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Robust Oscillations within the Interlocked Feedback Model of *Drosophila* Circadian Rhythm

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A mechanism for generating circadian rhythms has been of major interest in recent years. After the discovery of *per* and *tim*, a model with a simple feedback loop involving *per* and *tim* has been proposed. However, it is recognized that the simple feedback model cannot account for phenotypes generated by various mutants. A recent report by Glossop, Lyons & Hardin [*Science* **286,** 766 (1999)] on *Drosophila* suggests involvement of another feedback loop by *dClk* that is interlocked with *per–tim* feedback loop. In order to examine whether interlocked feedback loops can be a basic mechanism for circadian rhythms, a mathematical model was created and examined. Through extensive simulation and mathematical analysis, it was revealed that the interlocked feedback model accounts for the observations that are not explained by the simple feedback model. Moreover, the interlocked feedback model has robust properties in oscillations.

Introduction

Along evolution, organisms ranging from cyanobacteria to mammals have developed in themselves molecular clocks of circadian (about 24 hr) period (Dunlap, 1999). In *Drosophila* like other organisms, several genes are involved in sustaining circadian rhythm, namely *period* (*per*), *timeless* (*tim*), *Drosophila Clock* (*dClk*), *Cycle* (*Cyc*) and *doubletime* (*dbt*) (Hardin *et al*., 1990; Sehgal *et al*., 1994; Darlington *et al*., 1998; Allada *et al*., 1998; Rutila *et al*., 1998; Price *et al*., 1998; Kloss *et al*., 1998). Among them, three genes are rhythmically expressed: *per*, *tim* and *dClk*. *per* and *tim* mRNA levels peak early in the evening whereas the *dClk* mRNA level peaks late at night to early in the morning (Hardin *et al*., 1990; Darlington *et al*., 1998; Sehgal *et al*., 1995; Bae *et al*., 1998).

Analysis of *per* and *tim* oscillatory expression has revealed a negative feedback loop, "*per–tim* feedback loop". Transcription of *per* and *tim* is activated through E-box elements in *per* and *tim* promoters by a heterodimer of basic helix-loop-helix transcription factors, dCLK and CYC (Darlington *et al*., 1998; Allada *et al*., 1998; Rutila *et al*., 1998; Hao *et al*., 1997). Peaks of PER and TIM levels delay several hours from peaks of *per*

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and *tim* mRNA levels. PER and TIM proteins form complex. This complex enters into the nucleus (Saez & Young, 1996) and represses transcription of *per* and *tim* by forming a complex with a heterodimer of transcriptional activators dCLK and CYC (Darlington *et al*., 1998; Lee *et al*., 1999). PER proteins are periodically phosphorylated (Edery *et al*., 1994). This phosphorylation is mediated at least partly, if not entirely, by DOUBLETIME protein (DBT), which is closely related to human casein kinase Iε. PER phosphorylation by DBT is suggested to regulate stability of PER proteins because in the mutant (dbt^P) abolishing most *dbt* expression, PER proteins constitutively accumulate and remain hypophosphorylated whereas *per* mRNA levels are similar to that in the wild type (Price *et al*., 1998; Kloss *et al*., 1998).

Several models based on this delayed negative feedback has been proposed (Darlington *et al*., 1998; Sehgal *et al*., 1995; Leloup & Goldbeter, 1998), which are called here as "simple feedback model". The simple feedback model shows that a delayed negative feedback loop produces spontaneous oscillation (Leloup & Goldbeter, 1998). However, these models seem to contradict decrease of *per* and *tim* mRNA levels in mutants lacking PER (per^{01}) or TIM (tim^0) function (Hardin *et al*., 1990; So & Rosbash, 1997) and do not explain *dClk* oscillatory expression (Bae *et al*., 1998).

On the other hand, analysis of *dClk* expression has revealed another negative feedback loop, "*dClk* feedback loop". *dClk* mRNA level is decreased in per^{01} and tim^0 mutants, indicating that PER and TIM function as positive regulators probably in *dClk* transcription (Bae *et al*., 1998; Glossop *et al*., 1999). The decreased level of *dClk* mRNA in the per^{01} mutant is restored to the wild-type level in per^{01}; $dClk^{Jrk}$ and per^{01}; Cyc^0 double mutants, which suggests that dCLK and CYC have a direct or indirect repressive role in *dClk* transcription (Glossop *et al*., 1999).

Based on these analyses of *dClk* expression, another type of model has been proposed, which is called here as "interlocked feedback model". The interlocked feedback model is composed of two coupled negative feedback loops: a *per–tim* feedback loop, which is activated by dCLK–CYC and repressed by PER–TIM, and a *dClk* feedback loop, which is repressed by dCLK–CYC and derepressed by PER–TIM (Glossop *et al*., 1999).

Results and Discussions

To verify whether the interlocked feedback model explains the observations which the simple feedback model does not, we constructed a mathematical model based on the interlocked feedback model. The interlocked feedback model is schematized in Fig. 1. To consider kinetics of expression of *per*, *tim* and *dClk*, we formulate this scheme to ten-variable differential equations (Fig. 2).

These equations can be integrated numerically once numerical values for all system parameters and initial conditions are specified. Instead of converging toward a stable steady state, the system shows sustained oscillations [Fig. 3(a) and (b)]. The system follows the same trajectories regardless of initial conditions, indicating that our model provides a molecular basis for circadian rhythm of the limit-cycle type. *per* and *tim* oscillations are in phase [Fig. 3(c)], whereas the *dClk* oscillation is almost in antiphase to *per* and *tim* oscillations [Fig. 3(d)]. These results are consistent with observations that *per* and *tim* mRNA levels oscillate in phase to one another (Sehgal *et al*., 1995; Bae *et al*., 1998; So & Rosbash, 1997) and that the *dClk* mRNA level oscillates in antiphase to *per* and *tim* mRNA levels (Bae *et al*., 1998).

Parameter values in Fig. 3 are chosen to yield a period close to 24 hr in constant darkness conditions. Identical kinetic constants are taken for corresponding processes involving PER and TIM to produce in-phase oscillations of *per* and *tim* mRNA levels. These values are a possible set of kinetic constants to yield a circadian period oscillation and in-phase oscillations of *per* and *tim* mRNA levels. However, these values yield appropriate phenotypes in the wild type: the correct time course of mRNA and protein levels and the reasonable phase shifts by light. First, we measured the peak time of mRNA and protein levels. *per* (*tim*) mRNA level peaks at zeitgeber time (ZT) 15.0 given that *per* mRNA level bottoms at $ZT = 0$. The cytoplasmic PER (TIM), cytoplasmic PER–TIM and nuclear PER–TIM

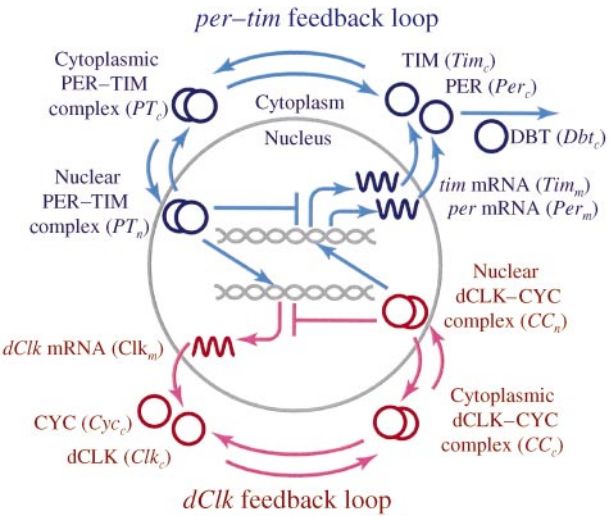

FIG. 1. The interlocked feedback model of *Drosophila* circadian rhythm. *per–tim* feedback loop: *per* (Per_m) and *tim* (Tim_m) mRNA are transcribed in the nucleus and translated to PER (Per_c) and TIM (Tim_c) in cytoplasm. Degradation of PER is promoted by kinase DBT (Dbt_c). Translated PER and TIM form a cytoplasmic PER–TIM complex (PT_c) and then enter the nucleus. A nuclear PER–TIM complex (PT_n) represses its own transcription activated by dCLK–CYC (CC_n). *dClk* feedback loop: *dClk* (Clk_m) mRNA is transcribed in the nucleus and translated to dCLK (Clk_c) in cytoplasm. Translated dCLK forms a cytoplasmic dCLK– CYC complex (CC_c) with CYC (Cyc_c) and then enters the nucleus. A nuclear dCLK–CYC complex (CC_n) represses its own transcription activated by dPER–TIM (PT_n).

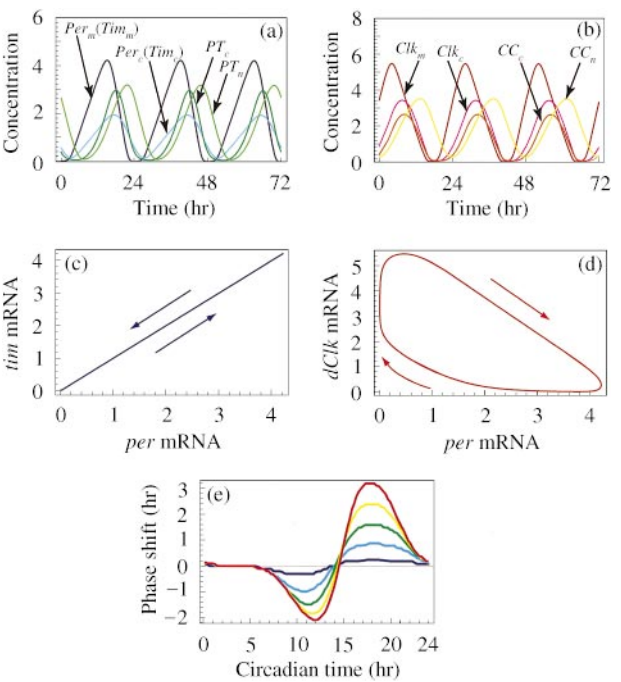

FIG. 3. Simulation of the wild type. (a)–(d) Time courses of *per* and *tim* expression (a) and *dClk* expression (b) are simulated. *tim* (c) and *dClk* (d) mRNA levels are plotted against *per* mRNA levels. *tim* and *per* mRNAs reach peak or bottom levels simultaneously (c) whereas *dClk* mRNA approaches the peak level when the *per* mRNA reaches the bottom level (d). Parameter values are: $Cyc_c = 1$ nM, $Dbt_c = 1$ nM. $C_1 = C_2 = C_3 = 0$ nM hr^{-1}, $S_1 = S_3 = 1.45$ nM hr^{-1}, $S_2 = S_4 = 0.48$ hr^{-1}, $S_5 = 1.63$ nM hr^{-1}, $S_6 = 0.47$ hr^{-1}, $r = 4$, $R_1 = R_2 = 1.02$ nM, $R_3 = 0.89$ nM, $a = 1$, $A_1 = A_2 = 0.45$ nM, $A_3 = 0.8$ nM, $B_1 = B_2 = 0$, $B_3 = 0.6$, $V_1 = 1.45$ nM^{-1} hr^{-1}, $V_2 = 1.45$ hr^{-1}, $V_3 = 1.63$ nM^{-1} hr^{-1}, $V_4 = 1.63$ hr^{-1}, $T_1 = 1.73$ nM hr^{-1}, $T_2 = 0.72$ nM hr^{-1}, $T_3 = 1.63$ nM hr^{-1}, $T_4 = 0.52$ nM hr^{-1}, $K_1 = 2$ nM, $K_2 = 2$ nM, $K_3 = 2$ nM, $K_4 = 2$ nM, $D_1 = D_3 = 0.94$ nM hr^{-1}, $D_2 = D_4 = 0.44$ nM hr^{-1}, $D_5 = 0.44$ nM hr^{-1}, $D_6 = 0.29$ nM hr^{-1}, $D_7 = 0.54$ nM hr^{-1}, $D_8 = 0.6$ nM hr^{-1}, $D_9 = 0.6$ nM hr^{-1}, $D_{10} = 0.3$ nM hr^{-1}, $L_1 = L_3 = 0.3$ nM, $L_2 = L_4 = 0.2$ nM, $L_5 = 0.2$ nM, $L_6 = 0.2$ nM, $L_7 = 0.13$ nM, $L_8 = 0.2$ nM, $L_9 = 0.2$ nM, $L_{10} = 0.2$ nM and $D_0 = 0.012$ hr^{-1}. (e) Phase shifts by light pulse, which is emulated by increasing TIM degradation rate (D_4) by two- (blue), four- (light blue), six- (green), eight- (yellow), ten- (red) folds for 1 hr. Phase shifts are plotted along subjective circadian time, at which the perturbation (increase of TIM degradation rate) is applied.

per–tim feedback loop

$$\frac{dPer_m}{dt} = C_1 + S_1 \frac{(CC_n/A_1)^a + B_1}{1 + (PT_n/R_1)^r + (CC_n/A_1)^a + B_1} - D_1 \frac{Per_m}{L_1 + Per_m} - D_0 Per_m,$$

$$\frac{dPer_c}{dt} = S_2 Per_m - V_1 Per_c Tim_c + V_2 PT_c - D_2 Dbt_c \frac{Per_c}{L_2 + Per_c} - D_0 Per_c,$$

$$\frac{dTim_m}{dt} = C_2 + S_3 \frac{(CC_n/A_2)^a + B_2}{1 + (PT_n/R_2)^r + (CC_n/A_2)^a + B_2} - D_3 \frac{Tim_m}{L_3 + Tim_m} - D_0 Tim_m,$$

$$\frac{dTim_c}{dt} = S_4 Tim_m - V_1 Per_c Tim_c + V_2 PT_c - D_4 \frac{Tim_c}{L_4 + Tim_c} - D_0 Tim_c,$$

$$\frac{dPT_c}{dt} = V_1 Per_c Tim_c - V_2 PT_c - T_1 \frac{PT_c}{K_1 + PT_c} + T_2 \frac{PT_n}{K_2 + PT_n} - D_5 \frac{PT_c}{L_5 + PT_c} - D_0 PT_c,$$

$$\frac{dPT_n}{dt} = T_1 \frac{PT_c}{K_1 + PT_c} - T_2 \frac{PT_n}{K_2 + PT_n} - D_6 \frac{PT_n}{L_6 + PT_n} - D_0 PT_n.$$

dClk feedback loop

$$\frac{dClk_m}{dt} = C_3 + S_5 \frac{(PT_n/A_3)^a + B_3}{1 + (CC_n/R_3)^r + (PT_n/A_3)^a + B_3} - D_7 \frac{Clk_m}{L_7 + Clk_m} - D_0 Clk_m,$$

$$\frac{dClk_c}{dt} = S_6 Clk_m - V_3 Clk_c Cyc_c + V_4 CC_c - D_8 \frac{Clk_c}{L_8 + Clk_c} - D_0 Clk_c,$$

$$\frac{dCC_c}{dt} = V_3 Clk_c Cyc_c - V_4 CC_c - T_3 \frac{CC_c}{K_3 + CC_c} + T_4 \frac{CC_n}{K_4 + CC_n} - D_9 \frac{CC_c}{L_9 + CC_c} - D_0 CC_c,$$

$$\frac{dCC_n}{dt} = T_3 \frac{CC_c}{K_3 + CC_c} - T_4 \frac{CC_n}{K_4 + CC_n} - D_{10} \frac{CC_n}{L_{10} + CC_n} - D_0 CC_n.$$

FIG. 2. A mathematical formulation of interlocked feedback model. We constructed ten-variable differential equations to investigate kinetics of concentration of *per* (Per_m), *tim* (Tim_m) and *dClk* (Clk_m,) mRNA, PER (Per_c), TIM (Tim_c) and dCLK (Clk_c) monomers, cytoplasmic (PT_c) and nuclear (PT_n) PER–TIM heterodimers and cytoplasmic (CC_c) and nuclear (CC_n) dCLK–CYC heterodimers. Concentrations of DBT (Dbt_c.) and CYC (Cyc_c) are supposed to be constant. To describe transcription, we used Hill equations with slight modification, which are characterized by six parameters representing the maximum velocity S_i ($i = 1, 3, 5$), two DNA binding constants of an activator (A_i, $i = 1, 2, 3$) and a repressor (R_i, $i = 1, 2, 3$), two Hill coeffcients for activation (a) and repression (r) and one constant B_i ($i = 1, 2, 3$), which indicates transcriptional activation by other transcription factors than PER–TIM or dCLK–CYC. Translation rate is proportional to the mRNA concentration with the proportional constant S_i ($i = 2, 4, 6$). Association and dissociation of PER–TIM and dCLK–CYC complex are subject to law of mass actions. These processes are characterized with the rate constant V_i ($i = 1, 2, \ldots$). Degradation and nuclear transportation are supposed to be mediated by degradation enzymes and transporters, respectively. We described these processes with Michaelis–Menten-type equations characterized by the maximum velocity D_i ($i = 1, 2, \ldots$) and T_j ($j = 1, 2, \ldots$), and the binding constant L_i ($i = 1, 2, \ldots$) and K_j ($j = 1, 2, \ldots$). Nonspecific degradation terms are proportional to each variable with the proportional constant D_0. For rescue experiments (described below), we introduced transcriptional activation from the constitutive promoter, which is characterized by the synthesis rate C_i ($i = 1, 2, 3$).

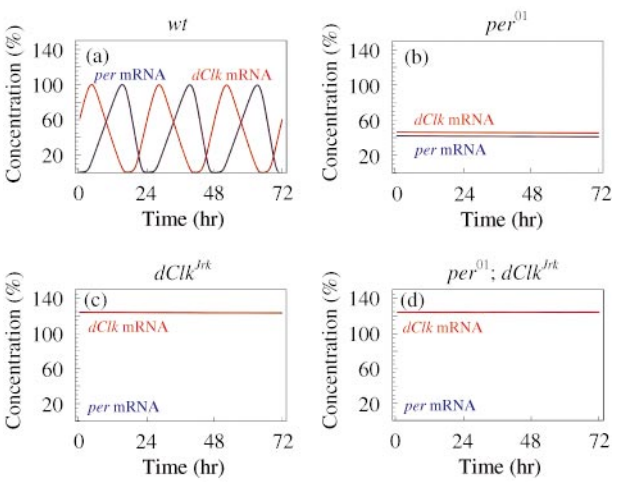

FIG. 4. Simulation of single and double mutants. Relative levels of *per* (blue) and *dClk* (red) mRNA are shown in the wild type (a) and per^{01} (b), $dClk^{Jrk}$ (c) per^{01}; $dClk^{Jrk}$ (d) mutants. The peak levels of *per* and *dClk* mRNA in the wild type are set to 100%. In per^{01} mutants (b), sustaining oscillations are abolished and *per* and *dClk* mRNA levels are decreased to about 42 and 46% of the peak levels in the wild type. In $dClk^{Jrk}$ (c) and per^{01}; $dClk^{Jrk}$ (d) mutants, sustaining oscillations are also abolished and the *per* mRNA level is severely decreased whereas the *dClk* mRNA level is restored to 124% of the peak level in the wild type. Parameter values are as in Fig. 3(a)–(d), except for $S_2 = 0\ \mathrm{hr}^{-1}$ for per^{01}, $S_6 = 0\ \mathrm{hr}^{-1}$ for $dClk^{Jrk}$ and $S_2 = S_6 = 0\ \mathrm{hr}^{-1}$ for per^{01}; $dClk^{Jrk}$.

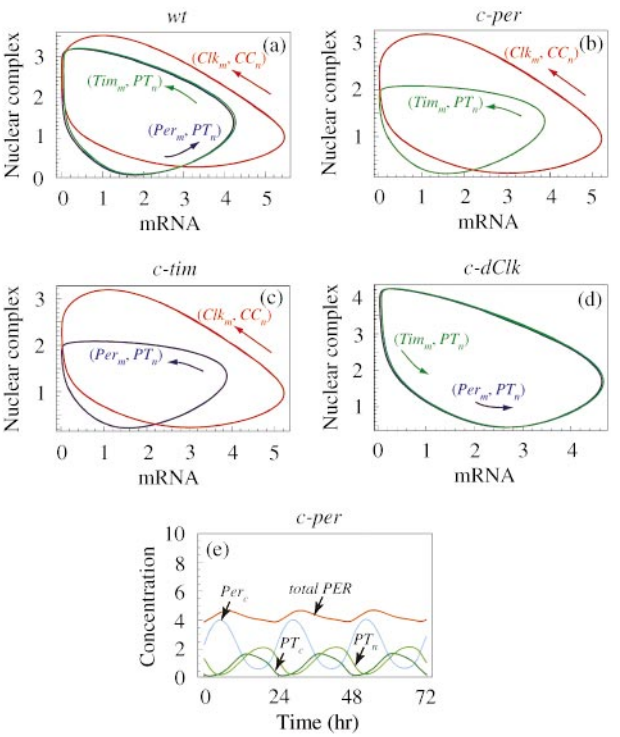

FIG. 5. Simulation of rescue experiments in single-mutant backgrounds. (a) *per* (blue), *tim* (green) and *dClk* (red) oscillations in the wild type. (b–d) Restored oscillations in transfected *c-per* (b), *c-tim* (c), *c-dClk* (d) single mutants. Rescued *per* oscillations (blue), *tim* oscillation (green) and *dClk* oscillation (red) are shown in Per_m–PT_n, Tim_m–PT_n and Clk_m–CC_n phase planes. (e) Simulated time courses of different forms of PER proteins in the *c-per* mutant. Parameter values are as in Fig. 3(a)–(d), except for $S_1 = 0\ \text{nM hr}^{-1}$, $C_1 = 0.846\ \text{nM hr}^{-1}$ for *c-per* mutant, $S_3 = 0\ \text{nM hr}^{-1}$, $C_2 = 0.846\ \text{nM hr}^{-1}$ for *c-tim* mutant and $S_5 = 0\ \text{nM hr}^{-1}$, $C_3 = 0.558\ \text{nM hr}^{-1}$ for *c-dClk* mutant.

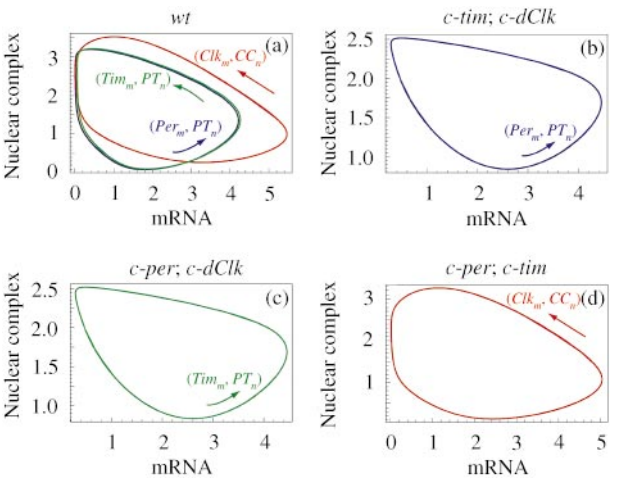

FIG. 6. Simulation of rescue experiments in double-mutant backgrounds. (a) *per* (blue), *tim* (green) and *dClk* (red) oscillations in the wild type. (b–d) Restored oscillations in transfected *c-tim*; *c-dClk* (b), *c-per*; *c-dClk* (c), *c-per*; *c-tim* (d) double mutants. Parameter values are as in Fig. 3(a)–(d) except for $S_3 = S_5 = 0\ \text{nM hr}^{-1}$, $C_2 = 0.846\ \text{nM hr}^{-1}$, $C_3 = 0.558\ \text{nM hr}^{-1}$ for *c-tim*; *c-dClk* mutant, $S_1 = S_5 = 0\ \text{nM hr}^{-1}$, $C_1 = 0.846\ \text{nM hr}^{-1}$, $C_3 = 0.558\ \text{nM hr}^{-1}$ for *c-per*-; *c-dClk* mutant and $S_1 = S_3 = 0\ \text{nM hr}^{-1}$, $C_1 = C_2 = 0.846\ \text{nM hr}^{-1}$ for *c-per*; *c-tim* mutant.

levels peak at $ZT = 17.3$, 17.9 and 21.6, respectively, while total amounts of PER (TIM) peak at $ZT = 18.8$. On the other hand, the *dClk* mRNA level peaks at $ZT = 4.0$. These results are consistent with observations that *per* and *tim* mRNAs reach peak levels early in the evening ($ZT =$ 13–16) (Hardin *et al*., 1990; Sehgal *et al*., 1994, 1995), that PER and TIM levels do not peak until late evening ($ZT = 18$–24) (Zerr *et al*., 1990; Edery *et al*., 1994) and that the *dClk* mRNA level peaks late at night to early in the morning (ZT 23 to ZT 4) (Darlington *et al*., 1998; Bae *et al*., 1998). Next, we investigated phase shifts by light pulse. It has been reported that TIM is rapidly reduced by light pulse (Hunter-Ensor *et al*., 1996; Zeng *et al*., 1996; Myers *et al*., 1996; Lee *et al*., 1996). Thus, we emulated light pulse by increasing the rate of TIM degradation by two-, four-, six-, eight-, or ten-fold for 1 h. We applied the same perturbation (promotion of TIM degradation) to the system from 100 different circadian time points and measured the following peak time of *per* mRNA. By comparing it with the peak time of *per* mRNA without perturbation, we can obtain phase shifts. Obtained phase shifts are plotted along circadian time at which perturbation is applied [Fig. 3(e)]. They show no phase shift in the middle of subjective day, maximum phase delays in early subjective night, and maximum phase advances in late subjective night. Shapes of the phase shifts–circadian time relationship are maintained in various intensity of perturbation although the magnitude of phase shifts becomes larger along the intensity. These results are also consistent with the observation that light-pulses delay the phase of the circadian activity rhythms during early subjective night and advance the phase during late subjective night whereas light pulses tend to cause minimal or no phase shifts during the subjective day (Hall & Rosbash, 1987).

To verify our model by comparing with phenotypes in reported mutants, we create mathematical "mutants" which correspond to per^{01}, tim^{0}, $dClk^{Jrk}$ and Cyc^{0} single mutants and per^{01}; $dClk^{Jrk}$ and per^{01}; Cyc^{0} double mutants. In per^{01} mutants, sustaining oscillations are abolished [Fig. 4(b)]. *per*, *tim* and *dClk* mRNA levels are decreased to about 42, 42 and 46% of the peak levels in the wild type. tim^{0} mutant shows similar phenotype to a per^{01} mutant. These results are consistent with reported observations that in per^{01} and tim^{0} mutants, the *per* and *tim* transcription is constitutive and *per*, *tim* and *dClk* mRNA levels are relatively low (Hardin *et al*., 1990; Sehgal *et al*., 1994; Allada *et al*., 1998; Bae *et al*., 1998; So & Rosbash, 1997; Glossop *et al*., 1999). In $dClk^{Jrk}$ and per^{01}; $dClk^{Jrk}$ mutants, sustaining oscillations are also abolished [Fig. 4(c) and (d)]. In these mutants, *per* and *tim* mRNA transcripts severely decreased whereas the *dClk* mRNA level is restored to 124% of the peak level in the wild type. Cyc^{0} and per^{01}; Cyc^{0} mutants show similar phenotypes to $dClk^{Jrk}$ and per^{01}; $dClk^{Jrk}$ mutants. These results are also consistent with previous observations that in $dClk^{Jrk}$ and Cyc^{0} mutants the *per* and *tim* mRNA levels are constant and significantly reduced (Allada *et al*., 1998; Rutila *et al*., 1998) and that in $dClk^{Jrk}$ and Cyc^{0} single mutants and per^{01}; $dClk^{Jrk}$ and per^{01}; Cyc^{0} double mutants, *dClk* mRNA levels are almost restored to the peak level in the wild type (Glossop *et al*., 1999). The mathematical model based on the interlocked feedback mechanism reproduces decrease of *per* and *tim* mRNA levels in mutants lacking PER (per^{01}) or TIM (tim^{0}) function, which seems to contradict the simple feedback mechanism. An intuitive explanation for these phenotypes is that PER and TIM protein knockout will inhibit the transcription of *dClk*, which will inhibit the transcription of *per* and *tim* resulting in low levels of *per* and *tim* messages.

per genomic fragments lacking a promoter (Frisch *et al*., 1994) or driven by constitutively active promoters (Ewer *et al*., 1988; Vosshall & Young, 1995) rescue locomotor activity rhythms in per^{01} flies. To test whether our model explains these observations, we create a mathematical mutant which corresponds a per^{01} fly carrying a transgene that constitutively expresses *per* mRNA (termed as *c-per*). In the *c-per* mutant, *tim* and *dClk* oscillations are restored [Fig. 5(b)]. We also find that cytoplasmic PER, cytoplasmic PER–TIM and total PER protein levels show oscillations in the *c-per* mutant [Fig. 5(e)]. Oscillations in cytoplasmic PER and PER–TIM levels occur because a *tim* feedback loop works and oscillatory expressed TIM proteins form PER–TIM complexes with PER proteins. These oscillations produce the oscillation of the total PER

protein level because of different degradation rates in different forms of PER and/or nonlinearity of degradation process. These results are consistent with a previously reported observation that the PER protein level oscillates in per^{01} files carrying a transgene that constantly expresses *per* mRNAs (Cheng & Hardin, 1998).

Similar rescue experiments in other single- or double-mutant backgrounds reveal robust oscillations within the interlocked feedback model. First, we transfect tim^0 or $dClk^{Jrk}$ single mutant with a transgene expressing *tim* or *dClk* mRNAs constitutively (termed as *c-tim* and *c-dClk*). *c-tim* mutant restores *per* and *dClk* oscillations [Fig. 5(c)] while *c-dClk* mutant restores *per* and *tim* oscillations [Fig. 5(d)]. Next, we transfect tim^0; $dClk^{Jrk}$, per^{01}; $dClk^{Jrk}$ or per^{01}; tim^0 double mutants with two transgenes expressing *tim*/*dClk*, *per*/*dClk* or *per*/*tim* mRNAs constantly (termed as *c-tim*; *c-dClk*, *c-per*; *c-dClk* and *c-per*; *c-tim*). In *c-tim*; *c-dClk*, *c-per*; *c-dClk* and *c-per*; *c-tim* mutants, *per*, *tim* and *dClk* oscillations are restored, respectively [Fig. 6(b)–(d)].

The experimental results summarized in Figs. 3–6 show that the interlocked feedback model provides a possible mechanism of *Drosophila* circadian rhythms. First, the interlocked feedback model provides the similar time course of mRNA and protein levels in the wild type and similar mRNA levels in single or double mutants to previously reported observations, including antiphase oscillations of *per* (*tim*) and *dClk* mRNAs and decreased *per* and *tim* mRNA levels in per^{01} and tim^0 mutants, which are not explained by one feedback model. The interlocked feedback model also provides light-pulse-type phase shifts induced by temporal promotion of TIM degradation, which is reported to be involved in light-pulse entrainment. Second, the interlocked feedback model shows highly robust oscillations in mutations, which abolish oscillations of one or two mRNAs among *per*, *tim* and *dClk* mRNAs.

One of the most attractive properties of circadian rhythms is the relative constancy of circadian period in the temperature range over 10°C, which is referred to as temperature compensation (Pittendrigh, 1954; Konopka *et al*., 1989). Temperature compensation poses an important constraint on models of the circadian rhythms. For, this phenomenon seemingly contradicts the intuitive prediction that increase of temperature by 10°C typically doubles the reaction rate (Segel, 1975), resulting in shortening the period of oscillations to half of the original one. One way to satisfy this constraint has been proposed by Leloup & Goldbeter (1997). They have found that the increase of one system parameter that represents one reaction rate of the circadian oscillator can either increase or decrease the period of oscillation and that magnitude of period change differs with altered parameters. They utilized this dependence of period on parameters and showed that if appropriate set of parameters is more sensitive (or insensitive) to temperature then the period of the circadian oscillators remains constant in the temperature range over 10°C. The interlocked feedback model presented here also shows various dependences of period on system parameters (unpublished data). Thus, in a way similar to that by Leloup and Goldbeter, the interlocked feedback model might show constancy of the period in some temperature range with several possible sets of parameters which are more sensitive to temperature than others. The challenge is to determine the correct parameter set among them and this remains for the future work.

Along evolution, various organism have developed molecular clocks, which seem more complex than simple negative feedback oscillators. In mammals, *per* homologues *Per1*, *Per2*, *Per3* and *dClk* functional homologue *Bmal1* mRNA levels oscillate in antiphase (Honma *et al*., 1998; Shearman *et al*., 2000). It has been shown that CLOCK and BMAL1 activate vasopressin gene transcription and that all three mouse PERs and TIM repress this activation (Jin *et al*., 1999). Although this simple negative feedback regulation may explain oscillatory expression of *Pers mRNA*, it does not explain how *BMAL1* mRNA levels oscillate in antiphase to *Pers*. In *Neurosopra*, FREQUENCY protein (FRQ) is periodically expressed and represses its own transcription. A positive regulator of *frq* transcription, WHITE COLOR 1 protein (WC-1) is also rhythmically expressed in antiphase to FRQ from constant *wc-1* mRNA by post-transcriptional regulation. WC-1 expression is regulated directly or indirectly by FRQ because driven FRQ expression

positively regulates WC-1 synthesis with similar lag time to that seen in the wild type (Lee *et al*., 2000). Although there are some molecular details which remain to be uncovered and some divergent twists among species, a conserved principal more complicated than simple negative feedback mechanism seems to exist. The mathematical model presented here would provide a means to investigate the design principal of seemingly complex molecular clocks.

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