Isolation of *timeless* by PER Protein Interaction: Defective Interaction Between *timeless* Protein and Long-Period Mutant PER^L

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The *period* (*per*) gene likely encodes a component of the *Drosophila* circadian clock. Circadian oscillations in the abundance of *per* messenger RNA and *per* protein (PER) are thought to arise from negative feedback control of *per* gene transcription by PER. A recently identified second clock locus, *timeless* (*tim*), apparently regulates entry of PER into the nucleus. Reported here are the cloning of complementary DNAs derived from the *tim* gene in a two-hybrid screen for PER-interacting proteins and the demonstration of a physical interaction between the *tim* protein (TIM) and PER in vitro. A restricted segment of TIM binds directly to a part of the PER dimerization domain PAS. PER^L, a mutation that causes a temperature-sensitive lengthening of circadian period and a temperature-sensitive delay in PER nuclear entry, exhibits a temperature-sensitive defect in binding to TIM. These results suggest that the interaction between TIM and PER determines the timing of PER nuclear entry and therefore the duration of part of the circadian cycle.

A circadian clock is a self-sustaining, endogenous oscillator that drives daily rhythms in physiology and behavior (*1*). Circadian clocks are remarkably widespread, having been documented in cyanobacteria, fungi, plants, invertebrates, and vertebrates (*2*). All such clocks share certain fundamental properties. Most important are an intrinsic period close to 24 hours, resetting of the clock in response to light-dark transitions, and temperature compensation of the period (*2*). Circadian clocks presumably evolved because an internal timing system of this sort makes possible the anticipation of daily environmental fluctuations, a capability that likely conferred a profound selective advantage (*3*).

Disturbances of circadian clock function can be inherited as monogenic traits (*4*), and screens for mutants exhibiting aberrant circadian periods have been successfully carried out in a variety of organisms (*5*, *6*). Previously, the only circadian clock genes to have been cloned were the *per* gene from *Drosophila* (*7*) and the *frequency* (*frq*) gene from the fungus *Neurospora crassa* (*8*). Despite recent advances in understanding the roles played by the *per* (*9*) and *frq* (*10*) genes in the fly and fungal circadian clocks, respectively, the molecular mechanism of circadian oscillations remains obscure.

The *per* gene almost certainly encodes a component of the *Drosophila* circadian clock. Both the *per* transcript (*11*) and the *per* protein (PER) (*12*) exhibit a circadian oscillation in abundance, and together genetic and biochemical studies (*9*, *11*) indicate that PER participates in negative feedback control of *per* gene expression. Despite this inferred role, PER has no known DNA binding activity or recognizable DNA binding motif. A suggestion that PER might act in association with a heterodimeric partner has come from the observation that PER contains a dimerization interface, termed PAS, that is conserved in several fly and mammalian transcription factors (*13*).

Regulated timing of PER nuclear translocation might be a critical determinant of clock period (*14*, *15*). At the time corresponding to the peak of PER abundance, PER immunoreactivity in lateral neurons, the probable circadian pacemaker cells (*16*), shifts from exclusively cytoplasmic to predominantly nuclear (*14*). It then remains nuclear for approximately 11 hours, after which time it disappears and the cycle recurs. In long-period mutant *per^L* flies, this shift of PER immunoreactivity from cytoplasm to nucleus is delayed compared with that in wild-type flies, and this delay is exacerbated by elevated temperature, as is the long-period behavioral phenotype (*14*).

The nuclear translocation of PER is likely regulated by a recently identified second *Drosophila* circadian clock locus, *timeless* (*tim*) (*6*). In *tim*$^-$ mutant flies [now referred to as *tim^{01}*, a presumptive *tim* null allele, see accompanying report (*17*)], loss of behavioral circadian rhythms is accompanied by a loss of circadian oscillations of *per* mRNA (*6*) and a failure of PER to undergo nuclear translocation (*15*). Examination of the subcellular localization of different reporter PER fusion proteins in *tim^{01}* flies suggested that a signal for cytoplasmic retention is contained within the PER PAS region and that a functional *tim* gene is required to overcome this cytoplasmic retention (*15*). These observations imply that the *tim* gene regulates the timing of the PER negative feedback loop.

PER oscillations almost certainly constitute a core element of the *Drosophila* circadian clock. These oscillations likely depend on the interaction of PER with other proteins, such as those predicted to regulate its subcellular localization or to constitute partners, effectors, or terminators of its transcriptional autoregulatory action. As a means of identifying previously unknown components of the circadian clock, we performed a yeast two-hybrid genetic screen (*18*) using a PER bait hybrid to isolate *Drosophila* head complementary DNA (cDNA) library clones encoding PER-interacting proteins (*19*). The library was introduced into the yeast reporter strain expressing the LexA-PER bait, and 2×10^7 resulting library transformants were screened for activation of the *HIS3* and *lacZ* (β-galactosidase) reporter genes (*20*). By 72 hours, 360 His$^+$ colonies had formed, and these were retested for histidine prototrophy and assayed for β-galactosidase activity by a filter-lift method (*20*). Twenty representative His$^+$ colonies from the screen are shown in Fig. 1A, all of which were confirmed as His$^+$ on retesting (−His plate). Of these His$^+$ colonies, three exhibited β-galactosidase activity (β-Gal$^+$), two having somewhat greater activity than the PER homodimerization positive control and one having less activity (Fig. 1A; compare β-gal–20 min and β-gal–120 min panels). Altogether, a total of 67 His$^+$ colonies had β-galactosidase activity, with 23 having greater activity than the positive control, 11 having roughly the same amount of activity, and 33 having less activity.

Library plasmids were purified from 59 of the His$^+$ β-Gal$^+$ colonies (*21*), and 48 library plasmids produced PER-dependent His$^+$ β-Gal$^+$ interaction signals (see Fig. 1C for examples). The other 11 did not produce interaction signals, most likely because, in these cases, an irrelevant, contaminating library plasmid was purified from the His$^+$ β-Gal$^+$ colony.

Because of the possibility that the functional interaction between the *tim* and *per* genes is mediated by direct interaction of a predicted *tim* protein (TIM) with PER, Southern (DNA) blots of the clones isolated in our two-hybrid screen were probed with a 4.5-kb cDNA derived from the *tim* locus (*17*). Altogether, inserts from 16 of

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the 48 plasmids producing PER-dependent interaction signals strongly hybridized to the probe at high stringency (Fig. 1B). The 16 hybridizing inserts ranged from 1.5 to 2.5 kb, and DNA sequences of the ends indicated that the set consisted of six different clones, with two clones represented four times, two represented three times, and two represented once.

To test reproducibility and specificity, we introduced each of the six library plasmids that hybridized to the *tim* cDNA back into yeast expressing the original LexA-PER bait or a negative control LexA-Lamin bait (*22*). The LexA-Lamin strain exhibited no detectable histidine prototrophy or β-galactosidase activity after transformation with each of the six library plasmids (Fig. 1C). In contrast, the LexA-PER strain showed robust histidine prototrophy after transformation with each of the six library plasmids, and it exhibited strong β-galactosidase activity relative to the PER homodimerization positive control when transformed with clones 2 or 4 and weaker β-galactosidase activity when transformed with clones 1, 3, 5, or 6 (Fig. 1C; compare β-gal–20 min and β-gal–120 min panels) (*23*). In addition, when expressed as LexA bait hybrids, clones 1 and 2 showed PER-dependent interaction signals with VP16-PER fragments (see below), as did clone 4 (*24*) .When yeast strains expressing LexA–clone 2 or LexA–clone 4 baits were transformed with a population of cDNA library plasmids encoding VP16 fusion proteins, none of the resulting ~1000 colonies showed detectable β-galactosidase activity after a 2-hour incubation in substrate, whereas colonies resulting from a parallel transformation with the VP16-PER plasmid exhibited marked β-galactosidase activity after 5 to 10 min (*25*). These experiments indicate that the interaction signals produced by these clones in the two-hybrid system are highly specific for PER.

Sequences obtained from *tim* cDNA and genomic clones revealed an open reading frame encoding a protein of 1389 amino acids (*17*). Sequences from both ends of each of the six hybridizing two-hybrid clones, constituting a total of 3 kb, were compared with the *tim* sequence, and all were found to match internal segments at intervals in accordance with the size of each insert (Fig. 2). In addition, the double-stranded 1.5-kb sequence of clone 6 matched a 1.5-kb stretch of the internal open reading frame. As determined by the VP16-insert fusion junctions, the reading frames of all six clones as translated in yeast agreed with the predicted open reading frame of the *tim* cDNA. All six clones overlap in the central portion of the open reading frame, indicating that a region sufficient for PER-dependent interaction signals lies within amino acids 505 to 906. This region does not include a PAS dimerization domain or any other sequence motif from which a function might be inferred, and it shows no significant similarity to any protein in the database (*17*, *24*). These experiments demonstrate that six independent, overlapping clones isolated a total of 16 times in a two-hybrid screen for PER-interacting proteins are identical to a *tim* cDNA isolated by positional cloning (*17*). We conclude that these cDNAs are derived from the *tim* gene and propose that the functional interaction between the *per* and *tim* genes is mediated by

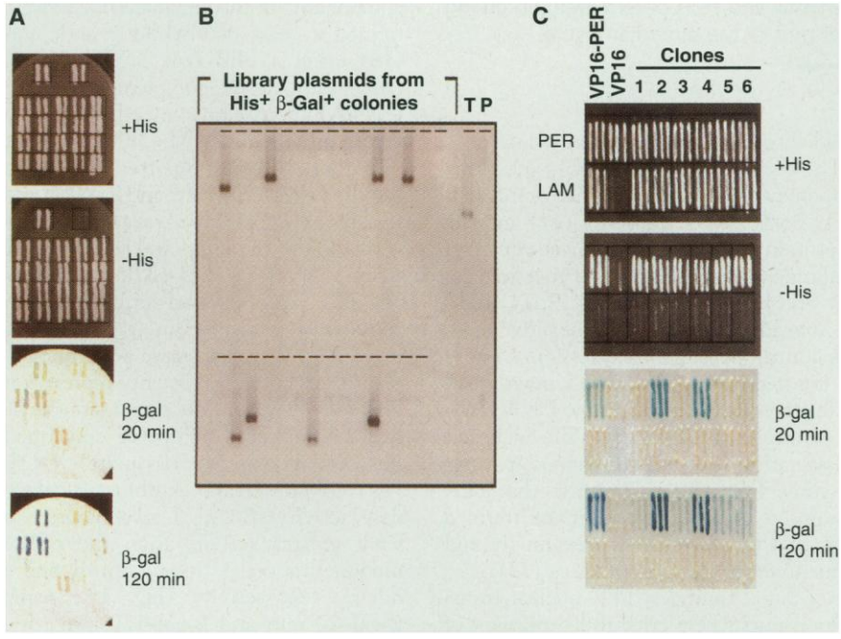

Fig. 1. Two-hybrid screen for PER-interacting proteins. (**A**) Example of screening for *HIS3* and *lacZ* reporter gene activation. Each panel shows duplicate yeast patches (4 by 5 grid) from 20 of the colonies that formed in the absence of histidine on the initial screening plates. Positive control PER homodimerization transformants are on the upper left (LexA-PER + VP16-PER), and negative control transformants are on the upper right (LexA-PER + VP16) in each panel. Top two panels: +His, control plate containing histidine (not selective for *HIS3* reporter gene activation) showing viability of the patches; and −His, plate lacking histidine (retest for the ability of transformants to grow in the absence of histidine). The bottom two panels show results of the β-galactosidase assay performed on a filter lifted directly from the control (+His) plate; blue color indicates cumulative β-galactosidase activity after 20 or 120 min, as indicated. The pink tinge of some patches in the bottom panels is due to the *ade2* mutation in the L40 strain. (**B**) Representatitive Southern (DNA) blot of library plasmids isolated from His$^+$ β-Gal$^+$ yeast colonies probed with a 4.5-kb *tim* cDNA (*17*). Short horizontal lines mark the positions corresponding to sample wells. T, positive control 1.5-kb insert from an independent, overlapping *tim* cDNA clone; P, negative control 1.4-kb insert from *per* cDNA. Approximately 100 to 200 ng of each plasmid was digested to completion with Not I to release the insert, and agarose gel electrophoresis and Southern blotting were performed by standard methods. The blot was washed at high stringency (0.1× standard saline citrate, 0.1% SDS, 65°C). (**C**) PER-dependent interaction signals from the six independent two-hybrid clones hybridizing to *tim* cDNA. Purified library plasmids were reintroduced into yeast to test for *HIS3* and *lacZ* reporter gene activation in a strain expressing the original LexA-PER bait (retest) and a strain expressing an irrelevant bait (specificity test). Each panel shows triplicate yeast patches of the LexA-PER strain (upper row) or LexA-Lamin strain (lower row) transformed with the positive control plasmid VP16-PER (PER), the negative control plasmid VP16, or one of the six independent positive two-hybrid library plasmids (clones 1 to 6) that hybridized to *tim* cDNA. Each triplicate represents three independent transformants. Panels arranged as in (A).

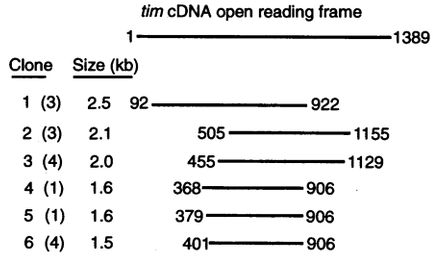

Fig. 2. Sequence alignments of clones encoding PER-interacting protein with *tim* cDNA. The bar at the top represents the 1389-codon open reading frame of full-length *tim* cDNA (*17*). Bars below show alignments of each of the six clones (numbered 1 to 6 as in Fig. 1C) to the open reading frame; each bar begins and ends, respectively, with the number of the first and last complete codon within the sequence. Although clones 4, 5, and 6 all end with codon 906, they do not all end at the same nucleotide. In parentheses next to the designation of each clone is the number of times that clone was isolated in the two-hybrid screen.

a heterotypic interaction between their respective protein products.

Experiments were done in which the arrangement of the elements in the two-hybrid system was reversed (Fig. 3A). We tested several VP16-PER hybrids for interactions with two LexA-TIM hybrids, TIM1 (designated clone 1 in Figs. 1C and 2; modest β-galactosidase activity) and TIM2 (designated clone 2 in Figs. 1C and 2; strong β-galactosidase activity). The LexA-TIM1 strain exhibited histidine prototrophy and modest β-galactosidase activity after transformation with VP16-PER(233–685) (corresponding to the original bait) and after transformation with VP16-PER(233–390), a PER fragment corresponding to the NH^2-terminal 158 amino acids of the 258–amino acid PAS domain and representing roughly one-eighth of the PER sequence. No histidine prototrophy or β-galactosidase activity was detectable after transformation of the LexA-TIM1 strain with VP16-PER(238–490), a fragment corresponding to a minimal PAS domain deleted of its five NH^2-terminal residues. The LexA-TIM2 strain exhibited histidine prototrophy and strong β-galactosidase activity after transformation with either VP16-PER(233–685) or VP16-PER(233–390) and weaker histidine prototrophy and β-galactosidase activity after transformation with VP16-PER(238–490). Like the experiment shown in Fig. 1B, the LexA-Lamin strain produced no sign of histidine prototrophy or β-galactosidase activity after transformation with any of these VP16-PER plasmids (*25*). These experiments indicate that a region sufficient to generate TIM-dependent interaction signals lies within PER(233–390). Because PER(233–685) and PER(233–390) produce indistinguishable TIM-dependent interaction signals, we infer from the weak signal produced by PER(238–490) that amino acids 233 to 237 contribute to but are not required for the interaction.

We also tested for direct association of TIM and PER polypeptide fragments in vitro (*26*) (Fig. 3, B to D). Glutathione-S-transferase (GST)–PER fusion proteins or GST alone were expressed in bacteria (Fig. 3B), purified on glutathione-agarose beads, and incubated with an in vitro–translated, ^{35}S-labeled TIM fragment [TIM(1–1003)]. SDS-PAGE analysis showed that TIM(1–1003) binds to GST-PER(1–640) and GST-PER(1–365) but not detectably to GST-PER(530–640) or GST alone (Fig. 3C). In addition, TIM(1–914) binds to GST-PER(1–640), whereas TIM(1–446) shows no detectable binding to GST-PER(1–640) (Fig. 3D). These experiments demonstrate that TIM and PER polypeptides directly associate in vitro. The differential in vitro binding of different PER and TIM fragments is consistent with the two-hybrid interaction results. Those fragments that bind in vitro include all or nearly all of the segments inferred to be sufficient for interaction in the two-hybrid assays (Figs. 1C, 2, and 3A), whereas those that fail to bind in vitro map entirely outside the segments sufficient for interaction in the two-hybrid assays. These results support the hypothesis that TIM regulates PER nuclear translocation through a direct protein-protein interaction.

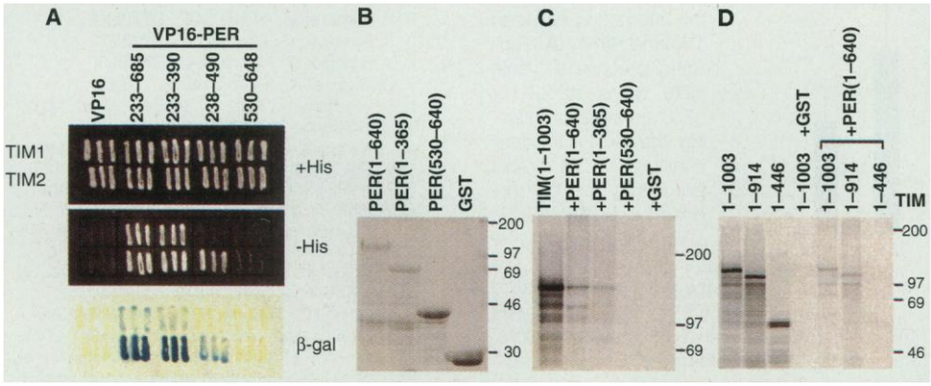

Fig. 3. (**A**) Restricted region of PER sufficient for TIM-PER interaction signals revealed by two-hybrid assays. Each panel shows triplicate patches of yeast expressing LexA-TIM1 (upper row) or LexA-TIM2 (lower row) transformed with the negative control plasmid VP16, the positive control plasmid VP16-PER(233–685), or the indicated VP16-PER plasmid. Each triplicate represents three independent transformants. TIM1 and TIM2 bait hybrids correspond to the clones designated 1 and 2, respectively, in Figs. 1C and 2. Top panel (+His), control plate containing histidine (not selective for *HIS3* reporter gene activation) showing viability of the patches; middle panel (−His), plate lacking histidine (tests for the ability to grow in the absence of histidine); bottom panel (β-gal), β-galactosidase assay performed on a filter lifted directly from the control plate in the top panel. Blue color in the β-gal panel indicates cumulative β-galactosidase activity after 120 min. The association of TIM and PER polypeptides in vitro is demonstrated in (B) to (D). (**B**) Coomassie-stained SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel of GST and GST-PER fusion proteins expressed in bacteria. The relative intensities of the major bands reflect the amount of GST fusion protein used in the in vitro TIM-PER binding assays; considerably more GST and GST-PER(530–640) were present than the other two GST-PER fusion proteins. (**C**) Autoradiograph of SDS-PAGE analysis showing differential binding of in vitro–translated, ^{35}S-labeled TIM(1–1003) to the indicated GST-PER fusion proteins. The far left lane shows the in vitro translation product before the binding reaction. (**D**) Autoradiograph of SDS-PAGE analysis of indicated in vitro–translated, ^{35}S-labeled TIM fragments bound to GST-PER(1–640). The first three lanes show the indicated in vitro translation products before the binding reaction. Molecular size markers are in kilodaltons.

Because PER^L exhibits a temperature-sensitive delay in nuclear entry, a process apparently regulated by the *tim* gene, we examined whether PER^L (V243D) (*27*) exhibits a temperature-sensitive phenotype in its interaction with TIM in two-hybrid assays. After transformation with LexA-TIM and VP16-PER or VP16-PER^L plasmids, patches of yeast were grown at different temperatures on X-Gal medium (*28*) and then assayed for β-galactosidase activity (Fig. 4A). At 22°C, the TIM interaction signals produced by VP16-PER^L(233–685) and VP16-PER^L(233–390) were stronger than (TIM1) or equal to (TIM2 and TIM4) those produced by the corresponding wild-type PER fragments. In contrast, at 37°C the TIM interaction signals produced by VP16-PER^L(233–685) and VP16-PER^L(233–390) were markedly weaker than those produced by the corresponding wild-type PER fragments in all three of the LexA-TIM strains. Liquid cultures of yeast expressing LexA-TIM1 and VP16-PER(233–685) or VP16-PER^L(233–685) were grown at different temperatures and assayed for β-galactosidase activity (Fig. 4B) (*29*). At 22°C the TIM interaction signal generated by the PER^L fragment was 3.4 times that of the wild-type PER fragment (probability $P < 0.05$, student's t test), at 28°C it was only marginally higher than that of wild-type PER (1.8 times as high, not significant), and at 35°C the signal was about one-fifth that generated by the wild-type PER ($P < 0.001$). Immunoblots with antibodies directed against PER or against VP16 showed no detectable difference in the abundance of VP16-PER and VP16-PER^L in extracts of the cultures grown at 35°C (*24*), consistent with the normal abundance of PER^L in vivo in *Drosophila* at elevated temperature (*30*). These results indicate that the interaction between TIM and PER^L is characterized by a grossly abnormal temperature-dependence. The enhanced interaction with PER^L at 22°C, evident only with the TIM1 fragment, could be peculiar to that fragment, but the much weakened interaction at 35° or 37°C is evident with all of the TIM constructs. These results suggest that a temperature-sensitive decrease in the affinity of PER^L for TIM could account for the temperature-sensitive delay of PER nuclear entry and the

temperature-sensitive lengthening of circadian period in per^L flies. These findings exclude a recent proposal (*30*), at least with regard to the PER-TIM interaction, that the PER^L mutation disrupts the association of PER with other proteins by means of a temperature-dependent, enhanced intramolecular association between PAS and the C-domain [PER(524–685)] because PER^L(233–390), entirely lacking the C-domain, shows the same temperature-sensitive abnormality of interaction with TIM as does PER^L(233–685) in all three LexA-TIM strains (Fig. 4A).

Oscillations of the *per* transcript and PER protein likely depend on negative feedback regulation of *per* gene transcription by PER (*9*, *11*, *12*). For any such self-sustaining oscillator, theory predicts that some mechanism must operate during each cycle to delay the onset of this negative feedback, or else the oscillations would damp out (*31*). The finding that PER accumulates exclusively in the cytoplasm and shifts to the nucleus only at a later time (*14*) suggests that cytoplasmic retention of PER contributes to this delay, because nuclear entry of PER is presumably required for its role in transcriptional autorepression. Given that nuclear translocation of PER requires a functional *tim* gene (*15*), we propose that the duration of this delay, and therefore the duration of the part of the circadian cycle in which *per* transcription is high, is determined by the protein-protein interaction between TIM and PER. This hypothesis is supported by our finding of a temperature-sensitive defect in the interaction between TIM and PER^L, the protein product of a *per* allele characterized by temperature-sensitive long-period behavioral rhythms (*32*), and an associated temperature-sensitive delay in PER^L translocation to the nucleus (*14*). It is also supported by the recent identification of two long-period alleles of *tim* (*33*).

It is not known in what way the interaction between PER and TIM, likely required for PER nuclear translocation, is dependent on the phase of the circadian clock, as it must be if this interaction itself contributes to the timing mechanism. The finding that the *tim* transcript exhibits a circadian oscillation with the same phase as that of the *per* transcript [see accompanying report (*34*)] suggests that TIM also undergoes a circadian oscillation in abundance. If so, one possibility is that the clock-dependent accumulation of both PER and TIM sets a fairly sharp point in the cycle when the interaction becomes favored, thereby setting the point at which PER nuclear translocation occurs. Another possibility is that clock-dependent posttranslational modification of PER (*35*) or TIM could make one or both available for interaction or make the PER-TIM complex competent for PER nuclear translocation, but our demonstration of binding between PER synthesized in bacteria and TIM translated in vitro makes it unlikely that any posttranslational modification is required for the interaction itself.

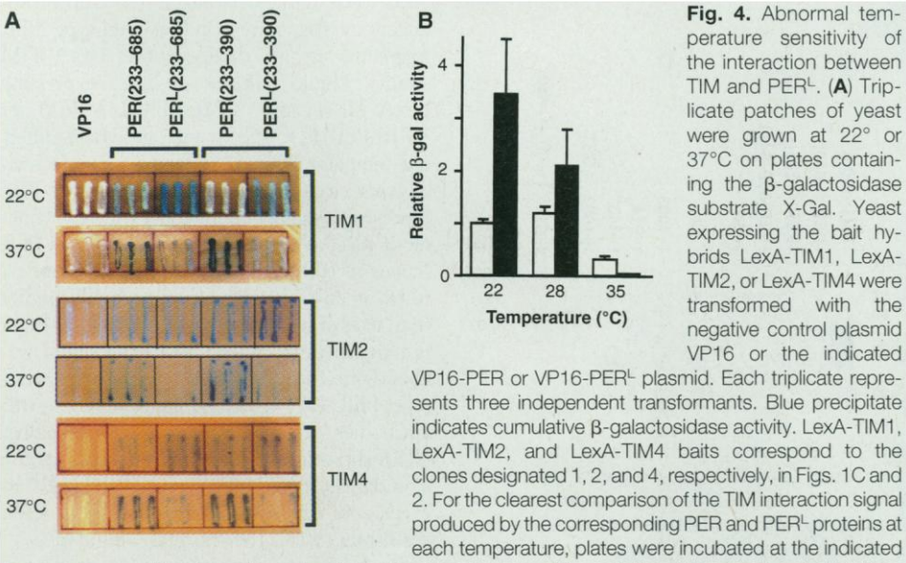

Fig. 4. Abnormal temperature sensitivity of the interaction between TIM and PER^L. (**A**) Triplicate patches of yeast were grown at 22° or 37°C on plates containing the β-galactosidase substrate X-Gal. Yeast expressing the bait hybrids LexA-TIM1, LexA-TIM2, or LexA-TIM4 were transformed with the negative control plasmid VP16 or the indicated VP16-PER or VP16-PER^L plasmid. Each triplicate represents three independent transformants. Blue precipitate indicates cumulative β-galactosidase activity. LexA-TIM1, LexA-TIM2, and LexA-TIM4 baits correspond to the clones designated 1, 2, and 4, respectively, in Figs. 1C and 2. For the clearest comparison of the TIM interaction signal produced by the corresponding PER and PER^L proteins at each temperature, plates were incubated at the indicated temperatures until a clear signal developed in at least some of the patches: in general, a shorter time for 37°C plates than for 22°C plates (because of the greater activity of β-galactosidase at the higher temperature) and a shorter time for TIM2 and TIM4 plates than for TIM1 plates (because of the stronger PER interaction signals produced by TIM2 and TIM4) (*28*). In this experiment, direct comparisons of signal strength are valid only for patches on a given plate, not across plates. (**B**) β-Galactosidase activity from liquid cultures of yeast expressing LexA-TIM1 and VP16-PER(233–685) (open bars) or VP16-PER^L(233–685) (solid bars) and grown at the indicated temperatures. Shown are the mean ± SE determined for at least 11 cultures derived from at least six independent transformants. For LexA-TIM1 and VP16-PER^L(233–685) at 35°C, the standard error is too small to be shown at this scale. Consistent with the plate assays shown in (A), the TIM1 interaction signal for PER^L is stronger than that of wild-type PER at 22°C ($P < 0.05$) and much weaker than that of wild-type PER at 35°C ($P < 0.001$). Specific β-galactosidase activity was calculated as the change in A_{420} per hour per milligram of protein. Within each independent experiment, specific β-galactosidase activities were normalized to that of the TIM1,PER (wild-type) control culture grown at 22°C (*29*).

REFERENCES AND NOTES

1. M. C. Moore-Ede, F. M. Sulzman, C. A. Fuller, *The Clocks That Time Us* (Harvard University Press, Cambridge, MA, 1982).
2. L. N. Edmunds, *Cellular and Molecular Bases of Biological Clocks* (Springer-Verlag, New York, 1988); T. Kondo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5672 (1993).
3. K. Hoffmann, *Proc. Dahlem Workshop* **20**, 63 (1976).
4. J. C. Dunlap, *Annu. Rev. Physiol.* **55**, 683 (1993).
5. Organisms in which circadian period mutants have been found include the cyanobacterium *Synechecoccus* [T. Kondo *et al.*, *Science* **266**, 1233 (1994)]; the freshwater alga *Chlamydomonas* [V. G. Bruce, *Genetics* **70**, 537 (1972)], the fungus *Neurospora crassa* [J. F. Feldman and M. Hoyle, *ibid.* **75**, 605 (1973)], *Drosophila* [R. J. Konopka and S. Benzer, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2112 (1971)], *Arabidopsis* [A. J. Millar, I. A. Carre, C. A. Strayer, N.-H. Chua, S. A. Kay, *Science* **267**, 1161 (1995)], and mouse [M. H. Vitaterna *et al.*, *ibid.* **264**, 719 (1994)].
6. A. Sehgal, J. L. Price, B. Man, M. W. Young, *Science* **263**, 1603 (1994).
7. W. A. Zehring *et al.*, *Cell* **39**, 369 (1984); T. A. Bargiello *et al.*, *Nature* **312**, 752 (1984).
8. C. R. McClung *et al.*, *Nature* **339**, 558 (1989).
9. X. Liu *et al.*, *Neuron* **6**, 753 (1991); L. J. Zweibel, P. E. Hardin, X. Liu, J. C. Hall, M. Rosbash, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3882 (1991); I. Edery, J. E. Rutila, M. Rosbash, *Science* **263**, 237 (1994); H. Zeng *et al.*, *EMBO J.* **13**, 3590 (1994).
10. B. B. Aronson, K. A. Johnson, J. J. Loros, J. C. Dunlap, *Science* **263**, 1578 (1994).
11. P. E. Hardin *et al.*, *Nature* **343**, 536 (1990).
12. K. K. Siwicki *et al.*, *Neuron* **1**, 141 (1988).
13. Z. J. Huang *et al.*, *Nature* **364**, 259 (1993).
14. K. D. Curtin *et al.*, *Neuron* **14**, 365 (1995).
15. L. B. Vosshall, J. L. Price, A. Sehgal, L. Saez, M. W. Young, *Science* **263**, 1606 (1994).
16. R. J. Konopka, S. Wells, T. Lee, *Mol. Gen. Genet.* **190**, 284 (1983); J. Ewer *et al.*, *J. Neurosci.* **12(9)**, 3321 (1992); B. Frisch, P. E. Hardin, M. J. Hamblen-Coyle, M. Rosbash, J. C. Hall, *Neuron* **12**, 555 (1994); L. B. Vosshall and M. W. Young, *ibid.* **15**, 345 (1995).
17. M. P. Myers, K. Wager-Smith, C. S. Wesley, M. W. Young, A. Sehgal, *Science* **270**, 805 (1995).
18. S. Fields and O.-K. Song, *Nature* **340**, 245 (1989).
19. The DNA binding bait hybrid was LexA(1–202)–PER(233–685) (*13*), corresponding to a segment of PER that is highly conserved among *Drosophila* species [H. V. Colot, J. C. Hall, M. Rosbash, *EMBO J.* **7**, 3929 (1988)]; it contains the PAS domain and all of the sites of single amino acid substitutions that lead to long-period or short-period phenotypes (*4*). *Drosophila* head cDNA library clones were expressed as VP16 transactivator hybrids, and the host yeast strain carried unlinked *HIS3* and *lacZ* (β-galactosidase) reporter genes downstream of LexA binding sites, as described (*36*). Because the PER fragment used as bait has been shown to homodimerize (*13*), a corresponding VP16-PER hybrid served as a positive control. For cDNA library construction, flies entrained to 12-hour light:12-hour dark cycles were collected at zeitgeber times 2, 8, 14, and 20, and total RNA was prepared from their heads (*6*). An aliquot of 45 μg of total RNA was taken from each time-specific sample and the aliquots were pooled, then polyadenylated RNA [poly(A)$^+$] RNA was prepared by oligo(dT) chromotagraphy (Oligotex-dT, Qiagen). Pooling of time-specific RNA samples was performed to maximize the likelihood that any transcripts exhibiting a circadian oscillation in abundance would be represented in the cDNA library.

Random hexamer-primed, double-stranded cDNA was prepared from 2 μg of poly(A)$^+$ RNA (Superscript Plasmid System, Gibco BRL) as suggested by the manufacturer, except that cDNAs were ligated to linkers containing Not I sites, digested with Not I, and were size-selected by agarose gel electrophoresis. Fragments ≥1.5 kb were ligated to Not I–digested dephosphorylated pVP16 (36), the resulting ligation products electroporated into *Escherichia coli* strain DH-10B (Gibco BRL), 2 × 10^6 transformants plated at 10^5 colonies per 15-cm dish, and plasmid DNA prepared from the bacterial colonies (Qiagen Plasmid Maxi Kit). Analysis of an arbitrarily chosen population of plasmids indicated that ≥98% were recombinant, and inserts ranged from 1.5 to 4 kb.

20. The L40 reporter yeast strain expressing the LexA-PER bait hybrid was transformed with the *Drosophila* head cDNA library (at efficiencies ≥10^4 per microgram of plasmid DNA) as described [D. Gietz, A. St. Jean, R. A. Woods, R. H. Shiestl, *Nucleic Acids Res.* **20**, 1425 (1992); J. Hill, K. A. Ian, G. Donald, D. E. Griffiths, *ibid.* **19**, 5791 (1991)]. For solid-phase amplification of the library, ~10^7 transformants were plated at 10^5 colonies per 15-cm plate on TULL , a standard medium lacking tryptophan and leucine (to select for the bait and library plasmids, respectively) and uracil and lysine (to maintain the genomic loci at which the reporter genes were integrated) (*36*). After 60 hours of growth at 30°C, colonies were removed from the plates with a sterile scraper, resuspended in 500 ml of sterile water, pelleted at 4000*g* for 10 min, and resuspended in 50 ml of sterile water. After thorough mixing, 20 μl of the suspension was diluted into 2 ml of liquid TULL medium, and the transformants were grown at 30°C to one doubling [final absorbance at 600 nm (A_{600}) of 0.8], pelleted, washed with sterile water, and a total of 2 × 10^7 transformants were spread onto 20 plates containing synthetic medium as above, but lacking histidine (THULL; to select additionally for activation of the *HIS3* reporter gene). This 10-fold over-screen of the cDNA library was performed to maximize the chance of detecting very rare clones. Plates were incubated at 30°C, and 360 His$^+$ colonies were patched to fresh THULL plates after 48 to 72 hours of growth for retesting of the His$^+$ phenotype and for β-galactosidase assays, which were performed by a filter-lift method [L. Breeden and K. Nasmyth, *Cold Spring Harbor Symp. Quant. Biol.* **50**, 643 (1985)].
21. Inocula from His$^+$ β-Gal$^+$ yeast colonies were grown in 2 ml of liquid THULL medium (*20*) for 24 to 48 hours at 30°C, and plasmid DNA was recovered after glass bead lysis of the cells [C. S. Hoffman and F. Winston, *Gene* **57**, 267 (1987)]. Plasmid DNA was electroporated into *E. coli* strain MC1066 [M. I. Chiu, H. Katz, V. Berlin, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12574 (1994)], which is auxotrophic for Trp and Leu, deficiencies that are complemented by the *Saccharomyces cerevisiae TRP1* and *LEU2* markers, respectively. For selective recovery of VP16–cDNA library plasmids (*LEU2* marker) rather than LexA-PER bait plasmid (*TRP1* marker), bacterial transformants were plated onto minimal medium plates (plus carbenicillin, tryptophan, and uracil) lacking leucine. Transformants were then checked for the absence of bait plasmid by demonstrating failure to grow on minimal medium plates (plus carbenicillin, leucine, and uracil) lacking tryptophan. Across different experiments, 1 to 5% of Leu$^+$ transformants were found to carry both plasmids; demonstration of a Trp$^-$ phenotype before plasmid purification is thus important for the reliability of subsequent specificity tests, which could be confounded by the presence of the original bait plasmid.
22. P. Bartel, C.-T. Chien, R. Sternglanz, S. Fields, *Biotechniques* **14**, 920 (1993).
23. The consistent differences in PER-dependent β-galactosidase activity produced by the six clones could result from differences in the affinity of their respective protein products for PER, but these differences could just as well result from other factors, such as differential hybrid protein stability or variability of transactivation activity in different VP16 hybrid proteins.
24. N. Gekakis and C. J. Weitz, unpublished observations.
25. K. Chua and C. J. Weitz, unpublished observations.
26. TIM fragments labeled with [^{35}S]methionine were synthesized in vitro by coupled transcription-translation (TNT Lysate System, Promega). GST or GST-PER fusion proteins were produced in *E. coli* with the pGEX vector (Pharmacia) and purified on glutathione-agarose beads. For in vitro binding reactions, glutathione-agarose beads with coupled GST or GST-PER fusion protein (50 μl, hydrated bead volume) were incubated at room temperature for 30 min with labeled TIM (1.5 × 10^5 cpm, Cerenkov) in binding buffer [200 μl (final volume) of 20 mM Hepes (pH 7.4), 100 mM KCl, 5 mM EDTA, 5 mM EGTA, 10% (v/v) glycerol, 5% (w/v) bovine serum albumin (BSA), 0.4% NP-40, and 1 mM dithiothreitol]. The beads were then washed at room temperature twice in binding buffer and twice in binding buffer lacking BSA (1 ml per wash). Beads were resuspended in an equal volume of 2× Laemmli buffer, incubated at 100°C for 5 min, and the entire sample was analyzed on 6% (Fig. 3C) or 7% (Fig. 3D) SDS-PAGE.
27. M. K. Baylies et al., *Nature* **326**, 390 (1987).
28. Each yeast transformant was patched to different plates (one to be grown at 22°C, and the other 37°C) containing TULL medium (*20*) supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Sigma) to a final concentration of 100 μg/ml (added to TULL medium as a 1000× stock in *N,N*-dimethylformamide). The transformants shown in Fig. 4A were grown as follows: TIM1: (22°C) for 6 days; TIM1 (37°C) for 5 days; TIM2 and TIM4: (22°C) for 1.5 days; TIM2 and TIM4 (37°C); for 1 day.
29. For each experiment, single TIM1,PER (wild-type) and TIM1,PER^L transformants were processed in parallel. Each transformant yeast colony was resuspended in 6 ml of TULL medium (*20*). The suspension was divided into three 2-ml cultures, which were then grown at 22°, 28°, or 35°C, respectively, for 24 hours. Cultures were diluted 100-fold with fresh TULL medium, and 2 ml of the diluted culture was grown (at the same temperature as the initial incubation) to a final A_{600} of 0.8 to 1.2. Extraction of β-galactosidase from yeast and assays of β-galactosidase activity were performed as described [F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1988)]. Specific β-galactosidase activity was calculated as the change in A_{420} per hour per milligram of protein. Within each independent experiment, specific β-galactosidase activities were normalized to that of the TIM1,PER (wild-type) control culture grown at 22°C.
30. Z. J. Huang, K. D. Kurtin, M. Rosbash, *Science* **267**, 1169 (1995).
31. W. O. Friesen, G. D. Block, C. G. Hocker, *Annu. Rev. Physiol.* **55**, 661 (1993).
32. R. J. Konopka, C. Pittendrigh, D. Orr, *J. Neurogenetics* **6**, 1 (1989).
33. A. Rothenfluh-Hilfiker and M. W. Young, unpublished observations.
34. A. Sehgal *et al.*, *Science* **270**, 808 (1995).
35. I. Edery, L. J. Zweibel, M. E. Dembinska, M. Rosbash, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2260 (1994).
36. A. B. Vojtek *et al.*, *Cell* **74**, 205 (1993).
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Participation of the Human β-Globin Locus Control Region in Initiation of DNA Replication

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The human β-globin locus control region (LCR) controls the transcription, chromatin structure, and replication timing of the entire locus. DNA replication was found to initiate in a transcription-independent manner within a region located 50 kilobases downstream of the LCR in human, mouse, and chicken cells containing the entire human β-globin locus. However, DNA replication did not initiate within a deletion mutant locus lacking the sequences that encompass the LCR. This mutant locus replicated in the 3′ to 5′ direction. Thus, interactions between distantly separated sequences can be required for replication initiation, and factors mediating this interaction appear to be conserved in evolution.

The human β-globin locus consists of five linked genes, ε, γ_A, γ_G, δ, and β, which exhibit erythroid-specific, developmentally regulated expression (*1*). Replication of this locus initiates from a region 5′ to the β-globin gene regardless of expression or replication timing (*2*). Gene expression, chromatin structure (*3*, *4*, *5*), and replication timing (*6*) are regulated by the LCR, an upstream element that contains five deoxyribonuclease (DNase) I hypersensitive sites (HSs) (*1*, *7*). The regulatory importance of the LCR in the native chromosomal context was established in naturally occurring deletions encompassing the LCR, the smallest of which (35 kb) deletes HSs 2 to 5 and 20 to 25 kb 5′ to HS5 (Hispanic thalassemia). Absence of this region prevents β-globin expression in erythroid cells, shifts replication timing from early to late S

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