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Period Protein Is Necessary for Circadian Control of Egg Hatching Behavior in the Silkmoth Antheraea pernyi

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Summary

We examined the molecular basis of the circadian control of egg hatching behavior in the silkmoth Antheraea pernyi. Egg hatching is rhythmically gated, persists under constant darkness, and can be entrained by light by midembryogenesis. The time of appearance of photic entrainment by the silkmoth embryo coincides with the appearance of Period (PER) and Timeless (TIM) proteins in eight cells in embryonic brain. Although daily rhythms in PER and/or TIM immunoreactivity in embryonic brain were not detected, a robust circadian oscillation of PER immunoreactivity is present in the nuclei of midgut epithelium. *per* antisense oligodeoxynucleotide treatment of pharate larvae on the day before hatching consistently abolishes the circadian gate of egg hatching behavior. *per* antisense treatment also causes a dramatic decrease in PER immunoreactivity in newly hatched larvae. The results provide direct evidence that PER is a necessary element of a circadian system in the silkmoth.

Introduction

Nowhere in the animal kingdom are circadian rhythms more evident than in insects. Circadian rhythms occur in many biological processes throughout the life histories of these animals. These rhythms are important, because they enable insects to perform critical behavioral and physiological events at the biologically advantageous time of the day or year (Saunders, 1982). The most studied circadian rhythms have been those of adult eclosion and locomotor activity in Drosophila.

In lepidoptera, the prolonged development of the embryo, compared with dipterans, provides a circadian behavior for study during early development. For example, in the pink bollworm moth Pectinophora gossypiella, the timing of egg hatching, a once-in-a-lifetime developmental event, was shown by Minis and Pittendrigh (1968) to be under precise circadian control. They showed that the population rhythm derives from an active circadian clock in the embryo. Moreover, the embryonic circadian clock is first entrainable by light midway through embryogenesis. It has also been shown in a preliminary report that egg hatching in the giant silkmoth Antheraea pernyi is under similar circadian control (Riddiford and Johnson, 1971).

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Genetic analysis of circadian clocks in Drosophila melanogaster has identified two clock genes, *period* (*per*) and *timeless* (*tim*), that are essential elements of a circadian timing mechanism that controls circadian rhythms in adult eclosion and locomotor activity (reviewed by Hall, 1995; Kay and Millar, 1995; Reppert and Sauman, 1995). The mRNA and protein products of both genes oscillate, and the cycling of each is dependent on PER–TIM association and nuclear entry. Thus, *per* and *tim* form interdependent autoregulatory transcriptional loops that are essential components of a Drosophila circadian clock. Recent studies show that TIM levels respond to light in a time-dependent manner, suggesting that photic manipulation of PER–TIM dimers may account for clock resetting effects of light in Drosophila (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996).

A discovery that now makes possible the study of the molecular basis of the egg hatching rhythm in lepidoptera is the cloning of a *per* cDNA from A. pernyi (Reppert et al., 1994). When the silkmoth *per* cDNA is expressed in arrhythmic per^0 flies, it rescues circadian behavior (Levine et al., 1995). This indicates that silkmoth *per* can function as a circadian clock element in Drosophila and that it most probably functions as a clock element in the silkmoth. Recent examination of PER-containing cells in A. pernyi brain shows that PER is restricted to eight neurosecretory cells (Sauman and Reppert, 1996b [this issue of *Neuron*]). These neurons appear to be circadian clock cells, because *per* protein and mRNA are colocalized and their levels oscillate in these cells. In addition, TIM-like immunoreactivity is coexpressed in each PER-positive neuron.

What distinguishes the PER-expressing clock cells in silkmoth brain from PER-expressing clock cells in Drosophila brain is that PER does not appear to enter the nucleus in the silkmoth (Sauman and Reppert, 1996b). A most exciting finding is the temporal expression of an antisense *per* transcript in PER-expressing cells that might be a necessary component of a circadian clock mechanism. Study of PER interactions in the silkmoth is thus providing important new insights into clock gene function (Sauman and Reppert, 1996b).

The present report examines the molecular basis of the circadian control of egg hatching behavior in A. pernyi. We test the hypothesis that PER is involved in this circadian event. The results provide compelling evidence that PER is indeed an essential molecular component of the circadian system regulating egg hatching behavior in the silkmoth.

Results

Defining a Diurnal Gate in Egg Hatching

To establish the diurnal character of egg hatching in populations of A. pernyi eggs, eggs were either placed into constant darkness (DD), or into one of two regimens of 12 hr of light followed by 12 hr of darkness (LD or DL) within 12 hr of being laid. The day on which the

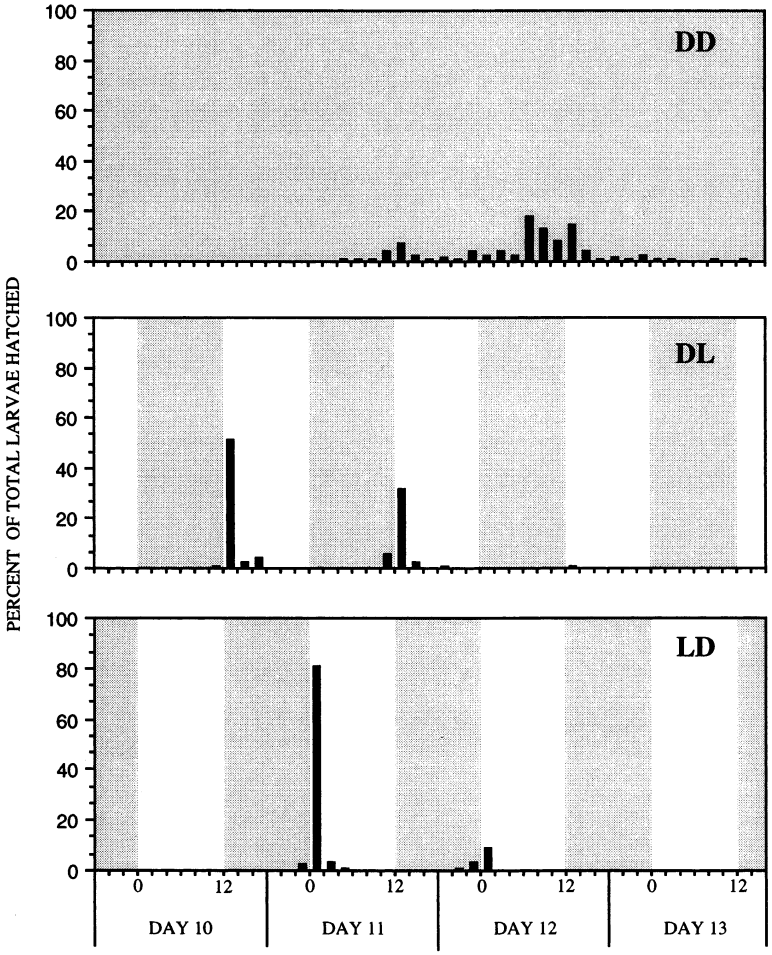

Figure 1. Defining a Diurnal Gate in A. pernyi Egg Hatching

Temporal profiles of hatching times studied under three lighting regimens (DD, DL, and LD). The vertical axes indicate the percentage of total hatching events for that lighting regimen recorded in a particular 2 hr interval. Number of eggs in each group: DD, 192; DL, 101; LD, 98. Over 90% of eggs in each group hatched. Stippled areas, dark periods. Open areas, light periods.

eggs were collected and placed under the appropriate lighting conditions was designated as day 1. The eggs were maintained under these three lighting regimens at approximately 23°C throughout their development. The normal duration of embryogenesis at this temperature in A. pernyi is 10–12 days. Thus, beginning at clock time (CkT) 10 on day 10 (CkT 0 = lights-on in LD and lights-off in DL), the eggs were examined every 2 hr to record the number of larvae hatched during the previous 2 hr period.

For both DL and LD, virtually all of the larvae emerged in one of two diurnal gates (Figure 1). In DL, the two gates exploited occurred at lights-on for days 10 and 11. Larvae in LD exploited the lights-on gate on days 11 and 12. The hatching in DL was more evenly distributed between the two most exploited diurnal gates than in LD.

When the hatching events were tabulated according to clock time by pooling bins across days on which hatching occurred, there is a synchronization of hatching in DL and LD centered at the 2 hr interval immediately following lights-on. In DL, 84% of all hatching events occurred during this interval, while 89% of LD animals hatched during this time. In both DL and LD, no hatching occurred outside of the time interval beginning 4 hr before and ending 6 hr after the light-to-dark transition.

In DD, larvae hatched in all 2 hr intervals throughout the day (Figure 1). No more than 22% of all hatching events occurred in any 2 hr interval. However, statistical analysis indicated that the hatching was not randomly distributed with respect to clock time ($X^2 = 199.2$, df = 11, $p < 0.01$).

The results clearly demonstrate that the time of egg emergence is tightly restricted (gated) to a specific portion of the light:dark cycle under LD or DL 12:12. Despite developmental synchronization by virtue of being laid on the same day, populations of larvae were capable of hatching at times dictated by the timing of the dark-to-light transition in both DL and LD lighting regimens (Figure 2).

A Circadian Clock Is Functioning by Midembryogenesis

Having demonstrated the diurnal character of egg hatching, we next assessed the endogenous nature of the egg hatching rhythm and estimated the time during embryogenesis when the lighting cycle could first synchronize the timing of hatching behavior. All eggs were placed into DL within 12 hr of being laid. During the dark phase on day 3 (between CkT 0 and CkT 12), group I eggs were transferred to DD; group II eggs were transferred to constant darkness during the dark phase on day 6; and group III eggs were transferred to constant darkness during the dark phase on day 9. The groups thus differed

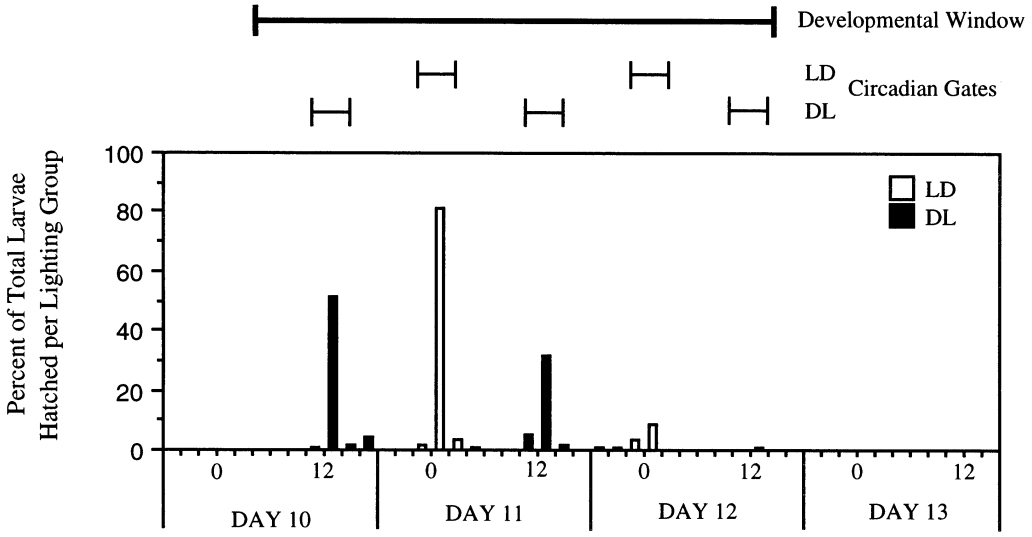

Figure 2. Interaction of Circadian and Developmental Gates for Egg Hatching Behavior in A. pernyi
The coincidence of circadian and developmental gates is shown. Data are from Figure 1.

by having experienced 3, 6, or 9 complete days of rhythmic lighting before transfer to DD.

No larvae under any of the three lighting conditions hatched before CkT 10 on day 10 or after CkT 12 on day 13 (Figure 3). Although the hatching gate was not as clearly defined as in the first experiment (compare Figures 1 and 3), both group II and group III eggs alternated between periods of high hatching activity and no hatching activity in DD with a period of approximately 24 hr (Figure 3), defining a circadian rhythm in hatching events. No obvious rhythm occurred for group I eggs; eggs moved to DD on day 3 exhibited hatching activity almost continuously from day 11 to day 13 (Figure 3) and thus were similar to animals reared in DD throughout embryogenesis (Figure 1, top).

The free-running period of egg emergence was very close to 24 hr. Group II eggs had been free-running for 3 days more than group III eggs, yet hatching activity peaks coincided between the two groups. Of all hatching events of both group II and group III, 73% took place within a 4 hr period centered on the time that lights-on would have occurred (in DL) based on the previous lighting cycles experienced by both groups (Figure 3). In group I, no more than 14% of the total larvae hatched within any 2 hr time period during the 24 hr day. Although a hatching rhythm was not obvious in group I, statistical analysis revealed a significant deviation from random hatching with respect to clock time ($X^2 = 23.9$, df = 11, $p < 0.05$). The difference in the timing of hatching between group I and group II (Figure 3) showed that the population rhythm was entrained by light by day 6 of embryogenesis.

PER Is Expressed in Developing Embryos

After demonstrating that the hatching of A. pernyi eggs was regulated by a circadian clock and entrained to the light:dark cycle, we examined whether PER provides a molecular correlate for this behavior.

PER was first detected in four pairs of cells in dorsolateral brain on day 6 of embryogenesis. The cells are in the corresponding location of brain as the eight PER-expressing cells in adult silkmoth brain (Figures 4A, 4C, and 4E). Staining in these cells was cytoplasmic with no detectable nuclear staining. PER staining did not vary, however, over the course of the day in either the cytoplasm or nucleus of brain cells (examined at 2 hr intervals throughout LD 12:12 on embryonic day 9; n = 2 experiments; data not shown). PER staining in axons was not detected during embryogenesis (Figures 4A, 4C, and 4E), but was first detected after hatching in first instar larvae. PER may be present in brain on day 5 of embryogenesis and earlier, but below the limit of sensitivity of our immunocytochemical method.

PER was also examined in the other tissues of the embryo. Specifically, PER staining was clearly detected in two other areas, the fat body and midgut epithelium. PER staining in fat body was first detected on embryonic day 6, persisted through the first larval instar, but was not examined at later times (Figure 5A). PER staining in fat body was limited to the cytoplasm and did not vary over the course of the day. In the midgut, PER staining was first detected in nuclei of epithelial cells lining this structure at embryonic day 7 and persisted throughout embryogenesis (Figure 5B). Surprisingly, from its first appearance, there was a diurnal oscillation of PER staining in the nuclei of midgut epithelium, with stained nuclei at CkT 0 and no staining at CkT 12 (Figures 5B and 5C). This oscillation persisted in DD identical to that shown in Figures 5B and 5C, showing that it is endogenously generated.

We also examined *per* mRNA levels in embryos by in situ hybridization and RNase protection assay. Using in situ hybridization with specific digoxigenin-labeled antisense and sense cRNA probes previously validated for localizing *per* hybridization signals in adult silkmoth brain (Sauman and Reppert, 1996b), we were unable to

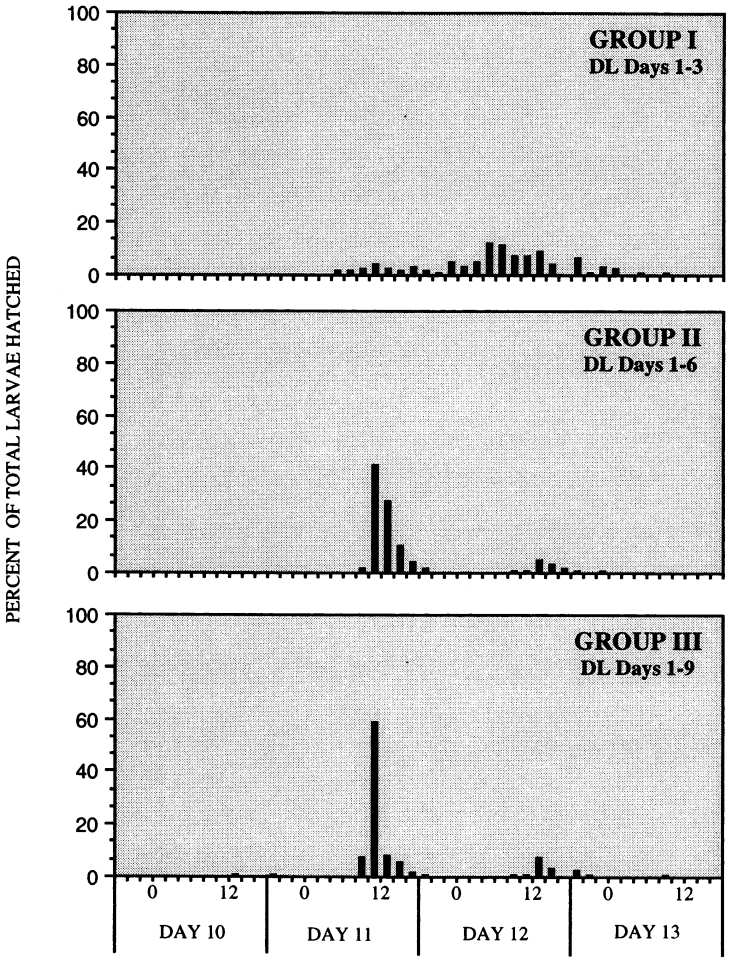

Figure 3. Egg Hatching in A. pernyi Is Circadian and Entrainable by Light by Day 6 of Embryogenesis

Temporal profiles of hatching times from eggs transfered from DL to DD on day 3 (group I), day 6 (group II), or day 9 (group III). The vertical axes indicate the percentage of total hatching events for that lighting regimen recorded in a particular 2 hr interval. Number of eggs in each group: group I, 129; group II, 132; group III, 132. Over 90% of eggs in each group hatched. Stippling indicates constant darkness.

detect a specific hybridization signal in silkmoth embryos. The lack of detection of hybridization signals was probably due to lack of sensitivity of this in situ procedure for *per* expression in embryonic tissue. RNA from embryos was also examined by RNase protection analysis using a *per* cRNA probe from nucleotides 1009 to 1215 of the silkmoth *per* cDNA. A *per* RNA fragment of appropriate size (207 nt) was protected by the antisense RNA probe, confirming that the *per* gene is in fact expressed on day 6 and day 9 of embryogenesis in the silkmoth embryo (Figure 6). However, no daily rhythm of mRNA levels was detected in embryo heads or body on the day before hatching or on the day after hatching (examined over 24 hr at 2–4 hr intervals in DD; n = 3 experiments; data not shown). Based on the intensity of PER staining within cephalic and body structures, *per* mRNA assessed by RNase protection assay is likely derived almost exclusively from fat body. The results of this study thus do not exclude rhythmicity of *per* RNA in specific embryonic tissues (e.g., brain and midgut).

PER and TIM Immunoreactivity Are Coexpressed in Brain during Embryogenesis

We previously showed that PER-expressing cells in adult A. pernyi brain coexpress TIM-like immunoreactivity (Sauman and Reppert, 1996b). Thus, we investigated whether the eight PER-expressing cells in the brains of A. pernyi embryos coexpress TIM using an antibody raised against Drosophila TIM (Myers et al., 1996). This is the same antibody that identified striking TIM-like immunoreactivity in PER-immunoreactive cells of adult silkmoth brain (Sauman and Reppert, 1996b).

The anti-TIM antibody stained four pairs of cells in embryonic brain (Figures 4B, 4D, and 4F). These cells were first detected on embryonic day 4. From the time of first appearance, TIM staining was intense in the cytoplasm without any detectable staining in the nucleus. In addition, axons from these cells were clearly stained for TIM beginning on embryonic day 4. TIM staining did not vary over the course of the day in either the cytoplasm or nucleus of these eight brain cells (examined at 2 hr intervals through LD 12:12 on embryonic day 9; n = 2 experiments).

The location of the TIM-immunoreactive cells suggested that they might be the same ones that express PER. Double-labeling experiments of embryonic day 9 embryos showed that PER and TIM immunoreactivities are colocalized in eight neurons (Figures 4G and 4H). TIM immunoreactivity was not detected at any time in fat body or midgut epithelium.

PER Is Necessary for the Circadian Gate of Larval Emergence

Because there is currently no genetic means of altering PER levels in lepidoptera, we used an antisense strategy to determine whether PER plays a role in the circadian

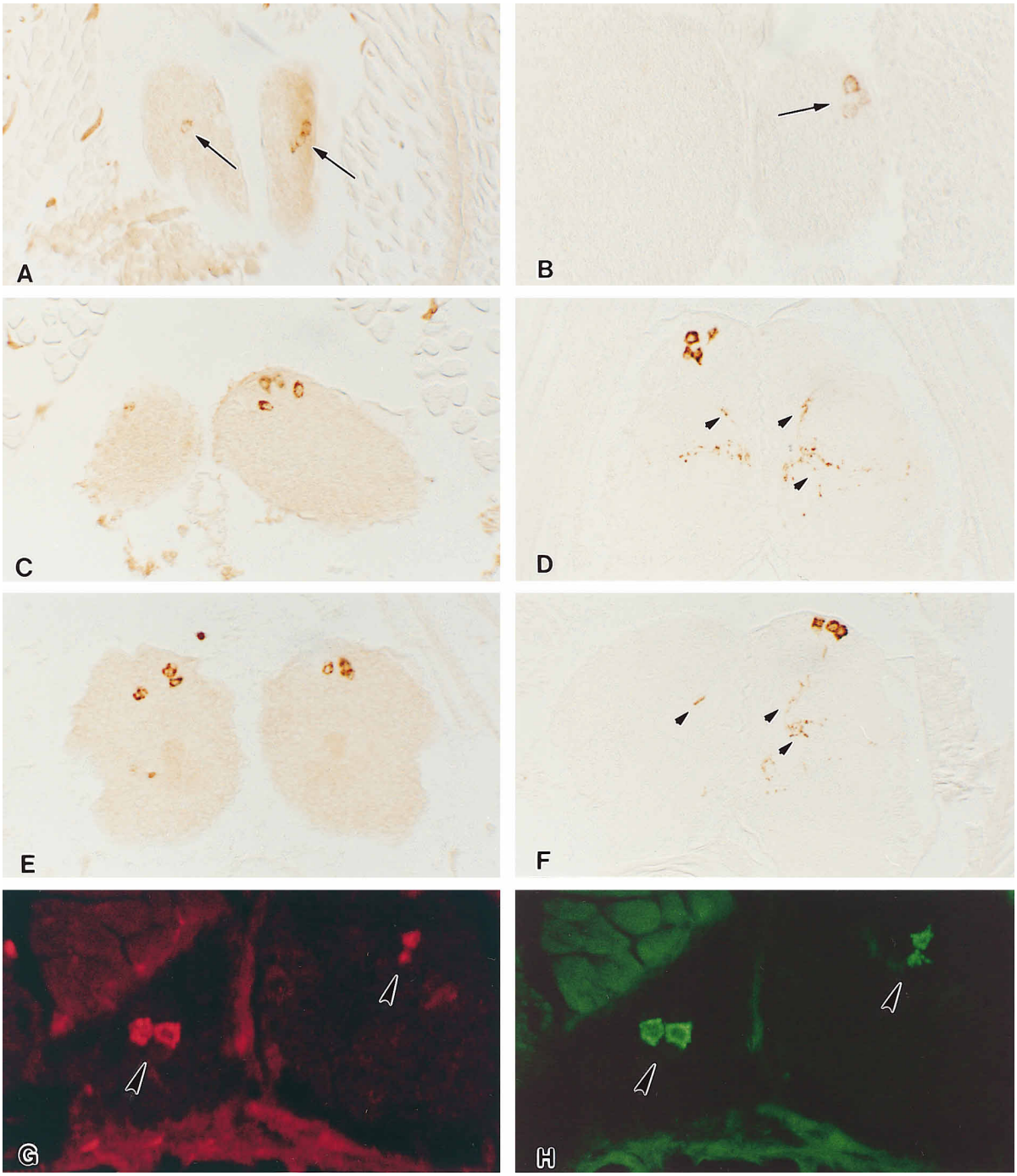

Figure 4. PER and TIM Are Colocalized and Expressed in A. peryni Brain during Embryonic Development

(A–F) PER immunoreactivity (A, C, and E) and TIM immunoreactivity (B, D, and F) in brains cells during embryogenesis. (A), embryonic day 6; (B), embryonic day 4; (C and D), pharate larvae; (E and F), day 1 larvae. Arrows, stained cells; arrowheads, stained axons.

(G and H) Double-labeling immunofluorescence of PER (G) and TIM (H) on embryonic day 9. Arrowheads, double-labeled cells.

Magnification: 170× (A–F); 300× (G and H).

system that gates egg hatching. A phosphorothioate antisense 25-base oligodeoxynucleotide (AS) that corresponded to the amino terminus of PER surrounding the initiation codon was injected into day 9 pharate larvae between CkT 0 and CkT 6 (Figure 7A). As controls, a reverse orientation phosphorothioate antisense 25-base oligodeoxynucleotide (RAS) or vehicle was injected into pharate larvae (Figure 7A). After injections, hatching was monitored every hour for 3 consecutive days. The mortality rates were 28% for the AS-injected group, 24% for the RAS-injected group, and 17% for the vehicle-injected group. All hatching events were confined to the 72 hr period following injections in AS-injected, RAS-injected, and vehicle-injected groups.

AS injections disrupted the circadian gate of egg hatching in the two experiments performed; the pattern

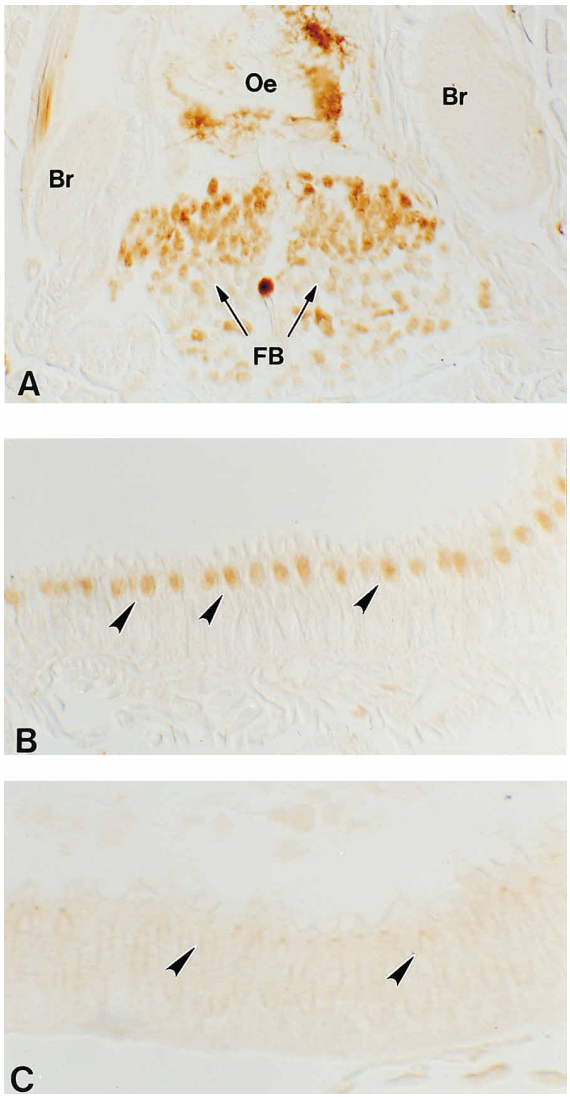

Figure 5. PER Is Expressed in Fat Body and Midgut Epithelium in A. pernyi Embryos

(A) PER staining in fat body (FB) on day 1 of first larval instar. Abbreviations: Br, brain; Oe, esophagus. Magnification, 175×.
(B and C) Oscillation of PER immunoreactivity in nuclei (arrowheads) of midgut epithelium (longitudinal sections) examined at CkT 0 (B) or CkT 12 (C) in day 1 larvae. Magnification, 300×.

of data from the two experiments was identical, and therefore results were combined. Hatching of AS-treated larvae was randomly distributed over the 24 hr day in LD ($X^2 = 13.8$, df = 23, $p > 0.7$; Figure 7B). RAS treatment, on the other hand, had little effect on the normal circadian gating of egg hatching behavior ($X^2 = 238.3$, df = 23, $p < 0.0001$; Figure 7B). The larvae from the RAS group exploited the three gates at lights-on over the 72 hr hatching interval. The gating of RAS-treated larvae was indistinguishable, when compared with vehicle-injected or uninjected animals (data not shown).

To determine whether the AS treatment altered PER expression, we examined newly hatched larvae using

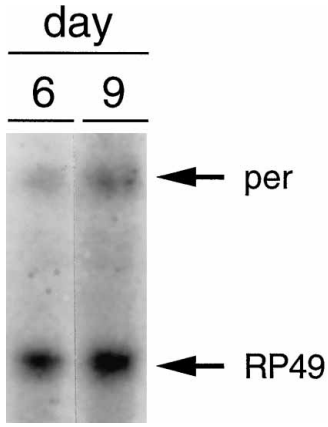

Figure 6. *per* mRNA Is Expressed in A. pernyi Embryos

RNase protection assay of *per* mRNA levels examined at embryonic days 6 and 9.

PER immunocytochemistry. In the four animals examined, PER levels were not detectable anywhere (brain, fat body, or midgut), whereas PER was readily detected in each of these areas in four RAS-injected larvae (Figure 8B). When the chromogen detection reaction was extended from 5 min to 24 hr, PER staining above background was barely detected in eight brain cells in the appropriate locations for PER-expressing cells (compare Figures 8A and 8B). Thus, the AS treatment reduced PER immunoreactivity in larvae to almost undetectable levels. In addition, TIM immunoreactivity in eight brain cells was decreased by about 90% in the AS-injected animals, compared with the RAS injections (n = 3 each group; Figures 8C and 8D). Finally, prothoracicotropic hormone (PTTH), which is heavily expressed in a pair of lateral neurosecretory cells in A. pernyi embryos and larvae, was avidly expressed in the brain in both AS- and RAS-injected larvae (Figures 8E and 8F). PTTH-expressing cells are adjacent to the lateral pair of PER–TIM expressing cells in silkmoth brain (Sauman and Reppert, 1996a).

Discussion

The results show that A. pernyi egg hatching is a circadian behavior. It is rhythmically gated, entrainable, and persists under constant conditions with a free-running period of about 24 hr. Like the adult eclosion rhythm, the egg hatching rhythm is determined by the combined effects of a developmental window and circadian gates (Figure 2). It is important to add that the events leading to egg hatching probably start several hours before the larvae actually emerge from the egg. Our unpublished observations of pharate larvae show that it takes them ~90 min to chew through the chorion and emerge from the egg.

By midembryogenesis, a circadian clock has formed in A. pernyi and is entrainable by light. The lack of entrainment by light during the first 3 days of development (Figure 3, group I) suggests that either the circadian pacemaker is not yet functional or that the embryos lack a functional photic entrainment pathway at this point in

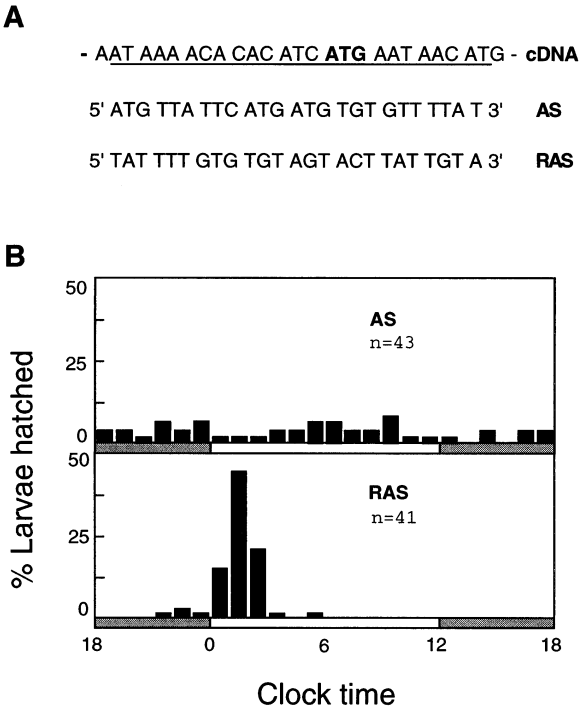

Figure 7. *per* Antisense Treatment of Pharate Larvae Disrupts the Circadian Gate of Egg Hatching Behavior

(A) Depicted are sequences of 5′ end of A. pernyi *per* cDNA (initiator codon in bold), phosphorothioate antisense 25-base oligodeoxynucleotide (AS), and reverse orientation phosphorothioate antisense 25-base oligodeoxynucleotide (RAS).

(B) Temporal profiles of hatching times from AS-treated (upper) or RAS-treated (lower) pharate larvae. Pharate larvae were injected between CkT 0 and 6 on embryonic day 9. For each panel, the pattern of data from two experiments were identical and therefore results shown were combined. Data are grouped according to clock time.

their development. In P. gossypiella, Minis and Pittendrigh (1968) used temperature as a nonphotic stimulus to address these two possibilities. They showed that photic entrainment was first possible at about the same time that entrainment to temperature cycles begins. This was taken as evidence that the circadian clock and photic entrainment pathway are functional at about the same time.

The time of appearance of photic entrainment by the silkmoth embryonic clock coincides with the appearance of PER and TIM immunoreactivities in brain. The colocalization of PER and TIM in eight cells in embryonic brain, which appear to be in the same regions as PER-expressing circadian clock cells in adult silkmoth brain, suggests that these brain cells might also function as circadian clock cells in the embryo. However, we were unable to detect a daily rhythm in the levels of PER or TIM immunoreactivity in the eight brain cells in the embryo. The possibility does remain that PER and TIM rhythms of small amplitude exist in embryonic brain, but were not detected by our immunocytochemical methods.

In striking contrast with the lack of a PER rhythm in embryonic brain, PER immunoreactivity in midgut epithelium exhibits a robust circadian oscillation. This oscillation in PER nuclear staining is reminiscent of the circadian oscillation in PER staining observed in photoreceptor nuclei of the adult silkmoth eye (Reppert et al., 1994; Sauman and Reppert, 1996b). Importantly, the midgut oscillation currently represents the only biochemical circadian oscillation detectable in the embryo. This PER oscillation was detected through the first larval instar and may thus provide a circadian oscillation for tracking the activity of a circadian clock during larval life.

Data from our AS experiments strongly suggest that PER is a necessary component of the circadian system that controls egg hatching behavior in A. pernyi. *per* AS treatment of pharate larvae on day 9 of embryogenesis consistently abolished the circadian gate of egg hatching behavior. The abolition of the circadian gate by AS treatment is not due to a generalized toxic effect of the treatment on the larvae, because the developmental window for expression of this activity was not altered. Furthermore, AS-treated larvae exhibit normal behavior during and after hatching. Significantly, *per* AS treatment and its effects on circadian behavior are correlated with a dramatic decrease in PER immunoreactivity throughout the larva. The AS-induced decrease in PER does not appear to be nonspecific, because the levels of PTTH in neurosecretory cells adjacent to PER-expressing cells, are unaffected by AS treatment.

An interesting aspect of the AS study is the decrease in TIM immunoreactivity in the presumed PER-expressing brain cells. In Drosophila, PER and TIM physically interact in the same cell, and each is important for the movement of the other into the nucleus (Hunter-Ensor et al., 1996; Myers et al., 1996). In A. pernyi adult and embryonic brain, PER and TIM do not appear to move into the nucleus, even though they are colocalized in the same cells (Sauman and Reppert, 1996b). The marked decrease in TIM levels in larval brain induced by *per* AS treatment suggests that PER and TIM are also interacting in A. pernyi brain. However, the exact nature of this interaction and its biological significance need to be clarified.

PER-positive cells in brain or midgut are likely sites of a circadian clock that controls egg hatching behavior. Even though egg hatching behavior involves activation of a coordinated neural program, this does not necessarily mean that the behavior is brain initiated. In fact, the circadian gate of gut purging behavior in fifth instar silkmoth larvae is clearly driven by a light-entrainable clock in prothoracic glands, not in brain (Mizoguchi and Ishizaki, 1982). Thus, it is possible that a circadian clock outside of brain (e.g., midgut) is important for circadian control of egg hatching behavior. Regardless of where the embryonic clock resides, the results presented here provide direct evidence that PER is necessary for expression of a circadian rhythm in the silkmoth and define another insect behavior whose circadian control requires PER.

Experimental Procedures

Animals

Diapausing pupae of A. pernyi were purchased from Worldwide Butterflies Limited (Sherborne, England) under United States Department of Agriculture permit number 929010. Cocoons were

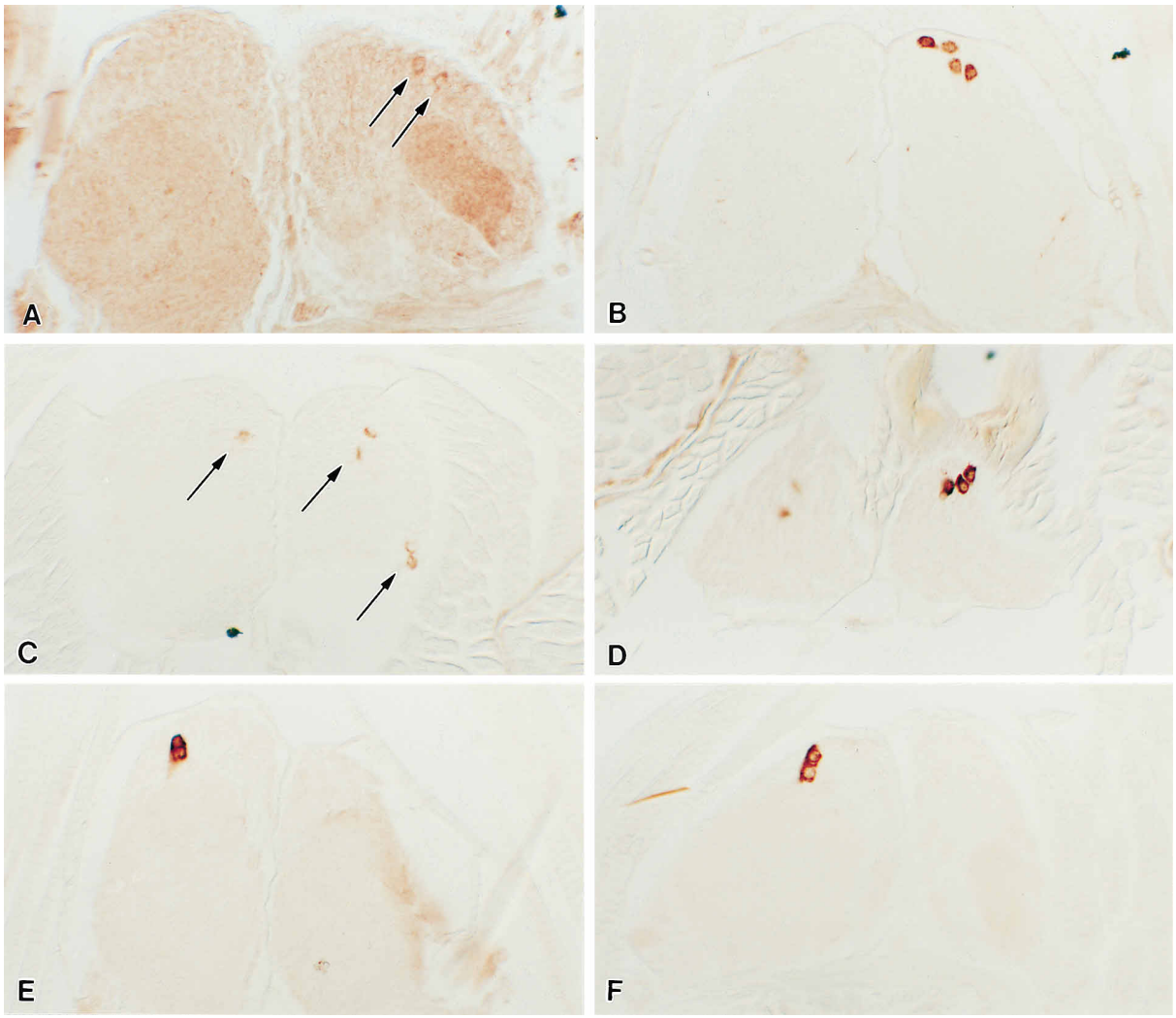

Figure 8. *per* Antisense Treatment Alters PER and TIM Expression

(A and B) PER staining in brain cells in AS-treated (A, arrowheads) or RAS-treated (B) animals. Identical results were obtained in four brains from each group. For the brain section in (A), the chromogen detection reaction was extended from 5 min (like in B) to 24 hr.

(C and D) TIM staining in brain cells in AS-treated (C) and RAS-treated (D) animals. Identical results were obtained in three brains from each group.

(E and F) PTTH staining in brain cells in AS-treated (E) and RAS-treated (F) larvae.

Magnification, 200×.

stored in darkness at 4°C until use. To terminate diapause and initiate adult development, pupae were removed from cocoons and placed in environmental compartments at 24°C with the daily lighting cycle consisting of LD 17:7 (Williams and Adkisson, 1964). Adult development was completed within 3–4 weeks, followed by adult eclosion.

Egg Collection

Male and female moths were allowed to mate at random after adult eclosion. The animals usually remained paired until late during the light period of the second day of adult life. At this time, the female was placed in a paper bag and returned to LD 17:7. Following the 7 hr of darkness, the bag was opened to collect the eggs that were laid on the inside overnight. The eggs were gently pried from the paper and placed one per well in a 96-well microtiter plate. Typically, one female laid 150–200 eggs on the first night. Eggs laid on subsequent nights were fewer in number (10–50). To have batches of eggs with developmental synchronization, only eggs laid on the first night were used.

Eggs were maintained at 23°C–25°C. The dark portion of the lighting cycles consisted of dim red light (wavelength >620 nm, 0.8 μW/cm^2) from special fluorescent tubes (Litho Light number 2, Chemical Products), which also remained on during the light phase. Egg hatching was examined by an observer at 1 or 2 hr intervals to record the number of larvae hatched during the previous interval. When eggs were examined in darkness, the same dim red light used for constant darkness aided visual inspection so that the eggs were not exposed to an additional light source during study in darkness.

RNase Protection Assay

Tissues were placed on dry ice and stored at −80°C until analysis. Total RNA was extracted from batches of tissues (10–15 at each timepoint) using an acid-phenol method (Chomczynski and Sacchi, 1987). [^{32}P]UTP-labeled *per* cRNA antisense and sense probes were generated by subcloning PCR-amplified *per* cDNA fragments into pBluescript followed by in vitro transcription driven from Sp6 and T7 RNA polymerase promoters, respectively. An antisense RP49 probe (153 bp) cloned from A. pernyi (Reppert et al., 1994) was

included in each RNase protection reaction as a control for the amount of RNA loaded in each lane. To equalize the signal intensity of the *per* and RP49 bands on the gel, the specific activity of the RP49 probe was decreased by diluting the [^{32}P]UTP 1:1000 with nonradioactive UTP in the in vitro transcription reaction.

RNase protection assays were performed using a kit from Ambion (RPAII) as previously described (Reppert et al., 1994). Quantitation was performed by directly counting radioactivity in the gel using a phosphorimager (Molecular Dynamics) and Image Quantitation (Molecular Dynamics) software (courtesy of S. Brown and R. Kingston, Massachusetts General Hospital). Each protection assay was performed twice with similar results.

Immunocytochemistry

Embryos and larvae were CO_2 anesthetized and immediately fixed in modified Bouin–Hollande solution (Levine et al., 1995) overnight at 4°C. Standard histological techniques were employed for tissue dehydration, embedding in paraplast, sectioning (4–7 μm), deparaffinization, and rehydration. To remove residual heavy metal ions from the fixed tissue, the sections were treated with Lugol's iodine followed by 5% sodium thiosulfate. After thorough washing with distilled water and PBS supplemented with 0.2% Tween 20 and 0.1% bovine serum albumin (PBS-TB), the sections were blocked with 10% normal goat serum in PBS-TB (30 min at room temperature) and incubated with the desired primary antibody (appropriately diluted in PBS-TB) in a humidified chamber overnight at 4°C. Following rinsing with PBS-TB (three times for 10 min at room temperature), samples were incubated with goat anti-rabbit IgG–horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immuno Research; 1:1,000 in PBS-TB, 1 hr at room temperature). The HRP enzymatic activity was stained with hydrogen peroxide (0.005%) and 3,3′-diaminobenzidine·4HCl (0.25 mM in 0.05 M Tris–HCl [pH 7.5]) as chromogen. Stained sections were dehydrated and mounted in AccuMount-60 mounting medium.

The antibodies used for immunocytochemistry included: rabbit anti–A. pernyi PER (57/10w at 1:500); rabbit anti–A. pernyi PER (58/10w at 1:500); rabbit anti-"alfa"PER (D. melanogaster Baculovirus recombinant protein from M. Young, Rockefeller University; dilution 1:11,000); rat anti-TIM #307 (D. melanogaster E. coli recombinant protein from M. Young [Myers et al., 1996], Rockefeller University; dilution 1:1,000); and rabbit anti-A. pernyi PTTH (274/IV-A [Sauman and Reppert, 1996a]; dilution 1:4,000).

For double-labeling experiments, the primary antibodies (rabbit anti-PER [Young] 1:1000 in PBS-TB and rat anti-TIM [Young] 1:11,000 in PBS-TB) as well as the corresponding secondary antibodies (goat anti-rabbit IgG–Cy3 conjugated and goat anti-rat IgG–Cy2 conjugated) were combined and applied to the brain sections under the same conditions as described above for single antibody labeling. The stained sections were mounted and viewed under a Nikon microscope equipped with epifluorescence and Nomarski optics.

In control experiments, the primary antibodies were replaced with normal goat serum. As an additional control for binding specificity, anti-PER antibodies (57/10w and 58/10w) were preincubated with 100 molar excess of antigen prior to immunological staining. In all cases, no significant staining was observed above background.

Oligodeoxynucleotide Injections

Between CkT 0 and CkT 6 on day 9 of embryogenesis, a ~100 μm diameter opening was made in the chorion with a 27 gauge needle. Approximately 200 nl of 100 μM oligodeoxynucleotide (diluted in sterile Ringer's solution) or Ringer's solution alone was injected through the opening in each egg using a positive displacement microinjection apparatus (Drummond) with attached double-drawn glass microcapillary tube (internal diameter = 10 μm). The injection was targeted inside the abdominal or thoracic cavity of pharate larvae. After injection, the opening in the chorion was sealed with a small drop of dental wax. Each experiment was performed twice, using a set of larvae from each of two different females. Phosphorothioate oliodeoxynucleotides (sequence in Figure 7) were purchased from Oligos Etc. Incorporated (Wilsonville, OR).

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