Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels

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Mutations in the *period* (*per*) gene of *Drosophila melanogaster* affect both circadian and ultradian rhythms. Levels of *per* gene product undergo circadian oscillation, and it is now shown that there is an underlying oscillation in the level of *per* RNA. The observations indicate that the cycling of *per*-encoded protein could result from *per* RNA cycling, and that there is a feedback loop through which the activity of *per*-encoded protein causes cycling of its own RNA.

CIRCADIAN rhythms influence many behaviours and physiological processes. These rhythms are generated by an endogenous circadian 'clock', persist ('free-run') under constant environmental conditions, and respond to environmental time cues. In *Drosophila melanogaster*, two well-studied phenomena—eclosion and adult locomotor activity—are under the control of the circadian clock[1].

The product of the *period* (*per*) gene in *D. melanogaster* is a candidate clock molecule[1-3]. The original three mutations described at the *per* locus either shorten (per^S), lengthen (per^{L1}) or essentially abolish (per^{O1}) circadian activity[4]. The *per* gene has been cloned and extensively analysed[1,5]. *In situ* hybridization and immunohistochemical analyses indicate that *per* is expressed in numerous adult tissues, including the eyes, antennae, lateral brain neurons and putative glia in heads, and the salivary gland, ovaries and gut in bodies[3,6,7]. Because the circadian oscillator has been mapped to the head[8], we consider that the *per* expression most relevant to clock function is located here.

In the adult visual system, the immunoreactivity of *per* gene product (Per protein) fluctuates[2]; intense staining is readily detectable in photoreceptor nuclei in the middle of the night, but essentially no staining is detectable in the middle of the day. The staining fluctuations persist in constant darkness. These

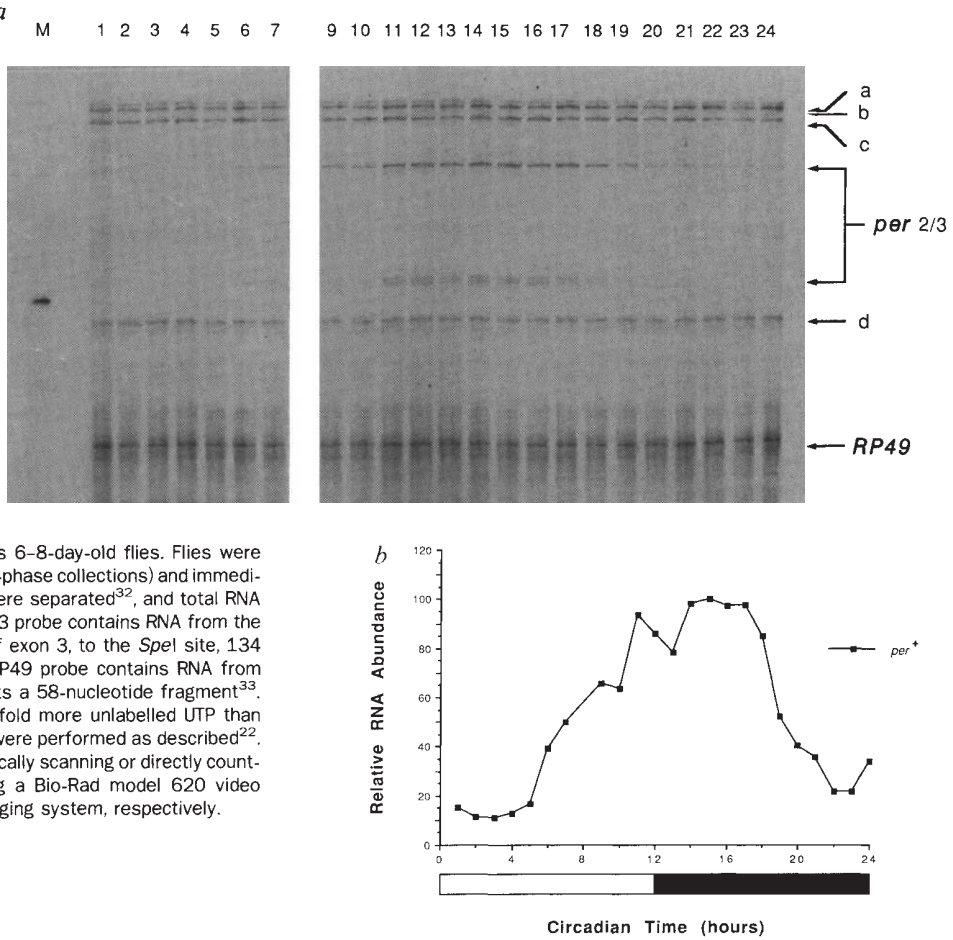

FIG. 1 Levels of per^+ cycle in flies under LD cycle conditions. *a*, RNase protections of RNA from flies collected during LD cycles. The number of hours after lights were turned on that the flies were collected are indicated above each RNase protection lane; lights were turned off after 12 h. M denotes the lane containing labelled 123-base pair markers (BRL); *per* 2/3 denotes the protected fragments from *per* RNA covering parts of exon 3 (upper) and exon 2 (lower). Bands a–d are due to incomplete digestions and full-length protections of the two probes by DNA. *b*, Quantitation of data shown in *a*. Relative RNA abundance refers to the values of *per*/*RP49*, where the peak value was adjusted to 100. The white and black bars represent lights on or off, respectively.
METHODS. Wild-type (Canton-S) adults (3–5-days-old) were exposed for 3 days to LD cycles before being collected as 6–8-day-old flies. Flies were then collected (in complete darkness for dark-phase collections) and immediately frozen on dry ice. Heads and bodies were separated[32], and total RNA was extracted from the heads[22]. The *per* 2/3 probe contains RNA from the *Bgl*II site, 259 nucleotides from the start of exon 3, to the *Spe*I site, 134 nucleotides from the end of exon 2. The RP49 probe contains RNA from the *Hin*dIII site to the *Pvu*II site and protects a 58-nucleotide fragment[33]. The RP49 probe was transcribed using 20-fold more unlabelled UTP than the *per*-specific probes. RNA hybridizations were performed as described[22]. Quantitation was done by either densitometrically scanning or directly counting the *per* exon 3 and RP49 bands using a Bio-Rad model 620 video densitometer or an AMBIS radioanalytic imaging system, respectively.

observations have recently been extended to show that there is only one peak of staining per day, that there are similar circadian fluctuations of Per immunoreactivity in additional adult central nervous system locations and that the various *per* mutations affect the antigen cycling in the same way as they affect behaviour[3].

Here we show that *per* messenger RNA levels in the fly head also undergo circadian fluctuations during both 12-h light/12-h dark (LD) cycles and constant darkness (DD), and that *per* mutations affect the cycling of their own mRNA. The observations indicate that the oscillations in Per immunoreactivity could be due to oscillations in Per protein levels, which, in turn, are due to changes in *per* mRNA levels. They also indicate that Per protein activity is involved in a feedback loop that influences the cycling of its own mRNA. We suggest that this feedback loop is a central feature of the circadian clock.

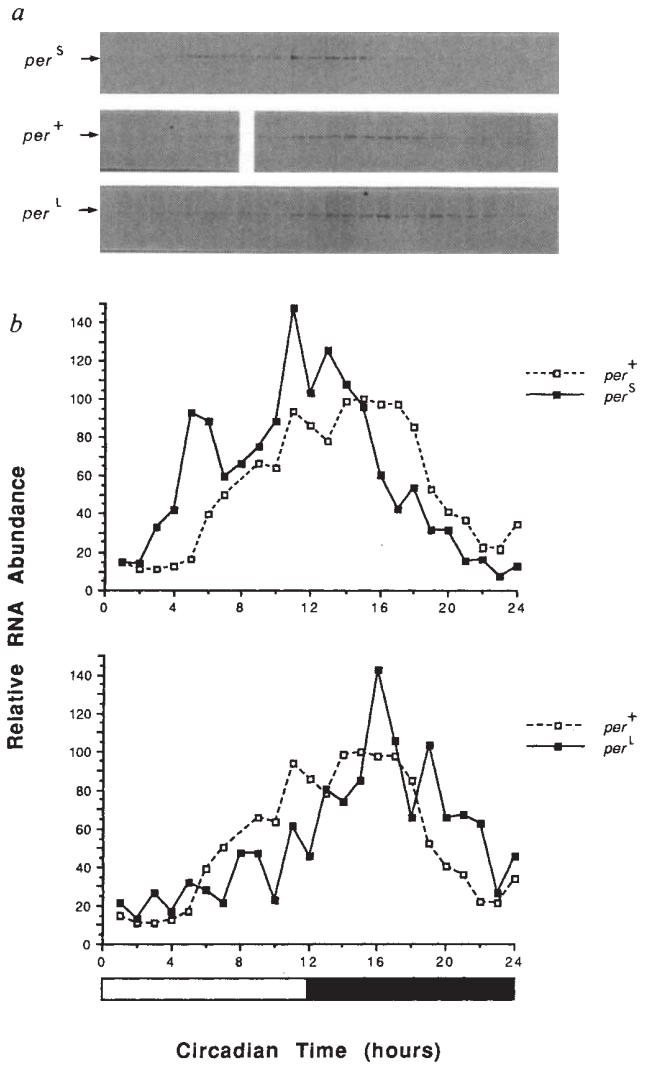

FIG. 2 Altered fluctuations of per^S and per^{L1} RNA under LD cycle conditions. *a*, Time courses of exon 3-protected fragments from per^S, per^+ and per^{L1}. Arrows point to the exon 3 *per* fragment. The times at which these collections were taken (in hours after the last time lights were turned on) are shown under the graphs in *b*. *b*, Quantitation of data shown in *a*. Relative RNA abundance and circadian time are as described in Fig. 1. The peak level of per^+ was set to 100. Both per^S (top graph) and per^{L1} (bottom graph) levels were adjusted relative to per^+ levels by comparing *per/RP49* ratios averaged from three peak time points. □, per^+ RNA levels; ■, per^S RNA levels (top graph) or per^{L1} RNA levels (bottom graph).
METHODS. The per^S and per^{L1} flies were descendants of the original short (~19-h) and long (~29-h) period mutants[4]. Both per^S and per^{L1} flies were exposed to the same light-dark cycles as in Fig. 1. Fly collections, RNA and probe preparations, and RNase protections were as in Fig. 1.

Analysis of per^+ RNA levels during LD cycles

Analysis of Per protein immunoreactivity in heads has shown that there is a stronger signal in the middle of the night than in the middle of the day[2,3]. To determine whether there is a similar fluctuation in *per* mRNA, we froze entrained wild-type (Canton-S) flies at different circadian times (CTs), extracted total RNA from heads and assayed for systematic changes in *per* mRNA levels.

RNA fragments protected by *per* antisense RNA covering parts of exons 2 and 3 (labelled *per* 2/3) fluctuated in abundance during LD cycles (Fig. 1*a*). These two exons are present in their entirety in the major (type A) and minor (types B and C) *per* RNAs[9]. Levels of *per* RNA were low when we turned lights on, started to increase about 5 h later, and peaked at about the time that we turned lights off. Maximum levels were maintained for 5–8 h before falling to minimum levels 1–2 h before we turned

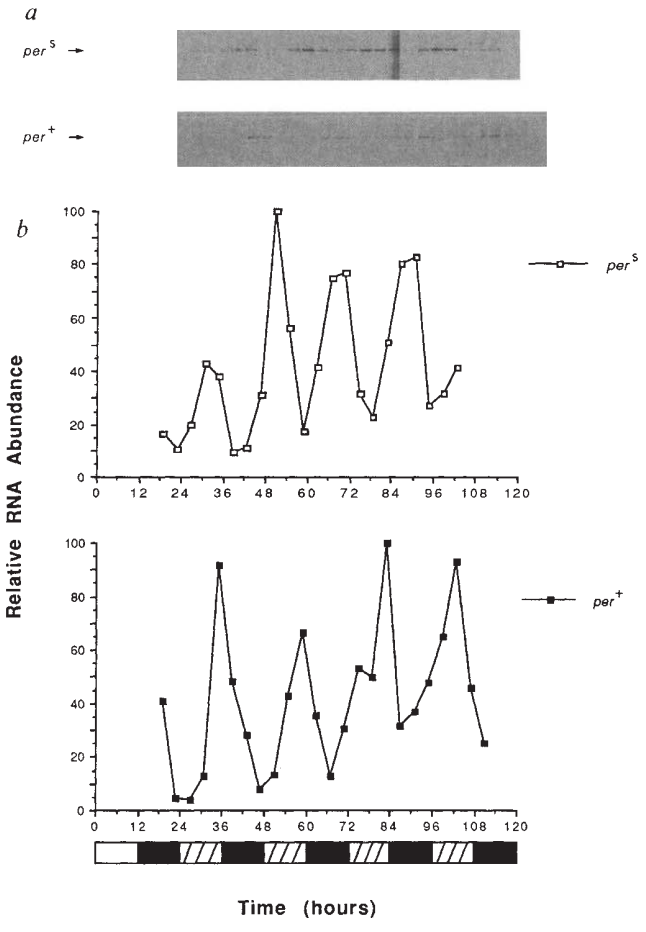

FIG. 3 The per^S and per^+ RNAs cycle with different periods under DD conditions. *a*, Protection of exon-3 fragments with time. Arrows point to the exon 3-protected fragments. These protections can be compared with those of the light regime shown at the bottom of *b*, where the white box indicates the last period when lights were on, the black boxes indicate when lights were off, and the hatched boxes indicate when the lights would have been on had the LD cycle been continued. *b*, Quantitation of data. Relative RNA abundance is as described in Fig. 1. Peak levels for both per^S (□) and per^+ (■) are set at 100. Time refers to the time after the start of the last period when lights were on.
METHODS. The per^S and per^+ flies were exposed to three complete LD cycles and then kept in complete darkness as a continuation of the dark phase of the fourth LD cycle. Flies were collected every 4 h starting 19 h after the start of the last period when lights were on and ending 84 h (per^S) and 92 h (per^+) later. All collections and RNA analyses were as in Fig. 1.

lights on. Quantitation of these data shows an amplitude (peak-to-trough ratio) of 5 to 10-fold (Fig. 1*b*). We replicated these measurements several times using RNAs from different fly collections, and consistently obtained amplitudes of 5 to 10-fold (data not shown). In addition, and to ensure that full-length *per* RNA was being assayed, we obtained identical results (although with fewer time points) with an RNase protection probe covering exon 7 and part of exon 8, and by northern blot analysis of poly(A)$^+$ head RNA (data not shown).

Levels of *per*^{S} and *per*^{L1} RNA during LD cycles

Two *per* mutations, *per*^{S} and *per*^{L1}, are single missense mutations of the *per* gene[10,11]. In addition to their effects on locomotor activity rhythms during DD cycles[3,12], these mutations also alter locomotor activity rhythms during LD cycles. Although mutant flies are rhythmic with a period of ~24 h under these conditions, the 'dusk' activity peaks of *per*^{S} and *per*^{L1} mutants occur earlier and later, respectively, than the wild-type *per*$^+$ dusk peak (M. Hamblen-Coyle and D. A. Wheeler, unpublished observations). To determine whether *per* RNA cycling is altered in *per*^{S} and *per*^{L1} mutants, we performed RNase protections using adult head RNAs from flies collected during LD cycles.

Like *per*$^+$ RNA, both *per*^{S} RNA and *per*^{L1} RNA levels displayed clear rhythmic fluctuations (Fig. 2). The patterns are very similar to that of *per*$^+$, but the *per*^{S} pattern is shifted to earlier times and the *per*^{L1} pattern to later times relative to the *per*$^+$ pattern. Both the peak levels of *per*^{S} and *per*^{L1} RNA and their 5- to 10-fold cycling amplitudes are indistinguishable from those of *per*$^+$ RNA (Fig. 2*b*, and data not shown).

Levels of *per*$^+$ and *per*^{S} RNA during DD cycles

The above results indicate that head *per* RNA levels cycle under LD cycle conditions. If this RNA cycling is like locomotor activity rhythms and not a driven rhythm (requiring the alternating light-dark cycles of the external environment), it should persist under free-running conditions. To test this prediction, we performed RNase protections on *per*$^+$ and *per*^{S} head RNA obtained from flies under DD cycle conditions.

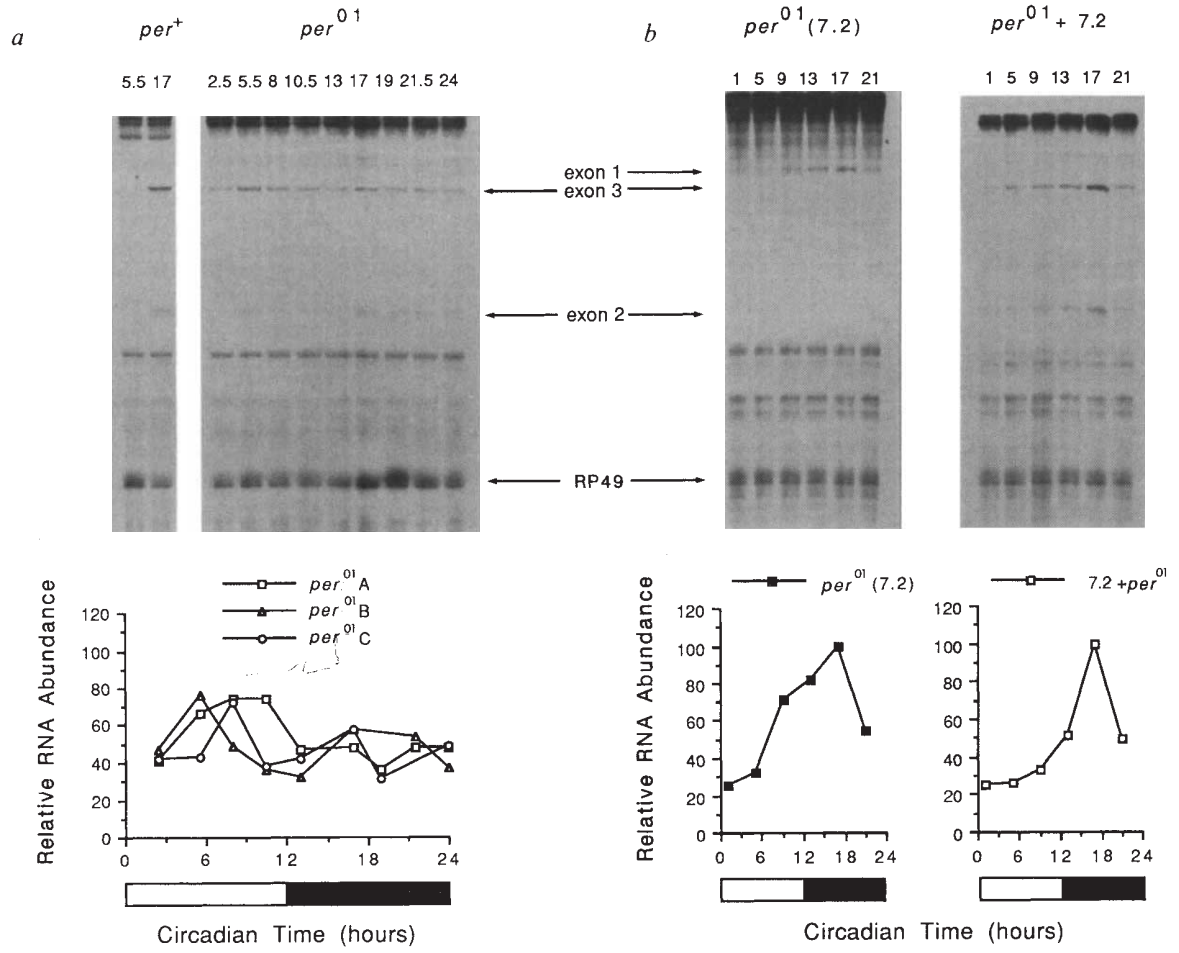

FIG. 4 A truncated *per* gene rescues the cycling of *per*^{01} RNA. *a*, RNase protection of *per*^{01} and *per*$^+$ RNAs. Times above each lane indicate the collection time, in hours, after lights were turned on. Arrows denote the positions of the *per* exon 3, *per* exon 2, and *RP49* protected fragments. The two lanes under the *per*$^+$ label and nine lanes under the *per*^{01} label contain RNase protections of wild-type and *per*^{01} head RNAs, respectively. A, B and C denote three independent *per*^{01} experiments. The level of *per*^{01} RNA is expressed as a proportion of the peak level of *per*$^+$ RNA, which is set to 100 (see Fig. 1). Relative RNA abundance and circadian time are as in Fig. 1. *b*, RNase protections of a behaviourally rescued *per* germline transformant. Times above each lane indicate the collection time, in hours, after lights were turned on. RNase protections of *per*^{01} RNA from *per*$^{01(7.2:9)}$ germline transformants using the exon 1 probe are referred to as *per*^{01}(7.2); *per*^{01} +7.2 refers to RNase protections of both the *per*^{01} and the 7.2-kb RNAs from the *per*$^{01(7.2:9)}$ germline transformants using the exon 2/3 probe. Arrows denote the positions of protected fragments from *per* exon 1, *per* exon 2, *per* exon 3 and *RP49*. (In some lanes, for example, lanes 17 and 19, there is a detectable smudge, just above the *RP49* band; this did not interfere with quantitation of the *RP49* protected fragment.) Relative RNA abundance and circadian time are as in Fig. 1. The peak levels of *per*^{01} (7.2) and 7.2 + *per*^{01} were set to 100.

METHODS. The *per*^{01} flies were descendants of the original arrhythmic strain[4]. Wild-type and *per*^{01} flies were exposed to the same light-dark regimes, collection procedures, and analyses as in Fig. 1. In addition, a *per* probe specific for exon 1 was used. This probe was transcribed from a *Pst*I site, 348 nucleotides from the start of exon 1, to a *Sal*I site, 28 nucleotides from the start of exon 1 (ref. 9).

Over 4 days, we observed four peaks in the level of per^+ RNA. The data indicate a period of ~24 h (Fig. 3*a*), indistinguishable from the average locomotor activity rhythm of wild-type flies measured during DD cycles[1,13]. These peaks were in phase with the LD peak shown in Fig. 1, although the last peak occurred 4 h earlier than predicted from an exact 24-h rhythm. The amplitude, initially similar to the LD value (Fig. 1), decreased noticeably with time. This was due to a 2- to 3-fold increase in the trough RNA level (Fig. 3*b*).

The per^S RNA also cycled under DD cycle conditions, with each successive peak occurring earlier than its per^+ counterpart (Fig. 3*a*). By measuring the time between peaks, we could estimate a period of ~20 h (Fig. 3*b*). This period is close to the per^S periods obtained with behavioural measurements[1]. As with the per^+ RNA, the per^S RNA amplitude fell during the timecourse because of an increase in the trough RNA levels.

We also performed RNAse protections on head per^{L1} RNA obtained from flies under DD cycle conditions. There was no discernible RNA rhythm in these flies (data not shown). This result could be related to the fact that the behavioural rhythms of per^{L1} mutants, as well as their protein immunoreactivity (in LD cycles), are weak[3,14].

RNase protection

The per^{O1} mutation is a nonsense mutation in the *per* gene at a codon corresponding to position 464 of the amino-acid sequence[10,11], and has little or no biological activity in flies under DD cycle conditions[1]. Under LD cycle conditions, flies are rhythmic with 24-h periods, but the rhythmic behaviour is distinct from that of wild-type flies. Rhythmic activity in the mutant seems to occur largely in response to the rhythmically applied external light cues rather than in response to an endogenous circadian clock[15–17]. To determine if per^{O1} RNA cycles, we used an LD-cycle timecourse of head per^{O1} RNA for RNase protections. Levels of per^{O1} RNA showed some variation (≤twofold) but no consistent rhythmic fluctuations; the level of per^{O1} RNA was ~50% of that of per^+ RNA at its peak (Fig. 4*a*). These data indicate that the per^{O1} flies contain a non-cycling median level of head *per* RNA. We cannot exclude the possibility, however, that in individual flies the cycling of per^{O1} RNA occurs, but that in the per^{O1}-mutant fly population it is not synchronized.

Germline transformation of arrhythmic per^{O1} flies with wild-type *per* DNA can restore rhythmic activity[9–11,13,17–19]. We exploited this observation to test whether the per^{O1} transcript undergoes circadian cycling in a rhythmic strain. To facilitate the analysis, we used a strain in which the per^{O1} RNA is distinguishable from the transcript derived from the transformed *per* DNA. This strain ($per^{O1(7.2:9)}$) carries a transformed 7.2-kilobase (kb) piece of *per* DNA that contains all of the *per* coding DNA but is missing much of the noncoding DNA including exon 1. It has weaker locomotor activity rhythms than the per^+ strain, with periods of 25–26 h[13].

Results of protections using the *per* 2/3 probe, which does not distinguish between the RNAs derived from the 7.2-kb fragment and per^{O1}, showed that *per* RNA levels fluctuated with an amplitude of ~4 (Fig. 4*b*). The phase is indistinguishable from that observed with wild-type flies. To examine specifically the per^{O1}-derived transcript, we used a probe specific for exon 1. Analysis of the same $per^{O1(7.2:9)}$ RNA samples showed that the per^{O1} RNA also cycles with an amplitude of ~4. We have also observed a similar but lower amplitude cycling of per^{O1} RNA in another independent transformed line, $per^{O1(14.6:21)13,18}$ (data not shown). We conclude that an active *per* gene rescues cycling of per^{O1} RNA as well as locomotor activity rhythms of the arrhythmic host strain.

Implications of *per* RNA cycling

The abundance of *per* RNA from adult heads fluctuates about 10-fold during a light–dark cycle in wild-type flies. We have observed similar results by analysing separately eye RNA and brain RNA (data not shown).

There are several likely reasons why this dramatic cycling was not noted in previous studies[20,21]. First, RNA was isolated from whole flies. The cycling amplitude is much lower for body RNA than for head RNA; as a consequence, the cycling amplitude of RNA isolated from whole flies is considerably lower than that of RNA isolated from heads. Second, most of our previous cycling experiments were performed under DD cycle conditions, where the RNA cycling is less dramatic. Third, the abundance of a transcript termed the '0.9-kb RNA' fluctuates more than 20-fold over the course of a day[20]. It has now been shown that apparent differences in 0.9-kb RNA levels were due to developmental gating during the process of eclosion; the abundance of this RNA does not cycle as a function of circadian time[22].

Although the results described here indicate that the *per* RNA fluctuations underlie the previously observed Per protein changes, the mutant analyses and the per^{O1} rescue experiment show that the reverse is also the case, that is, that Per protein levels or activities, or both, also affect the *per* RNA cycling. These observations are most easily explained by postulating that feedback of the *per* gene product regulates its own mRNA levels

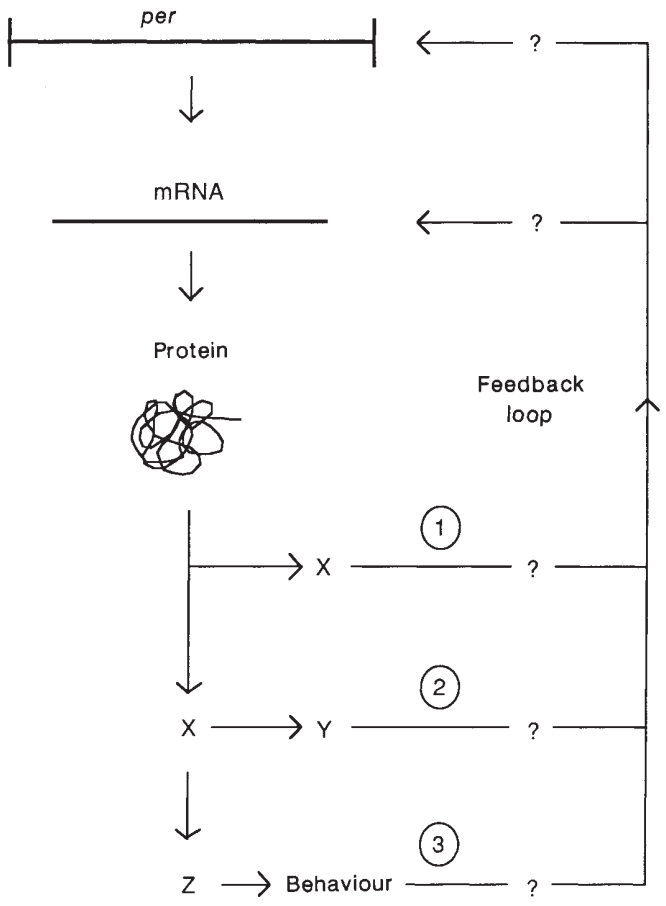

FIG. 5 Per protein feedback regulates the level of *per* RNA. The *per* gene (top thick line) is transcribed to yield *per* RNA (lower thick line) which is then translated to give Per protein (tangled line). X represents the result (unknown) of *per* activity, which is increased in per^S and decreased in per^{L1} mutants, as shown by Smith and Konopka[34]. The Per protein could feed back (1) directly, (2) through intermediate biochemical steps (represented by Y); or (3) through the behaviour of the animal, to regulate *per* RNA levels. Z represents additional components that could lie in the pathway between *per* activity and behaviour. The cycling of *per* RNA could be effected by transcriptional or post-transcriptional feedback, depicted by arrows pointing back to the gene and the mRNA, respectively.

(Fig. 5). We do not know at what level this regulation occurs (transcriptional or post-transcriptional), nor do we know whether the effect of *per* activity is direct or indirect. An indirect feedback loop could even include some aspect of rhythmic behaviour, as all of the experimental manipulations we have used so far affect locomotor activity and RNA cycling in parallel.

There is strong evidence that *per* gene activity influences the function of the circadian clock[1,4,5]. We suggest that the feedback loop described here is also an important component of the circadian pacemaker. The fluctuating protein levels are a consequence of, and contribute to, the fluctuating RNA levels. Both of these fluctuations underlie the behavioural and biochemical oscillations characteristic of circadian rhythms. Although *per* RNA cycling could be associated with the clock output pathway (as is probably the case in other systems where specific RNA levels cycle in a circadian manner[23-31]), the existence of such a feedback mechanism could make it difficult to distinguish clearly between clock function and clock output. □

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LETTERS TO NATURE

Young ages of two diogenites and their genetic implications

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THE achondrite meteorites known as eucrites and diogenites are thought to be igneous rocks derived from the crust and mantle, respectively, of a common parent planet or asteroid[1,2]. Several eucrites have been dated at 4.5–4.6 Gyr by the Rb–Sr method[4-6], and a Rb–Sr age of 4.45±0.18 Gyr ($\lambda = 1.42 \times 10^{-11}$ yr^{-1}) has been reported[3] for three diogenites. From these data, one cannot judge whether diogenites and eucrites are the same age. Here we present precise Rb–Sr data for two diogenites (Tatahouine and Johnstown) that yield an age of 4.394±0.011 Gyr—significantly younger than our age of 4.52±0.15 Gyr for the Juvinas eucrite. The low initial $^{87}Sr/^{86}Sr$ ratio of these diogenites, and their rare-earth element abundance patterns, are inconsistent with the interpretation that the young ages reflect secondary metamorphism. We suggest that our data raise doubts about a genetically close relationship between eucrites and these diogenites, and that there exist groups of diogenites that are genetically distinct from one another. Our results also suggest that igneous activity in the assumed parent body continued for about one hundred million years after its formation 4.52 Gyr ago. The parent body may thus have had a complicated evolutionary history.

Here we consider only the dates obtained using the Rb–Sr method so that we may discuss the relative ages of the meteorites irrespective of the accuracy of the decay constant of the parent nuclide. Strontium isotope ratios were measured with a VG-354 multi-collector mass spectrometer and the REE, Sr and Rb abundances were determined by the mass-spectrometric isotope-dilution method using a JEOL JMS-05RB mass spectrometer.

The ^{87}Rb–^{87}Sr analytical data and the Rb–Sr dates are given in Tables 1 and 2, respectively. Total blanks of Rb and Sr for 500–900-mg samples were 0.008 and 0.015 ng respectively, and because they never exceeded 0.05% of the sample values we did not make blank corrections. The Johnstown diogenite consists of coarse-grained clasts (centimetre-sized) and brecciated matrix. According to electron-probe microanalysis the coarse-grained clasts were orthopyroxene (opx; $En_{73-74}Fs_{23-24}$, where En is enstatite and Fs is forsterite) and the brecciated matrix contains mainly fragments of orthopyroxene, the composition of which is in the same range as that of the clasts. We analysed four samples from Johnstown: one sample containing several clasts and three samples from the brecciated portion. The Juvinas eucrite was separated into three fractions with heavy liquids (acetone, bromoform and methylene iodide saturated with iodoform).

As shown in Table 2, the eight diogenite samples (four from Tatahouine and four from Johnstown) define an age of 4.394± 0.011 Gyr, with quite a small uncertainty. The isochron for these samples is shown in Fig. 1. We note that the data of Tatahouine-1 and -2 lie very close together, as do the data of Tatahouine-3 and -4, but that they are different from one another. Also, the data points for the Johnstown samples lie far from those for the Tatahouine samples, but all the diogenite data is described by a single isochron. The dotted line in Fig. 1 is the isochron for the Juvinas eucrite, corresponding to an age of 4.52 Gyr. The age of Juvinas has an uncertainty of 0.15 Gyr (see Table 2) so that the lower limit is 4.37 Gyr. As previous workers have reported[4-6], the Rb–Sr ages obtained for Juvinas and other typical eucrites are in the range 4.51–4.54 Gyr (with uncertainties of 0.04–0.05 Gyr) so it is unlikely that the age of Juvinas lies in the range 4.37–4.39 Gyr. Given the ages of the Tatahouine and Johnstown samples (4.39 ± 0.01 Gyr), these diogenites are almost certainly younger than the eucrites. Birck and Allègre[3] reported that the Rb–Sr system of Tatahouine was disturbed on a small length scale. We do not believe that our results reflect such a