different protein targets, the PAS domain in PER exhibits great flexibility in mediating protein-protein interactions. This may have implications regarding the number of potential interacting targets for other PAS-containing proteins.

Molecular cloning of *tim* has allowed the detection of circadian cycles in *tim* RNA expression (26). Our combined molecular studies reveal a tight interplay between PER and TIM and suggest a rudimentary intracellular biochemical mechanism regulating circadian rhythms in *Drosophila*. Further analysis of *tim* and its interactions with *per* will likely shed new light on this central clock mechanism and may eventually provide clues about how the clock is linked to output paths that yield observable rhythmic behaviors.

REFERENCES AND NOTES

1. R. J. Konopka and S. Benzer, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2112 (1971).
2. M. K. Baylies, T. A. Bargiello, F. R. Jackson, M. W. Young, *Nature* **326**, 390 (1987); Q. Yu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 784 (1987).
3. P. E. Hardin, J. C. Hall, M. Rosbash, *Nature* **343**, 536 (1990); *EMBO J.* **11**, 1 (1992); *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11711 (1992); P. E. Hardin, *Mol. Cell. Biol.* **14**, 7211 (1994).
4. A. Sehgal, J. L. Price, B. Man, M. W. Young, *Science* **263**, 1603 (1994).
5. K. K. Siwicki, C. Eastman, G. Petersen, M. Rosbash, J. C. Hall, *Neuron* **1**, 141 (1988).
6. D. M. Zerr, J. C. Hall, M. Rosbash, K. K. Siwicki, *J. Neurosci.* **10**, 2749 (1990).
7. X. Liu *et al.*, *J. Neurosci.* **12**, 2735 (1992); L. Saez and M. W. Young, *Mol. Cell. Biol.* **8**, 5378 (1988).
8. I. Edery, L. J. Zwiebel, M. E. Dembinska, M. Rosbash, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2260 (1994).
9. L. B. Vosshall, J. L. Price, A. Sehgal, L. Saez, M. W. Young, *Science* **263**, 1606 (1994).
10. H. Zeng, P. E. Hardin, M. Rosbash, *EMBO J.* **13**, 3590 (1994).
11. F. R. Jackson, T. A. Bargiello, S. H. Yun, M. W. Young, *Nature* **320**, 185 (1986); Y. Citri *et al.*, *ibid.* **326**, 42 (1987).
12. S. T. Crews, J. B. Thomas, C. S. Goodman, *Cell* **52**, 143 (1988); E. C. Hoffman *et al.*, *Science* **252**, 954 (1991); K. M. Burbach, A. Poland, C. A. Bradfield, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8185 (1992).
13. H. Reyes, S. Reisz-Porszasz, O. Hankinson, *Science* **256**, 1193 (1992).
14. Z. J. Huang, I. Edery, M. Rosbash, *Nature* **364**, 259 (1993).
15. Z. J. Huang, K. D. Curtin, M. Rosbash, *Science* **267**, 1169 (1995).
16. N. Matsushita, K. Sogawa, M. Ema, A. Yoshida, Y. Fujii-Kuriyama, *J. Biol. Chem.* **268**, 21002 (1993); M. L. Whitelaw, J. A. Gustafsson, L. Poellinger, *Mol. Cell. Biol.* **14**, 8343 (1994).
17. J. L. Price, M. E. Dembinska, M. W. Young, M. Rosbash, *EMBO J.* **14**, 4044 (1995).
18. J. J. Sekelsky *et al.*, *Genetics* **139**, 1347 (1995).
19. A. Rothenfluh-Hilfiker and M. W. Young, unpublished data.
20. S. Cohen, personal communication.
21. Z. Shubo *et al.*, *EMBO J.* **14**, 2884 (1995).
22. C. S. Wesley, M. P. Myers, M. W. Young, *Nucleic Acids Res.* **22**, 538 (1994).
23. V. Pirrotta, in Drosophila: *A Practical Approach*, D. B. Roberts, Ed. (IRL, Oxford, 1986), pp. 83–110.
24. M. P. Myers, unpublished data.
25. S. Kidd, T. J. Lockett, M. W. Young, *Cell* **34**, 421 (1983); M. R. Kelley, S. Kidd, W. A. Deutsch, M. W. Young, *ibid.* **51**, 539 (1987).
26. A. Sehgal *et al.*, *Science* **270**, 808 (1995).
27. N. Gekakis *et al.*, *ibid.*, p. 811.
28. B. A. Hamilton, M. J. Palazzolo, E. M. Meyerowitz, *Nucleic Acids Res.* **19**, 1951 (1991).
29. R. E. Breitbart, A. Andreadis, B. Nadal-Ginard, *Annu. Rev. Biochem.* **56**, 467 (1987).
30. M. P. Myers, K. Wager-Smith, M. W. Young, data not shown.
31. K. Sogawa *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1936 (1995).

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Rhythmic Expression of *timeless*: A Basis for Promoting Circadian Cycles in *period* Gene Autoregulation

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The clock gene *timeless* (*tim*) is required for circadian rhythmicity in *Drosophila*. The accumulation of *tim* RNA followed a circadian rhythm, and the phase and period of the *tim* RNA rhythm were indistinguishable from those that have been reported for *per*. The *tim* RNA oscillations were found to be dependent on the presence of PER and TIM proteins, which demonstrates feedback control of *tim* by a mechanism previously shown to regulate *per* expression. The cyclic expression of *tim* appears to dictate the timing of PER protein accumulation and nuclear localization, suggesting that *tim* promotes circadian rhythms of *per* and *tim* transcription by restricting *per* RNA and PER protein accumulation to separate times of day.

The *tim* gene, which we recently cloned (*1*), is essential for the production of circadian rhythms in *Drosophila* (*2*, *3*). Molecular data indicated that TIM protein may be required at a specific time of day to allow accumulation and nuclear localization of the PER protein (*2*–*4*), so we determined if expression of the *tim* gene showed temporal regulation. We examined the expression in heads of adult *Drosophila* for the following reasons: (i) The clock is known to be located in the head (*5*). (ii) Oscillation of *per* RNA was first demonstrated in adult heads, although subsequently it was shown to occur in most body tissues as well (*6*, *7*). (iii) All effects of *tim* on *per* RNA and PER protein have been studied in adult heads (*2*–*4*).

Adult flies were maintained in the presence of 12-hour light: 12-hour dark cycles (LD 12:12), and the amount of *tim* RNA in the heads was measured at 4-hour intervals over 4 days (Fig. 1). We found that *tim* RNA quantities oscillated during the course of LD12:12, with peak quantities corresponding to the end of the day and lowest quantities to dawn. Oscillations of *per* RNA have the same phase (*2*, *6*, *7*). The amplitude of the variation in *tim* RNA quantities appeared similar to that reported for *per* (*2*, *6*). On some days there was as much as a 15-fold difference between peak and trough amounts (Fig. 1B, day 1).

Oscillations of *per* RNA persist in constant darkness and are, therefore, considered a circadian rhythm (*6*), and oscillations of the RNA encoded by the *frequency* (*frq*) clock gene in *Neurospora* also persist in the absence of environmental signals (*8*). We studied the expression of *tim* and *per* RNA in wild-type and per^{S} (short-period mutant) flies under free-running conditions and found that oscillations of both *per* and *tim* RNA persist in constant darkness with indistinguishable periods, phases, and amplitudes (Fig. 2). The plot of *tim* RNA oscillations is essentially superimposed on the curve displaying *per* RNA cycling. Whereas both RNAs cycle with a ~23-hour periodicity in the wild type, they cycle in per^{S} with a 17- to 18-hour period (Fig. 2). The amplitude of the oscillations in wild-type and per^{S} flies is, however, reduced in

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free-running conditions relative to the cycling observed in the presence of LD 12:12 (Fig. 1). The effects of constant darkness on amplitude of the *tim* RNA rhythm are comparable with those on *per* (Fig. 2) (6).

The effects of the per^S mutation indicated that PER protein functions in the cyclic expression of *tim* mRNA. We confirmed this by analyzing the effect of the per^{01} mutation on the cycling of *tim* mRNA. The per^{01} gene contains a nonsense mutation at amino acid position 464 (full-length PER is ~1200 amino acids) and is considered a null mutation because it does not express any functional gene product (9). Like *per* RNA oscillations, *tim* RNA oscillations were abolished in per^{01} flies maintained in LD 12:12 (*10*). Thus, PER protein is required to sustain oscillating amounts of *tim* mRNA as well as its own RNA.

The original *tim* mutation [(tim^{01}) (*1*)] eliminates *per* RNA oscillations and is a presumptive null mutation (*1*). It contains a deletion that removes coding sequences and produces a frame shift leading to a truncated protein of about half the size of full-length TIM (*1*). To determine whether TIM protein is required for the cyclic expression of its own mRNA, we measured *tim* RNA quantities at different times of day in tim^{01} flies maintained in LD12:12. Although *tim* RNA fluctuations occurred, they did not display a regularity suggesting a circadian or noncircadian rhythm (Fig. 3). Thus, regular oscillations of *tim* RNA depend on the presence of TIM protein, which indicates that products of the *tim* gene function in an autoregulatory feedback loop.

The *tim* and *per* genes appear to form a partnership that defines central elements of the *Drosophila* pacemaker. Because autoregulation of *tim* requires PER protein, and vice versa, it is likely that PER and TIM are components of, and are regulated by, the same feedback loop. That is, *tim* and *per* are both subject to autoregulation, and they regulate each other reciprocally. This interdependence is likely to be mediated by a direct physical association of the TIM and PER proteins (*11*). Additional evidence for action of *tim* as a component of the pacemaker comes from the discovery of multiple alleles of *tim* that display phenotypes ranging from long periodicity to arrhythmia (*12*). Aspects of *tim* function and expression fulfill several criteria proposed for a "state variable" of the circadian pacemaker [reviewed in (*8*, *13*)]. The only other genes known to satisfy these criteria are *per* and the *Neurospora* clock locus *frq*.

The accumulation of PER protein lags behind the synthesis of its mRNA by ~6 hours. PER accumulates in perinuclear regions about an hour before its transport to the nucleus (*14*). Because nuclear PER proteins may negatively regulate *per* transcrip-

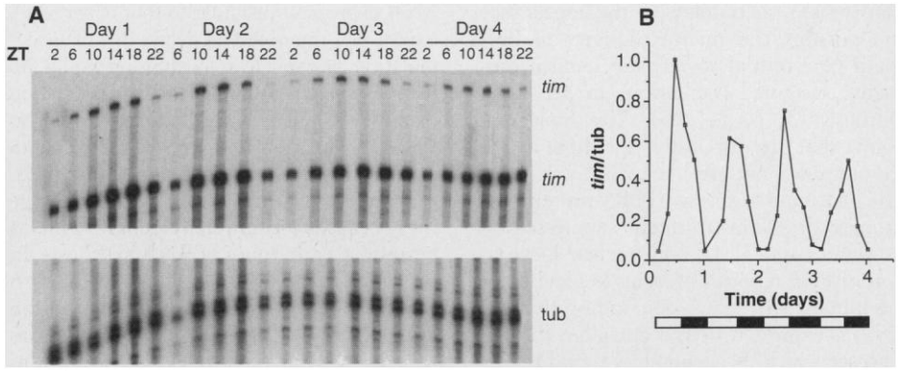

Fig. 1. Temporal expression of *tim* RNA in wild-type flies. (**A**) Adult flies maintained in LD12:12 for a minimum of three cycles were subjected to ribonuclease (RNase) protection assays as described (*2*). Total head RNA (10 μg) from these flies was hybridized to a *tim* RNA probe that corresponds to nucleotides 1971 to 2367 of the *tim* complementary DNA (cDNA) sequence (*1*) and also includes some plasmid vector sequences, and to a tubulin probe (tub) that is complementary to nucleotides 1 to 142 of the tubulin sequence (*18*). After RNase digestion, the fragments were run on a 5% denaturing polyacrylamide gel. The pattern of *tim* RNA expression was examined at 4-hour intervals during a 4-day period. Numbers at the top of the lanes correspond to zeitgeber time (ZT), which indicates the light:dark cycle that was used to entrain the flies (ZT 0, lights on; ZT 12, lights off). The ZT 2 time point is missing for the second day. The *tim* RNA probe protects two fragments, possibly because of the presence of a specific breakdown product of the probe, or because of a nucleotide polymorphism distinguishing the probe and the protected RNA, as these were derived from different *Drosophila* strains. Fragments protected by the tub RNA probes are also indicated. This image was generated by scanning the original autoradiograph with the UMax scanning program included in the Adobe Photoshop software. (**B**) The *tim* and tub bands were quantitated with a phosphorimager and the *tim*/tub ratio for each time point was plotted. All values are represented relative to the maximum *tim*/tub ratio, which is defined here as 1. Data shown here (and in Fig. 3) are for the top *tim* band, but a similar plot was obtained when the bottom band was quantitated (*19*). ZT 14 on day 4 was quantitated separately because the gel fractured adjacent to this lane before quantitation. Its value was normalized to the rest of the data by requantitating other time points from the gel at the same time. Open and closed boxes, duration of light and dark.

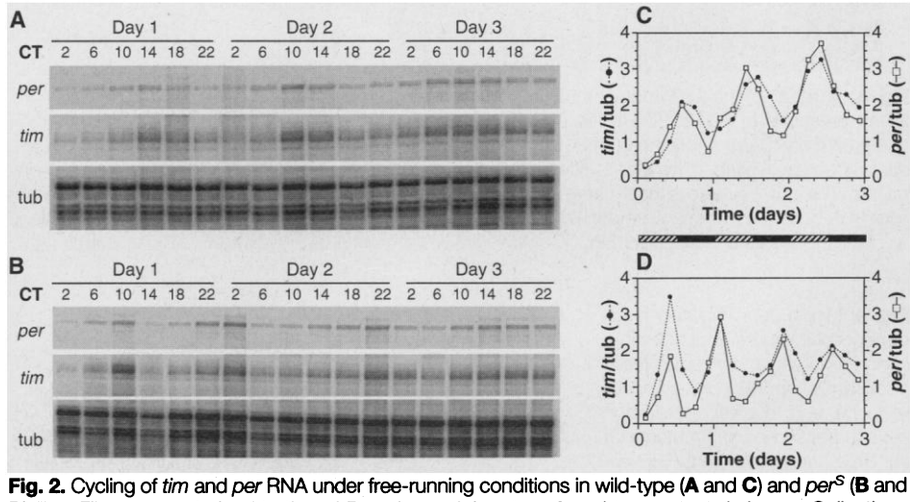

Fig. 2. Cycling of *tim* and *per* RNA under free-running conditions in wild-type (**A** and **C**) and per^S (**B** and **D**) flies. Flies were entrained to three LD cycles and then transferred to constant darkness. Collections were made at 4-hour intervals, starting 14 hours after "lights off." RNase protection assays (*20*) were performed on 15 μg of total head RNA with probes for *tim*, *per*, and tubulin. The *tim* probe protects nucleotides 4963 to 5192 of the *tim* cDNA sequence (*1*), the *per* probe is described in (*2*), and the tubulin probe is described in Fig. 1. A 3-day time course of *tim* and *per* RNA in wild-type and per^S flies in constant darkness is shown. Numbers above each lane indicate the circadian time (CT) of sample collection on three consecutive days. In free-running conditions, circadian time is used in lieu of ZT time to reflect the entrainment regimen. Gels were exposed in a phosphorimager cassette (Molecular Dynamics), and the images were transferred to Adobe Photoshop software for printout. In (C) and (D) the *tim*/tub (●) and *per*/tub (□) ratios were normalized such that the value of 1 corresponds to the mean relative *per* or *tim* RNA amount produced in wild-type flies reared in LD12:12. These values were determined by collecting time points at 12 consecutive 2-hour intervals in a single LD12:12 cycle. The *per* and *tim* RNA amounts for each time point were assessed in three separate experiments. The mean value of all time points was then set to 1. Wild-type ZT 2 and ZT 12 samples from the above experiments were also subjected to RNase protection and run on each gel for comparison to the experimental digests (*12*).

tion (6, 15), such delays in the appearance and possibly the nuclear activity of PER could be essential to any mechanism producing sustained oscillations in *per* RNA synthesis (3, 6, 14, 15). The molecular events that are responsible for these delays are unknown but are likely to involve *tim*. The accumulation of PER proteins is strongly and constitutively suppressed in tim^{01} mutants (3, 4), and nuclear localization of PER reporter proteins is blocked in the mutant (3). Thus, our finding that *tim* RNA is expressed with a circadian rhythm suggests that PER accumulation and nuclear localization are under temporal control that is dictated by the expression patterns of both *per* and *tim*.

In an accompanying report we have shown that the PER and TIM proteins physically interact with each other in vitro (11). Therefore, the dependence on *tim* function for PER accumulation and nuclear localization probably reflects a necessary interaction between the TIM and PER proteins in vivo. Because we reported in this study that the phase of *tim* RNA expression is similar to that of *per* RNA expression, the observed delays in PER accumulation and nuclear localization could reflect a concentration-dependent association of the TIM and PER proteins. Particularly as PER accumulation is suppressed in tim^{01} mutants (3, 4), PER proteins might fail to accumulate in step with *per* RNA in wild-type flies because of insufficient amounts of TIM protein at early times of RNA synthesis. In this model (Fig. 4), higher amounts of both RNAs would permit heterodimerization and stabilization of PER, but with a lag in relation to RNA synthesis. In addition, as suggested previously (3), TIM might stabilize PER by promoting nuclear as opposed to cytoplasmic localization.

Earlier work has shown that lowering *per* gene dosage or *per* RNA amounts lengthens the period (9), which supports our model. Our studies of the per^{L} mutant (11) are also consistent with this model. PER^{L} proteins, which confer long-period circadian behavioral rhythms (16), show delayed nuclear accumulation in vivo (14) and altered binding to TIM in a yeast two-hybrid assay (11). In the model proposed in Fig. 4, the time of PER accumulation and nuclear entry depend on (i) the concentrations of *per* and *tim* RNAs and (ii) the affinity of TIM for PER. It is also possible that other mechanisms and other, as yet unidentified, proteins influence the interaction between PER and TIM and affect the timing of PER accumulation and nuclear entry. Phosphorylation of PER, which has been shown to occur in a temporal manner, is one such mechanism (17).

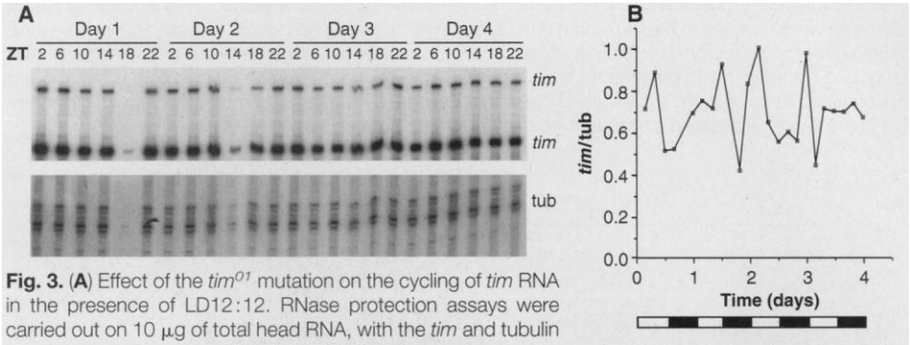

Fig. 3. (**A**) Effect of the tim^{01} mutation on the cycling of *tim* RNA in the presence of LD12:12. RNase protection assays were carried out on 10 μg of total head RNA, with the *tim* and tubulin riboprobes as described for Fig. 1. RNA determinations were made at 4-hour intervals over a 4-day period. The number above each lane indicates ZT. ZT 18 on day 1 and ZT 14 on day 2 were not quantitated, as these lanes were considerably underloaded relative to the other lanes. (**B**) Quantitations were performed as described for Fig. 1.

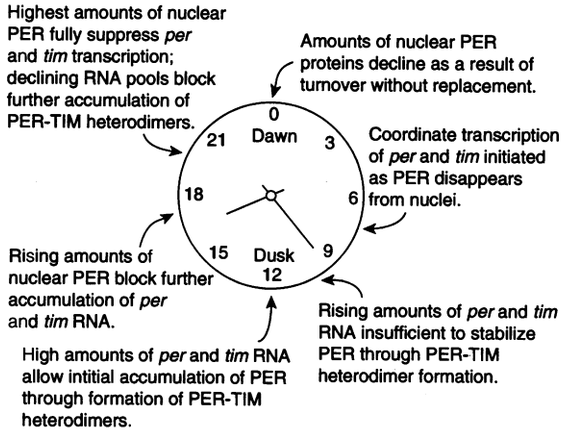

Fig. 4. Model depicting how interdependence of *per* and *tim* might generate circadian cycles in feedback regulation. PER protein accumulation and nuclear localization are suppressed in tim^{01} mutants (3, 4). Thus, we predict that PER will accumulate in wild-type flies only at times of the day when TIM proteins are present. PER proteins would accumulate in conjunction with *per* mRNA only if TIM proteins were amassed before *per* transcription. As *per* and *tim* RNAs accumulate with the same phase, delays in PER accumulation and nuclear localization are expected, probably reflecting times of PER-TIM heterodimer formation (11). If high amounts of PER protein suppress *per* expression (15), cycles in this regulation will result from separate temporal phases of *per* RNA accumulation and PER protein accumulation that are promoted by the pattern of *tim* expression. In the absence of a mechanism supporting such delays, feedback control should lead to constitutive gene and protein synthesis, albeit at intermediate levels.

REFERENCES AND NOTES

1. M. P. Myers, K. Wager-Smith, C. S. Wesley, M. W. Young, A. Sehgal, *Science* **270**, 805 (1995).
2. A. Sehgal, J. L. Price, B. Man, M. W. Young, *ibid.* **263**, 1603 (1994).
3. L. B. Vosshall, J. L. Price, A. Sehgal, L. Saez, M. W. Young, *ibid.*, p. 1606.
4. J. L. Price, M. E. Dembinska, M. W. Young, M. Rosbash, *EMBO J.* **14**, 4044 (1995).
5. R. J. Konopka, S. Wells, T. Lee, *Mol. Gen. Genet.* **190**, 284 (1983); J. Ewer, B. Frisch, M. J. Hamblen-Coyle, M. Rosbash, J. C. Hall, *J. Neurosci.* **12**, 3321 (1992); B. Frisch, P. E. Hardin, M. Hamblen-Coyle, M. Rosbash, J. C. Hall, *Neuron* **12**, 555 (1994); L. B. Vosshall and M. W. Young, *ibid.* **15**, 345 (1995).
6. P. E. Hardin, J. C. Hall, M. Rosbash, *Nature* **343**, 536 (1990); *EMBO J.* **11**, 1 (1992); *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11711 (1992).
7. P. E. Hardin, *Mol. Cell. Biol.* **14**, 7211 (1994).
8. B. D. Aronson, K. A. Johnson, J. J. Loros, J. C. Dunlap, *Science* **263**, 1578 (1994); S. K. Crosthwaite, J. J. Loros, J. C. Dunlap, *Cell* **81**, 1003 (1995).
9. M. K. Baylies, L. Wiener, L. B. Vosshall, L. Saez, M. W. Young, in *Molecular Genetics of Biological Rhythms*, M. W. Young, Ed. (Marcel-Dekker, New York, 1993), pp. 123–153.
10. A. Sehgal, unpublished data.
11. N. Gekakis *et al.*, *Science* **270**, 811 (1995).
12. A. Rothenfluh-Hilfiker and M. W. Young, unpublished data.
13. M. Zatz, *Discoveries Neurosci.* **8**, 67 (1992).
14. K. D. Curtin, Z. J. Huang, M. Rosbash, *Neuron* **14**, 365 (1995).
15. H. Zeng, P. E. Hardin, M. Rosbash, *EMBO J.* **13**, 3590 (1994).
16. R. J. Konopka and S. Benzer, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2112 (1971).
17. I. Edery, L. J. Zweibel, M. E. Dembinska, M. Rosbash, *ibid.* **91**, 2260 (1994).
18. W. E. Therkauf, H. Baum, J. Bo, P. C. Wensink, *ibid.* **83**, 8477 (1986).
19. A. Sehgal *et al.*, data not shown.
20. Total RNA was isolated from ~80 μl of fly heads with 1 ml of RNAzol B solution (TelTest, Friendswood, TX). RNase protections were performed according to the manufacturer's specifications (RPA II kit, Ambion, Austin, TX). RNA digestions were incubated for 1 hour at 37°C, and half the final sample volume was loaded and electrophoresed on a 5% acrylamide–8 M urea gel.

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