differences in single-channel conductance based on the subunit composition (*15*), the notion that conductance levels represent the conformational state of GluRs helps to explain why so many conductance levels are seen in natural single-channel currents at synapses (*16*).

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4. After patch excision, we used a rapid perfusion system [C. Rosenmund, A. Feltz, G. L Westbrook, *J. Neurosci.* **15**, 2788 (1995)] to produce rapid (200 to 400 μs) concentration changes at 0.1 to 0.2 Hz. The three-barreled squared capillary was filled with control, agonist, and antagonist. Switches were made from control → agonist → control and antagonist → agonist → antagonist. A version of this rapid switching method has been used previously to count binding sites [J. D. Clements, A. Feltz, Y. Sahara, G. L. Westbrook, *J. Neurosci.* **18**, 119 (1998)]. We tried several agonists (for example, S-AMPA, glutamate), but quisqualate was most commonly used because it dissociated most slowly (half-decay times: AMPA = 64 ± 8 ms, n = 5; glutamate 6.2 ± 2.6 ms, n = 5; quisqualate = 106 ± 21 ms, n = 6). Pipettes were filled with 150 mM CsF, 20 mM HEPES, 2 mM $MgCl_2$, 10 mM NaCl, 10 mM EGTA, and were adjusted to 320 mOsm and pH 7.3. The holding potential was −60 to −160 mV, and experiments were at room temperature. Currents were recorded with an Axopatch amplifier 200 B (Axon Instruments, Foster City, CA), low-pass filtered at 1 to 5 kHz, and digitized at 2 to 20 kHz. The extracellular medium contained 170 mM NaCl, 10 mM HEPES, 2 to 4 mM $CaCl_2$, 2 to 4 mM $MgCl_2$, and was adjusted to 330 mOsm and pH 7.25. Drugs were obtained from Tocris Cookson Ltd. (Bristol, UK) and dissolved in water- or dimethylsulfoxide-based stock solutions. Maximal final dimethylsulfoxide concentration was 0.1%.
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10. We assume: The receptor has four identical subunits with independent binding sites. Antagonist dissociation is rate limiting with a time constant τ, and the agonist concentrations are saturating. State C corresponds to binding of zero or one agonist; when two agonists are bound, the receptor enters state S; with three agonists bound, state M is entered; and the receptor is in state L when all four of its binding sites are occupied. After the antagonist/agonist switch, the mean waiting time t_1 for the $C \rightarrow S$ transition would be $t_1 = (\tau/4 + \tau/3)$. For the $S \rightarrow M$ transition, the waiting time t_2 would be $t_2 = \tau/2$. Finally, when only a single antagonist remains, the time t_3 for the final $M \rightarrow L$ transition is just $t_3 = \tau$. The total time for all three transitions is $(\tau/4 + \tau/3 + \tau/2 + \tau) = \tau(1/4 + 1/3 + 1/2 + 1) = 2.083\tau$ = 943 ms (measured), so τ is estimated to be 453 ms. Note that this is close to the time for the $M \rightarrow L$ transition (461 ± 20.3 ms), as predicted. The sum of the squared deviations of the predicted waiting times t_j from the observed values (measured in seconds) is used in Fig. 2D. This is a Poisson jump processes with probability distributions for waiting times (1: $C \rightarrow S$), $P_1(t) = 1 + 3e^{-4t/\tau} - 4e^{-3t/\tau}$; (2: $S \rightarrow M$), $P_2(t) = 1 - e^{-2t/\tau}$; and (3: $M \rightarrow L$), $P_3(t) = 1 - e^{-t/\tau}$, where $P_j(t)$ is the probability of waiting t or fewer seconds for the indicated jth transition and τ is the mean time for an antagonist dissociation. We also considered three alternative schemes. (i) Three identical binding sites with a single antagonist dissociation required for a transition. This is model 3. (ii) Five identical binding sites with three dissociations required for the $C \rightarrow S$ transition. This is model 5. (iii) Five identical binding sites with two dissociations required for the $C \rightarrow S$ transition and with a silent dissociation for the fifth site (that is, state L occurs if either 0 or 1 antagonists are bound). This is model 5(4 + 1). The mean waiting times for each transition are calculated as above with $\tau(1/3 + 1/2 + 1) = 943$ ms to give $\tau = 514$ ms for (i), $\tau(1/5 + 1/4 + 1/3 + 1/2 + 1) = 943$ ms to give = 413 ms for (ii), $\tau(1/5 + 1/4 + 1/3 + 1/2) = 943$ ms to give τ = 734 ms for (iii). Predicted values t_j for the three mean waiting times are used to quantify the goodness of fit for each model by calculating the sum of the squared deviations of the predicted waiting times from the observed values as was done above; these measures are presented in Fig. 2D. The probability distributions for waiting times predicted by these alternatives are: (i) $P_1(t) = 1 - e^{-3t/\tau}$, (ii) $P_1(t) = 1 - 6e^{-5t/\tau} + 15e^{-4t/\tau} - 10e^{-3t/\tau}$, and (iii) $P_1(t) = 1 + 4e^{-5t/\tau} - 5e^{-4t/\tau}$; these equations are used for the predictions in Fig. 2C with the values of given above.
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Closing the Circadian Loop: CLOCK-Induced Transcription of Its Own Inhibitors *per* and *tim*

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The circadian oscillator generates a rhythmic output with a period of about 24 hours. Despite extensive studies in several model systems, the biochemical mode of action has not yet been demonstrated for any of its components. Here, the *Drosophila* CLOCK protein was shown to induce transcription of the circadian rhythm genes *period* and *timeless*. dCLOCK functioned as a heterodimer with a *Drosophila* homolog of BMAL1. These proteins acted through an E-box sequence in the *period* promoter. The *timeless* promoter contains an 18–base pair element encompassing an E-box, which was sufficient to confer dCLOCK responsiveness to a reporter gene. PERIOD and TIMELESS proteins blocked dCLOCK's ability to transactivate their promoters via the E-box. Thus, dCLOCK drives expression of *period* and *timeless,* which in turn inhibit dCLOCK's activity and close the circadian loop.

In animals, plants, or prokaryotes, activities such as locomotion or photosynthesis do not occur with equal probability throughout the 24-hour day but are organized by an endogenous circadian oscillator. The oscillator allows the organism to anticipate daily environmental fluctuations rather than merely respond to them. In *Drosophila*, two essential oscillator components, *period* (*per*) and *timeless* (*tim*), have mRNA transcript levels that cycle with a circadian rhythm (*1*). Mouse homologs of *per* are also regulated in a circadian fashion (*1*). Thus, the core mechanism of the circadian oscillator is likely to be conserved between *Drosophila* and mammals.

In *Drosophila*, the *per* and *tim* mRNA oscillations are controlled in large part by transcriptional regulation (*2*) and some posttranscriptional processes (*3*). Point mutations in the coding region can change the length of the cycle or abolish it (*1*), indicating that PER and TIM proteins control their own oscillations. Overexpression of a

per transgene down-regulates the endogenous gene (*4*). PER and TIM proteins are nuclear at specific times of day, in such a way that nuclear entry coincides with a downturn in mRNA levels. As the nuclear protein levels fall, the mRNA levels rise (*5*). Thus, the manner in which PER and TIM regulate their transcription is thought to be through rhythmic repression in a negative feedback loop.

However, it is not known whether PER and TIM interact directly with transcription factors or influence transcription indirectly. Although neither protein has a recognizable DNA binding domain, PER shares with some transcription factors a region of homology termed the PAS domain (*6*), which functions as a protein-protein interaction interface (*1*) and often mediates heterodimerization between distinct bHLH-PAS transcription factors (*7*). TIM, which lacks the PAS domain, interacts with PER through the PER PAS domain (*8*). A mouse gene, *Clock*, encodes a bHLH-PAS protein whose wild-type function is necessary for the maintenance of circadian rhythms (*9*). An E-box sequence within the *per* promoter is required for high-level expression and robust cycling of transcription in transgenic flies (*10*). Because E-boxes are well-defined targets of bHLH-PAS protein heterodimers, we reasoned that *Clock* exists in flies and activates transcription of *per* and *tim*. In this model, PER and TIM would then abrogate the activity of CLOCK in a rhythmic fashion. This would provide the biochemical steps necessary to close the circadian loop.

We first determined whether a *Drosophila* homolog of the mouse *Clock* (*mClock*) gene existed and tested whether the protein product could activate *per* or *tim* transcription. A low-stringency screen of a cDNA library from adult heads with an *mClock* probe encoding the bHLH and PAS domains yielded overlapping clones with a high degree of homology to *mClock* (Fig. 1A) (*11*). Library screening did not reveal any clones with significant similarity to other bHLH-PAS proteins. At high stringency, a single band was seen on a Southern (DNA) blot of fly genomic DNA when a fragment encompassing *dclock* PAS B was used as the probe, which suggests that *dclock* is a single-copy gene (*12*). Sequence analysis revealed differential splicing. In variant A, the reading frame is intact and encodes a full-length protein of 115.7 kD. In variant B, the coding region goes out of frame after the bHLH domain (*13*). dCLOCK is highly homologous to both mCLOCK and NPAS2 (MOP4) (*14*, *15*). dCLOCK and mCLOCK share 60% identity in the bHLH domain, 43% in PAS A, and 70% in PAS B (Fig. 1A). Both dCLOCK (1023 amino acids) and mCLOCK (855 amino acids) are rich in glutamine and have long polyglutamine stretches. We mapped the chromosomal position of *dclock* to 66A (*16*).

A *dclock* probe hybridized to a single band at about 5 kb on a Northern (RNA) blot of whole fly RNA (Fig. 1B). *dclock* was detectable in RNA isolated from head, body, and appendage fractions, indicating that *dclock* was expressed widely (Fig. 1C). This correlates well with the observation that independent oscillators are present throughout the fly (*17*). To examine the temporal expression profile of *dclock*, we analyzed RNA isolated from fly heads harvested throughout 24 hours of a light-dark cycle. Unlike *per* and *tim*, *dclock* mRNA oscillated in a bimodal fashion (Fig. 1, C and D). It peaked at zeitgeber time 5 (ZT5) and at ZT23, whereas *per* and *tim* peak at ZT16 (*1*). Preliminary experiments indicated that the drop at ZT1 occurred even when the animals were kept in darkness (*12*). Whether this profile is reflected at the protein level is not known. To explore the possible significance of splice variant B, we measured its level of expression. Splice variant B was expressed weakly at all times of day, and it cycled in phase with the full-length form (Fig. 1, C and D).

To investigate the role of dCLOCK in the transcriptional regulation of *per* and *tim*, we used a transient transfection assay in *Drosophila* Schneider (S2) cells. Untransfected S2 cells did not express detectable levels of *dclock* mRNA (Fig. 1, B and C), PER, or TIM (*8*). We cotransfected a con-

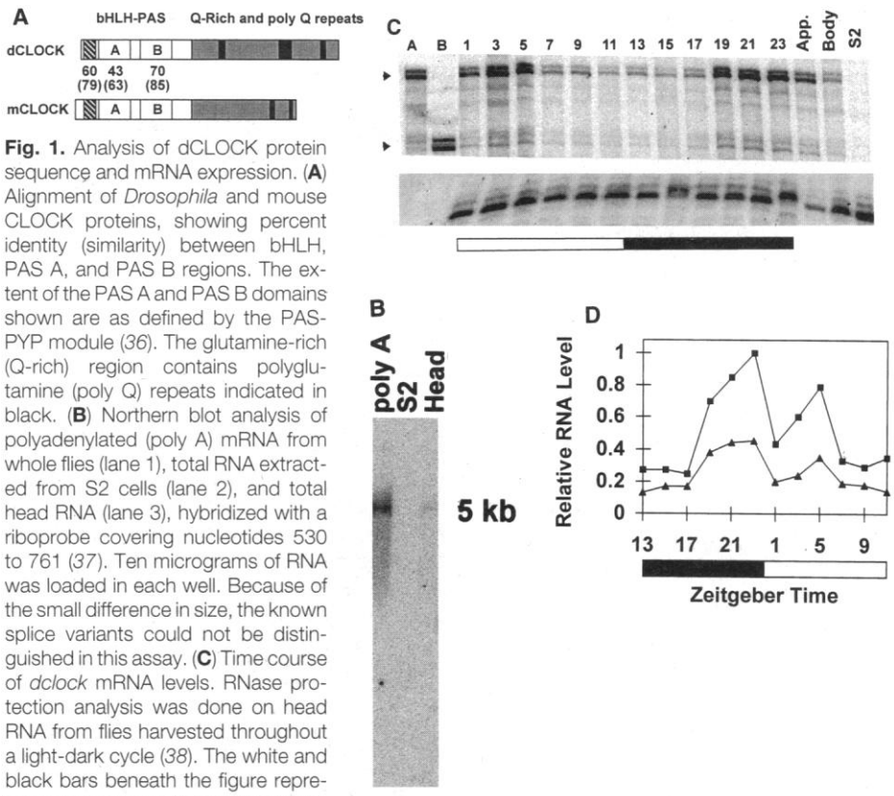

Fig. 1. Analysis of dCLOCK protein sequence and mRNA expression. (**A**) Alignment of *Drosophila* and mouse CLOCK proteins, showing percent identity (similarity) between bHLH, PAS A, and PAS B regions. The extent of the PAS A and PAS B domains shown are as defined by the PAS-PYP module (*36*). The glutamine-rich (Q-rich) region contains polyglutamine (poly Q) repeats indicated in black. (**B**) Northern blot analysis of polyadenylated (poly A) mRNA from whole flies (lane 1), total RNA extracted from S2 cells (lane 2), and total head RNA (lane 3), hybridized with a riboprobe covering nucleotides 530 to 761 (*37*). Ten micrograms of RNA was loaded in each well. Because of the small difference in size, the known splice variants could not be distinguished in this assay. (**C**) Time course of *dclock* mRNA levels. RNase protection analysis was done on head RNA from flies harvested throughout a light-dark cycle (*38*). The white and black bars beneath the figure represent the periods of light and dark, respectively, with lights on at ZT0 and lights off at ZT12. Lane A is a positive control for splice variant A, which gives rise to the upper doublet encoding the full-length protein. Lane B is a control for variant B. The probe hybridizing to this variant is clipped at a two-base mismatch and gives rise to the lower doublet encoding truncated proteins. The relevant bands are marked with an arrowhead. The band in the lower panel is a protection of RP49 RNA for reference. The zeitgeber time is indicated above the lanes. RNA from appendages (App.), bodies (Body), and S2 cells is also shown. (**D**) Variant A (squares) and B (triangles) *dclock* RNA levels from Fig. 1C were normalized to RP49 values and plotted with zeitgeber time on the *x* axis. Light and dark periods are reversed relative to Fig. 1C to highlight the bimodality. The assays were performed three times with indistinguishable results on independently obtained samples. A representative data set is shown.

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struct expressing *dclock* along with a construct driving luciferase expression from either the *per* or *tim* promoters (Fig. 2A). dCLOCK caused an approximately 20- and 200-fold induction of the *period* and *timeless* promoters, respectively.

To determine whether the effect of dCLOCK was specific for the *per* and *tim* promoters, we asked whether dCLOCK could induce transcription from the CNS midline element (CME). This element is a target for the bHLH-PAS partners dSIM and dARNT (TANGO) (*18*). dCLOCK was unable to induce transcription from this promoter element in S2 cells (Fig. 2A). Conversely, dSIM and dARNT were unable to induce the *per* or *tim* promoters, although they did induce expression from the CME. Thus, dCLOCK was not a promiscuous E-box activator but was rather a specific activator of the *per* and *tim* promoters. The sequence of splice variant B encoded two proteins that could act as dominant negative inhibitors (*13*). However, we did not see an effect of this splice variant on the ability of dCLOCK to induce expression (*12*). Although exogenous dARNT is necessary for high-level dSIM-mediated transactivation of the CME in S2 cells (*18*), dARNT did not cause a further increase in the transcriptional activity of dCLOCK (see below).

We also tested a 154–base pair (bp) fragment and a 69-bp subfragment of the *per* promoter, both of which confer robust expression and circadian cycling of mRNA in transgenic flies (*10*). Both fragments were sufficient for the dCLOCK-mediated induction (Fig. 2B). Furthermore, dCLOCK did not activate target constructs containing the 154-bp fragment with deletions in the E box (CACGTG), which abolish high-level mRNA cycling in transgenic animals (*10*) (Fig. 2B). Thus, dCLOCK's activity on this element of the *period* promoter closely correlates with the pattern observed in transgenic flies.

Sequence analysis of the *tim* promoter revealed a 10-bp consensus with the *per* E-box region (Fig. 2C). To test whether *per* and *tim* E-boxes supported dCLOCK activation, we tested multimerized E-boxes fused to a luciferase expression cassette. Both *per* and *tim* E-box targets were activat-

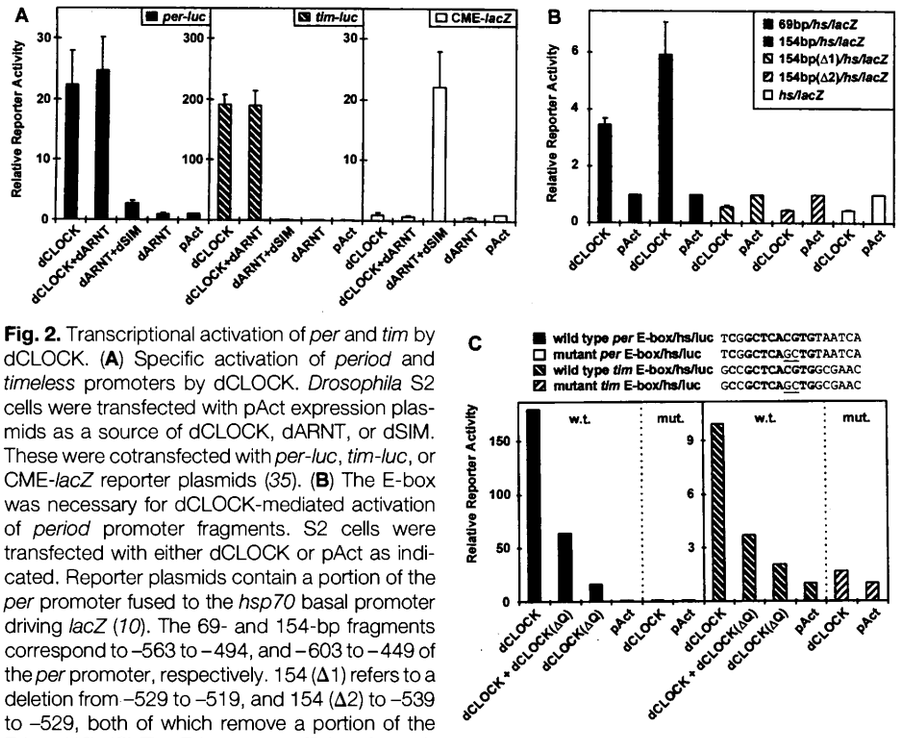

Fig. 2. Transcriptional activation of *per* and *tim* by dCLOCK. (**A**) Specific activation of *period* and *timeless* promoters by dCLOCK. *Drosophila* S2 cells were transfected with pAct expression plasmids as a source of dCLOCK, dARNT, or dSIM. These were cotransfected with *per-luc*, *tim-luc*, or CME-*lacZ* reporter plasmids (*35*). (**B**) The E-box was necessary for dCLOCK-mediated activation of *period* promoter fragments. S2 cells were transfected with either dCLOCK or pAct as indicated. Reporter plasmids contain a portion of the *per* promoter fused to the *hsp70* basal promoter driving *lacZ* (*10*). The 69- and 154-bp fragments correspond to –563 to –494, and –603 to –449 of the *per* promoter, respectively. 154 (Δ1) refers to a deletion from –529 to –519, and 154 (Δ2) to –539 to –529, both of which remove a portion of the E-box (*10*). (**C**) The E-box was sufficient for activation of *per* and *tim* promoters by dCLOCK. Reporters contained four copies of an 18-bp element from either the *per* or *tim* promoters fused to *hsp70* driving luciferase (*39*). Identity between the *per* and *tim* E-boxes and the surrounding sequence is indicated in bold. In mutant E-box targets, the central CG of the E-box was replaced with GC (underlined). Plasmids expressing dCLOCK or dCLOCK(ΔQ) were cotransfected alone or in combination. dCLOCK(ΔQ) contains amino acids 1 through 792 of dCLOCK, omitting two of the three glutamine repeats.

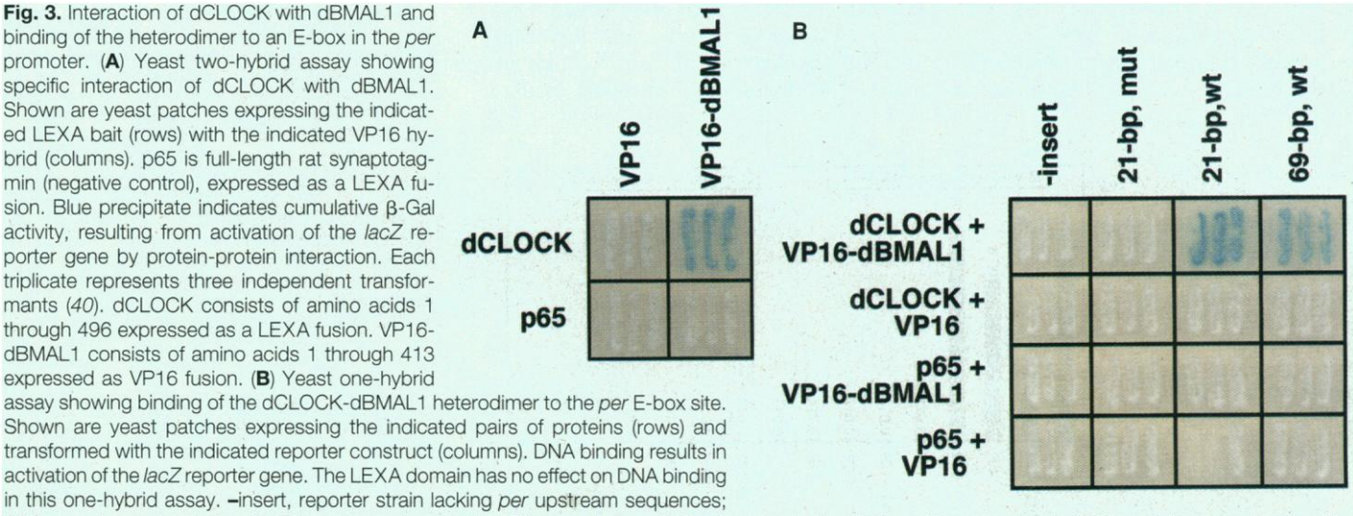

Fig. 3. Interaction of dCLOCK with dBMAL1 and binding of the heterodimer to an E-box in the *per* promoter. (**A**) Yeast two-hybrid assay showing specific interaction of dCLOCK with dBMAL1. Shown are yeast patches expressing the indicated LEXA bait (rows) with the indicated VP16 hybrid (columns). p65 is full-length rat synaptotagmin (negative control), expressed as a LEXA fusion. Blue precipitate indicates cumulative β-Gal activity, resulting from activation of the *lacZ* reporter gene by protein-protein interaction. Each triplicate represents three independent transformants (*40*). dCLOCK consists of amino acids 1 through 496 expressed as a LEXA fusion. VP16-dBMAL1 consists of amino acids 1 through 413 expressed as VP16 fusion. (**B**) Yeast one-hybrid assay showing binding of the dCLOCK-dBMAL1 heterodimer to the *per* E-box site. Shown are yeast patches expressing the indicated pairs of proteins (rows) and transformed with the indicated reporter construct (columns). DNA binding results in activation of the *lacZ* reporter gene. The LEXA domain has no effect on DNA binding in this one-hybrid assay. –insert, reporter strain lacking *per* upstream sequences; 21-bp, mut, reporter strain with E-box element scrambled; 21-bp, wt, same 21-bp fragment with wild-type E-box sequence; 69-bp, wt, larger region from the *per* promoter containing an intact E-box (*41*).

ed by dCLOCK (Fig. 2C). Constructs containing mutated E-boxes did not support transcriptional activation by dCLOCK. A truncated dCLOCK protein lacking two of the three polyglutamine repeats [dCLOCK(ΔQ)] only weakly activated *per* and *tim* E-boxes, which is consistent with the idea that this region corresponds to the transactivation domain. Furthermore, this protein antagonized full-length dCLOCK activation of *period* and *timeless* E-boxes. The activity of this truncation correlates well with the finding that the mouse circadian mutant *Clock*, in which part of the glutamine-rich region is deleted (*9*), behaves genetically as an antimorph (*19*).

bHLH-PAS transcription factors most often function as heterodimers (*18*, *20*). We initially predicted that the partner for dCLOCK was dARNT, a common partner for several bHLH-PAS proteins (*18*, *20*, *21*). When *dsim* is transfected into S2 cells, little activation of its target element is seen without cotransfection of dARNT (*18*). However, exogenous dARNT had no effect on the level of induction by dCLOCK (Fig. 2A), which suggests that dCLOCK acts either as a homodimer or with another partner endogenous to S2 cells. In an accompanying paper (*22*), evidence is provided that the bHLH-PAS transcription factor BMAL1 (MOP3) (*15*, *23*) is the partner for mCLOCK. To test whether this is true for *Drosophila*, we obtained a *Drosophila* homolog of BMAL1 through the expressed sequence tag (EST) database. Like *dclock*, *per*, and *tim* (*1*), *dbmal1* is expressed in the fly head as determined by Northern blot (*12*). However, when cotransfected with dCLOCK, this clone had no effect on the induction of the *per* promoter (*12*). Northern blot analysis showed *dbmal1* expression in naïve Schneider cells (*12*), in contrast to the absence of detectable signal when the same blot was probed with *dclock* (Fig. 1B). High levels of endogenous dBMAL1 may obscure any contribution of exogenous dBMAL1 (*24*). We employed both a two-hybrid and a one-hybrid yeast assay (*25*) as a heterologous system to test whether dCLOCK and dBMAL1 interact and bind the *per* E-box. dCLOCK and dBMAL1 specifically interacted with each other in yeast cells (Fig. 3A) and bind to a region encompassing the *per* E-box but not to an E-box mutant (Fig. 3B). Together with the results reported in mammals (*22*, *26*), this strongly suggests that dCLOCK and dBMAL1 form a complex that activates *period* and *timeless* transcription in vivo. We localized *dbmal1* to chromosomal position 76C (*27*).

To test the prediction that PER and TIM proteins prevent dCLOCK-induced expression from their E-boxes, we cotransfected *per* and *tim* cDNAs expressed from the *Drosophila actin 5C* promoter (Fig. 4). We observed a 4.5-fold reduction in dCLOCK-mediated reporter induction. Both *per* and *tim* were required to elicit this effect, probably because of their mutual dependence for nuclear localization (*8*). PER and TIM exhibited no negative effect on expression from the E-box multimer when dCLOCK was excluded or when the E-box was mutated. Thus, PER and TIM negated dCLOCK's transcriptional activity rather than repressing dCLOCK independent transcription. These data are in accord with a model in which either PER or TIM binds to either dCLOCK or dBMAL1, sequestering it in a nonfunctional complex.

When our results are placed in the context of the current understanding of the *Drosophila* circadian oscillator, dCLOCK closes the feedback loop. A dCLOCK-dBMAL1 complex drives expression of *per* and *tim* by binding an E-box that is present in their promoters. With time, PER and TIM heterodimers accumulate, translocate to the nucleus (*1*), and act as dominant negative inhibitors of dCLOCK-dBMAL1. As mRNA and protein levels fall, the inhibition is relieved, which allows dCLOCK-dBMAL1 to initiate a new round of synthesis. The position that dCLOCK holds in driving the expression of circadian oscillator components is reminiscent of the role played by the *white collar* genes in *Neurospora* (*28*). However, the ability of WC-1 and WC-2 to directly activate the *frequency* promoter has not yet been established.

It is tempting to speculate that the *Drosophila* four-component transcriptional feedback loop described here is sufficient to generate a rudimentary circadian rhythm. This oscillation would be amplified by other, unknown proteins that regulate RNA stability (*3*), protein stability (*29*), and phosphorylation (*30*) of the oscillator components.

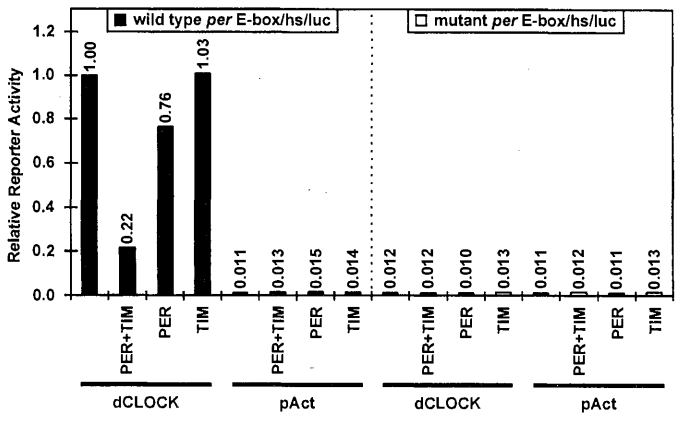

Fig. 4. PER and TIM inhibition of dCLOCK-mediated transactivation. S2 cells were transfected with combinations of pAct expression plasmids (dCLOCK, PER, and TIM) and wild-type or mutant *per* E-box reporters as indicated (*42*).

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11. *dClock* was cloned from a lambda ZapII head cDNA library. Plaques were screened with a fragment from the *mClock* clone YZ50 (*9*), which encodes the bHLH-PAS region. Filters were hybridized at 57°C in 6× saline sodium citrate, 5× Denhardt's solution, single-stranded salmon sperm DNA (0.1 mg/ml), and 0.1% SDS. A *dclock* fragment including the PAS B domain was used to rescreen the filters at high stringency.
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13. We amplified the region surrounding the splice site by polymerase chain reaction (PCR) from total RNA, using Gibco's Superscript Preamplification System, and from genomic DNA. Comparison of the sequence of the genomic and reverse transcription PCR products revealed a differentially spliced intron with two adjacent splice acceptors at position 569. In variant A, the second AG is used. In variant B, the first AG is used, introducing a frame shift. This isoform encodes two conceptual proteins. One initiates at the regular ATG sequence and consists only of the bHLH domain. The other initiates at an alternative Kozak consensus start site and consists of the PAS A and all COOH-terminal domains. When the clone is transcribed and translated in vitro, both polypeptides are observed (*12*). During the testing of these clones, a stretch of sequence identical to *dclock* was released in the EST database. Three clones were combined to construct the full-length version of *dclock* used in the transfection assays. The sequence of *dclock* has been submitted to GenBank (accession number AF067206).
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35. Expression plasmids contained the complete coding region of the indicated gene fused to the *Drosophila actin 5C* promoter in pAct (*18*). Reporter plasmids were as follows: *per-luc*, a 4.2-kb fragment of the *period* promoter fused to firefly luciferase (*32*); *tim-luc*, a 6.5-kb Bam HI–Sal I fragment of the *timeless* gene corresponding to the region upstream of the start of translation fused to firefly luciferase; and *CME-lacZ*, six *Toll* site 4 CMEs fused to *lacZ* (*18*). As a control for transfection efficiency, we used a plasmid containing the *actin 5C* promoter fused to *Renilla* luciferase in pRL-null (Promega). S2 cells in 12-well plates were transfected with Lipofectin (Gibco BRL) according to the manufacturer's recommendations. Each transfection contained 0.1 to 0.2 μg of each expression plasmid, 0.2 to 0.4 μg of reporter, 0.001 μg of control plasmid, and pAct to keep the amount of DNA per transfection constant. Cells were harvested 48 hours after transfection, and enzyme activity was measured with the Luciferase Assay, Dual-Luciferase Reporter Assay, and β-Galactosidase Enzyme Assay systems (Promega). For each sample, reporter activity was normalized to *Renilla* luc activity. Reporter activity was plotted relative to activity when cotransfected with empty expression vector pAct. Values are the mean ± SEM of two to four replicate experiments.
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37. Riboproprobes were generated with Ambion's Maxiscript kit (Austin, TX). Northern blots were performed by standard methods.
38. Frozen flies were vortexed and put through a tissue sieve. RNA was extracted from the fractions with RNAzolB (Tel-Test, Friendswood, TX). Ribonuclease (RNAse) protections were done on 100 μg of RNA with the RPAII kit (Ambion) according to the manufacturer's instructions. Positive control RNA and riboprobes were made with Ambion's Maxiscript kit.
39. Each multimer reporter consisted of four 18-bp elements containing the E box from either the *per* or *tim* promoter. Sequences shown in Fig. 2C are from the sense strand of the *per* promoter and the antisense strand of the *tim* promoter. To construct the reporters, the following synthetic oligonucleotides were annealed and inserted into a pGL3 vector (Promega) containing the *hsp70* basal promoter: wild-type *per* multimer, 5′-GTACCGCCGCTCACGTGGCGAACC CTCGAGCCGCTCACGTGGCGAA-3′, 5′- CCCGC GAGCCGCTCACGTGGCGAACAT TAATGCCGCT CACGTGGCGAACC-3′, 5′-CTCGCGGGTTCGCC ACGTGAGCGGCTCGAGGGTTCGCCACGTGA GCGGC-3′, 5′-TCGAGGTTCGCCACGTGAGCG GCATTAATGTTCGCCACGTGAGCGG-3′; mutant *per* multimer, 5′-GTACCGCCGCTCAGCTGGCGA ACCCTCGAGCCGCTCAGCTGGCGAA-3′, 5′- CC CGCGAGCCGCTCAGCTGGCGAACATTAATGCC GCTCAGCTGGCGAACC-3′, 5′-CTCGCGGGTT CGCCAGCTGAGCGGCTCGAGGGTTCGCCAG CTGAGCGGCG-3′, 5′-TCGAGGTTCGCCAGCTG AGCGGCATTAATGTTCGCCAGCTGAGCGG-3′; wild-type *tim* multimer, 5′-GTACCTGATTACACGT-GAGCCGAAGAGATTGATTACACGTGAGCCG-3′, 5′-AGGCGGTTGATTACACGTGAGCCGAAC-TAGTTGATTACACGTGAGCCGAC-3′, 5′-AACCG CCTCGGCTCACGTGTAATCAATCTCTTCGGC TCACGTGTAATCAG-3′, 5′ -TCGAGTCGGCTC ACGTGTAATCAACTAGTTCGGCTCACGTGTA ATC-3′; mutant *tim* multimer, 5′-GTACCTGA TTACAGCTGAGCCGAAGAGATTGATTACAGCTG AGCCG-3′, 5′- AGGCGGTTGATTACAGCTGAG CCGAACTAGTTGATTACAGCTGAGCCGAC-3′, 5′-AACCGCCTCGGCTCAGCTGTAATCAATCTCTT CGGCTCAGCTGTAATCAG-3′, 5′ -TCGAGTCGGC TCAGCTGTAATCAACTAGTTCGGCTCAGCTGTAA TC-3′. Replicate experiments were performed, and a representative data set is shown.
40. Two-hybrid assays were carried out on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) plates as described (*33*). For these assays, we used LEXA-dCLOCK(1–496) as bait, from which most of the COOH-terminal region was removed.
41. The indicated DNA fragments from the *per* gene clock control region were ligated into pBgl-lacZ, a one-hybrid reporter plasmid (*25*), and one-hybrid yeast reporter strains were constructed by insertion of recombinant pBgl-*lacZ* reporter plasmids into the *ura3* locus of YPH 499 (*34*). The negative control strain was constructed identically with nonrecombinant pBgl-*lacZ*. One-hybrid reporter strains were transformed with the indicated expression plasmids, and transformants were patched onto X-Gal plates (*33*) for detection of β-galactosidase (β-Gal) activity. The 21-bp fragment is part of the 69-bp circadian-regulated region (*10*) and contained the E-box with 8 bp of 5′ and 7 bp of 3′ flanking sequences. For the 21-bp fragment with the mutated E-box, the E-box sequence was scrambled by means of a random number table. E-box mutant: 5′-ATTCGC.
42. Plasmids expressing *per* and *tim* from the *actin 5C* promoter in pAct were constructed. Transfections and assays were performed as described (*35*) with minor modifications. For each well, expression plasmids were used at 0.001 μg for dCLOCK and 0.01 μg for PER and TIM. An additional plasmid with the *hsp70* promoter driving *lacZ* was used at 0.1 μg per well to control for transfection efficiency. For each sample, activity was normalized to β-Gal activity. For each reporter, activity was normalized to activity when transfected with pAct. The value for dCLOCK-activated wild-type reporter was set to one. Replicate experiments were performed, and a representative data set is shown.

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Teratogen-Mediated Inhibition of Target Tissue Response to *Shh* Signaling

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Veratrum alkaloids and distal inhibitors of cholesterol biosynthesis have been studied for more than 30 years as potent teratogens capable of inducing cyclopia and other birth defects. Here, it is shown that these compounds specifically block the Sonic hedgehog (Shh) signaling pathway. These teratogens did not prevent the sterol modification of Shh during autoprocessing but rather inhibited the response of target tissues to Shh, possibly acting through the sterol sensing domain within the Patched protein regulator of Shh response.

A striking aspect of *Shh* function is its role in developmental patterning of the head and brain, as revealed in $Shh^{-/-}$ mouse embryos by the occurrence of severe holoprosencephaly (HPE) (*1*). HPE is characterized by development of the prosencephalic derivatives as a single undivided vesicle that consists of the fused remnants of the dorsal telencephalic lobes, with an undivided eye field and an absence of ventral forebrain structures such as the optic stalks, the optic chiasm, and the pituitary (*1*, *2*). Externally, severe HPE is characterized by an absence of midline facial structures and development of a proboscis consisting of fused nasal chambers at a location overlying a cyclopic eye. Loss-of-function mutations at the human *Shh* locus are associated with a milder and more variable form of HPE that is inherited in autosomal dominant fashion, indicative of haploinsufficiency at the human *Shh* locus (*3*).

Hedgehog (Hh) proteins undergo an intramolecular autoprocessing reaction that

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