Interlocked Feedback Loops Within the Drosophila Circadian Oscillator

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Drosophila Clock (dClk) is rhythmically expressed, with peaks in mRNA and protein (dCLK) abundance early in the morning. dClk mRNA cycling is shown here to be regulated by PERIOD-TIMELESS (PER-TIM)–mediated release of dCLK- and CYCLE (CYC)–dependent repression. Lack of both PER-TIM derepression and dCLK-CYC repression results in high levels of dClk mRNA, which implies that a separate dclk activator is present. These results demonstrate that the Drosophila circadian feedback loop is composed of two interlocked negative feedback loops: a per-tim loop, which is activated by dCLK-CYC and repressed by PER-TIM, and a dClk loop, which is repressed by dCLK-CYC and derepressed by PER-TIM.

The circadian oscillators of eukaryotic and certain prokaryotic organisms are controlled through autoregulatory feedback loops in gene expression (1). In Drosophila, five genes have been identified that are necessary for circadian feedback loop function: *period* (per), *timeless* (tim), Drosophila Clock (dClk), Cycle (Cyc), and double-time (dbt) (2–9). Three of these genes—*per*, *tim*, and *dClk*—are rhythmically expressed: *per* and *tim* mRNA levels peak early in the evening [zeitgeber time (ZT) 13–16, where ZT 0 is lights on and ZT 12 is lights off], and *dClk* mRNA levels peak late at night to early in the morning (ZT 23 to ZT 4) (2–5, 10).

Regulation of *per* and *tim* expression has been characterized in certain detail. Activation of *per* and *tim* transcription is mediated by two basic helix-loop-helix–PAS transcription factors, dCLK and CYC, which form heterodimers that target E-box regulatory elements of the sequence CACGTG in the *per* and *tim* promoters (4, 6, 7, 11, 12). Although *per* and *tim* mRNAs reach peak levels early in the evening (ZT 13–16), PER and TIM levels do not peak until late evening (ZT 18–24) (13, 14). This delay results from the initial destabilization of PER by DBT–dependent phosphorylation, followed by the stabilization of PER by dimerization with TIM (8, 9). PER-TIM dimers then move into the nucleus and form a complex with dCLK-CYC activators (15), which results in transcriptional repression (by deactivation) of *per* and *tim* (4).

Comparatively little is known about the regulation of dClk mRNA cycling. The levels of dClk mRNA are low in mutants lacking PER (*per*01) or TIM (*tim*01) function, which suggests that PER and TIM activate dClk transcription in addition to their roles as transcriptional repressors (5). The mechanism of PER-TIM–dependent activation is not known, but three models have been proposed to account for this activation (5). In the first two models, PER and TIM promote dClk transcription by shuttling transcriptional activators into the nucleus (Fig. 1A) or by coactivating a transcriptional complex (Fig. 1B). In the third model, PER or TIM or both inhibit the activity of a transcriptional repressor complex (Fig. 1C).

To distinguish among these alternative models, we measured dClk mRNA levels in different clock gene mutant combinations. Because DCLK and CYC are both required for *per* and *tim* activation, we predicted that mutants lacking functional DCLK (dClk64) or CYC (Cyc0) would exhibit low levels of dClk mRNA because the concentrations of the PER and TIM activators (of *dClk*) would be low. We were surprised to find that the level of dClk mRNA was indistinguishable from the wild-type peak in both mutants (Fig. 2). The levels of dClk mRNA do not vary significantly over the circadian cycle in these mutants (*P > 0.05), which is consistent with the lack of a functional circadian oscillator (6, 7). The high level of dClk mRNA in the absence of DCLK-dependent PER accumulation indicates that PER-dependent dClk activation does not occur by nuclear localization of an activator or by coactivation (Fig. 1A and B). However, the possibility remains that low levels of *per* and *tim* transcripts in dClk64 or Cyc0 mutants (6, 7) lead to some active PER-TIM dimer formation and subsequent activation of dClk transcription. To eliminate this possibility, we measured dClk mRNA levels in *per*01;dClk64 and *per*01;Cyc0 double mutants. In both cases, the levels of dClk mRNA observed under light-dark (LD) or constant dark (DD) conditions were close to the peak level in wild-type flies (Fig. 3), indicating that PER-TIM activates dClk transcription through derepression (Fig. 1C).

The dClk repressor that is removed as a result of PER-TIM accumulation appears to be either dCLK-CYC itself or a repressor that is activated by dCLK-CYC. When comparing the levels of dClk between *per*01 flies and *per*01;dClk64, or *per*01;Cyc0 double mutants, the presence of active dCLK and CYC results in the repression of dClk transcript accumulation. In *per*01 mutants, dClk mRNA is at low but detectable levels (5) (Fig. 3). This suggests that in the absence of PER-TIM derepression, dClk transcription reaches a steady state in which activation and dCLK-CYC–dependent repression equilibrate to produce low levels of dClk mRNA transcripts and, hence, of dClk protein. In *per*01 and *tim*01 mutants, *per* and *tim* transcription is constitutive and *per* and *tim* transcripts are relatively low in abundance (2, 16). This result can be explained by the partial activation of *per* and *tim* by low levels of dCLK-CYC dimers in the absence of PER-TIM repression.

On the basis of these observations, we propose that interlocked negative feedback loops: A: shuttling transcriptional activators into the nucleus. B: Coactivating a transcription complex. C: Inhibiting a transcriptional repressor complex. Gray triangle, transcription complex that promotes dClk expression; black circle, transcription complex that represses dClk expression; striped bar, dClk transcriptional regulatory sequences. The diagram is based on fig. 5 of (5).
loops mediate circadian oscillator function in *Drosophila* (Fig. 4). Late at night, PER-TIM dimers in the nucleus bind to and sequester dCLK-CYC dimers. This interaction effectively inhibits dCLK-CYC function, which leads to the repression of *per* and *tim* transcription and the derepression of *dclk* transcription. As PER-TIM levels fall early in the evening (ZT 15), the levels of dCLK-CYC also fall (17), leading to a decrease in *per* and *tim* transcription and an increase in dClk mRNA accumulation. A new cycle then begins as high levels of PER and TIM enter the nucleus and dCLK-CYC dimers are released and repress dClk expression, thereby decreasing dClk mRNA levels so that they are low by the end of the day (ZT 12) (5). Concomitant with the drop in dClk mRNA levels (through dCLK-CYC–dependent repression) is the accumulation of *per* and *tim* mRNA (through E-box–dependent dCLK-CYC activation) (2, 3). As dClk mRNA falls to low levels early in the evening (ZT 15), the levels of dCLK-CYC also fall (17), leading to a decrease in *per* and *tim* transcription and an increase in dClk mRNA accumulation. A new cycle then begins as high levels of PER and TIM enter the nucleus and dCLK-CYC starts to accumulate late at night (17, 18).

These observations also fit well with the regulation of *Drosophila* cryptochrome (*cry*), whose mRNA cycles in phase with that of dClk (17). Like dClk, cry mRNA transcripts are constitutively low in *per*01 mutants and constitutively high in *dclkk* or *Cyc0* single mutants and in *per*01;*dclkk* or *per*01;*Cyc0* double mutants (19). These striking similarities between dClk and cry mRNA phases (in the wild type) and dClk and cry mRNA levels in circadian mutants suggest that the cry locus may be regulated by the same PER-TIM release of dCLK-CYC repression mechanism as dClk.

These results reveal the existence of a dClk feedback loop and its regulatory interactions with the well-characterized per-*tim* feedback loop. One clear prediction from these experiments is that there is a separate activator of dClk expression. Such an activator is indicated by the high levels of dClk mRNA in the absence of PER and of either dCLK or CYC. This observation is somewhat surprising because the presence of this activator is independent of factors that control the expression of other clock genes (that is, *PER*, dCLK, and CYC).

Data supporting the existence of interlocked *per*-*tim* and dClk feedback loops were obtained from whole heads, raising the possibility that dClk expression in small subsets of “clock-specific” cells such as the locomotor activity pacemaker cells (that is, lateral neurons) (20–22) could be masked by dClk.
expression in other tissues. However, the autonomy and synchrony of per expression in diverse tissues in the head and body suggest that the circadian feedback loop mechanism is the same in all tissues (23) and argue against fundamental tissue-specific differences in the feedback loop mechanism.

An important aspect of circadian biology is how the clock regulates clock-controlled genes (CCGs). In mammals, it has been shown in vitro that CLOCK and BMAL1 (the mammalian ortholog of CYC) activate vasopressin gene transcription and that all three mouse PERs and TIM repress this activation (24). Although this mode of regulation may be more general for CCGs whose mRNA transcripts peak in phase with per (or mPer), it does not explain how CCGs that cycle in antiphase are regulated. The results presented here provide a possible mechanism by which the clock regulates CCGs whose mRNAs cycle in antiphase to those of per. The similarities between dClk and cry mRNA profiles in the wild type and in several single and double circadian mutants suggest that PER-TIM release of dCLK-CYC repression may serve a more general role in regulating CCG mRNAs that cycle in antiphase to per mRNA.

**Light-Independent Role of CRY1 and CRY2 in the Mammalian Circadian Clock**

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Cryptochrome (CRY), a photoreceptor for the circadian clock in *Drosophila*, binds to the clock component TIM in a light-dependent fashion and blocks its function. In mammals, genetic evidence suggests a role for CRYs within the clock, distinct from hypothetical photoreceptor functions. Mammalian CRY1 and CRY2 are here shown to act as light-independent inhibitors of CLOCK-BMAL1, the activator driving Per1 transcription. CRY1 or CRY2 (or both) showed light-independent interactions with CLOCK and BMAL1, as well as with PER1, PER2, and TIM. Thus, mammalian CRYs act as light-independent components of the circadian clock and probably regulate Per1 transcriptional cycling by contacting both the activator and its feedback inhibitors.

Daily rhythms in physiology and behavior are driven by endogenous oscillators called circadian clocks (1). In all known cases, circadian timekeeping is cell-autonomous, generated at least in part by a feedback loop involving clock proteins that inhibit the transcription of their own genes (2). Regulation of the transcriptional feedback loop by light is thought to mediate setting of circadian clocks to light-dark cycles (2). In mammals, as in *Drosophila*, a negative feedback loop of Per gene transcription involving PER and TIM proteins is probably central to the clock (2-4). A heterodimeric activator consisting of the basic helix-loop-helix protein (bHLH)-PAS proteins CLOCK (5) and BMAL1 (6, 7) drives mouse Per1 (mPer1) transcription from E-box regulatory sequences (6), and the mPer1 protein in turn acts to inhibit CLOCK-BMAL1 activity (4). In *Drosophila*, as the circadian cycle progresses, PER-TIM dimers accumulate and enter the nucleus during midday (left), high levels of dCLK-CYC (in the absence of PER-TIM) serve to activate per transcription and repress dClk transcription (either directly or through intermediate factors). As the circadian cycle progresses, PER-TIM dimers accumulate and enter the nucleus during the late evening to start the next cycle. Dashes, maximal repression; plus signs, maximal activation; wavy lines, mRNA.