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8. BALB/c mice were inoculated in the right and left footpad with SLA (25 μg), SLA + IL-12 (0.5 μg), or IL-12 alone, and the popliteal LNs were harvested 3 days later. C3H/HeN mice were inoculated with SLA alone. LN cells were assayed for cytotoxic potential against ^{51}Cr-labeled YAC-1 cells in a 4-hour Cr-release assay at several effector to target ratios. For cytokine analysis, cells were cultured in 24-well plates at 5 × 10^6 per milliliter per well in tissue culture medium [Dulbecco's minimum essential medium with glucose (4.5 mg/ml), 10% fetal bovine serum, 2 mM glutamine, penicillin 6-phosphate (100 U/ml), streptomycin sulfate (100 μg/ml), 25 mM Hepes, 50 μM 2-mercaptoethanol] with or without SLA (50 μg/ml). Only the data from cells cultured with SLA are shown. Supernatants were harvested at 72 hours and IFN-γ and IL-4 measured by enzyme-linked immunosorbent assay (*7*). SLA was prepared from *L. major* promastigotes as described [P. Scott *et al.*, *J. Immunol.* **139**, 221 (1987)].
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10. BALB/c mice were immunized as described (*8*). CD4$^+$ T cells were cultured at 2 × 10^6 per milliliter with irradiated (3300 rad) normal spleen cells (3 × 10^6 milliliter) as antigen-presenting cells. CD4$^+$ T cells were positively selected on a magnetic cell separator (MACS; Miltenyi, Biotec, Sunnyvale, CA) as described (*7*). The CD4$^+$ T cell–enriched population contained greater than 98% CD4$^+$ T cells as assessed by cytofluorometric analysis. To deplete NK cells, we treated mice with anti–asialo GM1 (ASGM1) on day −4 (1.5 mg intravenously) and day 0 (350 μg intravenously) (*7*).
11. A requirement for the presence of IFN-γ for the in vitro development of T_H1 cells in response to IL-12 has been reported [S. E. Macatonia, C.-S. Hsieh, K. M. Murphy, A. O'Garra, *Int. Immunol.* **5**, 1119 (1993)], although in another in vitro system the capacity of IL-12 to initiate T_H1 cell development was found to be independent of IFN-γ [R. A. Seder, R. T. Gazzinelli, A. Sher, W. E. Paul, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10188 (1993)]. Our data suggest that NK cells may be one source of IFN-γ in vivo that augments T_H1 cell development. Similarly, recent findings in the SCID model with intracellular pathogens indicate that a common pathway for IFN-γ production is through the ability of IL-12 to activate NK cells [C. S. Tripp, S. F. Wolf, E. R. Unanue, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3725 (1993); R. T. Gazzinelli, S. Hieny, S. Wynn, S. Wolf, A. Sher, *ibid.*, p. 6115].
12. BALB/c mice were immunized in the right footpad with SLA (25 μg) or SLA + IL-12 (1 μg). Ten days later mice were given an intradermal injection in the flank with SLA (10 μg) or SLA + IL-12 (1 μg). Two weeks later mice were challenged in the left footpad with 10^5 purified metacyclic *L. major* promastigotes (*7*). In three experiments the mean lesion size of mice immunized with SLA + IL-12 at 10 weeks was 0.26 ± 0.59 mm, while unimmunized mice, SLA-immunized mice, or mice receiving only IL-12 exhibited lesions of 3 to 5 mm. One group of mice immunized with SLA + IL-12 was maintained for 20 weeks with no signs of resumption of disease.
13. Interleukin-12 when administered multiple times systemically to BALB/c mice during the infection enhances their resistance to *L. major* [F. P. Heinzel, D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, M. K. Gately, *J. Exp. Med.* **177**, 1505 (1993); J. P. Sypek *et al.*, *ibid.*, p. 1797]. This effect is distinct from the adjuvant effects of IL-12 observed in these studies since IL-12 by itself failed to exhibit any protective effects (Table 1).
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1 July 1993; accepted 26 October 1993

Phase Shifting of the Circadian Clock by Induction of the *Drosophila period* Protein

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Virtually all organisms manifest circadian (24-hour) rhythms, governed by an ill-defined endogenous pacemaker or clock. Several lines of evidence suggest that the *Drosophila melanogaster period* gene product PER is a clock component. If PER were central to the time-keeping mechanism, a transient increase in its concentration would cause a stable shift in the phase of the clock. Therefore, transgenic flies bearing a heat-inducible copy of PER were subjected to temperature pulses. This treatment caused long-lasting phase shifts in the locomotor activity circadian rhythm, a result that supports the contention that PER is a bona fide clock component.

A wide variety of organisms exhibit daily fluctuations in many biochemical, physiological, and behavioral phenomena that are governed by an endogenous circadian (≈24 hours) pacemaker or "clock" (*1*). Although no bona fide components of a circadian oscillator have been identified, a candidate is the *period* (*per*) gene product from *Drosophila melanogaster* (*2*, *3*). In the absence of *per* activity (per^{01} nonsense mutation), there is no observable rhythmicity of eclosion or of locomotor activity (*2*). Moreover, missense mutations shorten (per^s) to 19 hours or lengthen (per^L) to 29 hours the free-running periods of both rhythms (*2*). Consistent with the possibility that *per* is directly involved in the generation of these rhythms is the observation that PER itself is subject to circadian regulation; both *per* protein (PER) (*4*) and *per* mRNA (*5*) undergo daily fluctuations in the heads of adult flies. These two oscillations are connected, as PER may have a role in the circadian regulation of its own transcription (*5*, *6*).

If PER oscillations contribute directly to the clock mechanism, transient perturbations of its level or activity would cause a phase shift in the oscillator (Fig. 1A). Because identical stimuli applied at different phases in a circadian cycle can give rise to shifts with different magnitudes and directions, PER increases might advance or delay the rhythm of locomotor activity in an a priori unpredictable fashion (Fig. 1A).

To test this prediction, we generated transgenic flies bearing a heat-inducible copy of *per*, termed *hspcper* (Fig. 1B) (*7*). Several independent lines were obtained, and we assayed the biological activity of *hspcper* in a per^{01} arrhythmic background. As in previous results (*8*), some lines were rhythmic at 25° or 29°C, whereas others were not (*9*). We chose one completely arrhythmic line (per^{01};*hspc*-23a) (*10*) and reasoned that in a wild-type strain with this insert (per^+;*hspc*-23a), only the per^+ gene should contribute significantly to locomotor activity rhythms at 25°C. Indeed, the rhythms of per^+;*hspc*-23a flies were indistinguishable from those of control wild-type flies, including in their periods (*11*) and in their phases (*9*). With this strain, we then induced PER at various times throughout a circadian cycle and measured the phase of the resultant locomotor activity.

Because temperature pulses elicit phase shifts in many organisms (*12*), we first determined the effects of temperature on wild-type *D. melanogaster* locomotor activity rhythms. We administered short pulses of the heat stimulus throughout the circadian cycle and determined the magnitude and direction of the ensuing phase shift; plotting the average phase shift (*13*) as a function of the circadian time of the stimulus yielded a phase response curve (PRC) (Fig. 2A). This heat PRC is similar to results obtained with light pulses as the stimulus for phase-shifting *D. melanogaster* locomotor activity (*14*). Depending on when the temperature pulse was given, the activity phase was delayed, advanced, or unchanged. Both delays and advances reached a new steady state within one circadian cycle after treatment with heat (*9*).

We applied the identical temperature-pulse regime to per^+;*hspc*-23a flies and a different PRC was generated (Fig. 2, A and B). Expression of heat-induced PER (HSP-PER) converted the phase delay region

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[circadian time 9 to 17 (CT9–17) (Fig. 2A)] normally observed in wild-type flies into a strong phase-advance zone (Fig. 2B) (*15*). Expression of HSPPER in the normally unresponsive zone of wild-type flies (CT1–7) (Fig. 2A) produced phase delays (Fig. 2B). Although we observed significant delays only at CT3 and CT5, induction of HSPPER did phase-shift the clock at times at which temperature pulses are ineffective in wild-type flies.

Because it is likely that the PRC generated for *per*$^+$;*hspc*-23a flies (Fig. 2B) results from the cumulative effects of both temperature and HSPPER, we subtracted the values obtained for *per*$^+$;*hspc*-23a from those for wild-type flies to generate a PRC that should better reflect only HSPPER (Fig. 2C). Between CT9 and CT17, transient increases in HSPPER elicit phase advances much larger than those elicited by temperature alone (Fig. 2A). Furthermore, during certain times the clock is largely insensitive to increases in HSPPER (CT19–01) (Fig. 2C). We cannot eliminate the possibility that the analysis is complicated by synergistic effects (for example, that HSPPER affects the temperature sensitivity of the phase-shifting response). Nor is it certain that the two effects occur simultaneously or that the increase in PER activity is restricted to a 1-hour time window. Nevertheless, induction of HSPPER can advance the phase of the clock at times and under conditions identical to those that elicit phase delays in wild-type flies.

To test the possibility that gross differences in HSPPER induction or stability might account for the time-dependent differences in the HSPPER-induced phase shifts, we assayed HSPPER with antibodies to PER (Fig. 3) (*16–18*). The induction of HSPPER was indistinguishable at all circadian times. Also, the profiles of appearance and disappearance were indistinguishable, independent of the circadian time at which HSPPER was induced (9). Therefore, because the phase shifts are stable even after HSPPER is no longer detectable (*19*), the PRC (Fig. 2C) likely reflects the temporal sensitivity of the clock to the induction of HSPPER.

Although the 1-hour heat pulse generates HSPPER in excess of wild-type PER (Fig. 3), it is impossible to estimate from its biochemical profile the relative levels of the biological activity of HSPPER. For example, immunohistochemical staining of *per*$^+$;*hspc*-23a fly heads after a heat pulse of 1 hour at 37°C showed that HSPPER is expressed throughout the entire head (9). This pattern is very different from the highly restricted spatial expression pattern observed for wild-type (*per*$^+$) flies (*4*). Therefore, the protein immunoblot (Fig. 3) greatly overestimated the amount of HSPPER in the very limited number of (wild-type) PER-expressing cells. This compromises interpretations based solely on gross amounts of HSPPER.

A comparison of the PRCs (Fig. 2, A and B) in wild-type and *per*$^+$;*hspc*-23a flies raises the intriguing possibility that the fluctuations normally observed in PER amounts might underlie the fundamental light PRC of the wild-type *D. melanogaster* clock. The superposition of a classical light PRC and a curve describing the PER fluctuations (*20*) reveals that amounts of wild-type PER are greatest during the night, when phase shifts can be induced, and smallest during the middle of the day, when the clock is most refractory to phase-shifting stimuli (Fig. 4). The induction of HSPPER during the rise and fall in wild-

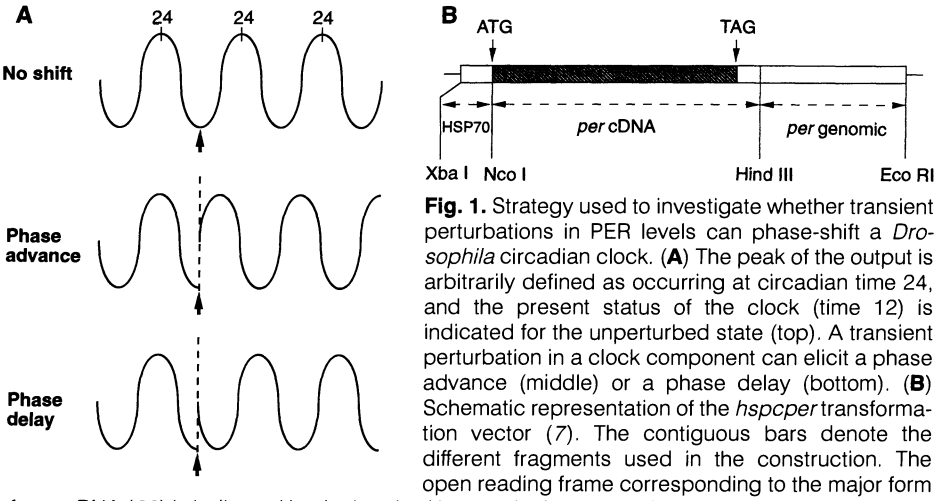

Fig. 1. Strategy used to investigate whether transient perturbations in PER levels can phase-shift a *Drosophila* circadian clock. (**A**) The peak of the output is arbitrarily defined as occurring at circadian time 24, and the present status of the clock (time 12) is indicated for the unperturbed state (top). A transient perturbation in a clock component can elicit a phase advance (middle) or a phase delay (bottom). (**B**) Schematic representation of the *hspcper* transformation vector (*7*). The contiguous bars denote the different fragments used in the construction. The open reading frame corresponding to the major form of *per* mRNA (*23*) is indicated by the hatched bar, and relevant restriction endonuclease sites are shown. The positions of Nco I (2835), Hind III (7212), and Eco RI (9302) are based on the sequence in (*23*).

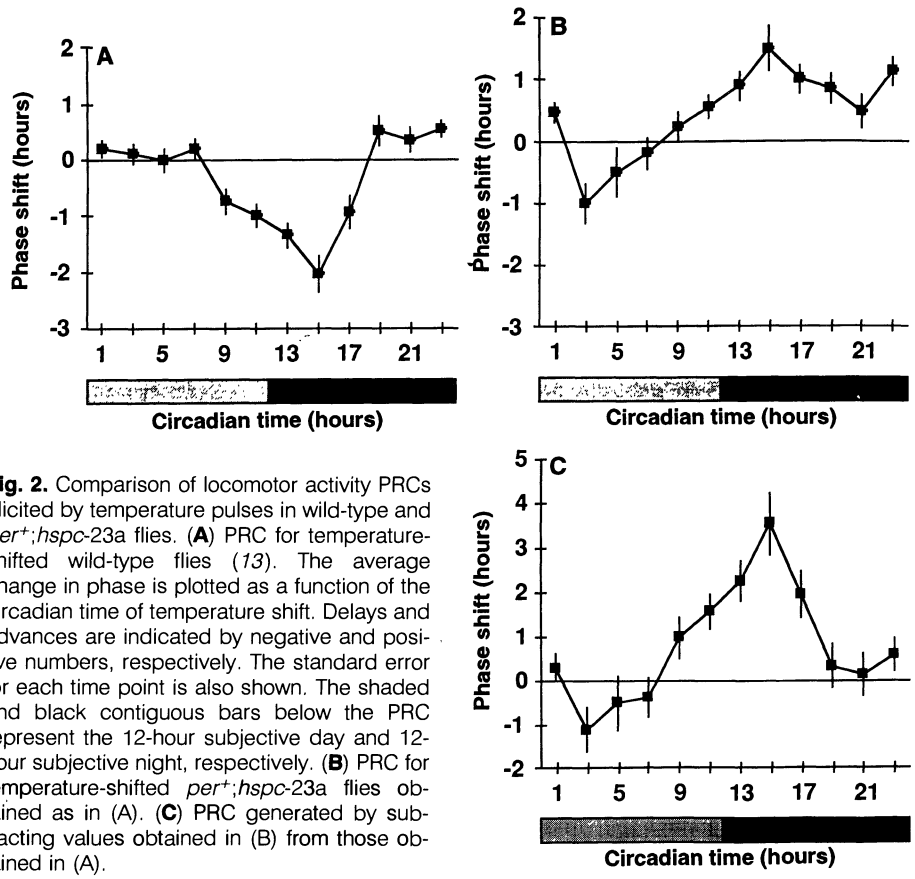

Fig. 2. Comparison of locomotor activity PRCs elicited by temperature pulses in wild-type and *per*$^+$;*hspc*-23a flies. (**A**) PRC for temperature-shifted wild-type flies (*13*). The average change in phase is plotted as a function of the circadian time of temperature shift. Delays and advances are indicated by negative and positive numbers, respectively. The standard error for each time point is also shown. The shaded and black contiguous bars below the PRC represent the 12-hour subjective day and 12-hour subjective night, respectively. (**B**) PRC for temperature-shifted *per*$^+$;*hspc*-23a flies obtained as in (A). (**C**) PRC generated by subtracting values obtained in (B) from those obtained in (A).

type PER amounts causes phase advances and delays, respectively. A reasonable scenario is that a premature increase in PER leads to a phase advance, whereas a retarded disappearance (due, for example, to induction of HSPPER at a time when the concentration of wild-type PER would be normally decreasing) leads to a phase delay. The use of reversible protein synthesis inhibitors in mollusks revealed a restricted time zone in the subjective night that results in delays (*21*), which is consistent with the view that the temporal synthesis of a critical protein or proteins is an integral property of circadian clocks.

Although its function in mediating normal phase-shifting responses remains speculative, experimental results over the past 20 years support a fundamental role for the *per* gene in the circadian time-keeping mechanism: *per* mutations effectively eliminate or dramatically alter normal circadian rhythms, and amounts of PER undergo daily oscillations. Our data strengthen this hypothesis. The accumulated evidence makes it likely that PER is a component of a *Drosophila* circadian clock.

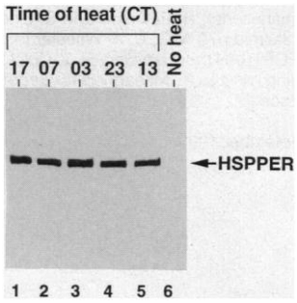

Fig. 3. Protein immunoblot of HSPPER in *per*$^+$;*hspc*-23a flies. Total head extracts were prepared from *per*$^+$;*hspc*-23a flies (*16*) that either received a 1-hour heat pulse at 37°C and then recovered for 4 hours at 25°C (lanes 1 to 5) or were not heated and were maintained at 25°C (lane 6). HSPPER (arrow) was visualized by protein immunoblot (*17*) in the presence of PER antibodies (*18*). Under these conditions, only large amounts of HSPPER were detected. The circadian time (CT) when the heat pulse was initiated is at the top.

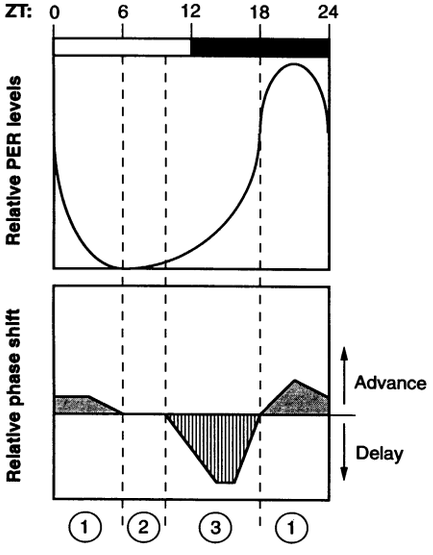

Fig. 4. Model for how the circadian fluctuations in PER levels (or activity) might underlie the classical *D. melanogaster* light PRC. Two curves are aligned with respect to a 24-hour cycle (top; the white and black contiguous bars represent the alternating periods of 12-hour day and 12-hour night, respectively). The top curve depicts the relative fluctuations in wild-type PER levels (*4, 20*). The bottom curve depicts the relative light PRC for *D. melanogaster* locomotor activity rhythms (*14*). Phase advances (shaded) and delays (vertical lines) are indicated to the right. ZT, Zeitgeber time. Delays occur when PER is accumulating. Advances occur when accumulation of PER starts to plateau (crossover point) and continues throughout its disappearance. The unresponsive zone occurs when PER amounts are at their lowest.

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7. To increase HSPPER expression relative to a previously described heat-inducible PER construct (*8*), we substituted the entire 5′ untranslated region (5′ UTR) of *per* with the entire hsp70 5′ UTR (*22*). We used the polymerase chain reaction (PCR) to amplify *D. melanogaster* hsp70 sequences (the plasmid p70 ATG Bam HI was used) and to create an Nco I site at the start of translation. The PCR-generated hsp70 fragment also includes promoter elements required for heat-inducible transcription and was digested with Xba I (position −252 base pairs relative to the hsp70 transcription initiation site) and Nco I. The digested product was subcloned upstream of *per* complementary DNA and genomic sequences derived from a previously constructed *per* DNA-containing transformation vector (pCDA) (*23*). We used *per* sequences from the Nco I site at the start of translation to an Eco RI site downstream of the transcription unit. Finally, the hybrid gene was subcloned into the *P* element transformation vector cp20.1 at the Xba I site, resulting in *hspcper* (Fig. 1). We used *per*^{01};*ry*^{506} flies as a host for *P* element–mediated transformation as described (*24*).
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10. Despite expression at least 10 times greater in adult heads than in our previous heat-inducible PER construct (I. Edery, unpublished data), flies from this strain were also completely arrhythmic after a single, 1-hour 37°C temperature pulse.
11. At 25°C, the average periods of locomotor activity in constant dark (*25*) for wild-type (n = 36) and *per*$^+$;*hspc*-23a (n = 44) flies were 23.8 ± 0.1 hours and 23.7 ± 0.1 hours, respectively.
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13. For each time point, at least five independent experiments were performed and data were pooled. Every time point consisted of 16 wild-type (Canton S) and 16 *per*$^+$;*hspc*-23a flies. Flies were first subjected to four cycles of 12 hours of light and 12 hours of dark followed by three continuous 24-hour dark periods. On the fourth dark period, flies were incubated for 1 hour at 37°C, and subsequent behavioral data were collected for each fly (*25*). Only data from those flies that fulfilled the following criteria were used for Fig. 2: (i) endogenous periods between 23.5 and 24.5 hours; (ii) no changes in endogenous periods greater than ±0.5 hour after the temperature shift; and (iii) strong rhythms [that is, a power setting greater than 10 (*25*)] before and after the temperature shift. These criteria ensured that PRCs (Fig. 2) were derived from flies with significant locomotor activity rhythms that also did not experience stable period changes after the temperature pulse. The circadian time is almost identical to Zeitgeber time, and we therefore use the term circadian time in this context. We determined the activity phase before and after the temperature pulse by identifying the activity offset for each activity peak, after the data had been filtered as described (*25*). The activity offset was defined as the time where the activity was 50% of the peak value, after that peak had occurred. The location where the next "average" phase offset would occur, had a temperature shift not been imposed, was then calculated by the method of least squares. The activity profile for the day when the temperature pulse was applied was not included in this average phase determination. The difference between the average phase offset before and after the heat pulse is the phase shift.
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15. For example, at CT15 81% of wild-type flies show a delay greater than 1 hour, whereas 76% of *per*$^+$;*hspc*-23a flies show an advance greater than 1 hour (*9*). Under the same conditions, two other independent lines bearing a heat-inducible copy of *per* also showed significant phase advances, in these cases at CT13 (*9*).
16. To visualize HSPPER, we subjected *per*$^+$;*hspc*-23a flies to conditions identical to those of flies used in our behavioral studies (Fig. 2). At the appropriate times (Fig. 3), flies were frozen and heads collected. Approximately 30 to 50 heads were sonicated in 200 μl of buffer I [100 mM KCl, 20 mM Hepes (pH 7.5), 5% glycerol, 10 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (20 μg/ml), leupeptin (2 μg/ml), and pepstatin A (1 μg/ml)]. Equal amounts of supernatant protein were mixed with SDS sample buffer, boiled, and analyzed by protein immunoblot (*17*).
17. All gels were 6% (29.6% acrylamide and 0.4% bis-acrylamide) polyacrylamide-SDS and cast in a Bio-Rad mini-PROTEAN II apparatus. After electrophoresis, the gels were electroblotted onto nitrocellulose for 23 min at 0.24 A with a semidry blotting apparatus according to the manufacturer's specifications (Bio-Rad). After the blot was washed in TBST [10 mM tris-HCl (pH 7.5), 140 mM NaCl, and 0.1% Tween-20], it was incubated for 1 hour in TBST containing 1% bovine serum albumin (BSA). An antibody to PER (*18*) was added at a dilution of 1:2000 in

1% BSA (TBST) for 3 to 5 hours. Extensive washing in TBST was followed by incubation with a 1:3000 dilution of an antibody to rat immunoglobulin G conjugated to horseradish peroxidase (Amersham) for 20 min in 1% BSA (TBST). After another extensive wash in TBST, the blots were visualized by chemiluminescence (Amersham).
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19. Approximately 24 to 30 hours after the heat pulse, HSPPER is essentially not detectable. However, the phase shifts are stable for at least 7 days after the heat pulse.
20. The biochemical profile of PER by protein immunoblotting is similar to that in the histochemical studies (*4*), except that in the blots PER is essentially not detectable between Zeitgeber time 06 and 10 (ZT06–10). Because the biochemical results are more reliable, we used these data to derive an approximate curve describing the fluctuations in PER levels (Fig. 4).
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1 September 1993; accepted 16 November 1993