga *Gonyaulax* seems to use two independent oscillators to control different outputs as well (Roenneberg 1996). Two different clock outputs have different free-running periods (von der Heyde et al. 1992) and phase shift differently in response to the same light stimulus, suggesting two different light input pathways for the underlying oscillators

The oscillation of different outputs with slightly different free-running period length might indicate the existence of multiple independent oscillators in multicellular organisms; for example, the oscillator regulating *CAB2* expression in *Arabidopsis* can be entrained to free run with different phases in different organs (Thain et al. 2000). However, since the period length of the above oscillator shows strong dependence on light quality and quantity (Somers et al. 1998a), it is easy to imagine that one oscillator could generate different period lengths in tissues differently exposed to light. A systematic analysis of the effects of *tej*, a period-altering mutant, on gene expression in *Arabidopsis* showed that this mutation lengthened the period length of all known clock-controlled genes, suggesting all overt rhythms are regulated by a single oscillator (S. Panda & S.A. Kay, unpublished data). However, a possible role of *tej* upstream of multiple oscillators cannot be ruled out.

There is some evidence that multiple clocks operate in mammals. Some strains of mice exhibit splitting of locomotor activity into two distinct phases after a few

days in constant light (Abe et al. 1998). Similarly, hamsters held in constant conditions for extended periods of time sometimes show two distinct bouts of activity rhythms within each circadian cycle. These activity rhythms may be up to 180° out of phase with each other. Correspondingly, clock components in the two halves of the SCN in these desynchronized hamsters, but not in normal animals, are found to oscillate with opposite phases (de la Iglesia et al. 2000). AVP and VIP rhythms in the rat SCN in vitro are generated by two separate sets of non-overlapping cells and maintain a constant phase relationship. However, they cycle with two distinct period lengths upon addition of an anti-mitotic agent, and NMDA treatment causes differential phase shifts of these two rhythms (Shinohara et al. 1995). Therefore, the same molecular oscillator can operate independently in different tissue types and a synchronization signal may normally keep these tissues in phase. There seems to be a hierarchical order of interaction between oscillators in different tissue types in mammals. Although resetting the SCN clock by administration of light can ultimately reset the peripheral clock, the peripheral clock can be reset independently of the SCN clock. This suggests that the SCN clock is a master oscillator, driving slave oscillators in peripheral tissue (although both oscillators contain the same components) (Yamazaki et al. 2000, Stokkan et al. 2001, Damiola et al. 2000). In *Arabidopsis*, two different molecular oscillators have been shown to have such a master-slave relationship. The CCR2 protein can inhibit the cycling transcription of its own gene and thus constitutes part of a molecular oscillator. However, the abolition of this rhythm does not affect other outputs, whereas mutations affecting *CCA1* expression do perturb the CCR2 oscillator (Heintzen et al. 1997, Wang & Tobin 1998).

EVOLUTIONARY RELATIONSHIPS

Despite the common mechanisms outlined above—the use of transcriptional feedback loops and extensive post-transcriptional controls to generate endogenous cell-autonomous oscillators—clock genes do not appear to have been conserved between higher taxa. It is therefore possible that these similar regulatory circuits arose by convergent evolution. However, common functional domains are found in clock genes from different taxa. The nature of these domains may help us to understand the evolutionary pressures that resulted in circadian clocks.

Cryptochromes are implicated in light input to the circadian clock in *Drosophila* and *Arabidopsis* and play an important role in the central oscillator in mice. However, phylogenetic studies suggest that the animal and plant proteins arose independently from the DNA repair enzyme photolyase in an example of repeated evolution (Cashmore et al. 1999). Similarly, PAS domains are found in clock genes in metazoa, fungi, and higher plants. However, they are widespread throughout the three kingdoms of life and are often found in proteins that play important roles in the detection of and adaptation to environmental stresses (Gu et al. 2000). Their presence in the clock proteins described above may be another case of evolutionary

convergence rather than common descent of these proteins from a primordial clock. Other common domains can be found in cyanobacteria and *Arabidopsis* clock genes. For example, the plant protein TOC1 and the cyanobacterial protein CikA both contain atypical response regulator receiver domains. Response regulator proteins play important roles in a wide variety of adaptive responses to environmental changes (Sakakibara et al. 2000). Similarly, the presumptive chromophore-binding domain of CikA is homologous to the chromophore-binding domains of higher plant phytochromes, with both types of proteins mediating light input to their respective central oscillators. However, for all of these cross-taxa comparisons, there is no well-defined homology outside the domains mentioned above.

Rather than reflecting common descent of clock proteins, the presence of these shared protein motifs may instead reflect common evolutionary influences that resulted in the independent evolution of circadian clocks in different higher taxa. All these functional domains are implicated in organismal responses to stress, notably stresses related to light exposure. For example, photolyases repair DNA damage induced by UV light, whereas PAS domains are often found in photoreceptor proteins. The recruitment of genes involved in light responses to the service of the circadian clock fits with speculation that a primary driving force for circadian clock evolution was a "flight from light" so that light-sensitive processes could be phased to occur at night (Pittendrigh 1993). Such speculation is reinforced by findings that cell division in the alga *Chlamydomonas* and regulation of a plant pathway involved in the production of photoprotective pigments are under clock control (Nikaido & Johnson 2000, Mori & Johnson 2000, Harmer et al. 2000). Conversely, a mechanism key to DNA damage repair in metazoans has been recruited to set the pace of the oscillator in plants (S. Panda & S.A. Kay, unpublished data).

SUMMARY

Circadian clock components in animals, plants, *Neurospora*, and cyanobacteria are structurally quite different yet show intriguingly similar functions. On the other hand, clock components are conserved among metazoa and yet appear to have acquired modified functions in different organisms. The disparate uses to which homologous genes have been put in the mouse and *Drosophila* clocks have been outlined above. Even more striking are the functional differences found between the mouse and fly *per* genes on one hand and their *Caenorhabditis elegans* homolog on the other. Steady-state mRNA levels encoding LIN-42, the apparent *C. elegans per* orthologue, oscillate in a rhythmic manner just as do *mper* and *dper*. However, the period length of *lin-42* oscillations is only about six hours, the length of time it takes *C. elegans* to progress from one larval stage to the next. Indeed, the relative timing of larval stage–specific developmental events is disrupted in *lin-42* mutants (Jeon et al. 1999). It appears as if clock machinery designed to measure daily time has been subverted to measure developmental time in *C. elegans*. This surprising finding suggests that clock genes are malleable components that can be put to diverse uses, presumably to best adapt organisms to their particular

environmental and temporal niches. Research in circadian rhythms continues at a dizzying pace; no doubt we will be treated to many further spectacular examples of clock proteins with converging and diverging functions.

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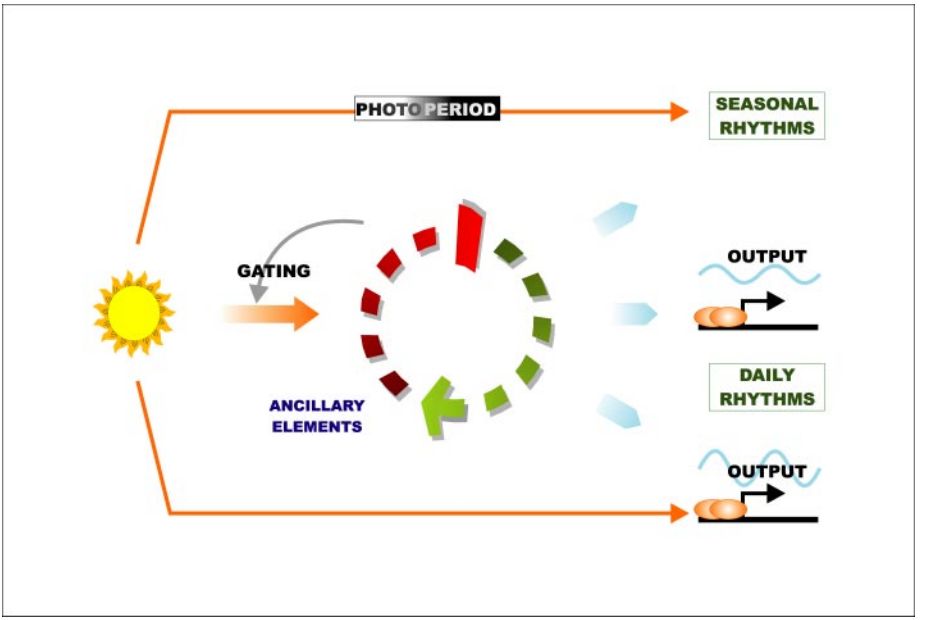

Figure 1 A schematic model of the circadian system in higher organisms. Positive and negative components constitute a feedback inhibition loop at the heart of the oscillator. Many ancillary components act on the central oscillator components to insert delays and to generate a stable near-24-h rhythm. Circadian photoreceptors and their downstream signaling components constitute the light input pathway to the clock that helps to maintain a stable phase relationship between the clock and the natural light:dark cycle. Some clock outputs may also participate in the light input pathway to fine-tune phase resetting. The clock generates daily rhythms, although light may also directly act on some clock outputs to generate robust rhythms. The clock also generates annual rhythms, as it measures seasonal changes in day length. See text for further details.

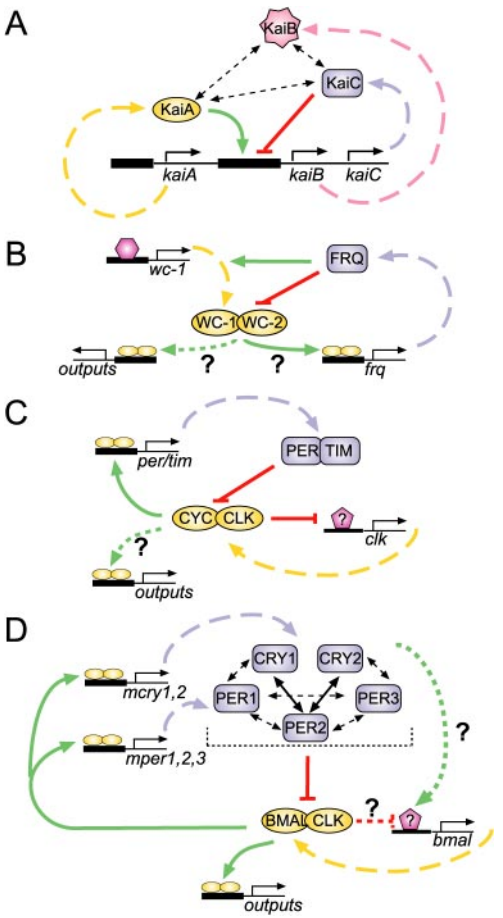

Figure 2 Transcriptional feedback loops function in circadian clocks. Colored dotted lines represent mRNAs and link genes with their respective proteins. Proteins with positive actions are in yellow, negative components are in blue. Protein-protein interactions are indicated with dotted black arrows. Green arrows indicate a positive effect of a component on some process or promoter, and red lines indicate an inhibitory action. (*A*) The cyanobacteria clock and (*B*) the *Neurospora* clock. The question marks indicate that the white-collar complex has not been shown to directly bind the promoters of *frq* or other clock-controlled genes. (*C*) The *Drosophila* clock. The question mark indicates that CYC and dCLK have not been shown to bind the promoters of output genes. Also, it is not known whether these proteins inhibit the activity of some activator of *dclk* transcription or act more directly. (*D*) The mouse circadian clock. The mPERs and mCRYs positively regulate *bmal1* transcription, possibly by inhibiting BMAL1/mCLK (similar to dPER/dTIM inhibition of CYC/dCLK), but evidence for this is inconclusive. The solid black lines indicate that the mCRYs stabilize or promote translation of mPER2 (Shearman et al. 2000b). See text for more details.

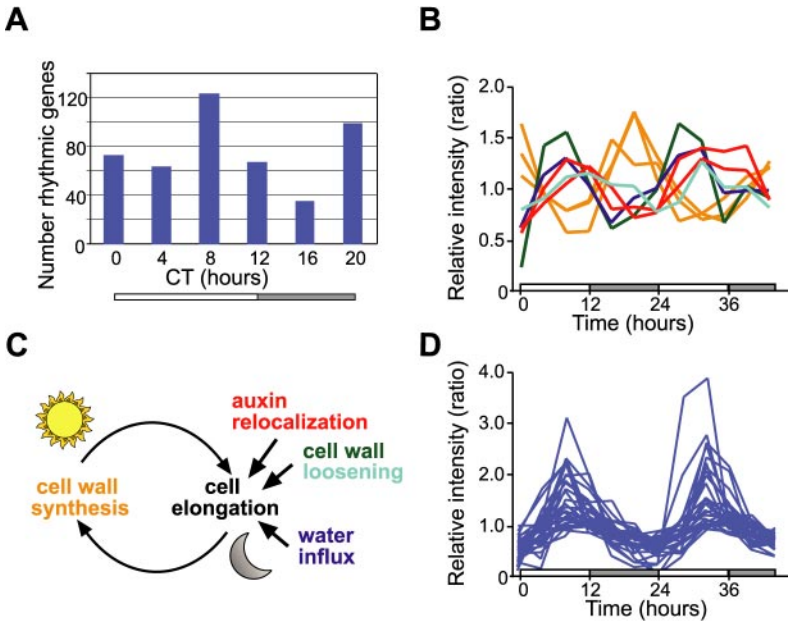

Figure 3 Regulation of clock-controlled genes in *Arabidopsis*. (*A*) Peak expression of clock-controlled genes occurs throughout the day. (*B*) Genes implicated in cell wall modification are clock-controlled. (*C*) Speculative model for function of genes depicted in (*B*). (*D*) All these evening-phased genes possess a common promoter motif. See Harmer et al. (2000) for further details. (Reprinted with permission from *Science* 290: 2110-2113. Copyright 2000 Am. Assoc. Adv. Sci.)