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 some 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 ($dClk^{Jrk}$) or CYC (Cyc^{0}) 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. 1, A and B). However, the possibility remains that low levels of *per* and *tim* transcripts in $dClk^{Jrk}$ or Cyc^{0} 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};$dClk^{Jrk}$ and per^{01};Cyc^{0} 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};$dClk^{Jrk}$ or per^{01};Cyc^{0} 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

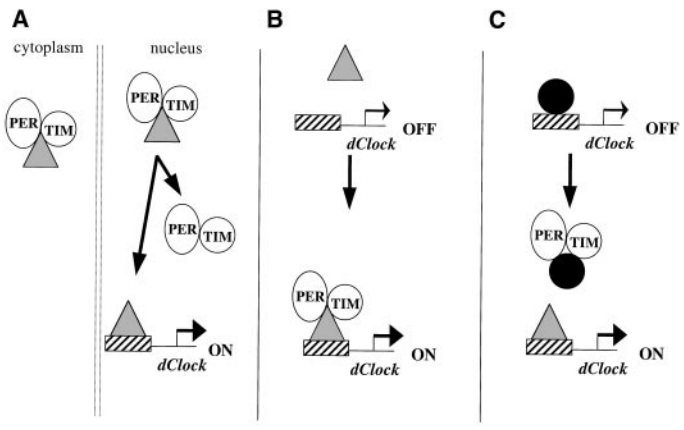

Fig. 1. Models for how PER and TIM might activate *dClk* expression. (**A**) Shuttling transcriptional activators into the nucleus. (**B**) Coactivating a transcription complex. (**C**) Inhibiting a transcriptional repressor complex. Gray triangle, transcription complex that promotes *dClk* expression; black circle, transcription complex that represses *dClk* expression; striped bar, *dClk* transcriptional regulatory sequences. The diagram is based on fig. 5 of (*5*).

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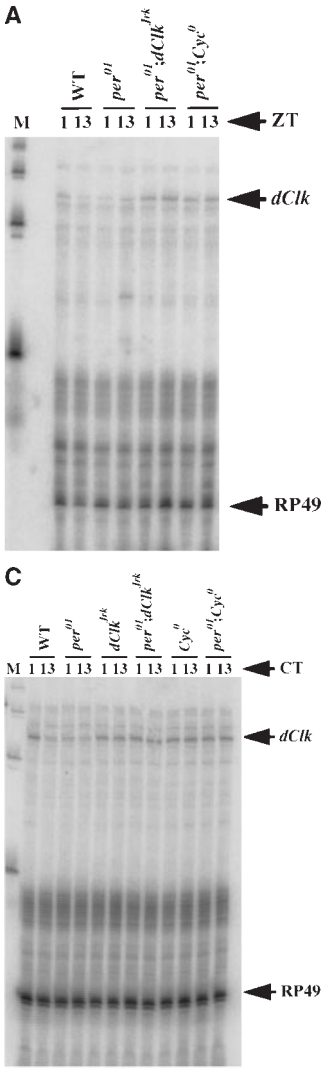
loops mediate circadian oscillator function in *Drosophila* (Fig. 4). Late at night, PER-TIM dimers in the nucleus bind to and sequester dCLK-CYC dimers. This interaction effectively inhibits dCLK-CYC function, which leads to the repression of *per* and *tim* transcription and the derepression of *dClk* transcription. As PER-TIM levels fall early in the morning (ZT 0–3), 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 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 $dClk^{Jrk}$ or Cyc^{0} single mutants and in per^{01};$dClk^{Jrk}$ or per^{01};Cyc^{0} 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*



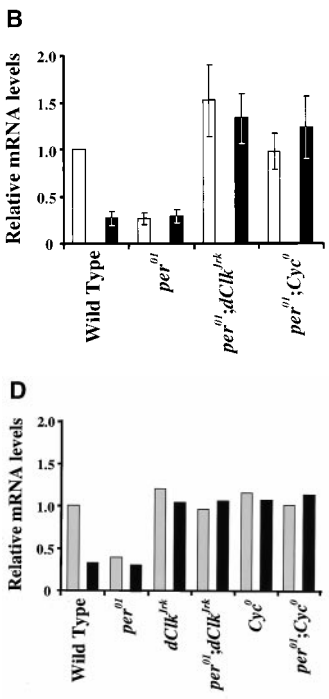
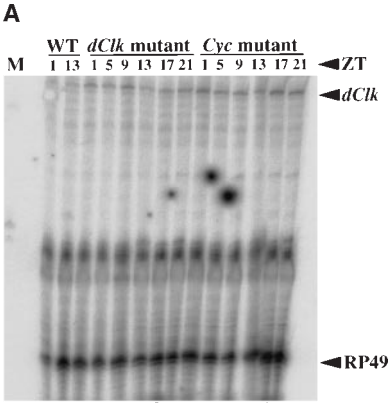


Fig. 3. Peak levels of *dClk* mRNA persist in per^{01};$dClk^{Jrk}$ and per^{01};Cyc^{0} double mutants. (**A**) Wild-type, per^{01}, per^{01};$dClk^{Jrk}$, and per^{01};Cyc^{0} flies were maintained, collected, and assayed as in Fig. 1. Numbers at the top of each lane correspond to the time of sample collection. M indicates the 123-bp marker lane. RP49 was included as a control for the total amount of RNA in each reaction. (**B**) *dClk* and RP49 protection products were quantified with a PhosphorImager and the *dClk*/RP49 ratios were plotted. The *dClk*/RP49 values at ZT 1 (open bars) or ZT 13 (solid bars) were normalized to the peak *dClk* level in the wild type at ZT 1, which was set to 1.0. Error bars represent standard deviations based on three independent experiments. (**C**) Wild-type, per^{01}, $dClk^{Jrk}$, per^{01};$dClk^{Jrk}$, Cyc^{0}, and per^{01};Cyc^{0} flies were entrained in LD12:12 for 3 days and collected at circadian times 1 (CT 1) and 13 (CT 13) during the first day of DD. RNase protection assays were performed as in Fig. 1. Numbers and symbols are the same as in (A). (**D**) *dClk* and RP49 protection products from (C) were quantified as described in (B). *dClk*/RP49 values are shown for CT 1 (gray bars) and CT 13 (solid bars). Normalization was performed as described in (B). This experiment was repeated with similar results.



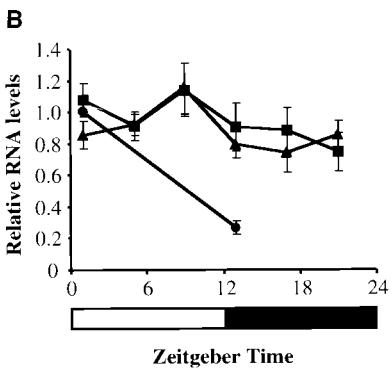


Fig. 2. Peak levels of *dClk* mRNA are present throughout the circadian cycle in $dClk^{Jrk}$ and Cyc^{0} mutants. (**A**) Wild-type, $dClk^{Jrk}$, and Cyc^{0} flies maintained in LD12:12 for a minimum of 3 days were collected and subjected to ribonuclease (RNase) protection assays as described (*2*). Total head RNA (12 μg) was hybridized to a *dClk* RNA probe that protects a 290-nucleotide (nt) fragment (*5*) and an RP49 probe that protects a 59-nt fragment (*2*). After RNase digestion, reactions were run on a 6% denaturing polyacrylamide gel. Numbers at the top of each lane correspond to the ZT of sample collection. M indicates the 123–base pair (bp) marker lane. RP49 was included as a control for the total amount of RNA in each reaction. (**B**) *dClk* and RP49 protection products were quantified with a PhosphorImager and the *dClk*/RP49 ratios were plotted. mRNA levels are shown for wild-type (●), $dClk^{Jrk}$ (▲), and Cyc^{0} (■) flies. The *dClk*/RP49 values were normalized to the peak *dClk* level in the wild type at ZT 1, which was set to 1.0. Error bars represent standard deviations based on five independent experiments. The light and dark bars represent times when lights were on or off, respectively. Analysis of these results by analysis of variance showed no significant differences in *dClk* mRNA over the day ($P > 0.05$) in $dClk^{Jrk}$ and Cyc^{0} mutants.

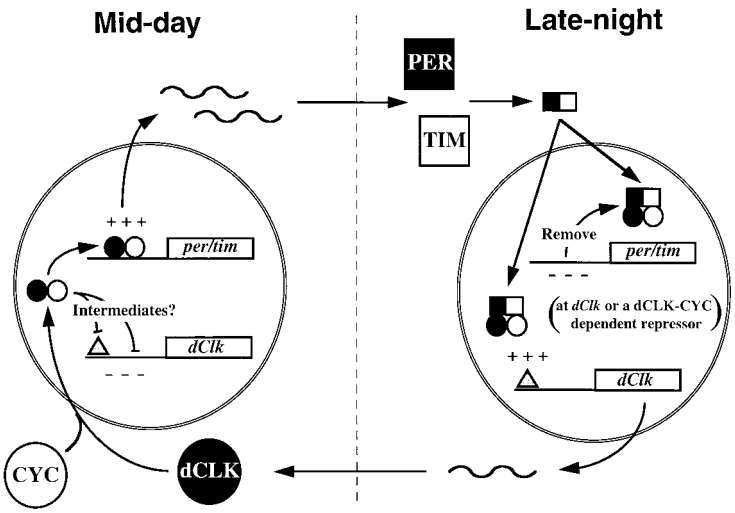


Fig. 4. Model for gene regulation within the *Drosophila* circadian oscillator. During the late evening (**right**), PER-TIM dimers (closed and open squares, respectively) enter the nucleus and bind dCLK-CYC dimers (closed and open circles, respectively), thereby repressing *per-tim* activation. Concurrently, the binding of PER-TIM dimers to dCLK-CYC releases dCLK-CYC–dependent repression of *dClk*, thus enabling *dClk* transcription via a separate activator or activator complex (triangle). By midday (**left**), high levels of dCLK-CYC (in the absence of PER-TIM) serve to activate *per-tim* 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.

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 BMAL-1 (the mammalian ortholog of CYC) activate vasopressin gene transcription and that all three mouse PERs and TIM repress this activation, resulting in peak vasopressin mRNA transcripts by midmorning (ZT 6) (*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.

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25. We thank I. Edery for the *dClk* RNase protection probe; M. Rosbash and J. Hall for $dClk^{Jrk}$ and Cyc^{O} single mutants and per^{01};$dClk^{Jrk}$ and per^{01};Cyc^{O} double mutants; B. Krishnan for statistical help; G. Cahill for comments on the manuscript; and A. Eskin, S. Dryer, G. Cahill, J. DeBruyne, and B. Krishnan for helpful discussions. Supported by National Institute of Neurological Disorders and Stroke grant NS-31214.

25 May 1999; accepted 6 August 1999

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 (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 *Dro-*

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