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The *period* gene of *Drosophila* carries species-specific behavioral instructions

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We have analyzed and compared the circadian locomotor activity rhythms of *Drosophila melanogaster* and *D.pseudoobscura*. The rhythms of *D.pseudoobscura* are stronger and the periods shorter than those of *D.melanogaster*. We have also transformed *D.melanogaster* flies with a hybrid gene containing the coding region of the *D.pseudoobscura period (per)* gene. Behavioral assays of flies containing this hybrid gene show that the *per* protein encoded by the *D.pseudoobscura per* gene is able to rescue the rhythmic deficiencies of arrhythmic, per^{o1} *D.melanogaster*. More important, the rhythms of some of these strains are stronger and the periods shorter than those of *D.melanogaster* (and those of transformants which carry the equivalent *D.melanogaster per* gene construct) and hence resemble those of *D.pseudoobscura*. The results suggest that the primary amino acid sequence of the *per* gene encodes species-specific behavioral instructions that are detectable when only the *per* gene is transferred to a different species.

Key words: locomotor behavior/*Drosophila melanogaster*/*Drosophila pseudoobscura*/circadian rhythms/transformation

Introduction

The *period (per)* gene of *Drosophila melanogaster* is the best characterized of all genes that control or influence the fly's circadian and ultradian rhythmic behavior (for review, see Konopka, 1987). *per* has been studied extensively at the molecular level, and within the last few years its gene structure and rather complicated pattern of expression have been described (for review, see Hall and Rosbash, 1988). Recently, it has been shown that expression of *per* in adults is necessary, and expression during development unnecessary, for maintaining rhythmic circadian locomotor activity (Ewer *et al.*, 1988). Thus, *per* may participate in ongoing oscillator functioning rather than in the construction of the fly's circadian pacemaker.

The X chromosomal *per* mutations affect both circadian locomotor activity rhythms and circadian eclosion rhythms in a similar fashion. Arrhythmic alleles (of which per^{o1} is the first and best studied example) essentially eliminate circadian rhythms, whereas the short period allele (per^S) and long period alleles (e.g. per^{L1}) shorten and lengthen the period, respectively (to 19 h and ~29 h, respectively) (Konopka and Benzer, 1971). The *per* gene also affects a high frequency fluctuation (period ~1 min) in the male courtship song. The mutant alleles influence this fast cycling (ultradian) rhythm in a manner similar to the effect on circadian rhythms; namely, per^{o1} flies are essentially arrhythmic, whereas per^S and per^{L1} flies have a courtship song rhythm of ~40 s and ~80 s, respectively (Kyriacou and Hall, 1980).

Yet there is reason to suspect that *per*'s effects on courtship song rhythms and circadian rhythms may not be identical. This suspicion is based on the fact that closely related *Drosophila* species manifest very similar circadian rhythms but different courtship song rhythms (Kyriacou and Hall, 1980, 1986). For *D.melanogaster* and *D.simulans*, the interspecific behavioral difference has been shown to map to the X chromosome (Kyriacou and Hall, 1986); this could mean that the *per* gene is responsible for the song rhythm difference between these two species. Also, we have previously shown that a region of the *per* gene can contribute differentially to circadian and ultradian rhythms; deletion of a Thr–Gly perfect repeat region gave rise to a marked effect on the male courtship song rhythm but little or no effect on the period of the circadian rhythm (Yu *et al.*, 1987). These data suggested that the *per* coding region might indeed contribute to species-specific features of behavior.

Recent work from this laboratory has tested one prediction of this hypothesis, namely, that the *per* coding region manifests substantial interspecific differences. In a comparison of the *per* coding regions of *D.melanogaster*, *D.virilis* and *D.pseudoobscura*, we have shown that only 60–70% of the *per* protein is well conserved. Interspersed are several non-conserved blocks, encoding 30–40% of the protein and including the *D.melanogaster* Thr–Gly repeat region, in which little similarity can be detected among these three species (Colot *et al.*, 1988).

A second prediction is that species-specific features of behavior can be transferred between species with *per* DNA. We have now performed the first experiments of this kind using *D.melanogaster*, *D.pseudoobscura* and *per* DNA cloned from them. There is no known courtship song difference between these two species, simply because singing rhythms have not yet been looked for in *D.pseudoobscura* (males of which sing a more complicated song than for *D.melanogaster*; Bennet-Clark and Ewing, 1970). Yet, we noticed that the characteristics of circadian behavioral rhythms are, by inspection, quite different for *D.melanogaster* versus *D.pseudoobscura* adults. To test whether the markedly diverged *per* genes in these two species could contribute to the rhythm variation, we first quantified features characteristic of *D.melanogaster*'s and *D.pseudoobscura*'s circadian locomotor behavior. We then transformed *D.melanogaster* with a *D.melanogaster*/*D.pseudoobscura* hybrid *per* gene containing the coding region from the *D.pseudoobscura per* gene. The behavioral

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results suggest that the primary amino acid sequence of the *per* gene encodes species-specific behavioral instructions.

Results and discussion

Activity assays of D.melanogaster and D.pseudoobscura flies

Both eclosion rhythms and locomotor activity rhythms of *D.melanogaster* have been extensively examined, whereas only eclosion rhythms of *D.pseudoobscura* have been well studied (Pittendrigh, 1954, 1967, 1974; Konopka, 1987). Although relatively little is known about circadian locomotor activity of individual *D.pseudoobscura* flies, it has been suggested that *D.pseudoobscura* contains two different oscillating systems: one controlling circadian locomotor activity with a period of ~22.5 h and the other regulating eclosion rhythms with a period of 24 h (Engelmann and Mack, 1978).

The first objective of this study was to compare the locomotor activity of these two species to determine whether there are significant differences in free running period and/or in other behavioral features. To this end we compared the flies' circadian behavior in free run (constant darkness = DD) following two different entrainment regimes. (i) The usual entrainment scheme previously used in this laboratory for *D.melanogaster* (Hamblen *et al.*, 1986): flies are raised in 12 h light:12 h dark cycles, then entrained by exposure to four cycles of alternating light and dark (again 12:12 LD, but with the beginning of lights-on 4 h later than in the rearing LD cycles), and then placed in constant darkness (DD) for activity monitoring (abbreviation for this scheme: LD−DD). (ii) A regime previously used only for *D.pseudoobscura* but not for *D.melanogaster* (Engelmann and Mack, 1978): flies are reared in constant light (LL) and, after a single transition to constant darkness, the activity is monitored for several days (LL−DD).

We applied a somewhat specialized procedure for analysis of these activity data, to gain insight into 'qualitative' aspects of free-running behavior (i.e. in addition to obtaining quantitative estimates of periodicity). The relevant program, called 'eduction' (Sulzman, 1982; F.M.Sulzman, personal communication), searches for the period by optimal alignment of activity per 0.5-h bin for a complete activity run (in our experiments, between 5 and 10 days). Figure 1 shows eduction profiles of representative flies from the two species, for both schemes [(LD−DD) and (LL−DD)]. Plotted are the number of activity events per bin; these are mean numbers (±SEM), i.e. obtained by averaging activity events from corresponding phases of successive cycles for the 5−10 days.

The *D.melanogaster* free running periods were 23.9 h in (LD−DD) and 23.8 h in (LL−DD). *D.pseudoobscura* periods were shorter: 23.3 h in (LD−DD) and 23.1 h in (LL−DD) (Table I, A−D). The interspecific difference [0.6 h (LD−DD) and 0.7 h (LL−DD)] is statistically significant ($P < 0.003$, Mann−Whitney U-Test; see Table I). The relatively short period of *D.pseudoobscura* is consistent with previous reports (Engelmann and Mack, 1978). There is no significant difference between the effects of the two regimes on the average period of a single genotype, although the free running periods after LD entrainment are slightly closer to the entraining period (i.e. 24 h). The fraction of arrhythmic wild-type flies was greater for *D.melanogaster* than for *D.pseudoobscura*, and greater without entrainment (LL−DD) than with entrainment (LD−DD) (Table I, and data not shown). All of these data are consistent with the notion that *D.pseudoobscura* has a stronger locomotor activity rhythm.

In addition to the difference in free running period, there was a striking difference in the activity distribution, both between the two species and between the two kinds of light/dark conditions to which the flies were exposed. With prior entrainment, *D.pseudoobscura* had essentially only a single, subjective evening ('evening') peak, whereas *D.melanogaster* had an almost bimodal activity distribution with two peaks, subjective morning ('morning') and 'evening' (Figure 1). Without prior entrainment, the *D.pseudoobscura* activity pattern did not change markedly, although it did sharpen somewhat (see below). The activity distribution of *D.melanogaster*, however, was strongly affected by the absence of prior entrainment. Under those conditions, the *D.melanogaster* flies exhibited weaker rhythms, without very sharp differences between periods of activity and inactivity. More specifically, these flies failed to manifest significant rest periods (0.5-h bins with few or no activity events), in contrast to *D.pseudoobscura* flies and to *D.melanogaster* flies that had been previously entrained (e.g. Figure 1). In addition, the *D.melanogaster* bimodal activity distribution was not as clear as with prior entrainment: 'morning' and 'evening' peaks were no longer separated by a clear, relatively inactive period but merged to give rise to a broad peak (e.g. Figure 1).

We have also estimated the relative strengths of the activity peaks using the eduction analysis. The average activities per 0.5-h bin have been calculated. Bins with activity below this value have been defined as inactive. Inactive 0.5-h bins have been counted accordingly and the degree of inactivity or resting time calculated as a percent of the period, thereby normalizing for differences in period length. For entrained flies these values are 35% for *D.melanogaster* and 55% for *D.pseudoobscura*, and, for non-entrained flies, 35% for *D.melanogaster* and 59% for *D.pseudoobscura* (Table I). Statistical analysis showed that the resting time is significantly different between the two genotypes ($P < 0.0001$).

We decided to use the LL−DD (i.e. without entrainment) regime in subsequent experiments for three reasons. First, the qualitative differences between the two species is greatest without prior entrainment. Second, the resting time value difference is also greater under these conditions, due in large part to the fact that *D.pseudoobscura* responds to the one light−dark-transition regime by generating stronger rhythms, with qualitatively sharper peaks and longer resting times (Figure 1 and Table I). Third, the average activity of these two species is more similar under these conditions than with prior entrainment (see Figure 1 legend).

We examined, in such LL−DD experiments, a transformant type previously analyzed only in LD−DD tests. The relevant *per* transforming fragment is 13.2 kb; it contains the entire *per* coding region with several kb of 5′ flanking and 3′ flanking DNA (Citri *et al.*, 1987; see Figure 2). With prior entrainment, this fragment rescued well the rhythmic deficits of arrhythmic, per^{o1} host flies, i.e. the rhythms of single insert 13.2 kb per^{o1} flies were robust and only slightly longer than those of wild-type flies, suggesting that this fragment provides near-normal *per* activity (Citri *et al.*, 1987; Yu *et al.*, 1987). In the present study, 16 per^{o1} flies containing two doses of this fragment were tested by eduction

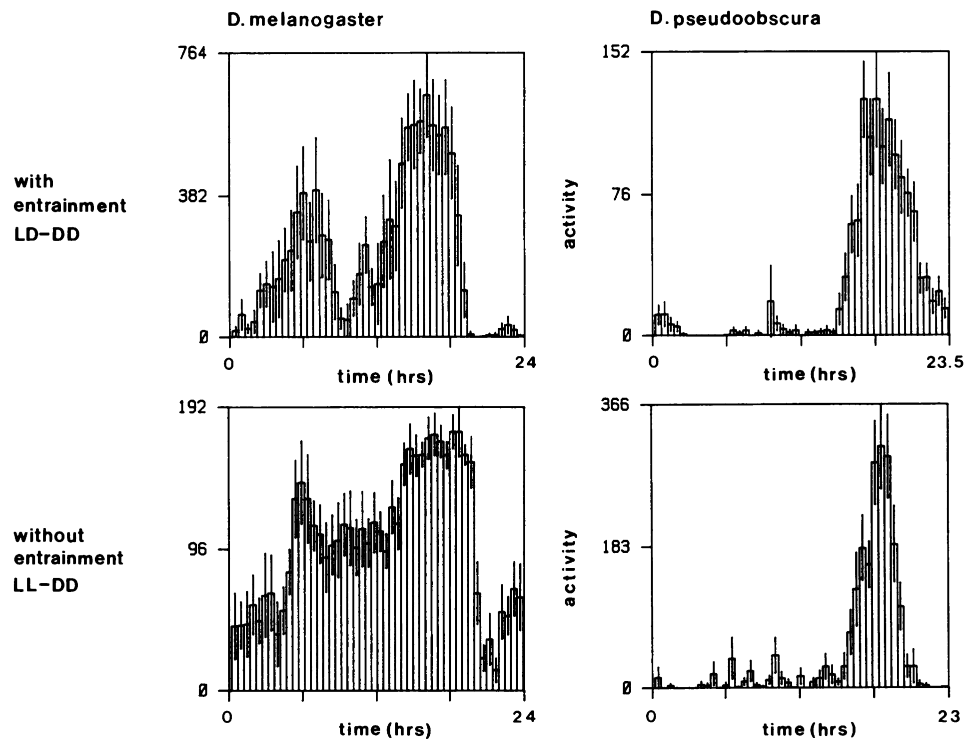

Fig. 1. Profiles of locomotor activity rhythms. Eduction plots (see Materials and methods) of the behavior monitored for four individual, wild-type flies during free run (constant darkness). *D.melanogaster* on the **left** (**top** and **bottom**), *D.pseudoobscura* on the **right**. The Y-axis shows the average (per period) activity (±SEM) and the X-axis gives the period in h, divided into bins of 0.5 h. The upper panel shows the activity distribution after entrainment (LD−DD); the lower panel shows the activity distribution without prior entrainment (LL−DD). Average activity events per 0.5-h bin (± SEM) were, for *D.melanogaster*, 173 ± 22 (LD−DD) and 41 ± 7 (LL−DD); for *D.pseudoobscura*, 27 ± 2 (LD−DD) and 27 ± 3 (LL−DD). For *D.pseudoobscura*, the behaviorally tested strain was the 'Ayala reference' wild-type; this is the strain from which the *D.pseudoobscura per* gene was cloned (Colot *et al.*, 1988). For *D.melanogaster*, flies from the Canton-S wild-type strain were tested. For the LD−DD runs, lights-on occurred at 8 h and lights-off at 16 h.

Table I. Summary of behavioral data

Genotype	Flies tested			Period (h ± SEM)	Resting time re average activity (% of period ± SEM)
	n	Arrhythmic	Rhythmic		
With entrainment LD−DD					
A *D.melanogaster*	24	3	21	23.9 ± 0.1	35 ± 2
B *D.pseudoobscura*	19	—	19	23.3 ± 0.2	55 ± 3
Without entrainment LL−DD					
C *D.melanogaster*	23	5	18	23.8 ± 0.2	35 ± 3
D *D.pseudoobscura*	27	—	27	23.1 ± 0.1	59 ± 3
E 2 doses 13.2 in *D.melanogaster* per^{01}	16	—	16	23.5 ± 0.1	38 ± 3
F 1 dose *D.pseudoobscura* in *D.melanogaster* per^{01}	64	58	6	29.3 ± 1.8	n.d.[a]
G 2 doses *D.pseudoobscura* in *D.melanogaster* per^{01}	34	25	9	29.6 ± 0.8	50 ± 6
H 13.2 *D.melanogaster* in *D.melanogaster*	30	2	28	23.7 ± 0.1	36 ± 2
I *D.pseudoobscura* in *D.melanogaster*	38	2	36	23.3 ± 0.1	48 ± 3

Results from testing flies from all genotypes under the two different conditions. LD−LL (lines A and B) means rearing in light:dark cycles followed by monitoring in constant darkness. LL−DD (lines C through I) means rearing in constant light followed by monitoring in constant darkness (i.e. after one light:dark transition). Rhythmicity was determined by χ^2 periodogram analysis and periods determined by the eduction program. The 'resting time' for a given fly's behavior was estimated by first computing the average activity per 0.5-h bin for the several days of monitoring (cf. Figure 1 legend); then the number of bins in the eduction plot for this fly that were less than the average activity value were counted. Statistical significance of differences in period or resting time between pairs of strains (e.g. line H versus I) was in all cases determined by the Mann−Whitney U-test. In the case of lines H and I, data from two different strains were pooled, since the behavioral results were homogeneous (i.e. for 13.2:2 versus 13.2:34 or hyb20 versus hyb26; see below). For line H, the strains (originally reported by Citri *et al.*, 1987) were: 13.2:2, 13 rhythmic flies, 23.8 h period, resting time 35%; 13.2:34, 15 rhythmic flies, 23.5 h period, resting time 38%. For line I, the strains (see Materials and methods) were: hyb20, 16 rhythmic flies, 23.2 h period, resting time 48%; hyb26, 20 rhythmic flies, 23.2 h period, resting time 48%.
[a]Resting time was not determined.

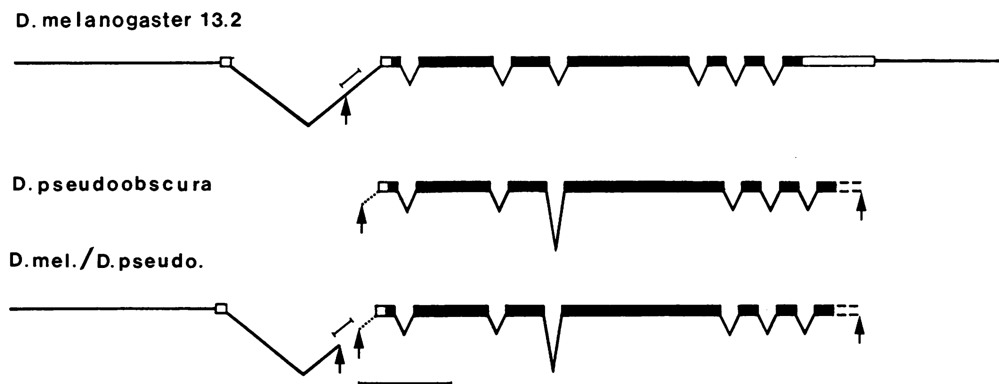

Fig. 2. *per* gene constructions. The structures of the 13.2 kb fragment of the *D.melanogaster per* gene (**top**) and the 5.6 kb fragment of the *D.pseudoobscura per* gene (**middle**) are shown. The thin, horizontal lines indicate untranslated 5′ and 3′ regions, the open boxes indicate non-coding transcribed DNA, the filled boxes indicate coding regions, and each 'V' designates an intron. Arrows indicate the exchange points that were used to generate the chimeric *D.melanogaster/D.pseudoobscura per* construct (**bottom**; also see text). The small bar above a portion of the first intron of the *D.melanogaster* gene indicates the portion deleted in the chimeric construct and the thin dotted line indicates a portion of the putative *D.pseudoobscura* first intron. Dashed lines at the 3′ end indicate *D.pseudoobscura* DNA which may or may not be transcribed. The bar below the bottom construct indicates the fragment used in the RNase mapping shown in Figure 3.

analysis. All 16 were rhythmic, with periods slightly shorter and resting times slightly longer than the wild-type control (Table I). The shorter periods and longer resting times probably reflect the extra dose of *per* present in this genotype as compared to the wild-type, male controls. [Extra doses of per^+ shorten the free running period (Smith and Konopka, 1982; Cote and Brody, 1986).]

Transformation and expression of the hybrid gene

Having determined that the LL−DD protocol can most readily allow a diagnosis of the behavioral differences between species, we sought to examine the circadian rhythms of *D.melanogaster* flies in which the *D.pseudoobscura* gene was present. To examine the relative efficacy of the *pseudoobscura per* coding region, a *D.pseudoobscura* construct comparable to the *D.melanogaster* 13.2 kb fragment was designed. However, to minimize potential problems associated with expression of the *trans*-gene or with interpretation of the data, a hybrid construct was engineered (Figure 2). Like the *D.melanogaster* 13.2 kb fragment, it includes 4 kb of 5′ flanking DNA, the first (untranslated) exon and the first, large intron up to the *Xba*I site (350 bp upstream of the second exon), all from the *D.melanogaster* gene. This is followed by ~190 bp of putative first intron, 50 bp of the non-coding portion of exon 2, and the entire protein coding region, including six introns, from *D.pseudoobscura*. The objective was to retain the putative *cis*-acting regulatory regions of *D.melanogaster*, so that behavioral properties encoded by the hybrid *trans*-gene that differ from those encoded by the *D.melanogaster trans*-gene could be attributed to the *per* coding region. With this construct in a cp20.1 vector (see Materials and methods), we generated five independent, transformed lines carrying the hybrid *D.melanogaster/D.pseudoobscura per* gene.

Because this hybrid construct contains a hybrid intron 1, we chose to use RNase mapping to assay simultaneously expression and proper splicing of the second exon. The probe used for the protection experiments (see Figure 2) covered the 3′ end of the putative first intron, the second exon, second intron and the 5′ half of the third exon such that fragments of 140 and 290 nt (the sizes of the second exon and the 5′ half of the third exon, respectively) were expected. No signal was detectable with pA^+ RNA from the background *D.melanogaster* per^{o1} strain, almost certainly because of the extensive sequence divergence between the *D.melanogaster* and *D.pseudoobscura* exons. Both expected fragments were observed with RNA from the transformant strains and from *D.pseudoobscura* wild-type flies, as shown in Figure 3, suggesting that the hybrid first intron does not adversely affect splicing. Within a factor of two, the RNA level was the same in the transformant as in the *D.pseudoobscura* wild-type flies, suggesting that gene expression from the *trans*-gene is comparable to that from a wild-type *per* gene. Also, the per^{o1} hybrid gene-containing flies synthesize *per* protein, as assayed histologically with an antibody reagent specific for the *per* product (data not shown; see Materials and methods).

Behavioral analysis of hybrid gene transformants

As described above, flies were tested according to the scheme that showed the most prominent behavioral differences between the two species with comparable activity levels, i.e. free run without prior entrainment (LL−DD). Of all flies carrying only one dose of the hybrid gene, 90% were arrhythmic. In the remaining 10% a very weak rhythm could be detected by χ^2 periodogram analysis (Table I, F). Clear rescue of rhythmicity was only observed in transformants with two doses of the hybrid gene, although the proportion of rhythmic flies was relatively low, 30% for the summary data from both lines (Table I, G). The free running periods were variable and long (ranging from 27 to 33 h). Nevertheless, analysis of eduction profiles revealed an activity distribution comparable to that of *D.pseudoobscura*: one major peak of activity (which is not quite as sharp as the *D.pseudoobscura* wild-type peak), with the activity values approaching zero at several points, and long rest times, 50% on average (see Table I, G).

The results show that the *D.pseudoobscura per* protein functions in *D.melanogaster*. However, it cannot perform all functions properly, as indicated by the inability of a single gene dose to rescue rhythmicity and by the low penetrance (30%) and abnormal period length of even the double dose-containing flies. On the other hand, the resemblance of the activity distribution and rest time values to those of

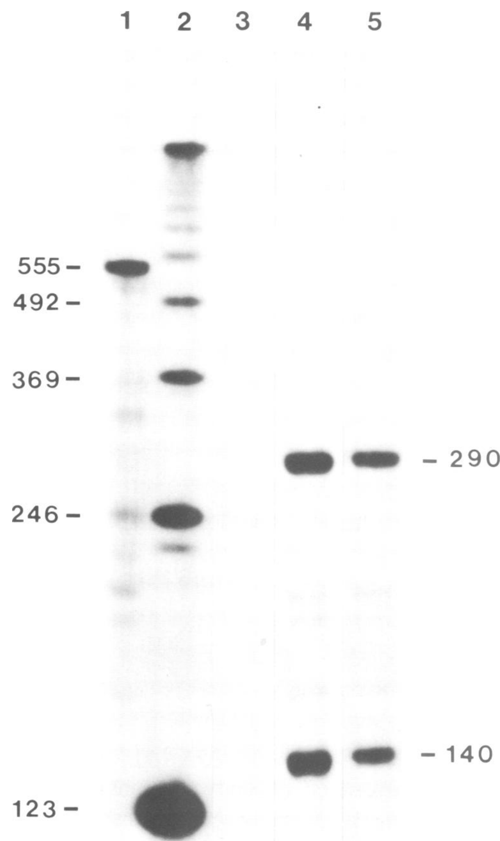

Fig. 3. RNase protection experiment. Autoradiogram of an RNase protection experiment with pA^+ RNA from total flies and a ^{32}P-labeled antisense RNA generated from the *D.pseudoobscura per* DNA fragment indicated in Figure 2 is shown. **Lane 1**, undigested probe (555 nt); **lane 2**, size marker (fragment length is given in nt); **lane 3**, hybridization with *D.melanogaster* per^{o1} RNA; **lane 4**, hybridization with hybrid gene/per^{o1} transformant RNA; **lane 5**, hybridization with *D.pseudoobscura* wild-type RNA. RNA from a second, independent transformant strain was assayed, with a result identical to that shown in lane 4 (data not shown).

D.pseudoobscura suggests a transfer of these species-specific behavioral features.

To test further the activity of the *D.pseudoobscura per* gene product, we have crossed the transformant autosome into a *D.melanogaster* per^+ background. [Although the *per* primary transcript is differentially spliced and probably produces multiple products (Citri *et al.*, 1987), we refer here, for simplicity's sake, to a single *per* product.] As a control we also crossed the 13.2 *per trans*-gene from *D.melanogaster* into a per^+ background. Flies were tested without prior entrainment (LL−DD), and the resulting eduction profiles of two flies are shown in Figure 4. For many of these behavioral records, the presence of the hybrid gene clearly changed the activity distribution (to one sharp peak, and long stretches of zero activity with an occasional relatively minor 'morning' peak) so that it resembled that of *D.pseudoobscura*, whereas the control flies showed the characteristic activity profile of *D.melanogaster*. Free running periods for the control transformants were 23.7 h, marginally shorter than for wild-type *D.melanogaster* (Table I). For the hybrid gene transformants, the periods were even shorter, 23.3 h, the same as for *D.pseudoobscura* wild-type flies (Table I). Statistical analysis (Table I, legend) revealed a significant difference in period for the two transformant types ($P < 0.02$). Resting times were 36% for the controls and 48% for the flies expressing the *D.pseudoobscura per* protein (Table I). Thus, an additional dose of the *D.melanogaster per* gene marginally lengthened the resting time (compare Table I, A and H). The presence of the *D.pseudoobscura per* product increased the resting time substantially, to 48%, although it did not reach *D.pseudoobscura* wild-type values (compare Table I, B and I). There is a significant difference in resting time between the 13.2 kb transformants and the hybrid gene transformants ($P < 0.004$). Importantly, two independently transformed strains were analyzed for each group; there was no statistically significant difference between the two strains either for period length or resting time (Table I, legend).

The flies' activity distributions have also been determined in 12 h light:12 h dark cycles. Under these LD conditions, flies manifest rhythmic behavior with periods of 24 h as their clocks couple to the external oscillator. The fact that all flies have the same period allowed us to generate summary (superimposed) eduction profiles for a given genotype, thereby expressing the activity present in any single bin as the percent of total activity for all flies tested.

Under these conditions, even per^{o1} flies manifested rhythmic behavior, i.e. although arrhythmic in free run, this mutant was 'forced' into a quasi-rhythmic behavior which is triggered by and follows the light/dark cycle (Figure 5, cf. Hamblen *et al.*, 1986; Dushay *et al.*, 1988). Of their activity, 80% was during the light phase and, except for occasional startle responses to lights-off that caused high levels of activity in the first 0.5 h of the dark phase, they were almost completely inactive thereafter. In contrast, the two transformant genotypes (two-dose homozygous 13.2 kb transformants and two-dose homozygous hybrid gene equivalents, both in a per^{o1} background) shifted almost all their activity into the dark phase (75 and 85% of total activity, respectively). Both genotypes had a very sharp peak immediately following lights-off. The 13.2 kb transformants also seemed to have an additional, smaller peak of activity in the morning before lights-on. This peak, less prominent in the activity profiles from the hybrid gene transformants, suggests an anticipation of the light transition and recalls the *D.melanogaster* bimodal activity distribution in the free run, absent from the transformants expressing the hybrid gene (e.g. Figures 1 and 4).

To augment these comparisons, we have also tested under these LD conditions both wild-type flies and wild-type transformants (Figure 6). Both *D.melanogaster* and *D.pseudoobscura* were inactive during most of the day but anticipated lights-off by becoming active shortly before the light/dark transition. The eduction summaries for both genotypes have a major peak of activity at this transition point, although *D.melanogaster* was somewhat more active after lights-off and *D.pseudoobscura* before lights-off. Thereafter, *D.pseudoobscura* flies remained quite inactive

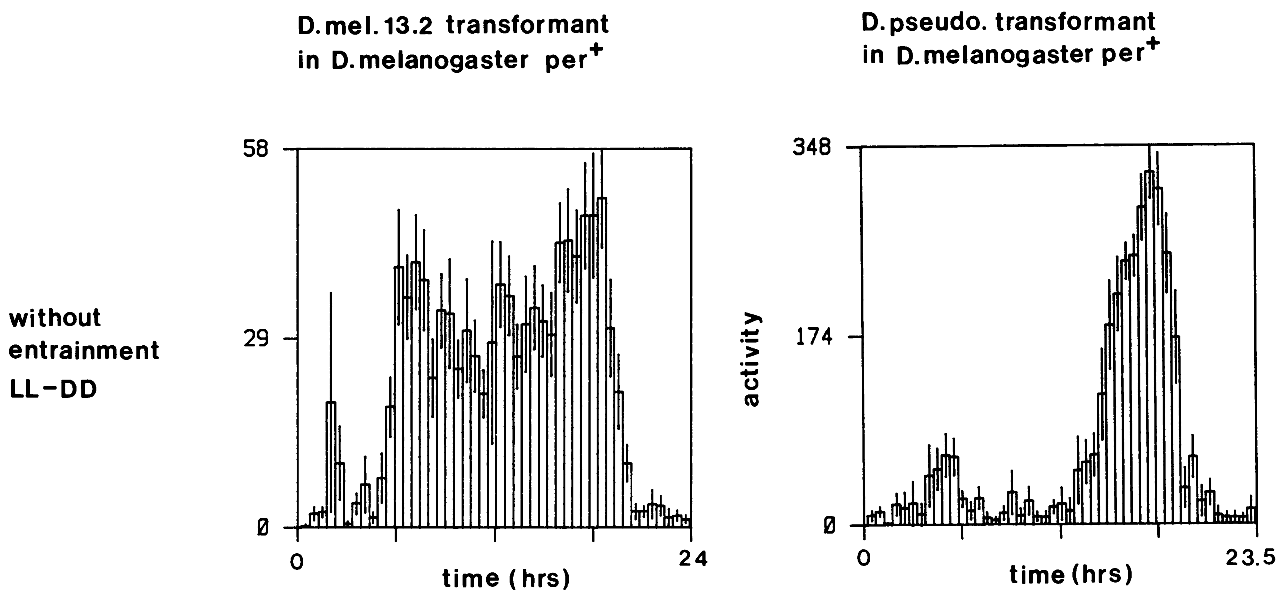

Fig. 4. Activity of wild-type flies carrying transformed *per* genes. Eduction profiles of *D.melanogaster* wild-type flies carrying, on an autosome, the 13.2 kb *per* DNA fragment of *D.melanogaster* (**left**) or the hybrid *per* gene (**right**). Activity distribution is shown for free running behavior (in constant darkness, DD) for flies raised in constant light (LL). The Y-axis is activity (mean numbers of activity events per period ± SEM), and the X-axis is circadian period in h, divided into bins of 0.5 h.

for the rest of the dark cycle. Occasionally a *D.pseudoobscura* fly became startled by lights-on and was active during the first 0.5 h of the light phase, but in general the behavior under these LD conditions resembles closely the unimodal activity pattern observed in free run (e.g. Figure 1). In contrast, *D.melanogaster* showed a bimodal activity distribution with two activity peaks. After the evening peak, the activity level fell but not as far as for *D.pseudoobscura*, i.e. the night time basal level was higher for *D.melanogaster* than for *D.pseudoobscura*. Of the total activity of *D.melanogaster*, 70% occurred during the night compared to 30% for *D.pseudoobscura*. *D.melanogaster* not only anticipated the transition from day to night in the evening but, in contrast to *D.pseudoobscura*, also from night to day in the morning; activity during the 2 h before lights-on comprises about 22% of the total activity for *D.melanogaster* compared to 2% for *D.pseudoobscura*. This difference in activity during the 2 h (the four 0.5-h bins) prior to lights-on is significant ($P < 0.0001$).

To examine the influence of the *D.pseudoobscura per* protein on this activity distribution, we examined the *per* transformants in a per^+ background (Figure 6). The activity distribution pattern of a per^+/*D.melanogaster* 13.2 kb transformant was comparable to the *D.melanogaster* wild type. As expected, additional doses of *per* appeared to strengthen the rhythm, in that the basal level of activity at night was decreased and the activity more concentrated, giving rise to somewhat sharper 'morning' and 'evening' peaks. Of all activity, 70% still ocurred during the night with 16% of the total activity during the 2 h prior to lights-on. The genotypes expressing the *D.pseudoobscura per* gene, on the other hand, were less active during the night (50% of all activity). During the 2 h prior to lights-on, activity comprised only 7% of the total activity. Like *D.pseudoobscura* wild-type flies, the hybrid gene transformants were occasionally startled by lights-on and exhibited a peak of activity during the first 0.5 h thereafter, but they anticipated relatively poorly the dark/light transition. The two transformants were significantly different in their activity distributions during the 2 h prior to lights-on ($P < 0.0016$). Thus, the hybrid gene transformants resemble more closely the *D.pseudoobscura* wild-type both in the total level of night time activity and in the relatively poor anticipation of lights-on.

Conclusions

Our previous analyses showed that the coding regions of the *per* genes of *D.virilis* and *D.pseudoobscura* are dramatically different from that of *D.melanogaster*; ~30% (~400 amino acids) of the *D.melanogaster* coding sequence is nearly unrecognizable when compared to the genes of these two species (Colot *et al.*, 1988). In this context, it is remarkable that the hybrid gene can function and manifest biological activity in a *D.melanogaster* host. Yet we note that, by standard criteria, the hybrid gene activity is weak by comparison with the *D.melanogaster per* gene. This conclusion is drawn from the failure of a single dose of the hybrid gene to rescue arrhythmic, per^o hosts (Table I) and from the poor penetrance and long periods of two-dose per^o flies (Table I). In other words, the hybrid gene did not restore strong rhythmic properties, those which are well rescued by the comparable 13.2 kb *D.melanogaster* fragment.

There are several possible interpretations for these observations. The *D.pseudoobscura per* product may be intrinsically weak. Alternatively, the hybrid construction may give rise to unusually weak *D.pseudoobscura per* activity. This latter possibility could be due to the absence of important *D.melanogaster cis*-acting regulatory information that may normally be present in the missing 350 bp of the first intron sequence or in the coding region. However, we suspect that these possibilities are incorrect, based on the strength of the *D.pseudoobscura* activity rhythms (Figure 1), on the behavioral experiments which assay hybrid gene

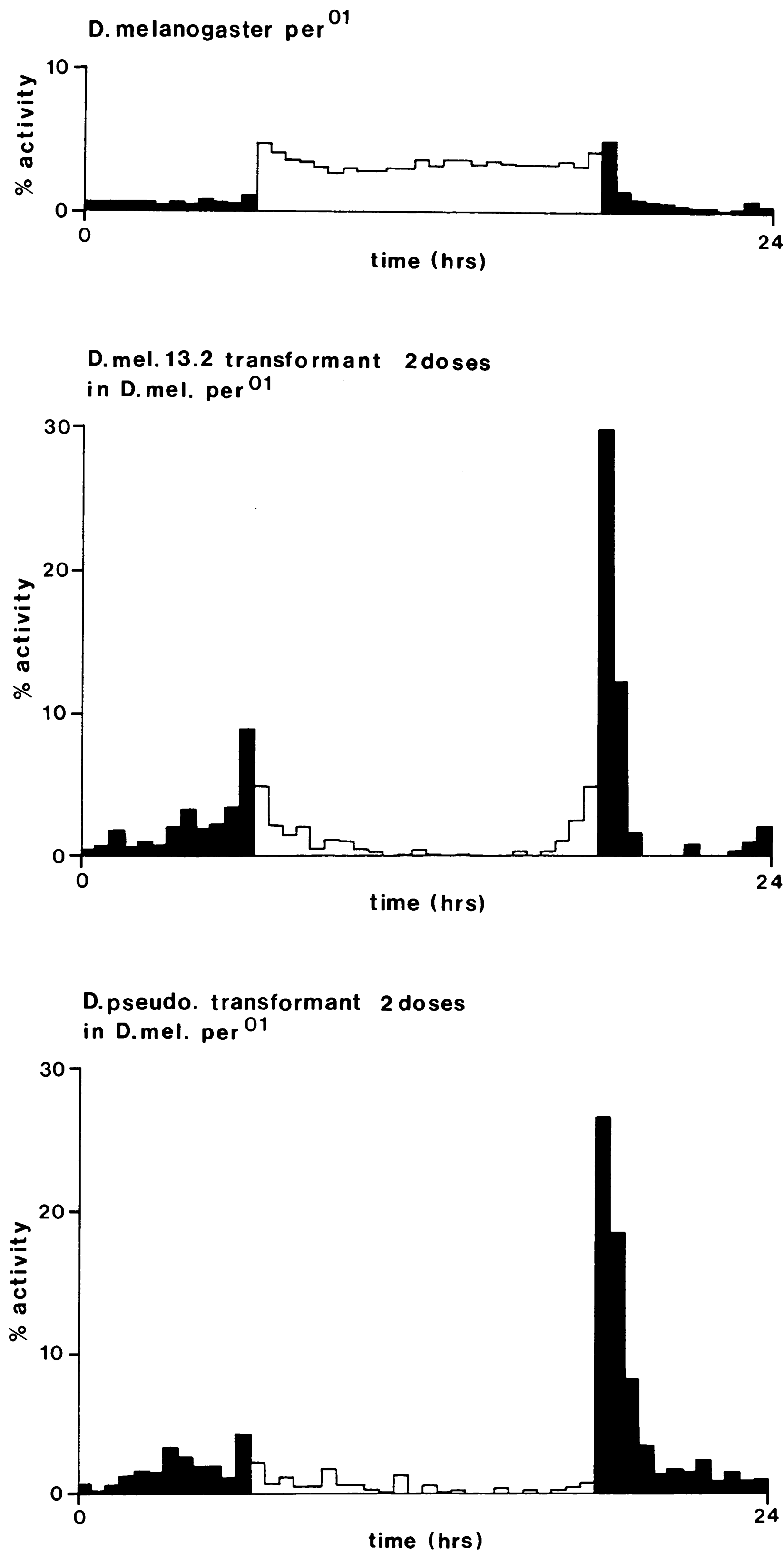

Fig. 5. Activity profiles of *per^{01}* and transformed flies during light:dark cycling. Comparison of activity distribution under conditions of light:dark cycling; 12 h light (white areas), 12 h dark (shaded areas) per cycle. The flies had been raised to adulthood and then entrained under these conditions as well. Each plot is a 'summary eduction' i.e. a merging of 6–8 individual fly eductions (cf. Figures 1 and 4) for each genotype. The activity on the Y-axis is given as percent of total activity. The periods—which were indeed 24.0 h for each fly tested in these LD conditions—are shown on the X-axis, divided into 0.5-h bins. The **upper panel** shows *D.melanogaster per^{01}* flies (six flies), the **middle panel** transformants homozygous for the 13.2 kb *D.melanogaster per* DNA (eight flies), and the **lower panel** shows transformants homozygous for the hybrid gene (seven flies).

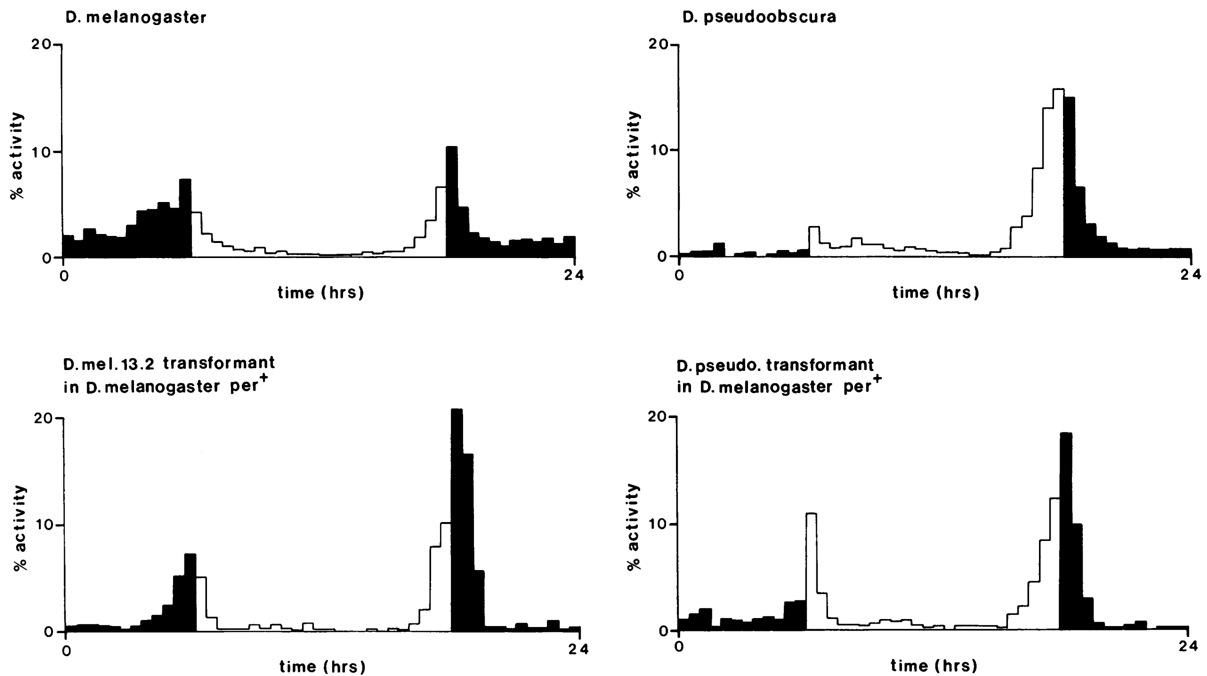

Fig. 6. Activity profiles of per^+ and transformed flies during light:dark cycling. Comparison of activity distribution under light:dark cycling conditions; 12 h light (white areas), 12 h dark (shaded areas) per cycle. Shown are summary eductions (cf. Figure 5) for nine flies (*D.pseudoobscura*) or eight flies (all others). The activity is given on the Y-axis in percent of the total activity. The periods, which were 24.0 h for each fly, are shown on the X-axis, divided into 0.5-h bins. The **upper panel** shows the wild types, *D.melanogaster* (**left**) and *D.pseudoobscura* (**right**). The **lower panel** shows *D.melanogaster* wild type carrying, on an autosome, an additional 13.2 kb per DNA fragment of *D.melanogaster* (**left**) or the hybrid *per* DNA fragment (**right**).

activity in a *D.melanogaster* per^+ background (Figure 4), and on the molecular experiments which assay directly hybrid gene expression (Figure 3). A more likely explanation, in our view, is the failure of the *D.pseudoobscura per* product to interact properly with other *D.melanogaster* clock components. We presume that such interactions depend, at least in part, on features of the *per* primary amino acid sequences which are different between *D.melanogaster* and *D.pseudoobscura* (Colot *et al.*, 1988).

Yet the presence of the hybrid *per* gene in a wild-type *D.melanogaster* background has a pronounced effect on rhythmic behavior and strengthens the rhythm of the wild-type host fly, even more than the comparable *D.melanogaster* gene (Figure 4, Table I). This is true by analysis of the resting time as well as of the period. By both of these criteria (which may be manifestations of the same phenomenon), the flies with the hybrid gene behave more like *D.pseudoobscura* than do those with the comparable *D.melanogaster* gene. This is also true under entrainment conditions (LD) where the wild-type fly with the hybrid gene behaves more like *D.pseudoobscura*, in that it transfers less of its activity to the 'night' and anticipates less well lights-on, by comparison to the wild-type fly with the 13.2 kb *D.melanogaster* gene (Figure 6). We interpret these observations to indicate that, once the intraspecific requirements have been fulfilled (e.g. in a wild-type background), the intrinsic 'strength' and qualitative features of the *D.pseudoobscura per* product can be manifest and superimposed on those of the host, *D.melanogaster per* gene. A more specific interpretation awaits some insight into the biochemical function(s) of the *per* product.

If the differences apparent between the per^+ flies that carry the *D.melanogaster* gene and those that carry the hybrid gene truly reflect behavioral properties of these two species, it suggests not only that the *per* gene encodes species-specific behavioral properties but also that some of these can be expressed in the absence of other species-specific factors. The *per* gene may be special in this regard. However, the similarity in behavior between the per^+/hybrid gene flies and *D.pseudoobscura* could still be a coincidence rather than a true reflection of species-specific behavioral instructions, the above arguments notwithstanding. Characterization of a third species, e.g. *D.virilis*, and transfer of its *per* gene to a *D.melanogaster* host should help to clarify this issue.

Although the experiments presented here suggest that the *per* coding region is the source of the observed behavioral differences, they do not prove that this is the case, nor do they indicate where in the *per* sequence the species-specific instructions reside. Recent experiments, in which the male courtship song differences between *D.melanogaster* and *D.simulans*, rather than locomotor activity, were assayed, are consistent with the coding region interpretation and suggest further that it is the nonconserved portions of the coding region that are responsible for these interspecific differences (C.P.Kyriacou, D.A.Wheeler, Q.Yu and J. Rutila, personal communication). Locomotor activity assays of additional chimeric constructs should be able to localize, in a similar manner, the regions of the *D.pseudoobscura*/*D.melanogaster per* gene responsible for at least some of the behavioral differences described here.

Materials and methods

Molecular experiments

A 13.2 kb DNA fragment from the *D.melanogaster per* gene, cloned in cp20.1 (Citri *et al.*, 1987), and a 5.6 kb DNA fragment from the *D.pseudoobscura per* gene, cloned in pTZ18U (Colot *et al.*, 1988), were digested with various restriction enzymes; fragments were gel purified and ligated to generate the chimeric clone depicted in Figure 1. In the construction, the entire *per* protein coding region of *D.melanogaster* was replaced by that of *D.pseudoobscura*, while the upstream regions of *D.melanogaster* were retained. A 350 bp section of the first intron of *D.melanogaster* was deleted and the 3' portion replaced by 190 bp of the putative first intron of *D.pseudoobscura*.

A *Hin*dIII−*Xba*I fragment containing part of the putative first intron of *D.pseudoobscura*, the second exon, the second intron and part of the third exon (see Figure 2) was cloned into transcription vector pSP64. After digestion with *Eco*RV, ^{32}P-labeled antisense RNA was transcribed with SP6 polymerase according to the manufacturer's protocol (Promega Biotec) to generate a probe for RNase protection experiments. The radioactive transcript was gel purified from a 4% acrylamide−urea gel.

pA^+ RNA was isolated from adult flies as described previously (Reddy *et al.*, 1984). Amounts of 1 μg were used for the RNase protection experiments, which were performed following the protocol of Zinn *et al.* (1983).

per^{o1} hybrid gene transformant adults were also sectioned and stained with a specific anti-*per* antibody reagent, in a manner identical to that described by Siwicki *et al.* (1988). As in the case of the *D.melanogaster per* protein (Siwicki *et al.*, 1988) and the *per*−β-galactosidase fusions (Liu *et al.*, 1988), staining in sections of these transformants was clearly detectable in the photoreceptor cells and only at night (cf. Siwicki *et al.*, 1988). Staining was much weaker than for wild-type *D.melanogaster* or for control 13.2 kb/per^{o1} transformants (see below), perhaps because there is a single amino acid change between *D.melanogaster* and *D.pseudoobscura* in the 14 amino acid sequence against which the antibody reagent was generated (compare Citri *et al.*, 1987, and Siwicki *et al.*, 1988, to Colot *et al.*, 1988).

Transformation and flies

Transformation was performed according to standard methods (for review, see Rubin, 1985), and five independently transformed strains were identified by segregational analysis of the ry^+ marker included in the vectors. The basic behavioral effects of all these inserts (in a per^{o1} genetic background) were essentially the same, so two of the strains—called hyb20 (with the chimeric gene inserted on *D.melanogaster*'s chromosome *2* in region 38A) and hyb26 (insert location, *2*, 55E)—were chosen arbitrarily for use in the further, extensive experiments (whose results appear in Table I). Other strains used were (all from *D.melanogaster*): Canton-S wild type, $per^+\ ry^{506}$, $per^{o1}\ ry^{506}$; two '13.2' transformants [*melanogaster*-only *per* DNA inserted on chromosome *2* (region 55−56) or *3* (89A); strains originally reported by Citri *et al.* (1987); also see Yu *et al.* (1987); *D.pseudoobscura*: 'Ayala reference' strain. The $per^{o1}\ ry^{506}$ strain was used as the original host in experiments involving isolation of ry^+ transformants; the $per^+\ ry^{506}$ strain was used to produce ry^+ transformed individuals that had their per^{o1} mutation replaced by the wild-type allele.

Behavior

Locomotor activity was monitored automatically, as described previously (Hamblen *et al.*, 1986). All flies tested were adult males. 'Eduction' analyses were performed using software from F.M.Sulzman (Sulzman, 1982, and personal communication). The relevant program superimposes the numbers (the computed mean and SEM) of locomotor activity events (per time bin) for the several successive circadian cycles of a 'run' (run durations were 5−10 days in our experiments). Therefore, the entire run is depicted in a plot whose abcissa defines ~1 day, i.e. the length of the rhythm period for this fly. Thus, the eduction program—in addition to superimposing activity values for corresponding parts of each successive cycle—also searches for the best period estimate by optimal (lowest variance) alignment of activity per 0.5-h bin for the complete run.

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