

33. Standard genetic techniques and media were as described or referenced [L. C. DeVeaux, N. A. Hoagland, G. R. Smith, *Genetics* **130**, 251 (1992); C. Grimm, J. Bähler, J. Kohli, *ibid.* **136**, 41 (1994)].
34. D. E. Lea and C. A. Coulson, *J. Genet.* **49**, 264 (1949).
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PER Protein Interactions and Temperature Compensation of a Circadian Clock in *Drosophila*

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The periods of circadian clocks are relatively temperature-insensitive. Indeed, the *per^L* mutation in the *Drosophila melanogaster period* gene, a central component of the clock, affects temperature compensation as well as period length. The *per* protein (PER) contains a dimerization domain (PAS) within which the *per^L* mutation is located. Amino acid substitutions at the *per^L* position rendered PER dimerization temperature-sensitive. In addition, another region of PER interacted with PAS, and the *per^L* mutation enhanced this putative intramolecular interaction, which may compete with PAS-PAS intermolecular interactions. Therefore, temperature compensation of circadian period in *Drosophila* may be due in part to temperature-independent PER activity, which is based on competition between inter- and intramolecular interactions with similar temperature coefficients.

Circadian rhythms, nearly ubiquitous in eukaryotes, are self-sustaining, have periods of approximately 24 hours, and can be entrained by environmental signals such as light. Unlike many physiological processes that are dependent on temperature, the period lengths of circadian clocks are nearly constant over a wide range of physiological temperatures (1). Such temperature compensation is crucial to the construction of a circadian clock; otherwise, the period length of the clock would fluctuate with ambient temperature and would be unreliable.

Although a number of hypotheses have been proposed (2), there is no experimental information on the molecular basis of temperature compensation in any system. However, mutations that compromise temperature compensation exist in the *D. melanogaster period* (*per*) gene and in the *Neurospora frequency* (*frq*) gene (3–5). For example, the period length of wild-type *Drosophila* varies less than 0.5 hour from 15°C to 30°C, but the periods of *per^L* flies increase from about 27 hours to 33 hours over this temperature range, a change of more than 20% (4, 6). Because both *per* and *frq* genes have been shown to be central components of circadian pacemakers (7), temperature compensation is likely to derive directly from intrinsic properties of

clock components or their interconnection.

The *per* mRNA and protein levels undergo robust circadian oscillations (8–10), and there is a feedback loop in which PER affects the circadian transcription of its own gene (8, 11). PER contains a PAS domain, a protein dimerization motif present in several basic helix-loop-helix transcription factors (12, 13). The *per^L* mutation is a valine to aspartic acid missense mutation (14) in the PAS domain, which affects PAS-mediated PER-PER homodimerization in vitro (12) as well as period length and temperature compensation in vivo (4, 6).

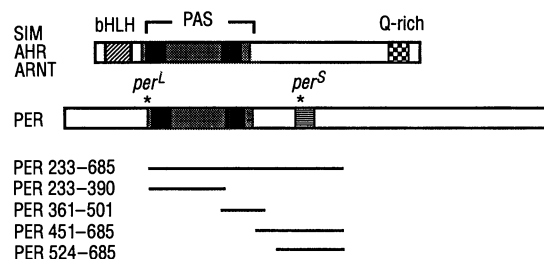
To perform a structure-function analysis of the PER PAS domain, we reconstituted PER dimerization in a yeast two-hybrid system (Fig. 1) (15). Consistent with our previous in vitro results, the large PAS-containing fragment (PER 233–685) dimerized well in this assay (Fig. 2A). Reporter gene activation was dependent on PER interaction, because PER 233–685/LexA with the prey vector alone did not produce any de-

tectable β -galactosidase. The *per^S* mutation, a serine to asparagine substitution at position 589 that shortens the circadian period from 24 to 19 hours (3, 14, 16), dimerized as well (Fig. 2A). Surprisingly, the *per^L* mutation also showed dimerization nearly indistinguishable from that of the wild-type (Fig. 2A), in contrast to our previous in vitro results that indicated a significant decrease in dimerization (12). Because the *per^L* mutation not only lengthens circadian period but also compromises temperature compensation (4, 6), we repeated the yeast assay at higher temperatures. PER^L dimerization was essentially undetectable at 37°C (Fig. 2A), which indicates that some aspect of the interaction is temperature-sensitive.

To substantiate this correlation between circadian behavior in flies and dimerization in yeast, we made another nonconservative change at amino acid 243 (valine to arginine) (PER^LR) and introduced this mutant *per* gene into flies by P element-mediated transformation (17). The two independent homozygote mutant lines showed lengthened and temperature-sensitive circadian periods that were very similar to those of the original *per^L* mutant (Table 1). We also compared the dimerization phenotype of PER^LR in yeast to that of PER^L and of wild-type PER. By growth on a plate without Leu as well as by β -galactosidase activity measurements in liquid media, both the PER^L 233–685 and PER^LR 233–685 bait led to similar decreases in dimerization that were also sensitive to temperature (Table 2). When the preys also carried the same point mutations, dimerization was further reduced and even more sensitive to temperature.

To determine whether the temperature sensitivity of PER^L dimerization might be peculiar to the yeast system, we also tested PER^L dimerization in an in vitro chemical crosslinking and immunoprecipitation assay. PER 233–685/H [which carries a hem-

Fig. 1. The basic helix-loop-helix (bHLH) PAS proteins (SIM, AHR, and ARNT) and the full-length PER protein. The bHLH motif, PAS domain, the two 51-amino acid PAS repeats, and the glutamine-rich (Q-rich) region are indicated. The asterisk indicates the *per^L* and *per^S* missense mutations. Numbers represent the first and last amino acids of the PER protein fragments. The C-domain is PER 524–685. The shaded box within the C-domain indicates the highly conserved region between fly PER and moth PER (21).



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Fig. 2. (A) PER^L dimerization is temperature-sensitive in the yeast two-hybrid system. Wild-type and mutant PER 233–685 baits and wild-type PER 233–685 or control preys were introduced into the yeast strain EGY48-pSH1834 (15, 26). Transformants were streaked onto a Ura-His-Trp-glucose plate. They were then replica-plated onto two Ura-His-Trp 2% galactose–1% raffinose plates containing X-gal and incubated either at 30°C or 37°C overnight. **(B)** PER^L dimerization is temperature-sensitive in vitro. In vitro translations of the ³⁵S-labeled wild-type (WT) (lanes 1, 2, 7, and 8), *per^L* (lanes 3, 4, 9, and 10), or *per^LR* (lane 5, 6, 11, and 12) versions of PER 233–685/H were incubated at either 16°C or 25°C for 2 hours. They were further incubated at the same temperature either in the presence (+) or the absence (–) of 0.005% glutaraldehyde (GA) in phosphate buffer (pH 7.4) for 30 min. They were then immunoprecipitated with antibody to HA and analyzed on a 10% SDS-polyacrylamide gel (30). The arrowhead on the left indicates the crosslinked homodimers. The doublet probably reflects crosslinking at two different locations, giving rise to different covalent dimers with different cross-sectional areas. In this gel, wild-type PER 233–685/H appears to give rise to more dimers at 16°C (lane 2) than at 25°C (lane 8). This is due to higher local background in lane 2 and was not seen in other experiments. PER^L233–685/H migrates more slowly on SDS-PAGE, as described (12).

agglutinin (HA) epitope at its COOH-terminus] dimerized well and with nearly identical efficiencies at 16°C, 25°C, and 30°C (Fig. 2B) (12). Consistent with the yeast two-hybrid data, PER^L 233–685/H in vitro dimerization was temperature-sensitive: it was undetectable at 30°C (12) and at 25°C but was detectable (albeit poorly) at 16°C (Fig. 2B). PER^LR 233–685/H dimerization was undetectable at all three temperatures.

To examine whether the temperature dependence of dimerization was due to a detectable change in PER stability in yeast, we assayed amounts of PER by protein immunoblot. The results showed that amounts of mutant and wild-type PER were indistinguishable from each other and were inde-

Table 1. The period length of *per^LR* is sensitive to temperature (17). Periods were measured as described (27). CS, Canton S strain.

Genotype	Period (± SEM) (hours)	Flies tested (n)
18°C		
CS	23.8 ± 0.2	11
<i>per^L</i>	27.1 ± 0.1	12
<i>per^LR-25</i>	26.7 ± 0.3	6
<i>per^LR-27</i>	27.4 ± 0.4	6
25°C		
CS	24.3 ± 0.1	15
<i>per^L</i>	29.6 ± 0.1	11
<i>per^LR-25</i>	28.7 ± 0.4	7
<i>per^LR-27</i>	28.6 ± 0.3	8
29°C		
CS	24.2 ± 0.1	11
<i>per^L</i>	31.4 ± 0.3	10
<i>per^LR-25</i>	30.3 ± 0.3	12
<i>per^LR-27</i>	30.1 ± 0.2	8

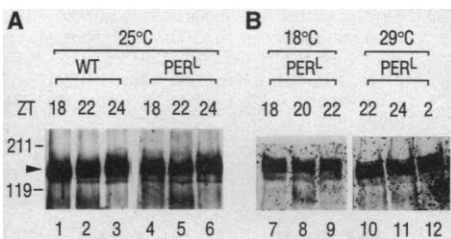
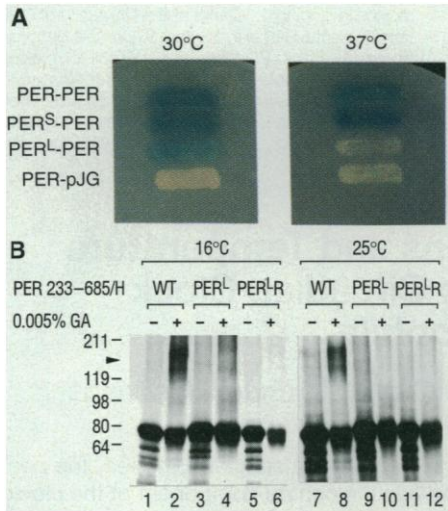


Fig. 3. The amount of PER^L protein in adult heads is similar to that of the wild-type protein **(A)** and is not affected by temperature **(B)**. Wild-type (WT) or *per^L* (PER^L) flies were entrained with three cycles of light and dark at the indicated temperatures and frozen at the indicated times. Total head extracts were prepared and analyzed for PER by protein immunoblotting with antibodies to PER (37). The zeitgeber time (ZT; in hours) of fly collection is shown. The arrowhead indicates the PER protein band.

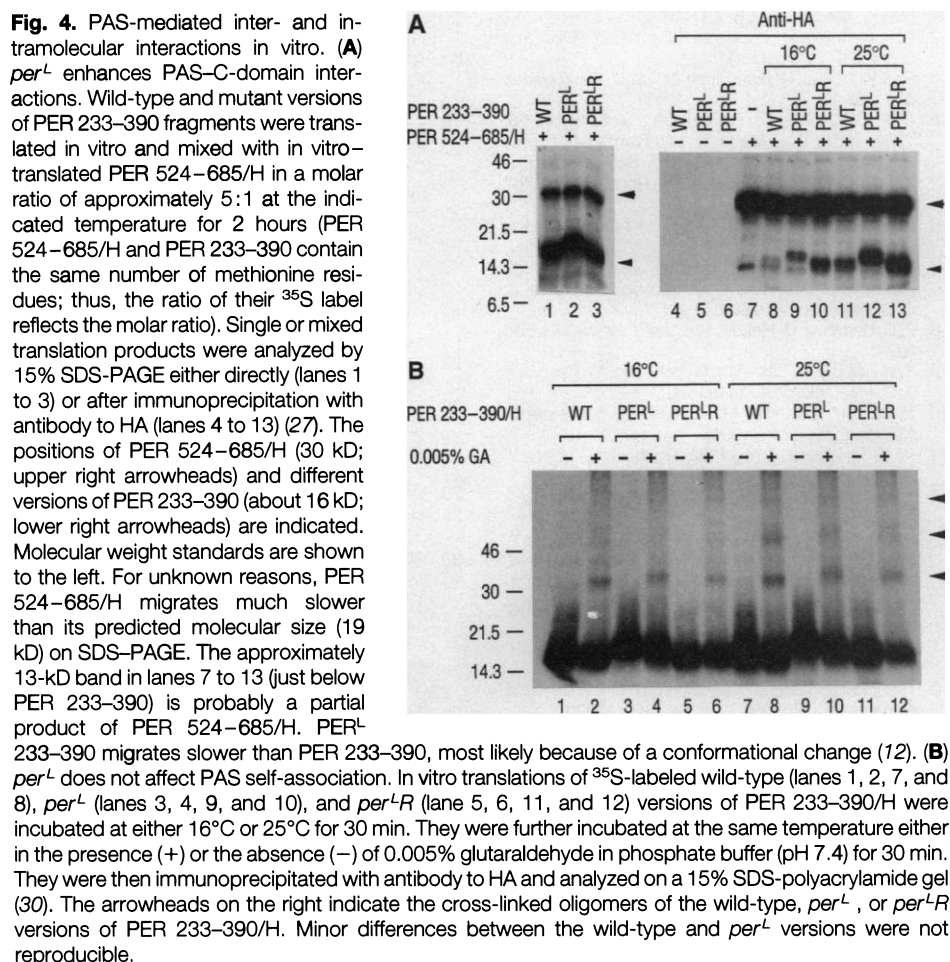
pendent of temperature (assayed at 25°C, 30°C, and 37°C) (18). Because increases and decreases in *per* gene copy number can shorten and lengthen circadian periods in flies (19), we also assayed amounts of PER in head extracts from flies grown at different temperatures. At 25°C, peak amounts of PER^L [at about zeitgeber time (ZT) 24] are comparable to those of wild-type PER (at about ZT 18) (Fig. 3A); there is at most a twofold difference. Peak amounts of PER^L were also unaffected by temperature, as extracts taken at 18°C were not different from extracts taken at 29°C (Fig. 3B). Thus, the minor differences between the amounts of PER^L and PER are unlikely to be solely responsible for the more than 5-hour lengthening of the circadian period, as it has been estimated that a 5-hour change in period requires about a 50-fold change in PER gene dose (20).

To understand further the strong effect of the substitutions at amino acid 243, we

tested interactions between PER 233–390 (which contains only part of the PAS domain, including amino acid 243 and the first PAS repeat) and the entire PER 233–685 fragment. This interaction was even stronger than PER 233–685 homodimerization (Table 3), which raised the possibility that sequences on the COOH-terminal side of the first PAS repeat (or COOH-terminal to PAS) may suppress PAS-mediated dimerization through an intramolecular interaction or by binding to a heterologous cellular protein. We tested the first possibility with the yeast two-hybrid assay. A domain COOH-terminal to PAS (PER 524–685, here termed the C-domain) associated with PER 233–390 (Table 3), which suggests that an intramolecular interaction may contribute to PER function. We verified this interaction in the in vitro assay and also examined the effects of the *per^L* mutations as well as those of temperature (Fig. 4A). Although only about 0.4% of PER 524–685/H (the PER 524–685 fragment with an HA epitope at its COOH-terminus) associated with PER 233–390 at 16°C, an intramolecular interaction might be expected to be weak when tested in an

Table 2. PER^L dimerization is temperature-sensitive in the yeast two-hybrid system. Plasmids that direct the synthesis of the listed bait and prey proteins were introduced into EGY48. The background refers to β-galactosidase activity when PER 233–685 bait was introduced with the prey vector pJG4–5. All interactions are presented as the percentage of the PER 233–685 homotypic interactions at 30°C. Three independent isolates were tested for each interaction, and the average value is given here.

Bait	Prey	Extent of dimerization at					
		25°C		30°C		37°C	
		Leu	β-gal (%)	Leu	β-gal (%)	Leu	β-gal (%)
PER 233–685	PER 233–685	+++	109 ± 11	+++	100	+++	112 ± 10
PER ^L 233–685	PER 233–685	+++	59 ± 9	++	39 ± 6	–	12 ± 2
PER ^L 233–685	PER ^L 233–685	++	43 ± 9	–	6 ± 2	–	4 ± 2
PER ^L R 233–685	PER 233–685	++	34 ± 5	++	18 ± 3	–	7 ± 2
PER ^L R 233–685	PER ^L R 233–685	++	26 ± 3	–	8 ± 1	–	8 ± 2



intermolecular assay. The *PER^L* mutation led to a fourfold increase in the association of PER 233–390 with PER 524–685/H (Fig. 4A, lanes 8 and 9). In addition, the interactions between the C-domain and the wild-type, the *per^L*, and the *per^LR* versions of PER 233–390 all increased at 25°C (Fig. 4A, compare lanes 8 and 11, lanes 9 and 12, and lanes 10 and 13). In the cases of wild-type and *per^L*, the increases were fourfold

(3.8 ± 0.4) and sixfold (5.7 ± 1.0), respectively (numbers are averaged from three experiments). Therefore, the *per^L* mutation also increased the temperature coefficient of the interaction. In contrast, the *per^S* mutation in PER 524–685/H had no effect—that is, it was indistinguishable from the wild-type fragment (18). The increase in the PAS-C-domain interaction by the *per^L* mutation indicates a direct association be-

Table 3. The C-domain interacts with PAS in yeast. Assays were performed at 30°C on three independent isolates for each combination. Values shown are the average of the three individual values, which varied by less than 10%. β -Gal activity represents the activation above background (expressed in fold increase). Background is the β -galactosidase activity of PER 233–685 paired with prey vector pJG4–5.

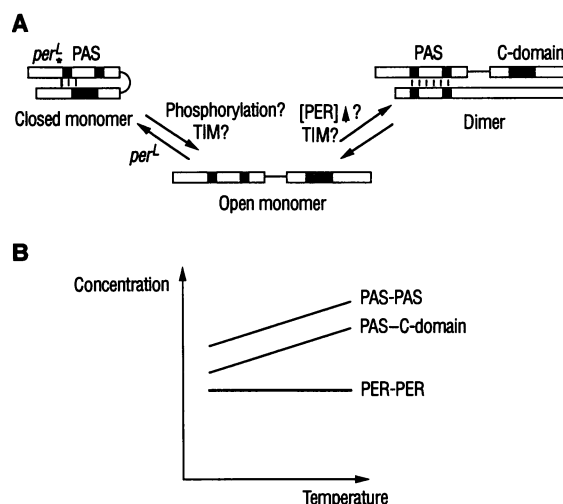
Bait	Prey	C-domain interaction	
		Leu	β -gal activity (fold activation)
PER 233–685	PER 233–685	+++	68.9
PER ^S 233–685	PER 233–685	+++	70.4
PER 233–390	PER 233–685	++++	85.6
PER 233–390	PER 361–501	–	1.4
PER 233–390	PER 451–685	+	10.8
PER 233–390	PER 524–685	+	12.4

tween the two fragments.

To determine whether the *per^L* mutation also decreases intermolecular interactions in the absence of the C-domain, we performed chemical crosslinking and immunoprecipitation experiments with the short PER 233–390/H fragment (Fig. 4B). As expected, wild-type PER 233–390/H underwent self-association. This interaction increased about threefold at higher temperatures (25°C as compared to 16°C). In contrast to its strong negative effect on dimerization of the large PER 233–685 fragment (12), the *per^L* mutation had little effect on PER 233–390/H self-association at either temperature. A larger PER fragment (PER 233–485) containing the complete PAS domain interacted even more efficiently in this assay and resulted predominantly in higher molecular weight oligomers, but the *per^L* mutation also had no effect on these interactions (18). Taken together with the effect of *PER^L* on the PAS-C-domain interaction, these data suggest that the *per^L* mutation may decrease PER 233–685 dimerization by increasing the strength of an inhibitory intramolecular interaction between the PER 233–390 and the PER 524–685 regions.

Our study reveals that *per^L* point mutations render temperature-sensitive the homotypic interactions of a large PAS-containing fragment. In addition, our results show that a subregion of the PER PAS domain can associate with another region of PER (the C-domain) both in vitro and in yeast. This interaction is stronger at higher temperatures. The *per^L* mutations increase the PAS-C-domain interaction and its temperature coefficient, with little or no effect on self-association of fragments that contain only PAS. We consider it unlikely that the PAS-C-domain interaction reflects only an inter-

Fig. 5. (A) A hypothetical equilibrium between the PER closed monomer, the open monomer, and the dimer. The PAS domain, C-domain, and *per^L* mutation are indicated. A putative PAS-containing protein is shown to dimerize with PER. TIM, putative *timeless* protein. (B) A model for PER PAS-mediated intra- and intermolecular interactions that may underlie temperature-compensated PER dimerization. "Concentration" refers to the hypothetical concentration of PAS-only dimers (PAS-PAS), PER monomers (PAS-C-domain), and PER dimers (PER-PER); it is an indication of the relative interaction strength.



molecular interaction that contributes positively to PER 233–685 dimerization. This is because PER^L increases the PAS–C-domain interaction, which would then increase rather than decrease PER 233–685 dimerization. An important role for the C-domain is also indicated by its high degree of phylogenetic conservation (21).

We propose that the PER PAS domain not only engages in intermolecular interactions but also undergoes an intramolecular interaction with the C-domain (Fig. 5A). The intramolecular interaction would favor a “closed” conformation and would compete with PAS-mediated intermolecular associations. Because both sets of interactions share one or more components, they may change with temperature in a similar fashion; as a consequence, PER dimerization should manifest temperature compensation (Fig. 5B). The *per^L* mutation interferes with this balance as it enhances the intramolecular interaction as well as its temperature coefficient with little effect on PAS-delimited homotypic interactions. The *per^L* mutation may thus give rise to two different temperature coefficients for the two competing reactions, resulting in temperature-sensitive PER^L intermolecular interactions. A conformational switch also helps to explain how a single amino acid substitution in a large fragment (PER 233–685) has such a strong effect on dimerization.

Our proposed intramolecular interaction might contribute to the gating of molecular events during the circadian cycle. This likely includes nuclear translocation; the *per^L* mutation results in temperature-sensitive nuclear entry, which is correlated with its effects on circadian period (22). A similar role for an intramolecular interaction in the regulation of protein oligomerization or nuclear entry has been reported for several transcription factors (23). An intramolecular interaction should be independent of concentration and thus should predominate at low PER concentrations early in the accumulation cycle. PER phosphorylation (24) and the *timeless* gene product (25) may also affect a competition between intra- and intermolecular interactions (Fig. 5A). Although this model is unlikely to explain all aspects of temperature compensation (6), it suggests that a temperature-compensated activity could derive from two antagonistic protein-protein interactions (one intermolecular, the other intramolecular), each with a similar temperature coefficient.

REFERENCES AND NOTES

- C. S. Pittendrigh, *Proc. Natl. Acad. Sci. U.S.A.* **40**, 1018 (1954); J. W. Hastings and B. M. Sweeney, *ibid.* **43**, 804 (1957).
- P. Lakin-Thomas, G. G. Coté, S. Brody, *Crit. Rev. Microbiol.* **17**, 365 (1990); C. S. Pittendrigh, in *Neurosciences: Third Study Program*, F. O. Schmitt and F. G. Worden, Eds. (MIT Press, Cambridge, MA, 1974), pp. 437–458.
- R. J. Konopka and S. Benzer, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2112 (1971).
- R. J. Konopka, C. Pittendrigh, D. Orr, *J. Neurogenet.* **6**, 1 (1989).
- G. F. Gardner and J. F. Feldman, *Plant Physiol.* **68**, 1244 (1981); D. L. Mattern, L. R. Forman, S. Brody, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 825 (1982); J. J. Lorosa and J. F. Feldman, *J. Biol. Rhythms* **1**, 18767 (1986).
- J. Ewer, M. Hamblen-Coyle, M. Rosbash, J. C. Hall, *J. Neurogenet.* **7**, 31 (1990).
- I. Edery, J. E. Rutilla, M. Rosbash, *Science* **263**, 237 (1994); B. D. Aronson, K. A. Johnson, J. J. Loros, J. C. Dunlap, *ibid.*, p. 1578.
- P. E. Hardin, J. C. Hall, M. Rosbash, *Nature* **343**, 536 (1990).
- K. K. Siwicki, C. Eastman, G. Petersen, M. Rosbash, J. C. Hall, *Neuron* **1**, 141 (1988).
- D. M. Zerr, J. C. Hall, M. Rosbash, K. K. Siwicki, *J. Neurosci.* **10**, 2749 (1990).
- P. E. Hardin, J. C. Hall, M. Rosbash, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11711 (1992).
- Z. J. Huang, I. Edery, M. Rosbash, *Nature* **364**, 259 (1993).
- J. R. Nambu, J. O. Lewis, K. A. Wharton, S. T. Crews, *Cell* **67**, 1157 (1991); K. M. Burbach, A. Poland, C. A. Bradfield, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8185 (1992); E. C. Hoffman *et al.*, *Science* **252**, 954 (1991).
- M. K. Bayliss, T. A. Bargiello, F. R. Jackson, M. W. Young, *Nature* **326**, 390 (1987).
- The yeast two-hybrid interaction assays were done as described (26). We constructed all the bait plasmids by inserting complementary DNAs (cDNAs) coding for different PER fragments in-frame downstream of the *lexA* gene in plasmid plexA202+PL (26). We constructed all the prey plasmids by inserting the same cDNAs in-frame downstream of the activation domain in plasmid pJG4–5. Each pair of bait and prey plasmid was introduced into the yeast host strain EGY48(Mata, *trp1 ura3 his3 LEU2::plexop6-LEU2*) for the interaction experiments. The *lacZ* reporter used was pSH18–34. The interactions between bait and prey peptides were detected as the activation of either of the two reporter genes: *LEU2* as growth in the absence of leucine and β -galactosidase as blue color on x-gal plates. The liquid β -galactosidase assay was performed as described (26). Expression of bait and prey fusion proteins was monitored by protein immunoblotting with LexA and HA antibodies.
- Q. Yu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 784 (1987).
- To create the *per^LR* mutation for germline transformation, we used a subclone of the *per* gene for mutagenesis from a 13.2-kb *per* genomic fragment coding an HA tag at the COOH-terminus cloned into cp20.1 (27). An Xba I fragment extending from nucleotide 2445 [numbering is as in (28)] in this clone through the 3' end of the *per* gene (including some cp20.1 sequences) was subcloned into pBluescript KS⁺. Clones of one orientation were chosen and used to subclone a Sac I fragment from nucleotide 4935 through the 3' end of the *per* gene and into the polylinker. This 2.5-kb Sac I fragment was subcloned into pBluescript KS⁺ and subsequently used to generate single-stranded DNA for site-directed mutagenesis. A 30-bp oligonucleotide was used to introduce an asparagine-coding triplet into the position coding amino acid 243 centered at nucleotide 3624. Mutagenesis was as described (29). Mutant clones were confirmed by dideoxy sequencing and were subsequently recombined in two steps back into the starting vector. The *per⁰¹;ry⁵⁰⁶* flies were transformed with the mutant *per* P-element construct as described (27). Two lines were recovered and designated *per^LR*. Wild-type and *per^LR* flies containing one copy of the *per* gene were entrained in a cycle of 12 hours of light:12 hours of dark for 2 to 3 days before being assayed in constant darkness at the indicated temperatures as described (27). Data were collected for 5 days, and activity periods determined by χ^2 square periodogram analysis (27).
- Z. J. Huang and M. Rosbash, unpublished results.
- R. F. Smith and R. J. Konopka, *Mol. Gen. Genet.* **189**, 30 (1982).
- G. G. Coté and S. Brody, *J. Theor. Biol.* **121**, 487 (1986).
- S. M. Reppert, T. Tsai, A. L. Roca, I. Sauman, *Neuron* **13**, 1167 (1994).
- K. Curtin, Z. J. Huang, M. Rosbash, *ibid.* in press.
- E. S. Klein, D. M. Simmons, L. W. Swanson, M. G. Rosenfeld, *Genes Dev.* **7**, 55 (1993); S. K. Rabin, R. I. Haroun, J. Clos, J. Wisniewski, C. Wu, *Science* **259**, 230 (1993); H.-C. Liou, G. P. Nolan, S. Ghosh, T. Fujita, D. Baltimore, *EMBO J.* **11**, 3003 (1992); V. Blank, P. Kourilsky, A. Israël, *ibid.* **10**, 4159 (1991); F. C. De La Brousse and S. L. McKnight, *Trends Genet.* **9**, 151 (1993).
- I. Edery, L. J. Zwiebel, M. E. Dembinska, M. Rosbash, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2260 (1994).
- L. B. Vosshall, J. L. Price, A. Sehgal, L. Saez, M. W. Young, *Science* **263**, 1606 (1994).
- J. Gyuris, E. Golemis, H. Chertkov, R. Brent, *Cell* **75**, 791 (1993).
- J. E. Rutilla, I. Edery, J. C. Hall, M. Rosbash, *J. Neurogenet.* **8**, 101 (1992).
- Y. Citri *et al.*, *Nature* **326**, 42 (1987).
- T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985).
- We generated cDNA templates for in vitro transcription and translation by the polymerase chain reaction (PCR) from the full-length *per* cDNA [pSP65AT*per* (12)] and gel-purified them. The 5' primers contained a T7 promoter, the 5' leader and ATG from the human β -globin gene (12), and the corresponding *per* cDNA sequences; the 3' primer contained, from 5' to 3', a TAG stop codon, the sequence corresponding to the HA peptide [YPYDVPDVAL; abbreviations for the amino acid residues are Y, Tyr; P, Pro; D, Asp; V, Val; A, Ala; S, Ser; and L, Leu (12)], and corresponding *per* cDNA. Coupled in vitro transcription and translation were performed with rabbit reticulocyte lysate in the presence of ³⁵S-labeled methionine according to the manufacturer's instructions (TNT, Promega). Immunoprecipitation was essentially as described (12), except that the HND buffer contained 0.1% NP-40. For chemical crosslinking, 2 μ l of the above translation reaction was incubated in 30 μ l of 0.1 M phosphate buffer (pH 7.4) and glutaraldehyde at different temperatures for the indicated times. We terminated the crosslinking by adding 2 M Tris (pH 7.5) to a final concentration of 100 mM. Samples were diluted in 250 μ l of modified HND buffer as above. Subsequent immunoprecipitation procedures were as described (12).
- Flies were entrained with three cycles of 12 hours of light:12 hours of dark, collected at the indicated times, and frozen at –80°C. Protein extract was made from 20 heads for each time point as described (24), with minor modifications. Each sample was homogenized in 20 μ l of ice-cold extraction buffer [20 mM Hepes, pH 7.5, 100 mM KCl, 10% glycerol, 50 mM NaF, 10 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, aprotinin (20 μ g/ml), leupeptin (5 μ g/ml), and pepstatin (5 μ g/ml)]. Another 10 μ l of extraction buffer was then used to rinse the tip of the homogenizer, then was collected and combined with the extract. Because no internal control was included in the assay, care was taken not to lose any protein from the starting material. We added 10 μ l of 4× SDS sample buffer, and the sample was boiled for 5 min and microfuged to pellet the undissolved debris. The supernatant was loaded on a 5.7% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Protein immunoblotting was done as described (24). For quantitation of the chemiluminescence (ECL, Amersham), the blot was exposed to a chemiluminescence screen (Bio-Rad) for 0.5 to 3 hours and quantified in a Bio-Rad phosphorimager.
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