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Review

Regulation of clock genes

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Abstract. A recent explosion in the identification of new clock components in cyanobacteria, fungi, insects, mammals as well as potential candidates in plants has uncovered common themes among the structure, function and regulation of these components. Positive and negative interactions that are organized in negative feedback loops have been found crucial for clock function. Both transcriptional and posttranscriptional mechanisms appear to be important for circadian rhythm generation in all of these organisms.

Key words. Circadian; *frq*; *per*; *Neurospora*; *Drosophila*; mouse.

Introduction

Among the many rhythmic phenomena observed in organisms, those activities with a period length of about 24 h are the most widespread and probably most important. After all, these activities reflect the crucial adaptation of life to the daily light/dark (LD) and temperature cycles on the earth. To name just a few of these adaptations, there is nitrogen fixation in cyanobacteria, photosynthesis and photoperiodic flower induction in many plants, rhythmic conidiation in fungi, locomotor activity and eclosion rhythms in flies, and the sleep-wake and activity cycles in vertebrates (for reviews see [1–3]). Since these rhythmic phenomena persist under constant conditions, they are endogenous. These endogenous rhythms have characteristic period lengths that deviate slightly from exactly 24 h; hence, they were termed circadian (Latin for 'about a day') rhythms. In real life, circadian rhythms are entrained to exact 24-h cycles by the resetting action of light and temperature. Since circadian rhythms are considered adaptations to rhythmic changes of the environment, they can be found in almost all organisms, and they influence many different processes at every level of organization. In contrast to the enormous variety of clock-regulated processes, the identification of the molecular components of circadian clocks in mammals, flies, fungi and other systems indicates that the clocks in these organisms are composed of only a few central clock components. These components seem to comprise the molecular core of the oscillators and are part of a network of positive and negative interactions that establish a negative feedback cycle generating the basic oscillation.

Although transcriptional regulation seems to play a major role in rhythm generation, it is becoming more and more evident that various posttranscriptional mechanisms also have a major influence on rhythm generation and the shaping of the characteristics of the oscillation. Phosphorylation and subsequent protein degradation are important regulatory mechanisms of the *Drosophila* and *Neurospora* clock proteins. Nuclear entry of clock proteins has been shown to be essential for clock function in both *Drosophila* and *Neurospora*. In *Drosophila* and *Neurospora*, heteromeric protein-protein interactions have been shown to be important for the formation of transcriptionally active complexes and in *Drosophila* for nuclear entry and protein stabil-

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ity. In *Neurospora*, the use of alternative translational start sites of the *frq* messenger RNA (mRNA) generates functionally different clock proteins, and the use of alternative splice sites within the 3′ untranslated region (UTR) of *per* may affect transcript stability in *Drosophila*. Future research in these systems will doubtlessly reveal more aspects of such regulations (for reviews see [4–8]). The focus of this review will be on the regulation of the clock genes in two of the best-characterized clock systems, *Drosophila* and *Neurospora*. We will also briefly summarize the progress that has been made towards the identification of clock components in several other model systems.

Common themes of the clock mechanisms: negative feedback loops

In theory, a continuous oscillation of a given process requires at least two elements: a negative element which can feed back on itself to slow down the rate of the process, and a positive element which can reactivate the process after it has been repressed. The rather long period ($\sim$24 h) of a circadian oscillator relative to other biological oscillations requires some built-in delay mechanisms to prevent immediate feedback. At the molecular level, nearly all the evidence to date (from prokaryotic cyanobacteria to mammals) is consistent with clocks based on negative feedback loops in which some rhythmically expressed clock genes encode proteins that act to shut off their own expression. Various transcriptional and posttranscriptional regulations are crucial to maintain such molecular oscillations.

Figure 1 is a simplified illustration of a circadian oscillator with some of the clock elements identified in different circadian model systems. The simplicity of the figure should not be taken as suggesting that circadian oscillators will only have one simple feedback loop. In fact, it only represents what we currently think is the core feedback loop in each system based on the available molecular and genetic evidence. A circadian system can and often will be made of one or more interconnected feedback loops [9]. In addition, another interconnected loop can be added [10] if the core loop regulates the components of its input, for instance a photoreceptor [11], or if a clock-controlled output component influences the input or a component of the core loop [12]. Furthermore, a circadian system might also employ suboscillators that are under control of a master oscillator which serves to enhance and/or rephase their oscillations [13]. Despite the involvement of different clock genes and different regulations in each organism, a variety of data now suggest that they may share some common features. First, the core feedback loop involves both positive and negative elements and is based on the transcription and translation of the clock genes and clock proteins. The positive elements in the loop activate the transcription of clock genes which encode clock protein products that act as the negative elements in the loop. The known positive elements include KaiA in *Synechococcus* [14], WC-1 and WC-2 in *Neurospora* [15], dCLOCK and CYCLE in *Drosophila* [16–18], CLOCK and BMAL1 in mice [19–21]. In these four systems, the negative elements are KaiC in *Synechococcus* [14], FRQ in *Neurospora* [22, 23], PER and TIM in *Drosophila* [18, 24, 25], PER1, PER2, PER3 and TIM in mouse [26–30]. In the eukaryotic systems of *Neurospora*, *Drosophila* and mouse, the positive elements are all transcriptional activators that contain PAS (shared regions among the proteins PERIOD, ARNT and SIM) protein-protein interaction domains as well as DNA binding domains. These transcriptional activators may form protein-protein heterodimers via the PAS domain and then activate the transcription of a clock gene(s). In *Synechococcus*, the activation of *kaiC* may go through KaiA. In all four cases, the activation of a clock gene gives rise to a message which encodes the negative element(s). These negative elements then feed back to block the activation of their own transcription so that their steady-state levels decline. After the amount of the clock protein (s) decreases to a certain level, they will no longer repress their own synthesis, and positive elements

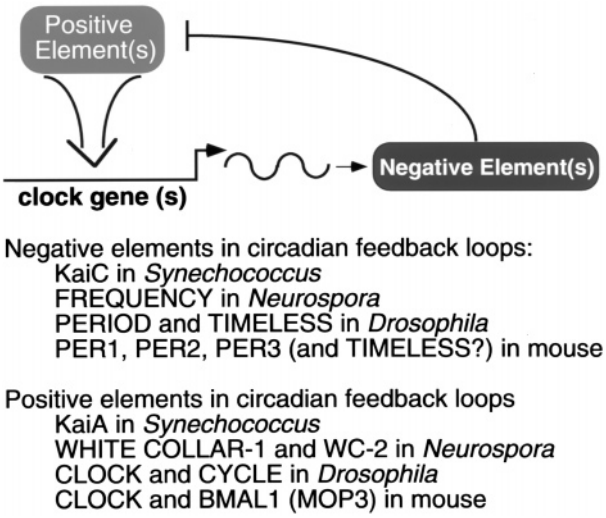

Figure 1. Common themes among different circadian oscillators. A general regulatory scheme seen in the four best-understood circadian systems, cyanobacteria (*Synechococcus*), fungi (*Neurospora*), insects (*Drosophila*) and mammals. Negative elements in a circadian feedback loop, acting to negatively regulate the activation of the positive elements. The functionally similar elements in different systems are listed. This figure is modified from [1].

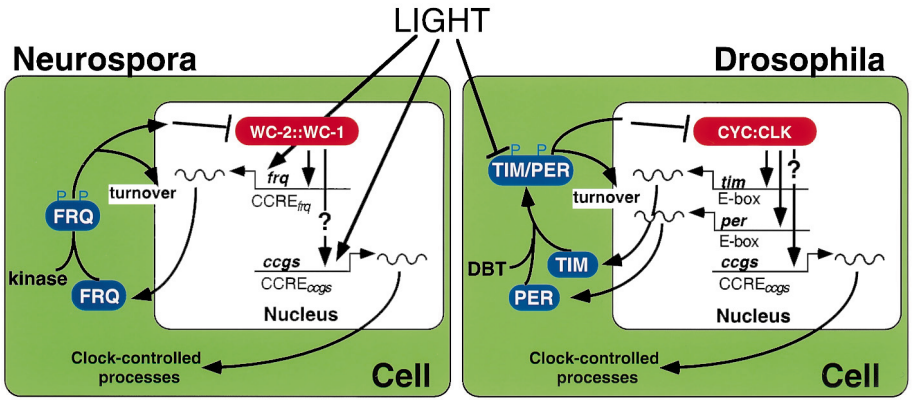

Figure 2. Negative feedback loops in *Neurospora* and *Drosophila* circadian clocks. The details of the molecular core loops are shown. A feedback loop in *Neurospora* includes the *frq* gene and protein and WC-1 and WC-2 proteins. In *Drosophila*, it contains *per* and *tim* genes and PER, TIM, CYC and CLK proteins. All these proteins are thought to act in the nucleus, but the kinase(s) (DBT in *Drosophila*) act in the cytoplasm. Light acts through the WC proteins to reset the *Neurospora* clock by inducing *frq* and to induce the transcription of other clock-controlled genes (*ccgs*). In *Drosophila*, light resets the clock by triggering the degradation of TIM, which destabilizes PER. *ccgs* are output genes of the clock. They are controlled by the clock, but are not components of the feedback loop. This figure is modified from [1].

can then reactivate their transcription. This repeated activation and repression process generates a robust daily cycling of the levels of mRNA and protein of the negative elements which is a common feature in all circadian systems [14, 22, 24, 31–36]. Although not all of the details of feedback loops have been revealed, the threads of similarity among all systems suggest that this emerging theme may reflect a common mechanistic core for circadian oscillators in different organisms.

Two clocks: the *Neurospora* and *Drosophila* stories

Neurospora and *Drosophila* are the two best-studied and understood systems with respect to the molecular mechanisms of circadian clocks. The first clock mutants were isolated in these two organisms in the early 1970s [1, 37, 38], and two clock genes, the *period* gene in *Drosophila* and the *frequency* gene in *Neurospora*, were cloned in the 1980s [39–41]. Within the last 4–5 years, six more clock genes have been cloned: *timeless*, *dClock*, *cycle* and *double-time* in *Drosophila* [18, 42–44], and *white collar-1* and *white collar-2* in *Neurospora* [15, 45, 46]. Most of our current knowledge of how circadian clocks work has been through the understanding of how these genes are regulated.

The *Neurospora clock*

The *Neurospora* oscillator comprises an autoregulatory negative feedback cycle [22] in which *frq* mRNA and FRQ protein are the central components [4, 22, 47]. Deletion of the *frq* locus results in loss of rhythmicity. Mutations at the *frq* locus have a variety of clock phenotypes: long and short period length (from 16 to 29 h), arrhythmia and loss of temperature compensation of the clock [47]. The sense transcript of *frq* encodes two FRQ protein forms, a long form of 989 amino acids and a shorter form of 890 amino acids, due to alternative initiation of translation from an internal AUG codon [32]. Levels of both *frq* mRNA and FRQ protein cycle in a daily fashion, and FRQ protein acts to repress its own transcript abundance [22, 23, 32]. Importantly, rhythmic expression of *frq* transcript is essential for the loop, because constitutive expression of *frq* results in the loss of the overt rhythm and step changes in *frq* expression reset the phase of the clock [22]. Since this rhythmic autoregulated expression is required for the clock, *frq* mRNA and FRQ protein are not just components but state variables of the circadian oscillator whose values truly define biological time. Their levels and kinetics define the oscillation of the clock [32].

If the progress of a *Neurospora* clock cycle is followed starting from ~CT18 (fig. 2), then the *frq* mRNA and FRQ levels are low. Gradually, *frq* mRNA level begins to rise, a process that takes 10–12 h to reach its peak. This increase in *frq* is possibly the result of transcriptional activation by a dimeric pair of transcription factors encoded by *white collar-1* (*wc-1*) and *wc-2* [15]. Both WC-1 and WC-2 are positive elements in the *Neurospora* oscillator because they are required for maintaining *frq* level in both light and darkness. In strains with lesions of either *wc-1* or *wc-2*, the levels of *frq* mRNA and FRQ are extremely low, and these low levels of *frq* and FRQ expression are unable to support the overt rhythmicity. WC-1 and WC-2 are both PAS domain-containing transcription factors, and they transcriptionally activate target genes, including *frq*, by

forming heterodimers with each other through their PAS regions and binding to the cis-acting elements in the promoter of these genes [45, 46, 48].

As the *frq* mRNA is translated, the level of FRQ protein starts to accumulate with a delay of several hours when compared with peak levels of *frq* mRNA [32, 49]. *frq* mRNA levels peak early in the morning [circadian time (CT) 2–6], about the same time as nuclear FRQ levels, but several hours before total FRQ protein levels peak in the early afternoon (CT 8–10). While in the nucleus, FRQ may interact with WC-1 and WC-2, an interaction which could lead to the repression of its own transcription. In addition, FRQ might also directly or indirectly regulate the expression of many other clock-controlled genes [50, 51]. The negative feedback process can occur rather fast, as known from experiments where *frq* is put under the control of the quinic acid-inducible promoter (in an *frq* null background). In these experiments the time from the onset of *frq* transcription until the complete decline of *frq* mRNA levels can take place in just 6 h [23]. In contrast, nearly 14 h are required for FRQ to become phosphorylated and to be turned over, so that *frq* steady-state levels stay low for the most of the day, whereas FRQ protein levels are relatively high [32]. During the afternoon, the FRQ protein becomes increasingly phosphorylated, and its steady-state levels decline. This process can last 8–10 h before FRQ protein levels drop below a certain threshold that allows *frq* transcription to become reactivated during midnight and hence reinitiate a new cycle.

Superimposed on this basic principle of rhythm generation are several layers of posttranscriptional regulations that allow proper oscillator function in real-life conditions. First, after *frq* transcript is made, it is subject to translational regulation which is important for keeping the clock running properly at different physiological temperatures [32, 52]. Like all other clocks, the *Neurospora* circadian clock can only function within certain temperature limits (16–34 °C), and the molecular basis for this phenomenon lies in part in the translational regulation of FRQ. As mentioned, two forms of FRQ are produced due to the alternative initiation of translation from two different start sites of the *frq* transcript. The large form initiates from AUG # 1, and the small form initiates from AUG # 3 at codon 100. Although both forms of FRQ are required for proper clock function within the physiological temperature range, at higher temperatures (~ 30 °C), the first AUG is favored, whereas at lower temperatures (~ 18 °C), there is relatively more of the small FRQ form produced [52]. In strains lacking AUG # 1, rhythmicity is lost at higher temperatures, whereas a strain with the third AUG mutated becomes arrhythmic at low temperatures. This temperature-regulated alternative translational initiation represents a novel adaptive mechanism to allow the *Neurospora* clock to function over a wide range of physiological temperatures.

Temperature not only regulates the choice of the initiation codon, but it also determines the total amount of FRQ protein by a posttranscriptional mechanism. As temperature increases, the total amount of FRQ also increases despite little increase in the levels of *frq* mRNA [53]. This mechanism may also contribute to the mechanism that allows the *Neurospora* clock to be entrained by temperature (see below).

Another posttranscriptional phenomenon is the nuclear entry of FRQ. Soon after their synthesis, both forms of FRQ enter the nucleus where they are required for the clock function [49]. The deletion of the nuclear localization signal (NLS) renders FRQ unable to enter the nucleus and abolishes overt circadian rhythmicity, whereas reinsertion of the NLS at another novel site restores its nuclear entry and the overt rhythmicity.

Protein phosphorylation is yet another mode of regulation. As soon as FRQ is made, it is progressively phosphorylated over time, a process that coincides with increasing turnover. Thus, FRQ is differentially phosphorylated over the course of a day [32]. Although the identity of the phosphorylating kinase(s) is still unknown, current evidence is consistent with a model in which FRQ phosphorylation may trigger its own turnover. First, FRQ starts to degrade only after it has been extensively phosphorylated. Second, when phosphorylation is blocked by certain kinase inhibitors, the turnover rate of FRQ decreases, which in turn leads to a longer period length of the clock (Y. Liu et al., unpublished results). This is similar to what happens with PER in *Drosophila*, where *dbt*, a type of casein kinase I, phosphorylates PER, thereby regulating PER degradation [43, 44].

Another possible step of posttranscriptional regulation could be achieved through the use of an antisense *frq* transcript which arises within the *frq* locus and overlaps with the 3′ end of the sense transcript [54]. Although no long open reading frame has been identified, it might play a role in regulating sense *frq* mRNA by hybrid formation. Interestingly, a similar situation might exist in silk moth, where a *per* antisense transcript is synthesized out of phase to the *per* sense transcript [55].

One of the basic characteristics of any functional circadian clock is its ability to be entrained by environmental time cues, among which light and temperature are the two most important. In the real world, light and temperature reinforce each other to keep clocks synchronous with the outside environment. In *Neurospora*, both light and temperature reset the clock by changing the level of the central clock components, *frq* mRNA and FRQ protein. Light acts rapidly through WC-1 and WC-2 proteins to induce *frq* transcription, which then

results in the increase of FRQ protein [56]. Since *frq* mRNA and FRQ protein levels normally cycle with a defined phase (i.e. subjective night always corresponds to low levels of *frq* and FRQ, and *frq* levels are high in the morning), any abrupt changes in *frq* and FRQ result in an abrupt and apparent change in time. Thus, the new phase of the clock after the light treatment will always correspond to the phase when *frq* and FRQ levels are high. When a light pulse is given in the late night or early morning when *frq* mRNA is rising, induction of *frq* rapidly advances the clock to a point corresponding to midday. During the subjective evening and early night when *frq* is falling, induction of *frq* by light rapidly sends the clock back to the peak point of *frq* accumulation (corresponding to midday), causing a phase delay. Similar results are seen in mammals, where light also rapidly induces the transcription of the two putative clock genes, *mper1* and *mper2* [57–59].

The other major entraining factor is temperature. Resetting the *Neurospora* clock by temperature steps is at least partially the result of posttranscriptional regulation [53]. Both *frq* and FRQ levels are rhythmic at different temperatures, but FRQ amounts oscillate at higher levels at higher temperatures, whereas the level of *frq* mRNA oscillations does not show any significant increase or decrease as temperature rises. At 28 °C, the trough point of the FRQ protein oscillation is higher than the peak of FRQ amount at 21 °C, so that a certain amount of FRQ corresponds to a different phase at different temperatures. After a temperature step, the levels of *frq* and FRQ either increase (for temperature steps up) or decrease (for temperature steps down) to adjust to the new temperature, causing rapid and proportional phase shifts. When *Neurospora* is shifted from 21 to 28 °C, FRQ levels increase, and the new phase of the clock is always reset to the point corresponding to the nadir of the new cycle ($\sim$ CT 0). Since at 28 °C, FRQ level at every point of the cycle is higher than the peak FRQ level at 21 °C, every point of the cycle at 21 °C was treated as the trough of the new cycle. After a temperature step down (from 28 to 21 °C), the opposite happens: FRQ levels decrease, and the new phase is reset to the zenith of the new cycle (around CT 12). In a direct comparison between the entraining strength of light and temperature, nonextreme temperature changes can have a stronger influence on clock resetting than light, contrary to the general belief that light is the single most important factor [53].

The *Drosophila* clock

In *Drosophila*, five clock components have been identified which include *period* (*per*), *timeless* (*tim*), *dClock* (*dClk* or just *Clk*, the *Drosophila* homolog of mouse *Clock*), *cycle* (*cyc*) and *double-time* (*dbt*) [5, 16–18, 42–44]. Mutations in these loci result in mutants which have various period length or arrhythmic phenotypes. Moreover, these mutations also alter the molecular oscillations in a fashion which corresponds to their behavioral rhythmicity. In terms of the *Drosophila* clock feedback loop, *per* and *tim* sit at the center of the loop [18, 25]. Although the *Drosophila* oscillator has a reversed phase from that of *Neurospora* [60], it follows a similar pattern of events. *per* and *tim* mRNA begin to rise in the morning and reach their peak in the early evening [24, 33], and this increase is largely the result of transcriptional activation by the heterodimer comprised of dCLK and CYC [16, 17]. Mutants of *dClk* and *cyc* have low levels of PER and TIM due to low transcript levels [16, 17]. Both dCLK and CYC are bHLH-PAS transcription factors and can form heterodimers that bind to the circadian enhancer elements, the E boxes, within the promoter of *per* and *tim* to activate their transcription [18, 61].

After PER and TIM are synthesized (there is a 4–6-h lag between steady-state levels of mRNA and protein [60]), PER and TIM form heterodimers via the PAS domain of PER and a non-PAS domain of TIM and subsequently enter the nucleus [62–65] (fig. 2). The formation of the heterodimer is required for PER's nuclear entry [64, 66, 67]. Once in the nucleus, they probably bind to dCLK [68] and block the transcriptional activation of dCLK and CYC, depressing the expression of *per* and *tim* transcription [18]. This part of the feedback loop has been reconstructed in insect S2 cultured cells. In these cells, CYC is expressed normally, but coexpression of dCLK serves to activate the expression of *per* and *tim* genes. When PER and TIM are simultaneously overexpressed in these cells, the activation of dCLK and CYC is depressed, suggesting either PER, TIM or the PER-TIM complex binds to dCLK or CYC, rendering them nonfunctional. This conclusion is supported by the latest evidence, which demonstrated that dCLK indeed interacts with PER and TIM in vivo [68]. PER and TIM also become progressively phosphorylated after their synthesis, and PER protein is known to be phosphorylated by DBT (a type of *Drosophila* casein kinase I) [43, 44, 60]. Levels of PER and TIM reach their maximum around midnight and become increasingly phosphorylated through the rest of the night into the early morning, which correlates with an increasing turnover rate. In this feedback loop, negative elements within the loop (PER and TIM) act on positive elements to downregulate their own transcription.

To be consistent with the feedback model, the expression of negative elements (*per* and *tim*) should be rhythmic, whereas the positive elements (dCLK and CYC) and *dbt* do not necessarily have to be rhythmically expressed. Most of the evidence supports this notion—there is a robust rhythm for both *per* and *tim* transcripts

in wild-type flies, and no rhythm is detected at the transcript level for *cyc* and *dbt* (*dClk* and dCLK are rhythmically expressed) [16–18, 44, 68]. However, a number of studies suggest that this transcription based feedback loop is not the entire story for the *Drosophila* oscillator. For example, a *per* construct lacking its promoter was shown to be able to partially rescue rhythmicity in a *per*-null strain; and in another study, PER cycling persists in the *Drosophila* eye when *per* is constitutively expressed from the *rhodopsin* promoter [69, 70]. These studies suggest that additional posttranscriptional regulation exists in addition to the transcription-based feedback loop.

In order to possess a fully functional circadian oscillator, other regulatory mechanisms are required. As seen for the *frq* gene products in *Neurospora*, *per* and *tim* are subject to several steps of posttranscriptional regulation.

The first step of posttranscriptional regulation of *per* and *tim* is at the transcript level. The comparison of the *per* transcription profile and mRNA level suggests that posttranscriptional regulation contributes to the cycling of *per* transcript. Moreover, temporal regulation of mRNA stability should also play a role, since in a construct without the *per* promoter, *per* mRNA is still cycling and was able to rescue the rhythmicity of *per*-null flies to some degree [71]. In another study, an intragenic element of *per* was shown to affect *per* expression [72].

Another step of posttranscriptional regulation is nuclear entry via the protein-protein interaction between PER and TIM. The NLS and cytoplasmatic localization domain (CLD) on both PER and TIM play major roles in determining the cellular distribution of PER and TIM [65, 67]. Formation of the PER-TIM heterodimer has dual roles: the promotion of the nuclear entry of the complex and the stabilization of PER (which is unstable in the absence of TIM) [66, 67]. Last, the time lag between the synthesis of PER and TIM and their nuclear localization suggests that their nuclear entry is also under temporal control [63].

The third aspect of regulation is protein phosphorylation and turnover. Both PER and TIM are phosphorylated in vivo, and their phosphorylation appears to be progressive (like FRQ), with extensive phosphorylation occurring just before protein levels decline [60, 73]. The expected link between phosphorylation and protein turnover was only recently confirmed when the *Drosophila* clock mutant *double-time* (*dbt*) was cloned [43, 44]. Mutations in this locus give rise to long, short or arrhythmic phenotypes. Beside changes in period length, the PER phosphorylation pattern is also altered in these mutants. The molecular cloning of *dbt* revealed that it is a type of *Drosophila* casein kinase I closely related to the human casein kinase Iε. In a strain which has a largely nonfunctional allele of *dbt*, PER appears to be hypophosphorylated and accumulates to high levels. In addition, biochemical studies demonstrate that PER and DBT physically interact with each other [44]. Taken together, these data support the model that DBT phosphorylates PER, and PER phosphorylation leads to its own degradation. The degradation of clock proteins is a very important part of clock function because it constitutes half of the molecular cycle. The function of DBT may promote the crucial time delay between *per*/*tim* transcription and the formation of functional PER-TIM complex, which is essential for the generation of stable circadian rhythms.

Although phosphorylation-mediated degradation may play a major role in determining the half-life of PER, other evidence also suggests additional regulation of turnover. Levels of *dbt* mRNA are nearly constant throughout the day, contrasting with evidence that suggests that PER half-life is not the same at different times of the day [74, 75]. This may indicate the existence of temporal control of PER protein half-life.

Like the *Neurospora* clock, the *Drosophila* clock can be entrained by both light and temperature. The light-entraining mechanism in *Drosophila* is similar to that in *Neurospora*, such that light changes the levels of the central clock components, but with an interesting variation. Unlike in *Neurospora*, where light rapidly induces a central clock component, in *Drosophila* light triggers protein turnover [34, 73, 76, 77]. TIM is light-sensitive, so its level is low in the light, and the PER level is also low since its monomeric form is unstable without TIM. When the clock is running in constant darkness, a light pulse triggers rapid TIM degradation, leading to a lower level of PER and resetting of the cycle. Light pulses cause phase delays when they are given between subjective dusk and midnight, whereas advances occur when the pulses are administered between subjective midnight and dawn, and the size of the phase shifts is also dependent on the amount and quality of the light given [78, 79]. This response acts mainly posttranscriptionally, since light hardly alters the level of *tim* and *per* transcript initially [77]. Furthermore, PER appears to play only a passive role in this process: TIM is the initial response element, and the changes in PER appear to be secondary responses to light. This is in contrast to the light responses of *mper1* and *mper2* in mice, where light rapidly induces the levels of the transcripts of these two mouse *per* homologs [57–59], suggesting that the resetting mechanism in the mouse clock may be quite different from that in *Drosophila*, even though they share some of the common elements.

Very recently, a putative photoreceptor for *Drosophila* light entrainment was identified as CRY, a protein with homology to the plant blue light photoreceptor cryptochrome [80, 81]. In cry^b mutants (a point mutation of

a highly conserved flavin-binding residue of CRY), the clock cannot be entrained by pulses of light, and PER and TIM are not cycling in constant darkness or in an LD cycle. Moreover, the *cry*-overexpressing strain shows higher circadian light sensitivity. However, the behavioral rhythms can still be entrained by temperature cycles, and while the mutation abolishes rhythmic expression of PER and TIM in photoreceptor and glial cells, it does not eliminate TIM and PER cycling in the lateral neurons in the fly brain. These and other data suggest that CRY is the circadian photoreceptor for body clocks and may mediate entrainment by light pulses, whereas the lateral neurons receive photic information both through the CRY pathway and through the eye-mediated rhodopsin pathway.

Although small changes (< 2 °C) of ambient temperature (within the physiological temperature range) can entrain the *Drosophila* clock, the molecular mechanism of temperature entrainment is currently unknown. Investigations have indicated that heat pulses (37 °C) cause a rapid decrease of the levels of PER and TIM at all times, which result in small phase delays in the early night but have little effect on the phase at late night [82].

Clock genes in mammals, plants and cyanobacteria

Besides the pioneering work in *Drosophila* and *Neurospora*, recently studies of other model organisms have made giant leaps towards the understanding of how clocks work. Several clock and clock-associated genes in these organisms have been identified by forward and reverse genetics, and these works have benefited greatly from rapid progress in genome sequencing. The cloning of the mouse *clock* gene by Takahashi and co-workers represented the first clock gene cloned in mammals [19, 20]. Together with the identification of mouse *per* homologs, they triggered an avalanche of clock studies in mammals [26, 35, 36]. In cyanobacteria and plants, independent forward and reverse genetic approaches led to the identification of clock genes or clock-associated genes. So far, they do not show any similarities to the previously described clock components; however, their mode of operation seems to follow a common theme, that is an assembly of interconnected positive and negative feedback loops [14, 83, 84]. Due to the length limit of this review, the following is only a brief summary of the situation found in plants, cyanobacteria and mammals.

Plants

Historically plants have always played a crucial role in circadian rhythm research. However, in the era of molecular genetics the lack of a readily screenable clock phenotype for plants significantly slowed down further progress. A breakthrough came when an 'artificial' clock phenotype was introduced into *Arabidopsis* plants (using a bioluminescent luciferase reporter fused to the clock-regulated *cab2* gene promoter), and transgenic *Arabidopsis* plants could be easily screened for mutant rhythm phenotypes [85]. Indeed, several mutants with an altered period of *cab* promoter activity were recovered [85]. One of the identified short period *toc* mutants (timing of *cab* expression), *toc1*, has been shown to effect clock control of all the molecular and physiological rhythms examined at the time [85, 86]. The cloning of the *toc* gene(s) in the future will likely be a major step towards the understanding of plant circadian clocks.

Another interesting clock mutant is *elf3* (*early flowering 3*), isolated for its photoperiod-insensitive early-flowering phenotype. Since photoperiodism is under clock control, it was interesting that circadian leaf movement and *cab2* mRNA rhythms are also abolished in these mutants. However, since the mutant phenotype is only seen under constant light conditions but not in constant darkness, ELF3 probably operates upstream of the oscillator [87]. It is also interesting to mention the possibility of having similar putative blue-light photoreceptors in plants and mammals feeding into the circadian systems of these organisms [88, 89]. If true, it might indicate common signal transduction pathways with structurally and functionally similar components that tie the plant circadian system to the other eukaryotic clock systems.

Recently, the first two plant candidate clock components, LHY (late elongated hypocotyl) and CCA-1 (circadian clock associated1), were isolated from *Arabidopsis* [83, 84, 90]. Both proteins influence a variety of physiological (flower induction, leaf movement) and molecular rhythms (transcript oscillations of *cab2* and *Atgrp7*/*ccr2*). In fact, both proteins encode two closely related MYB-like transcription factors. The transcripts and proteins (only shown for CCA-1) strongly oscillate. Furthermore, both factors downregulate their own transcript abundance, and overexpression of CCA-1 also downregulates the level of *lhy* mRNA, indicating that they form negative feedback loops that are interconnected.

This resembles a situation that was already known for the products of the clock-controlled *Atgrp7*/*ccr2* gene [13]. In transgenic *Arabidopsis* plants that constitutively overexpress this RNA-binding protein, oscillations of the endogenous *Atgrp7* transcript are severely depressed, suggesting a significant contribution to the generation/maintenance of its own rhythmicity by negative feedback regulation. However, in contrast to CCA-1 and LHY overexpressors, most of the other clock-controlled genes and physiological clock pheno-

types remain unaffected in the *Arabidopsis* plants that constitutively overexpress AtGRP7. Hence, a model was proposed where the *Atgrp7*/AtGRP7 feedback loop comprises a subordinated 'slave' oscillator—as originally proposed by Pittendrigh [91, 92]—that is in turn entrained by a 'master' oscillator [9]. Whether CCA-1 and LHY are components of a slave oscillator such as AtGRP7, or the core itself, must await further studies, in particular the analysis of null mutants.

The importance of posttranscriptional mechanisms within the plant clockwork is underlined by the identification of a casein kinase II which phosphorylates CCA1 and influences its binding to the *cab* promoter [93]. However, whether the phosphorylation status of CCA1 is of relevance to its clock function is not known yet.

Despite the lack of evidence that plants share one of the 'classical' clock components (*per*, *tim*, *Clk*, *cyc* and *frq*), forward and reverse genetic approaches in *Arabidopsis thaliana* have led to a rapid advance in our understanding of the plant circadian clock. As with other model organisms, it may turn out that the formation of negative feedback loops plays an essential role in rhythm generation in plants (for a detailed review see [9]).

Cyanobacteria

Rapid progress in the identification of clock genes from the cyanobacterium *Synechococcus* has been made possible only recently, when (as mentioned for *Arabidopsis*) an artificial circadian-regulated bioluminescent reporter system was introduced into *Synechococcus* [94]. An enormous variety of different clock phenotypes were isolated, ranging from extremely short to very long period mutants as well as arrhythmic phenotypes [95]. It turned out that most of the mutations mapped to only one locus. This locus was cloned and found to contain three ORFs named *kaiABC* [14]. As with plants, there seems to be no homology to any known clock genes. Both *kaiA* and *kaiBC* mRNAs are rhythmically expressed, and all three *kai* genes are required for the clock to function, since their deletions abolish the rhythmicity and reduce the promoter activity of *kaiBC*. Since KaiA enhances the expression of KaiBC, and the overexpression of KaiC represses the promoter activity of *kaiBC*, this simple gene cluster seems to contain all the positive and negative elements necessary for proper clock function.

Furthermore, by studying the relative fitness among different period mutants of *kai*, Ouyang et al. were able to show that strains with a period similar to that of the environmental cycle has higher reproductive fitness than the arrhythmic strain or those strains with period lengths different from the environmental cycle [96]. This study demonstrates the adaptive significance of the circadian clock in *Synechococcus*, and it also suggests that the evolution of the circadian clock may be a result of the natural selection.

Mammals

The groundwork for the isolation of the first mammalian clock gene was laid when the mouse *clock* gene was identified about 5 years ago [97]. Only a couple of years later, the gene was cloned and shown to encode a bHLH-PAS transcription factor [19, 20]. The existence of a PAS domain in mouse CLK and the previous identification of PAS domains within the *Drosophila* PER protein and in the *Neurospora* clock proteins WC-1 and WC-2 gave the first hint that common structural and functional themes might emerge among eukaryotic clock proteins [15, 46, 98]. The existence of human and mouse homologs of the *Drosophila per* and *tim* genes [27–30, 35, 36] strongly suggest that the clock components in flies and mammals are conserved. To date three mouse *period* homologs have been identified (*mPer1*, *mPer2*, *mPer3*), which all show circadian oscillations in transcript levels in the supra-chiasmatic nucleus (SCN). The regulation of these *mper* genes shows interesting differences in terms of peak levels and tissue distribution [29, 30, 57–59, 99]. Both *mPer1* and *mPer2* mRNA, like *frq* in *Neurospora*, are rapidly induced by light pulses and the size of the light-induced phase shift is proportional to the size of the mRNA induction [57–59]. This is different from the situation in *Drosophila*, where light does not have an immediate effect on *per* mRNA, and light phase shifts the clock by triggering the degradation of TIM, which then leads to the turnover of PER. Moreover, as mentioned above, the mouse homolog to the *Drosophila* protein *cycle (cyc)*, BMAL1(MOP3), was identified [21, 100]. Interestingly, CLOCK and BMAL1 form heterodimers that bind E box motifs (the same E box as in *Drosophila per* promoter) within the *mper* 1 promoter and activate transcription. Somewhat similar to its counterpart in fly, mammalian TIM also interacts with mPER and dampens transcriptional activation by BMAL1/CLOCK. Additionally, mTIM confers the nuclear entry of dPER in insect cells [27, 28]. However, unlike in *Drosophila*, the abundance of mTIM mRNA cycles only weakly if at all, and mPER-mPER interactions appear much stronger than mPER-mTIM interactions, suggesting that heterodimeric PER-PER interactions may play an important role in the mammalian clock.

Although it has not been shown that all these genes (except for *clock*) are indeed part of the mammalian oscillator (this awaits the identification of clock phenotypes when these genes are mutated in mice), the similarity to the situations found in *Drosophila* and the clock phenotypes found in *clock* mice make their central

role as clock components very likely. In addition, it has been found that among the two blue-light photoreceptors, cryptochromes 1 and 2 (*mCry1* and *mCry2*) that were recently discovered in mammals, *mCry1* is expressed at high level in the SCN and oscillates in this tissue in a circadian manner [101]. Most recently, Thresher et al. have shown that *mCry2* may have a role in circadian photoreception in mice [89].

Finally, the recent discovery of serum shock-induced circadian rhythms of gene transcription in mammalian cell culture system represented a major technical breakthrough for circadian clock research and should provide a great tool for future research [102].

Conclusions

Neurospora and *Drosophila* studies have paved the way in many aspects of clock research. The general principles of how circadian clocks work at the molecular level have been outlined in these organisms. Rapid progress in the identification of clock components (related or not) in other model organisms will now allow for a more specific and detailed understanding of how circadian clocks function. In the future, detailed studies of transcriptional and posttranscriptional processes that regulate clock components will eventually uncover the kinetics of the network of feedback loops that make the clock tick with a period of about a day.

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1 Dunlap J. C. (1999) Molecular bases for circadian clocks. Cell **96:** 271–290
2 Johnson C. H., Golden S., Ishiura M. and Kondo T. (1996) Circadian clocks in prokaryotes. Mol. Microbiol. **21:** 5–11
3 Takahashi J. S. (1995) Molecular neurobiology and genetics of circadian rhythms in mammals. Ann. Rev. Neurosci. **18:** 531–553
4 Dunlap J. C. (1996) Genetic and molecular analysis of circadian rhythms. Annu. Rev. Genetics **30:** 579–601
5 Hall J. C. (1995) Tripping along the trail to the molecular mechanisms of biological clocks. Trends Neurosci. **18:** 230–240
6 Rosbash M., Allada R., Dembinska M., Guo W. Q., Le M., Marrus S. et al. (1996) A *Drosophila* circadian clock. Cold Spring Harbor Symp. Quant. Biol. **61:** 265–278
7 Young M. W. (1998) The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*. Annu. Rev. Biochem. **67:** 135–152
8 Wilsbacher L. D. and Takahashi J. S. (1998) Circadian rhythms: molecular basis of the clock. Curr. Opin. Genet. Dev. **8:** 595–602
9 Staiger D. and Heintzen C. (1999) The circadian system of *Arabidopsis thaliana*. Chronobiol. Int. **16**: 1–16
10 Roenneberg Y. and Merrow M. (1998) Molecular circadian oscillators: an alternative hypothesis. J. Biol. Rhythms **13:** 167–179
11 Fleissner G. and Fleissner G. (1992) Feedback loops in the circadian system. Disc. Neurosci. **8:** 79–84
12 Mrosovsky N., Reebs S. G., Honrado G. I. and Salmon P. A. (1989) Behavioral entrainment of circadian rhythms. Experientia **45:** 696–702
13 Heintzen C., Nater M., Apel K. and Staiger D. (1997) AtGRP7, a nuclear RNA-binding protein as a component of a circadian-regulated negative feedback loop in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **94:** 8515–8520
14 Ishiura M., Kutsuna S., Aoki S., Iwasaki H., Anderson C. R., Tanabe A. et al. (1998) Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. Science **281:** 1519–1523
15 Crosthwaite S. C., Dunlap J. C. and Loros J. J. (1997) *Neurospora wc-1* and *wc-2*: transcription, photoresponses, and the origins of circadian rhythmicity. Science **276:** 763–769
16 Allada R., White N. E., So W. V., Hall J. C. and Rosbash M. (1998) A mutant Drosophila homolog of mammalian *CLOCK* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell **93:** 805–814
17 Rutila J. E., Suri V., Le M., So W. V., Rosbash M. and Hall J. C. (1998) *CYCLE* is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila *period* and *timeless*. Cell **93:** 805–813
18 Darlington T. K., Wager-Smith K., Ceriani M. F., Stankis D., Gekakis N., Steeves T. et al. (1998) Closing the circadian loop: CLOCK induced transcription of its own inhibitors, *per* and *tim*. Science **280:** 1599–1603
19 Antoch M., Soog E., Chang A., Vitaterna M., Zhao Y., Wilsbacher L. et al. (1997) Functional identification of the mouse circadian *CLOCK* gene by transgenic BAC rescue. Cell **89:** 655–667
20 King D., Zhao Y., Sangoram A., Wilsbacher L., Tanaka M., Antoch M. et al. (1997) Positional cloning of the mouse circadian *CLOCK* gene. Cell **89:** 641–653
21 Gekakis N., Stankis D., Nguyen H. B., Davis F. C., Wilsbacher L. D., King D. P. et al. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science **280:** 1564–1569
22 Aronson B., Johnson K., Loros J. J. and Dunlap J. C. (1994) Negative feedback defining a circadian clock: autoregulation in the clock gene *frequency*. Science **263:** 1578–1584
23 Merrow M., Garceau N. and Dunlap J. C. (1997) Dissection of a circadian oscillation into discrete domains. Proc. Natl. Acad. Sci. USA **94:** 3877–3882
24 Hardin P. E., Hall J. C. and Rosbash M. (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature **343:** 536–540
25 Zeng H., Hardin P. E. and Rosbash M. (1994) Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling. EMBO J. **13:** 3590–3598
26 Reppert S. M. (1998) A clockwork explosion!. Neuron **21:** 1–4
27 Sangoram A. M., Saez L., Antoch M. P., Gekakis N., Staknis D., Whiteley A. et al. (1998) Mammalian circadian autoregulatory loop: a timeless ortholog and *mPer1* interact and negatively regulate CLOCK-BMAL1-induced transcription. Neuron **21:** 1101–1113
28 Zylka M. J., Shearman L. P., Levine J. D., Jin X., Weaver D. R. and Reppert S. M. (1998) Molecular analysis of mammalian timeless. Neuron **21:** 1115–1122
29 Takumi T., Taguchi K., Miyake S., Sakakida Y., Takashima N., Matsubara C. et al. (1998) A light-independent oscillatory gene *mPer3* in mouse SCN and OVLT. EMBO J. **17:** 4753–4759
30 Takumi T., Matsubara C., Shigeyoshi Y., Taguchi K., Yagita K., Maebayashi Y. et al. (1998) A new mammalian

period gene predominantly expressed in the suprachiasmatic nucleus. Genes Cells **3:** 167–176
31 Edery I., Rutila J. and Rosbash M. (1994) Phase shifting of the circadian clock by induction of the *Drosophila period* protein. Science **263:** 237–240
32 Garceau N., Liu Y., Loros J. J. and Dunlap J. C. (1997) Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell **89:** 469–476
33 Sehgal A., Rothenfluh-Hilfiker A., Hunter-Ensor M., Chen Y., Myers M. P. and Young M. W. (1995) Rhythmic expression of *timeless*: a basis for promoting circadian cycles in period gene autoregulation. Science **270:** 808–810
34 Myers M., Wager-Smith K., Rothenfluh-Hilfiker A. and Young M. (1996) Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. Science **271:** 1736–1740
35 Sun S., Alsbrecht U., Zhuchenko O., Bailey J., Eichele G. and Lee C. (1997) RIGUI, a putative mammalian ortholog of the *Drosophila period* gene. Cell **90:** 1003–1011
36 Tei H., Okamura H., Shigeyoshi Y., Fukuhara C., Ozawa R., Hirose M. et al. (1997) Circadian oscillation of a mammalian homologue of the *Drosophila period* gene. Nature **389:** 512–516
37 Konopka R. J. and Benzer S. (1971) Clock mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **68:** 2112–2116
38 Feldman J. F. and Hoyle M. (1973) Isolation of circadian clock mutants of *Neurospora crassa*. Genetics **75:** 605–613
39 Bargiello T. A. and Young M. W. (1984) Molecular genetics of a biological clock in *Drosophila*. Proc. Natl. Acad. Sci. USA **81:** 2142–2146
40 Reddy P., Zehring W. A., Wheeler D. A., Pirrotta V., Hadfield C., Rosbash M. et al. (1984) Molecular analysis of the *period* locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. Cell **38:** 701–710
41 McClung C. R., Fox B. A. and Dunlap J. C. (1989) The *Neurospora* clock gene *frequency* shares a sequence element with the *Drosophila* clock gene *period*. Nature **339:** 558–562
42 Myers M. P., Wager-Smith K., Wesley C. S., Young M. W. and Sehgal A. (1995) Positional cloning and sequence analysis of the *Drosophila* clock gene *timeless*. Science **270:** 805–808
43 Price J. L., Blau J., Rothenfluh A., Adodeely M., Kloss B. and Young M. W. (1998) *double-time* is a new *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell **94:** 83–95
44 Kloss B., Price J. L., Saez L., Blau J., Rothenfluh A. and Young M. W. (1998) The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. Cell **94:** 97–107
45 Ballario P., Vittorioso P., Magrelli A., Talora C., Cabibbo A. and Macino G. (1996) *White collar-1*, a central regulator of blue-light responses in *Neurospora crassa*, is a zinc-finger protein. EMBO J. **15:** 1650–1657
46 Linden H. and Macino G. (1997) White collar-2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. EMBO J. **16:** 98–109
47 Aronson B. D., Johnson K. A. and Dunlap J. C. (1994) The circadian clock locus *frequency*: a single ORF defines period length and temperature compensation. Proc. Natl. Acad. Sci. USA **91:** 7683–7687
48 Ballario P., Talora C., Galli D., Linden H. and Macino G. (1998) Roles in dimerization and blue light photoresponses of PAS and LOV domains of *Neurospora crassa* white collar proteins. Mol. Microbiol. **29:** 719–729
49 Luo C., Loros J. J. and Dunlap J. C. (1998) Nuclear Localization is Required for Function of the Essential Clock Protein FREQUENCY. EMBO J. **17:** 1228–1235
50 Loros J. J., Denome S. A. and Dunlap J. C. (1989) Molecular cloning of genes under the control of the circadian clock in *Neurospora*. Science **243:** 385–388
51 Bell-Pedersen D., Shinohara M., Loros J. and Dunlap J. C. (1996) Circadian clock-controlled genes isolated from *Neurospora crassa* are late night to early morning specific. Proc. Natl. Acad. Sci. USA **93:** 13096–13101
52 Liu Y., Garceau N., Loros J. J. and Dunlap J. C. (1997) Thermally regulated translational control mediates an aspect of temperature compensation in the *Neurospora* circadian clock. Cell **89:** 477–486
53 Liu Y., Merrow M. M., Loros J. J. and Dunlap J. C. (1998) How temperature changes reset a circadian oscillator. Science **281:** 825–829
54 Johnson K. A. (1994) Ph.D. thesis, Dartmouth Medical School
55 Sauman I. and Reppert S. (1996) Circadian clock neurons in the silkmoth *Antherea pernyi*: novel mechanisms of period protein regulation. Neuron **17:** 889–900
56 Crosthwaite S. C., Loros J. J. and Dunlap J. C. (1995) Light-induced resetting of a circadian clock is mediated by a rapid increase in *frequency* transcript. Cell **81:** 1003–1012
57 Albrecht U., Sun Z., Eichele G. and Lee C. (1997) A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. Cell **91:** 1055–1064
58 Shearman L. P., Zylka M. J., Weaver D. R., Kolakowski L. F. Jr. and Reppert S. M. (1997) Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron **19:** 1261-1269.
59 Shigeyoshi Y., Taguchi K., Yamamoto S., Takeida S., Yan L., Tei H. et al. (1997) Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript. Cell **91:** 1043–1053
60 Edery I., Zweibel L., Dembinska M. and Rosbash M. (1994) Temporal phosphorylation of the *Drosophila period* protein. Proc. Natl. Acad. Sci. USA **91:** 2260–2264
61 Hao H., Allen D. L. and Hardin P. E. (1997) A cycling element in the *per* promoter. Mol. Cell. Biol. **17:** 3687–3693
62 Liu X., Zweibel L. J., Hinton D., Benzer S., Hall J. C. and Rosbash M. R. (1992) The *period* gene encodes a predominantly nuclear protein in adult *Drosophila*. J. Neurosci. **12:** 2735–2744
63 Curtin K., Huang J. and Rosbash M. (1995) Temporally regulated entry of the *Drosophila period* protein contributes to the circadian clock. Neuron **14:** 365–372
64 Gekakis N., Saez L., Delahaye-Brown A. M., Myers M. P., Sehgal A., Young M. W. et al. (1995) Isolation of timeless by PER protein interaction: defective interaction between timeless protein and long-period mutant PER^L. Science **270:** 811–815
65 Saez L. and Young M. W. (1996) Regulation of nuclear entry of the *Drosophila* clock proteins *period* and *timeless*. Neuron **17:** 911–920
66 Sehgal A., Price J., Man B. and Young M. (1994) Loss of circadian behavioral rhythms and *per* oscillations in the *Drosophila* mutant *timeless*. Science **263:** 1603–1606
67 Vosshall L., Price J., Sehgal A., Saez L. and Young M. (1994) Specific block in nuclear localization of *period* protein by a second clock mutation, *timeless*. Science **263:** 1606–1609
68 Lee C., Bae K. and Edery I. (1998) The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex. Neuron **21:** 857–867
69 Frisch B., Hardin P. E., Hamblen-Coyle M. J., Rosbash M. and Hall J. C. (1994) A promoterless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the *Drosophila* nervous system. Neuron **12:** 555–570
70 Cheng Y. and Hardin P. E. (1998) *Drosophila* photoreceptors contain an autonomous circadian oscillator that can function without *period* mRNA cycling. J. Neurosci. **18:** 741–750
71 So W. and Rosbash M. (1997) Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. EMBO J. **16:** 7146–7155

72 Stanewsky R., Jamison C. F., Plautz J. D., Kay S. A. and Hall J. C. (1997) Multiple circadian-regulated elements contribute to cycling *period* gene expression in *Drosophila*. EMBO J. **16:** 5006–5018

73 Zeng H., Qian Z., Myers M. and Rosbash M. (1996) A light-entrainment mechanism for the *Drosophila* circadian clock. Nature **380:** 129–135

74 Stanewsky R., Frisch B., Brandies C., Hamblen-Coyle M. J., Rosbash M. and Hall J. C. (1997) Temporal and spatial expression patterns of transgenes containing increasing amounts of the *Drosophila* clock gene *period* and a *lacZ* reporter: mapping elements of the PER protein involved in circadian cycling. J. Neurosci. **17:** 676–696

75 Dembinska M., Stanewsky R., Hall J. and Rosbash M. (1997) Circadian cycling of a *period-lacZ* fusion protein in *Drosophila*: evidence for an instability cycling element in PER. J. Biol. Rhythms **12:** 157–172

76 Lee C., Parikh V., Itsukaichi T., Bae K. and Edery I. (1996) Resetting the *Drosophila* clock by photic regulation of PER and a PER-TIM complex. Science **271:** 1740–1744

77 Hunter-Ensor M., Ousley A. and Sehgal A. (1996) Regulation of the *Drosophila* protein TIMELESS suggests a mechanism for resetting the circadian clock by light. Cell **84:** 677–685

78 Yang Z., Emerson M., Su H. S. and Sehgal A. (1998) Response of the *timeless* protein to light correlates with behavioral entrainment and suggests a nonvisual pathway for circadian photoreception. Neuron **21:** 215–223

79 Suri V., Qian Z., Hall J. C. and Rosbash M. (1998) Evidence that the TIM light response is relevant to light-induced phase shifts in *Drosophila melanogaster*. Neuron **21:** 225–234

80 Stanewsky R., Kaneko M., Emery P., Beretta B., Wager-Smith K., Kay S. A. et al. (1998) The *cryb* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell **95:** 681–692

81 Emery P., So W. V., Kaneko M., Hall J. C. and Rosbash M. (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell **95:** 669–679

82 Sidote D., Majercak J., Parikh V. and Edery I. (1998) Differential effects of light and heat on the *Drosophila* circadian clock proteins PER and TIM. Mol. Cell. Biol. **18:** 2004–2013

83 Wang Z. Y. and Tobin E. M. (1998) Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. Cell **93:** 1207–1217

84 Schaffer R., Ramsay N., Samach A., Corden S., Putterill J., Carre I. A. et al. (1998) The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. Cell **93:** 1219–1229

85 Millar A., Carré I. A., Strayer C. S., Chua N.-H. and Kay S. (1995) Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. Science **267:** 1161–1163

86 Somers D. E., Webb A. A., Pearson M. and Kay S. A. (1998) The short-period mutant, *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. Development **125:** 485–494

87 Hicks K., Millar A., Carre I., Somers D., Straume M., Meeks-Wagner R. et al. (1996) Conditional circadian dysfunction in the *Arabidopsis early-flowering* 3 mutant. Science **274:** 790–792

88 Somers D. E., Devlin P. F. and Kay S. A. (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. Science **282:** 1488–1490

89 Thresher R. J., Vitaterna M. H., Miyamoto Y., Kazantsev A., Hsu D. S., Petit C. et al. (1998) Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. Science **282:** 1490–1494

90 Wang Z. Y., Kenigsbuch D., Sun L., Harel E., Ong M. S. and Tobin E. M. (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis* Lhcb gene. Plant Cell **9:** 491–507

91 Pittendrigh C. S. (1960) Circadian rhythms and the circadian organization of living things. In: Cold Spring Harbor Symp. Quant. Biol. XXV: Biological Clocks, pp. 159–184, Chovnick A. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, New York

92 Pittendrigh C. S. (1981) Circadian organization and the photoperiodic phenomena. In: Biological Clocks in Seasonal Reproductive Cycles, pp. 1–35. Follett B. K. (ed.), John Wright and Sons, Bristol

93 Sugano S., Andonis C., Green R., Wang Z. Y. and Tobin E. M. (1998) Protein kinase CK2 interacts with andphosphorylates the *Arabidopsis circadian clock-associated 1* protein. Proc. Natl. Acad. Sci. USA **95:** 11020–11025

94 Kondo T., Strayer C., Kulkarni R., Taylor W. R., Ishiura M., Golden S. et al. (1993) Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc. Nat. Acad. Sci. USA **90:** 5672–5676

95 Kondo T., Tsinoremas N., Golden S., Johnson C. H., Kutsuna S. and Ishiura M. (1994) Circadian clock mutants of cyanobacteria. Science **266:** 1233–1236

96 Ouyang Y., Andersson C. R., Kondo T., Golden S. S. and Johnson C. H. (1998) Resonating circadian clocks enhance fitness in cyanobacteria. Proc. Natl. Acad. Sci. USA **95:** 8660–8664

97 Vitaterna K. W., King P. Y., Chang A. M., Kornhauser J. M., Lowrey P. L., McDonald J. D. et al. (1994) Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science **264:** 719–725

98 Huang Z. J., Edery I. and Rosbash M. (1993) PAS is a dimerization domain common to *Drosophila* period and several transcription factors. Nature **364:** 259–262

99 Sakamoto K., Nagase T., Fukui H., Horikawa K., Okada T., Tanaka H. et al. (1998) Multitissue circadian expression of rat period homolog (*rPer2*) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. J. Biol. Chem. **273:** 27039–27042

100 Hogenesch J. B., Gu Y.-Z., Jain S. and Bradfield C. A. (1998) The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc. Natl. Acad. Sci. USA **95:** 5474–5479

101 Miyamoto Y. and Sancar A. (1998) Vitamin B2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. Proc. Natl. Acad. Sci. USA **95:** 6097–6102

102 Balsalobre A., Damiola F. and Schibler U. (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell **93:** 929–937