A NEW MUTATION AT THE PERIOD LOCUS OF DROSOPHILA MELANOGASTER WITH SOME NOVEL EFFECTS ON CIRCADIAN RHYTHMS

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A new period mutation has been induced and characterized in D. melanogaster. It causes flies to be apparently arrhythmic in tests of locomotor activity and thus is superficially similar to the original per" mutant. Yet, the new "zero" allele, per", has some novel properties and effects: Behaviorally, per" adults often exhibit weak, long-period rhythms of locomotor activity in constant darkness; this low-frequency rhythmicity usually was not obvious in the analog behavioral records but was readily revealed by spectral analyses. These treatments of the data also extracted hidden high-frequency (ultradian) rhythms in many of the behavioral records, of the type associated with per" and other per-nulls. The wide range of periodicities exhibited by different per"-expressing flies implies the expression of multiple oscillatory modes by this mutant. The new mutation also leads to a tendency for flies to be hyperactive during activity monitoring and is thus dissimilar to the other arrhythmic variants in the per gene but similar to the effects of a deletion of the locus. During light:dark cycling, per" adults once more behave differently from other per"s and in fact tend to resemble wild-type flies in these conditions. The new mutation is not caused by the same nucleotide substitution that created a stop codon in the original arrhythmic per mutant and, as it turns out, per" and per" as well. per" is also not a null variant at the transcriptional level; but it leads to an anomalous form of per mRNA, which is smaller than the normal 4.5 kb species encoded by this clock gene.

Keywords: clock mutants, activity/rest cycles, arrhythmicity, periodograms, spectral analyses, Southern and Northern blots

Mutations in the period clock gene of D. melanogaster have been extremely useful in analyses of the flies’ biological rhythms (reviews: Konopka, 1987a, b). In addition, these variants and a collection of chromosome aberrations have allowed the relevant genetic locus to be cytogenetically well defined; this in turn permitted per to be cloned and investigated with regard to its molecular structure and expression (reviews: Hall & Rosbash, 1987, 1988).

The “zero” per alleles are apparently null mutations, in that they cause arrhythmicity which is similar to that associated with a deletion of the locus (Konopka & Benzer, 1971; Smith & Konopka, 1981; Hamblen et al., 1986). However, rather than being utterly devoid of periodically fluctuating behavior, the per0 mutations and the per" deletion cause adult flies to be cryptically, though genuinely, rhythmic. Specifically, this means that ultradian (ca. 5–15 h) rhythms are extractable from records of these flies’ locomotor activity (Dowse et al., 1987; Dowse & Ringo, 1987). Yet, there are differences between per"s and per". For example, the latter seems to make the flies

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more active in locomotor tests than do the previously reported \( \text{per}^0 \) "point" mutations (Hamblen et al., 1986). Molecular studies of the \text{period} locus have shown that \( \text{per}^- \) flies are devoid of three RNA species, i.e., the \( \text{per} \) transcript (a 4.5 kb mRNA) and two smaller ones (Bargiello & Young, 1984; Reddy et al., 1984). Nucleic acid sequencing data revealed that the original zero allele, \( \text{per}^0 \) (Konopka & Benzer, 1971), is accounted for by a single nucleotide substitution, which creates a nonsense codon in the fourth coding exon of the 4.5 kb \( \text{per} \) RNA (Yu et al., 1987a; Baylies et al., 1987).

It would be useful to have additional \( \text{per}^0 \) alleles, at least to compare their effects to those of the \( \text{per}^- \) deletion and to the similarly null point mutation. As we report here, only one \( \text{per}^- \) null mutant seems to have been isolated previously. With additional mutants, one could ask: Will all arrhythmic variants at this locus lead to the same array of behavioral subtleties? Might a new allele of this kind contain a missense mutation, which could lead to apparent inactivity of \( \text{per} \)'s product and thus end up being more informative than the absence of it, with regard to relationships between primary protein structure and function? Perhaps a new arrhythmic mutation would be transcriptionally silent, thus putatively defining a regulatory region within the \( \text{per} \) locus.

We now report that a newly induced \( X \)-chromosomal mutation causing largely arrhythmic locomotor activity is a \( \text{per} \) allele and that this variant does have some unusual effects and molecular characteristics.

**MATERIALS AND METHODS**

*Drosophila Strains, Including Rearing and Mutagenesis of them*

Most of the flies used in these studies were grown on a cornmeal, agar, molasses, and yeast medium, at 25°C, in 12:12 light:dark (LD) cycles (lights on at 8 am). Some of the starting strains for this study were maintained on the medium of Lewis (1960), which contains sugar instead of molasses.

To search for newly induced rhythm mutants, a mixture of wild-type males from Oregon-R and Hikone strains was treated with ethyl methanesulphonate (EMS), using the method of Lewis & Bacher (1968). [Using parental males of mixed types was an oddity, i.e., was not done for strategically interesting or useful reasons.] These mutagenized flies were crossed to attached-\( X (X^X y f) \) females. Individual F1 males were backcrossed to the same kinds of \( X^X \) females to establish a series of ca. 1500 putatively mutant stocks, in which only males would exhibit effects of any rhythm mutation that was induced—unless it turned out to be an autosomal dominant factor. Sample males from these stocks were monitored for locomotor activity as described in Smith & Konopka (1981, 1982). The resultant activity records ("actograms," or cartesian coordinate plots of digitized activity events vs. time) were examined for any noticeable rhythm defects (weak or no rhythmicity, altered periodicity).

In this screen, males in one of the stocks were noticed to be apparently arrhythmic. They were eventually (1) retested, to accumulate data on large numbers of putatively mutant individuals [for this, similar (but independently constructed) activity monitoring devices were employed (cf. Hamblen et al., 1986)]; and (2) crossed to flies carrying the following \( \text{per} \) genotypes, to determine the elementary genetic properties of the new mutation: (a) a \( \text{per}^- \) deletion called \( DF(1)w^d \) (Smith & Konopka, 1981), (b) the original \( \text{per}^0 \) mutation (Konopka & Benzer, 1971), and (c) \( \text{per}^+ \) (derived from a Canton-S wild-type strain). The activity of females heterozygous for the new mutation and each of these three genotypes was then monitored (as in Hamblen et al., 1986; and see below).
Other genetic variations involving the *period* gene, tested as above, were: (1) *per*<sup>62</sup> (Smith & Konopka, 1982) and *per*<sup>63</sup> (Hamblen et al., 1986); (2) homozygous *per*- females, obtained (cf. Smith & Konopka, 1981) by crossing flies carrying *Df(1)64j4* or *Df(1)62d18* to those carrying *Df(1)TEM 202*; any of these three deletions is itself a recessive lethal, so a given *Df* of this type was heterozygous with a balancer chromosome in the female parents and covered by a *w*<sup>+</sup> *Y* chromosome in males; the *64j4/TEM202* or *62d18/TEM202* females are deficient for ca. 10–15 kb of *per*-locus DNA, including the source of the 4.5 kb *per* mRNA (Bargiello & Young, 1984; Reddy et al., 1984); and (3) certain germ-line “transformed” flies involving *per*<sup>64</sup> and DNA cloned from *per*<sup>+</sup> (see last section of Materials and Methods).

**Behavioral Tests**

All such testing involved continuous monitoring of adults’ locomotor activity “events,” using glass-tube fly containers flanked by infrared light emitters and detectors (cf. Smith & Konopka, 1981; Hamblen et al., 1986). The monitors were placed in incubators maintained at 25°C. Newly emerged adults to be monitored were entrained by exposure to four 12 h:12 h light:dark (LD) cycles, for which lights-on was at noon, i.e., a “re-entrainment,” referring to the phase of LD cycles during rearing (see above). [The re-entrainment was effected so that subjective lights-on, in “free-running” conditions (see below), would be well displaced from times when extraneous environmental cues (commencing between 8–9 am on a given day) might leak into the incubators and cause spuriously “forced” activity to occur.]

The flies were monitored in constant darkness (DD) for their free-running activity, as in Hamblen et al. (1986); that report describes the Apple Ile-based data collection and analysis system which we used in the current study (also see Sulzman, 1982). Numbers of activity events (infrared beam breakages) for a given fly were written to disc every half hour, over the course of 7–10 days of monitoring. The software associated with this system was augmented to perform elementary calculations on the average numbers of activity events per half hour, per fly (see Table II in Results). The analytical programs applied to the digitized activity data involved Chi-square periodograms, Very Efficient Spectral Analysis (VESTAL), Maximum Entropy Spectral Analysis (MESA), and autocorrelations (cf. Hamblen et al., 1986; Dowse et al., 1987; Dowse & Ringo, 1989b; Dushay et al., 1989). MESA and autocorrelation have routinely been applied in searches for significant periodicities over a wide range (1–40 or 1–50 h). In the current study, instructions involving application of the periodogram and VESTAL programs were, for the first time (for this organism), altered similarly to attempt detection of significant rhythms well outside of the circadian range (see Results, Table I).

In some experiments, the flies’ activity was monitored during 12:12 LD cycling, as in Dushay et al. (1989); the rather widely varying light intensities (at different locations within a given incubator) during “L” were as indicated in this report. “Short-term” assessments of general locomotor activity were performed on certain of the relevant genotypes (re *per*<sup>64</sup> and controls, cf. Table I in Results), by counting the number of times a fly, which was placed in a small transparent chamber, crossed a line that bisected the chamber (see Kulkarni & Hall, 1987, for details).

**Molecular Materials and Procedures**

A series of germ-like transformants, involving DNA fragments cloned from the *per* locus (Zehring et al., 1984; Hamblen et al., 1986; Citri et al., 1987) was used in
**TABLE 1**

Locomotor activity rhythms affected by *per<sup>mut</sup>*. Adults were entrained in LD and then had their locomotor movements monitored for ca. 7–10 days in DD (as in Hamblen et al., 1986). Chi-square periodogram, VESTAL, and MESA programs were applied to the digitally collected activity data, whereby determinations of significant rhythmicity—if any—and best-estimates of periods resulted. "Significant" means at the 5% level for periodograms and VESTALS; and, for MESAs, as described below (also see Fig. 2). All non-integer values in the table are mean periods (*T's*) in h ± SEM; the integers (in parentheses except for the arrhythmic cases) are the numbers of flies in a given category. This classification follows the scheme of Dowse et al. (1989), which leads to the sets of four columns under each of the three analytical headings. Analyses that led to "dual periodicities" (see Dowse & Ringo, 1987, and text of the current report) were tabulated based on the most prominent and the longest-period peak (see footnotes to this table). The so-called "short/normal" category encompasses values that were between ca. 19-25 h; for the 4th and 5th rows of genotype (the only cases involving per<sup>+</sup>) the great majority of *T'S* were in the circadian range (ca. 23-25 h).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Periodogram</th>
<th>VESTAL</th>
<th>MESA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ultradian</td>
<td>Short/normal</td>
<td>Long</td>
</tr>
<tr>
<td><em>per&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td>7.4 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36</td>
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<tr>
<td>(7)</td>
<td></td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>per&lt;sup&gt;mut&lt;/sup&gt;/per&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td>3.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>28</td>
</tr>
<tr>
<td>(1)</td>
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<td></td>
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<tr>
<td><em>per&lt;sup&gt;mut&lt;/sup&gt;</em>/Df</td>
<td>12.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>39.5&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>(1)</td>
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<td></td>
<td>(1)</td>
</tr>
<tr>
<td><em>per&lt;sup&gt;mut&lt;/sup&gt;</em>/+</td>
<td>-</td>
<td>24.7 ± 0.1</td>
<td>-</td>
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<tr>
<td>(9)</td>
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<td>(2)</td>
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<tr>
<td><em>per&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td>-</td>
<td>23.9 ± 0.1</td>
<td>-</td>
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<tr>
<td>(52)</td>
<td></td>
<td>(53)</td>
<td></td>
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<tr>
<td><em>per&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td>6.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>39.0&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>(1)</td>
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The genotypic designations are self-explanatory, except for Df (an X-chromosomal deletion that removes the *per* locus, *Df(1)w<sup>11</sup>*; see Materials and Methods) and *per<sup>+</sup>* = Canton-S wild-type adults or flies from a variety of other *per*-normal strains carrying markers (48 males and 5 females were tested). In recent tests of some additional *per<sup>+</sup>*-bearing flies, 24/27 Oregon-R and 34/34 Hikone adults (one of which strain type was, at an earlier time, the source of *per<sup>mut</sup>*<sup>+</sup>) were strongly rhythmic. Of the *per<sup>mut</sup>* adults tabulated above, 34 were males, and 10 were females. For the high-resolution spectral analyses (right-hand group of 4 columns), the behavioral files were analyzed by autocorrelation and MESA (see text and legend to Fig. 2). The periodic components were determined as described in detail by Dowse & Ringo (1989b). Briefly, if a significant rhythm (at the 5% level, cf. Dowse et al., 1987; Dowse & Ringo, 1989b) was detected by autocorrelation its (*r*) value was noted and the MESA-determined periodicity (which was usually very close to the autocorrelation result was used for tabulation.

a, by VESTAL, 4 of these were ultradian, 1 was long, and 2 were arrhythmic; by MESA, all were ultradian.
b, VESTAL: dual period (circadian + long); long by MESA.
c, VESTAL: arrhythmic; MESA: short.
d, VESTAL: short/normal; MESA: arrhythmic.
e, both VESTAL and MESA: long.
f. by Periodogram, 2 were ultradian, 5 were arrhythmic; by MESA, 6 were ultradian, 1 arrhythmic.
g. Periodogram: arrhythmic; MESA: short/normal.
h. for these long-period VESTALs, 4 also had an ultradian peak, 2 also had a short/normal peak, and 1 (with predominant peak at 36.0 h) also had a strong peak at 30.0 h; by Periodogram, 2 ultradian, 1 long, 17 arrhythmic; by MESA, 3 ultradian, 5 long, 5 ultradian + long (i.e., dual peak; 1 of these had the same result from VESTAL), and 6 arrhythmic.
i. Periodogram: all arrhythmic; MESA: 3 ultradian, 1 long.
j. for 2 of these VESTALs, there was an ultradian peak also (not harmonics of these circadian T's); Periodogram: all arrhythmic; MESA: 2 short/normal, 1 dual peak (ultradian + long).
k. 1 of these VESTALs was dual peak (ultradian + long); Periodogram: all arrhythmic; MESA: 2 ultradian, one short normal, 4 long, 2 arrhythmic.
l. Periodogram: 1 short, 1 arrhythmic; MESA: 1 short/normal, 1 arrhythmic.
m. 2 of these VESTALs were dual peak (ultradian + long); Periodogram: 1 long, 4 arrhythmic.
n. 2 of these MESAs were dual peak (ultradian + long); by VESTAL, 1 of these had this property, 1 arrhythmic; Periodogram: both arrhythmic.
o. 1 of these MESAs was dual peak (ultradian + long); VESTAL: same; Periodogram, arrhythmic.
p. 1 MESA dual peak (ultradian + long); VESTAL: same; Periodogram: arrhythmic.
q. 24.5 h and 25.0 h period estimates, by Periodogram and VESTAL, respectively.
r. was also ultradian by VESTAL and by MESA.
s. by VESTAL: triple-peak (ultradian + short/normal + long); MESA: short/normal.
t. 1 was dual peak (ultradian + weak circadian); by Periodogram, I had an ultradian peak, and 5 were arrhythmic.
u. by Periodogram and by MESA, both were arrhythmic.
v. 3 were dual-peak (ultradian + long); 2 were triple-peak (cf. s); by Periodogram, 1 was long, and 7 were arrhythmic; by MESA, 5 were ultradian, 1 short/normal, 1 long, and 1 arrhythmic.
w. by Periodogram, arrhythmic; by VESTAL, long.
behavioral tests (monitoring of free-running rhythms in DD, see above) of males
hemizygous for per^dd. The "transduced insert"-bearing flies were crossed to per^dd
females, who also carried an appropriate marker (Adhnull or rosynull)—one what would
be covered by a “marker-plus” stretch of DNA engineered into the insert in question,
along with the given piece of per DNA (see Table III in Results): a 7.2 kb piece
inserted on chromosome 3 at 62B: 8.0 kb (2, 26F-27A); 10.2kb (2, 37A); 13.2 (3, 89A); and
two independently isolated inserts of a 14.6 kb fragment (each on chromosome
2, at 35D-E for strain #21 and 42C-D for #63). Marker-plus, male transformant
progeny of the crosses just noted were tested to see if a given per^+ insert would restore
overt rhythmicity to flies hemizygous for per^dd.

Southern blotting experiments, performed to compare per-locus DNA from per^dd
flies vs. those expressing the other “zero” alleles, were initiated by preparing genomic
DNA from approximately 10 flies of a given genotype, as described in Hamblen et al.
(1986). These DNA preps were digested with restriction endonucleases (see Fig. 5 in
Results), according to the manufacturers’ instructions, for 3–5 hours. The resultant
fragments were electrophoresed on 1% agarose gels and transferred to nitrocellulose
filters by the method described in Maniatis et al. (1982). These filters were then
probed with an 8.0 kb EcoR I fragment from the per locus (cf. Hamblen et al., 1986; and see Fig.
5 of the current report). Labeled DNAs generated in an overnight reaction (according
to Feinberg & Vogelstein, 1983) were separated from unincorporated nucleotides by
passage over a Sephadex G-50 spin column. All filters were hybridized under high-
stringency conditions, i.e., 50% formamide, 5X SSC, at 40°C; and washed several
times with 0.1x SSC, 0.5% SDS, at 68°C. The filters were then exposed to X-ray
film, with an intensifying screen, at −70°C, overnight.

Northern blotting experiments, performed to determine whether per^dd leads to
abnormalities in the level or quality of transcription controlled by per, were carried
out by first preparing total RNA from adult flies. This was done by (1) a simplification
of the method in Barnett et al. (1980) [after homogenization and phenol-chloroform
extraction, the RNA was precipitated and used for isolation of polyadenylated
{p(A)+} RNA without further purification (see Results, Fig. 6A)]; or (2) by the
method of Chirgwin et al. (1979) [Fig. 6B]. 5–10μg of p(A)^+ RNA were then
fractionated on formaldehyde agarose gels, transferred to Biotrans (ICN Radio-
chemicals, Fig. 6A) or nitrocellulose (Fig. 6B) and hybridized to per probes, as
described in Colot et al. (1982). The probes were used (1) a Hind III-BamH I fragment
from a per cDNA called “type A” (Citri et al., 1987), which includes 200 bp that are
upstream of the transcription start site, exons 1 through 4, and most of exon 5 (this
probe was applied to the blot in Fig. 6A), or (2) a Hind III-EcoR I fragment (Fig. 6B)
corresponding to a portion of the 3’ untranslated region of the 4.5 kb per transcript
and the entirety of a 0.9 kb transcript, whose genomic source is just proximal to per
on the X chromosome (Reddy et al., 1984; Bargiello & Young, 1984). The two kinds
of fragments were labeled and purified as described above.

RESULTS

The New Rhythm Mutation Causes Near Arrhythmicity and is a per Allele

By observing “actograms” that display the free-running locomotor behavior of adult
flies, the newly induced, X-chromosomal mutant seemed to be arrhythmic (example,
Fig. 1A, top; also see Fig. 1D in Hall & Rosbash, 1987). This phenotype is similar to
that associated with “null” alleles at the period locus on this chromosome (Konopka
FIGURE 1  Locomotor activity of *per*° flies in constant darkness. Movement of adults were monitored automatically, after prior entrainment in 12:12 LD cycles, as in Hamblen et al. (1986). The animals' breakage of infrared light beams led to the actograms shown here, which are "double plots," modulo 24 h (cf. Konopka and Benzer, 1971): days 1–2 of DD activity are plotted on the top line, days 2–3 on the second line, etc. A. Records from three *per*° females (homozygous for this mutation); for the top actogram, the Chi-square periodogram indicated arrhythmicity; application of the VESTAL program led to a significant periodicity at 34.0 h; by standard MESA, there was only a weak, insignificant peak in the ultradian range (see Fig. 2A, top), but when a low pass filter was incorporated into the analysis (see text), a substantial peak at 31 h appeared in the plot; middle actogram: periodogram, VESTAL, and MESA each led to significant periodicities at 9.5, 9.0, and 9.0 h, respectively, and this periodicity can be seen in the double-plotted, modulo 9 h actogram (inset to right; see Dowse et al., 1987, 1989, for explanation of these filtered actograms) [Fig. 2A (middle) displays the MESA and autocorrelation results corresponding to this middle actogram]; bottom actogram: periodogram, VESTAL, and MESA each led to significant periods at 8.0 h, as is obvious in the filtered, modulo 8 h actogram (inset) [see Fig. 2A (bottom) for the strong ultradian peaks determined by MESA and by autocorrelation, for the data shown in this bottom actogram]. B. *per*°/*Df(1)w°* females (the Df here is an X-chromosomal deletion that is missing the *per* gene; see text); top: the periodogram was arrhythmic, VESTAL led to significant periodicities at 6.0 and 36.0 h, and there was a principal MESA peak at 6.0 h (see Fig. 2B, top); bottom: periodogram arrhythmic, VESTAL 23.0 h, MESA 18.5 h [see Fig. 2B, bottom]. C. *per*°/*per*° females; top: periodogram arrhythmic, VESTAL 29.0 h; principal MESA peak 5.5 h (see Fig. 2C, top); bottom: periodogram arrhythmic, VESTAL 12.0 and 25.0 h (i.e., two significant peaks), MESA 11.5 and 36.0 h (see Fig. 2C, bottom). D. three *per*° records, (top two, males; bottom, female), each arrhythmic by periodogram and VESTAL; but MESA for the top case revealed a principal peak at 5.0 h (with many other subsidiary peaks) and for the middle actogram a single sharp peak at 8.0 h; the bottom case was arrhythmic by MESA.
& Benzer, 1971; Smith & Konopka, 1981, 1982; Hamblen et al., 1986). Also, disconnected mutations, which map at another locus on the X chromosome (Steller et al., 1987), have recently been shown to cause per<sup>o</sup>-type arrhythmia (Dushay et al., 1989). The newly induced factor could therefore be in either of these genes (or at a locus separate from per or disco).

In complementation tests, the effects of the new mutation were found to be uncovered by the original arrhythmic per allele or by a deletion of the locus (Table I; examples: Figs. 1B, C). These tests were validated by showing that the new allele—hereafter called per<sup>04</sup>—is, like per<sup>01</sup> (cf. Konopka & Benzer, 1971), recessive for overt rhythmicity (Table I). However, the new mutation is semi-dominant, in that the circadian rhythms of per<sup>04</sup>/+ females were noticeably longer than those of wild-type (Table I). This period-lengthening effect is routinely observed when a “null” or nearly null variant involving the per locus is heterozygous with a normal allele (e.g., Smith & Konopka, 1982; Coté & Brody, 1986; Citri et al., 1987).

Hidden Periodicities are Present in per<sup>04</sup> Activity Records

The new mutant is not a thoroughgoing “circadian arrhythmic” variant. Though inspection of per<sup>04</sup> actograms implied no periodic components in this mutant’s locomotor activity, and only 13% of the tested individuals expressing the new mutation were statistically rhythmic by periodogram analysis (Table I), other treatments of these data revealed significant periodic components in a relatively high proportion of the per<sup>04</sup>, per<sup>04</sup>/per<sup>01</sup>, and per<sup>04</sup>/per<sup>0</sup> flies.

The VESTAL program (see Materials and Methods) showed that 60% of the mutant flies were rhythmic (Table I). This method of searching for periodic components in these kinds of data has previously been noted to be more “sensitive” than Chi-square periodogram (Hamblen et al., 1986; Dushay et al., 1989). The current cases of significant VESTAL periodicities for the new mutant were usually either (1) ultradian [ca. 10 h, and in these cases the periodogram results were much the same (Table I)]; or (2) much longer than the ca. 24 h values routinely found in the free-running activity rhythms of per<sup>+</sup>-expressing D. melanogaster (e.g., Smith & Konopka, 1981, 1982; Hamblen et al., 1986; and Table I of this report). There were also so-called “short/normal” periodicities (ca. 19–23 h) determined for a few of these mutant records (Table I; also see discussion of MESA results, below). Essentially the same array of results was found, by VESTAL, for flies expressing the three per<sup>04</sup>-associated genotypes noted above. The “weakness” of these rhythms is underscored by the fact that cyclical patterns of obvious activity vs. rest are usually not apparent in the analog records of these behaviors (Fig. 1), though in some instances a long-period pattern can be discerned by inspection (see bottom part of Fig. 3B, below). Also, these very high- or quite low-frequency rhythms were mostly clustered at the short- or long-period values, respectively (Table I), as opposed to being arrayed arbitrarily throughout the entire range. That kind of scattering had been found for the weakly rhythmic cases detected, by periodogram or VESTAL, in 3–5% of the activity records for the per<sup>01-03</sup> mutants (Hamblen et al., 1986).

In other earlier studies of Drosophila’s locomotor behavior, ultradian rhythms exhibited by supposedly aperiodic animals have been consistently found to lurk in the DD activity records, by MESA and autocorrelation (Dowse et al., 1987, 1989; Dowse & Ringo, 1987; and see Dowse & Ringo, 1989b, for detailed descriptions of these analyses and a summary of the relevant findings stemming from application of them to the locomotor data from per-null flies). We analyzed the DD records of flies expressing per<sup>04</sup> to see if high proportions of these animals would exhibit ultradian
rhythmicity. We found by MESA (and autocorrelation; see legend to Table I) that these mutant adults can have ultradian rhythms analytically extracted (1) more readily than in the case of applying the periodogram or VESTAL treatments (Table I), and (2) from at least as high a proportion of the tested animals as previously reported for per^null and per^- (cf. Dowse et al., 1987; Dowse & Ringo, 1987). However, in contrast to previous analyses of activity data from these pernull flies (Dowse et al., 1987; Dowse & Ringo, 1987), MESA-determined results for per^mutant were distributed over four categories (a) ultradian rhythms (examples, Fig. 2A, middle and bottom), (b) short/normal (example, Fig. 2B, bottom), (c) much longer than circadian (example, Fig. 2C, bottom), and (d) arrhythmic (example, Fig. 2A, top). The first three (rhythmic) categories summed to 73% of the per^mutant's tested.

For per^control groups, extending the VESTAL-based period searches downward to 1 h and upward to 40 h resulted in a fair number of ultradian and/or very long-period cases (Table I). The MESA results on this set of per^mutants (Table I) were essentially as in previous reports: The phenotypes exhibited by per^null, per^mutant, and per^- have been rhythmic, in the ultradian range, or arrhythmic (Dowse et al., 1987; Dowse & Ringo, 1987).

There were appreciable numbers of per^mutant's and even per^null's exhibiting “almost circadian” rhythms (category b, above). But this phenotype was not wild-type-like, as these ca. 19–23 h rhythms were barely if at all detectable by inspection (see Fig. 1B, bottom, whose MESA plot is in Fig. 2B, bottom). Period estimates falling into the long (category c) were rather frequent for these mutants, especially for per^null (Table I). The proportions of results of this type were, however, lower as a result of MESA vs. VESTAL (also see below).

For control groups (per^null/ + and per^null/+), the high-resolution analyses revealed few significant ultradian components, and almost all peaks were in the circadian range (Table I, cf. Dowse et al., 1987). As found by periodgram and VESTAL, MESA revealed the mutant heterozygotes to have slower clocks than for the homozygous wild-types (Table I).

The MESAs and correlograms obtained from the per^mutant activity records generally indicated a more robust rhythmicity, with less attendant random noise, than in the majority of per^null, per^mutant, and per^- behavioral data sets analyzed in this manner (Dowse et al., 1987). Some notable examples are in Fig. 2A (middle and bottom). The corresponding actograms are in Fig. 1A (middle and bottom), including filtered analog records in which the ultradian rhythms for two adults expressing the new mutation are overt.

Another difference between per^mutant and the three per-nulls just referred to is that several MESAs from the new mutant were indistinguishable from those published for the long-period per^mutant (Dowse & Ringo, 1987), i.e., with considerable power in the ultradian range as well as a strong peak at ca. 30 h (example, Fig. 2C, bottom; and see legend to Fig. C; note: these types are tabulated under “long” throughout Table I). This kind of “dual period” result was also found in several of the VESTALS but almost never in the periodograms (detailed in the legend to Table I).

When comparing the results from the different kinds of analytical treatments of the activity data (Table I), fairly consistent patterns emerged: If a per^mutant or per^null fly had an ultradian rhythm by periodogram, it usually did by VESTAL and MESA (examples, Fig. 1A, middle and bottom). Period estimates in these cases were usually the same or within 0.5 h of one another (but sometimes as much as 2 h discrepant). MESA (and autocorrelation) performed best with regard to extraction of ultradian components (examples, Figs. 2B and 2C, top plots in each; cf. Figs. 1B and 1C), whereas VESTAL did so for the long-period rhythms (e.g., legend describing top part of Fig. 1A).
FIGURE 2A  CAPTION ON P.00
Overall, 5 of 44 pero\textsuperscript{4d} flies were totally arrhythmic, by all three methods; the corresponding numbers for pero\textsuperscript{4d}/per\textsuperscript{01} were 3/29; and for pero\textsuperscript{4d}/per\textsuperscript{−}, 1/12. For the 41 per\textsuperscript{01} flies tested here (Table I), a relatively high proportion (N = 12) was totally arrhythmic by all three analytical criteria.

There were several cases of rhythm decisions that were not very close to each other from the different treatments (e.g., VESTAL vs. MESA, see legend to Table I). We do not believe that these inconsistencies are matters of analytical chaos. First, it needs to be kept in mind that overtly periodic behaviors are simply not common in the activity records for pero\textsuperscript{4d} (or the per-null mutants either). Thus, one is dependent on these high-resolution and/or spectral analyses to detect rhythms from these mutant data; and yet the programs are not performing tricks, because when data of these kinds are “shuffled” then MESA treated, no significant rhythmicities have ever been detected (Dowse et al., 1987; Dowse & Ringo, 1989b). Second, the observation that applications of VESTAL and MESA algorithms do not always agree is in a sense expected. Techniques such as these do not have flat spectral responses, so any innate “filtering” effect will affect the output of the analysis. Based on empirical observations (Table I), (1) VESTAL appears to have a filtering effect that “passes” lower frequencies (i.e., longer periods) preferentially (Table I, Fig. 1); (2) MESA, which is arguably able to produce the least biased estimate of the spectrum (Ulrych & Bishop, 1975), nevertheless has been fitted with a low-pass Butterworth digital filter (Hamming, 1983; Dowse & Ringo, 1989b), which passes periods of 4 h with an attenuation of 3 db, calling off rapidly with lower periods; this sharpens the ultradian peaks of interest, on which the various reports of MESA-analyzed Drosophila behavior have concentrated (e.g., Dowse et al., 1987; Dowse et al., 1989; and Table I of the current study). We replaced this filter with another low-pass one that has its 3 db cutoff period at 12 h. Then, 12 records, which were arrhythmic by this analysis as originally applied but long-period by VESTAL, were re-analyzed by MESA: 6 were still arrhythmic, but 3 were long-period (for an example, see legend describing Fig. 1A); and the remaining 3 had periodicities appear in the 16–19 h range.

The pero\textsuperscript{4d} Mutant is Hyperactive in Free-running Conditions

Actograms, based on monitoring the locomotor behavior of pero\textsuperscript{4d} flies in DD, seemed...
THE perミュANT

Activity during long-term monitoring of behavior as affected by per variants. From the data summarized (regarding rhythmicities) in Table I, and from locomotor activity records of adults expressing additional genotypes (see text), the numbers of detected movements (infrared beam breakages) per half hour, per fly were computed. Using these means, genotypic averages (hence means of means, unweighted) were arrived at, and are here quoted ± SEM. N = number of flies tested

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Activity/0.5 h</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. per뮤</td>
<td>204 ± 16</td>
<td>44</td>
</tr>
<tr>
<td>2. per뮤/peP</td>
<td>232 ± 24</td>
<td>29</td>
</tr>
<tr>
<td>3. per뮤Df</td>
<td>196 ± 27</td>
<td>12</td>
</tr>
<tr>
<td>4. perquam/ +</td>
<td>126 ± 19</td>
<td>9</td>
</tr>
<tr>
<td>5. per+</td>
<td>121 ± 9</td>
<td>53</td>
</tr>
<tr>
<td>6. perquam</td>
<td>113 ± 13</td>
<td>41</td>
</tr>
<tr>
<td>7. perquam</td>
<td>102 ± 29</td>
<td>4</td>
</tr>
<tr>
<td>8. perquam</td>
<td>126 ± 23</td>
<td>14</td>
</tr>
<tr>
<td>9. perquam [64j4/TEM202]</td>
<td>249 ± 29</td>
<td>34</td>
</tr>
<tr>
<td>10. perquam [62d18/TEM202]</td>
<td>255 ± 49</td>
<td>4</td>
</tr>
</tbody>
</table>

From statistical tests performed on these data, one way ANOVA indicated, overall, a highly significant difference among these genotypes; yet, the values from the perquam, perquam, and peP flies were the same, even though the "genetic background" differs among these three stocks [the first two being derived from Canton-S wild-type, albeit 10 years apart (see text); and the last from either Oregon-R or Hikone (Hamblen et al., 1986)]. The recent tests of Oregon-R's and Hikone's activity rhythms (see legend to Table I) revealed no hyperactivity associated with these two wild-type strains (Oregon-R: ca. 85% of the activity levels exhibited per + and perquam-1, this table; Hikone: ca. 90% of these levels). perquam (line 1) behaved statistically the same as when this mutation was uncovered by either perquam (line 2) or the Df (line 3, = Df(1)w autonomous); in addition, the last two types listed here (lines 9 and 10; see text) were homogeneous. By a posterior comparison (Scheffe, 1959), perquam was more active (α = 0.05) than wild-type and all three of the other peP types (yet the latter were the same as wild-type as well as each other; see above). Similarly, the two perquam-uncovers types (see above) were more active than per+ or the three other peP's, and the same was so when the homozygous per cases were compared to wild-type or these peP's. Comparing the data from peP/+ to those from wild-type and the peP-1, types indicated no differences; the activity level for his heterozygous type also, however, just missed being significantly less than those recorded from either straight perquam, this allele uncovered (two genotypes), or the per+ types.

To reveal denser activity "markings" than we commonly see in the same kinds of analog displays of other arrhythmic mutants’ movements (e.g., Hamblen et al., 1986; Citri et al., 1987; Dushay et al., 1989). Examples are shown in Fig. 1A–C (also see Fig. 1D in Hall & Rosbash, 1987). Similar hyperactivity has been noticed in the actograms of thoroughly per-"minus" flies, i.e., females carrying separate, overlapping per- deletions on each of their X chromosomes (Fig. 6E in Hamblen et al., 1986).

These impressions led us to quantify the amounts of activity, in the context of these relatively long-term experiments (see below), manifested by flies expressing various mutant alleles of per and/or deletions of the locus. Indeed, perquam and per- flies tend to be numerically hyperactive (Table II, lines 1, 9, and 10—the last of these indicating data from a per- type partly independent of that in line 9). There is, however, a good deal of variation of these activity scores (reflected in the large standard errors throughout most of Table II). The perquam mutation is nominally recessive in this character (Table II, line 4 vs. 5; though see caveat in table legend). Its hyperactivity is essentially uncovered by other per-null genotypes (Table II, lines 2 and 3). Thus, this behavioral anomaly maps to the per locus, as opposed to the phenotype being some sort of gratuitous "strain difference." Note, in this regard, that the other per mutations are not appreciably different in their gross levels of activity from the per+ expressing flies (Table II, lines 6–8, compared to lines 4–5; and see legends to Tables.
Figure 3. Locomotor activity monitored during light:dark cycling. Adult movements were recorded as described in Fig. 1, except that lights cycled on and off: 12 h light (open portions of horizontal bars accompanying these actograms) followed by 12 h darkness (filled portions of the bars), then 12 h light again, etc. The periodicities in LD for all these flies' behavior were 24.0 h (by Chi-square periodogram analysis). A. two per\textsuperscript{04} records, each from monitoring a male. B. per\textsuperscript{04}/Df(1)w\textsuperscript{11} female, whose behavior was recorded in LD for about the first 10 days of this record, followed by continuation of this monitoring in DD, after the time indicated by the arrow; the periodogram for these DD data indicated a significant rhythm, $\tau = 36.0$ h. C. wild-type male, monitored in LD, then DD (see B); DD $\tau = 24.0$ h. D. two per\textsuperscript{04} female records. E. per\textsuperscript{04}/Df(1)w\textsuperscript{11} female, monitored in LD, then DD (see above); there was no significant rhythmicity (by periodogram) for the DD portion of this record.
The $per^{04}$ Mutant was repeated (three months later), and the values for these three genotypes (in the same order as just listed; $N = 12$ in each case) were: 158 ± 10, 106 ± 11, and 142 ± 10. Therefore, this pattern of results, while equivocal, seem reproducible.

$per^{04}$ Flies are Different from Other $per$-nulls During Light:Dark Cycling

Tests of the $per^{04}$ mutant during light:dark cycling (LD) revealed it to behave differently from flies expressing other $per$ alleles. In LD, normal adults exhibit both morning and evening peaks of activity (Fig. 3C), whereas $per^{01}$ tends to be more active throughout the light phase than in darkness (Fig. 3D, E; cf. Petersen et al., 1988; Dushay et al., 1989). In this feature, the new $per^{04}$ mutant (Fig. 3A, B) behaves more like wild-type than $per^{01}$. These results are presented more quantitatively in Fig. 4, which also indicates (see legend) that the relative day- vs. night-time activity levels (computed as in Table II) for most of the $per^{04}$ flies tested in LD, were more similar to wild-type than to $per^{01}$. Yet, a few of the LD records from $per^{04}$ looked more like those of $per^{01}$ than wild-type. Such relatively uncommon $per^{04}$ cases are plotted in Fig. 4C, and the numerical L vs. D activity levels were $per^{01}$-like as well (see legend to Fig. 4). The absolute, overall activity levels in these experiments did not precisely parallel the DD results. In LD, $per^{01}$ flies were twice as active as $per^{04}$'s, which were 80% more active than $per^{+}$. Yet, when 9 $per^{-}$ adults had their behavior monitored in LD (data not shown), they were 60% more active than $per^{01}$; this result is similar to that found in the DD experiments (Table II). The $per^{-}$ cases were ca. three times as active during L vs. D, i.e., the same relatively high level of daytime behavior as exhibited by $per^{01}$ (see legend to Fig. 4).

Another characteristic of LD activity records for wild-type Drosophila is an appreciable “phase-lead,” whereby the flies seem to anticipate light-on by becoming quite active on the order of 1–2 h in advance of this environmental change (discussed by Hamblen et al., 1986; also see Helfrich & Engelmann, 1987, Petersen et al., 1988, and Dushay et al., 1989, for examples). This phenomenon is revealed in the summary of our LD records for wild-type (Fig. 4B) as increasing activity between 1000 and 1200 h (the latter being the time of lights-on). $per^{04}$ flies, however, did not exhibit any more pre-light-on activity than did $per^{01}$ (Figs. 4A, C vs. D). Thus, the new mutant is not entirely wild-type-like in its LD activity behavior.

Rescue of $per^{04}$ by DNA-mediated Transformation

Transformants, involving $per$-locus DNA cloned from wild-type, have been generated, resulting in “rescue” of the arrhythmicity caused by the original three $per^{0}$ mutations or $per^{-}$ (Bargiello et al., 1984; Zehringer et al., 1984; Hamblen et al., 1986; Citri et al., 1987; Yu et al., 1987a, b). Certain of these studies were aimed in part at determining, at least roughly, the locations of the “inactivating” mutations. This kind of question was asked with regard to $per^{04}$, namely: Does the factor responsible for its phenotypic defects map to this locus, i.e., to the coding (and intron-containing) portion of it or to the “S’ flanking” region—as opposed to involving (in addition or instead) a neighboring subset of the locus that encodes transcripts other than $per$'s 4.5 kb mRNA (see below)? If $per^{04}$ is a $per$ mutant per se, then the rescuing DNA fragments determined in the studies cited above should restore overt rhythmicity to flies carrying the new mutant allele. [Note in this regard that a regulatory vs. coding-region etiology for $per^{04}$'s defects cannot be discriminated here: Even if the putative site change responsible for this mutation is 5' to a piece of rescuing DNA, that fragment could still effect rescue, given previous results showing that $per$ DNA devoid
FIGURE 4 Summary of LD activity experiments. These panels display results of analyses in which the first step involved averaging the numbers of activity events occurring in light:dark cycling conditions (cf. Fig. 3) from corresponding portions of successive cycles; this resulted in a single cartesian-coordinate plot for a given fly, which displays its entire 7–10 days' worth of behavioral data (cf. Petersen et al., 1988). The generation of such an activity histogram also involves determination of the "best fit" circadian period for this animal, so that the average activities, per cycle, for the 0.5-h bins in successive cycles will be correctly aligned. After the individual activity histograms were prepared for the several LD records of flies expressing a given genotype, the periods turned out to be 24.0 h in each case (not only within, but also among genotypes), i.e., the same time span as that of the light:dark cycle. Thus, summary histograms, which merge (and average) the data for each 0.5 h bin's worth of events for the several flies of a type, could be readily produced and are displayed here. Before such merging, the activity value for a given bar in the histogram for each individual-fly histogram was converted to a fraction of the total activity for this animal; then, to create a bar in the summary histograms shown, the average of the several fractions from all flies of a given type, for a given portion of the cycle, were computed. Dots above the histogram bars designate SEMs (re fly-to-fly, as opposed to day-to-day, variation). The levels of activity (normalized as just described) that occurred during the light phases are depicted by open vertical bars and behavior occurring in darkness by black bars. Note that lights-on was at noon (see abscissas of these modulo 24 h plots), as is also shown in actograms of Fig. 3. The genotypes of the groups are indicated with each panel. A. There were 14 flies tested; the mean per bin, per fly activity value (cf. Table II) during the day (L) was ca. 45% higher than during the night (D) for these perΔ's. B. 16 flies; D activity was ca. 60% higher than during L for these wild-types. C. 3 flies expressing perΔ (cf. A), whose actogram data (cf. Fig. 3) resembled those from LD testing of perΔ; L activity levels were ca. 5 times higher than during D for these particular perΔ's. D. 11 flies; L activity levels were ca. 3 times higher than during D for these perΔ's.

of all 5′ flanking material—as in the 7.2, 8.0, and 14.6 kb fragments indicated in Table II of this report—can restore rhythms even to flies entirely deleted of the per locus (Hamblen et al., 1986).
on locomotor activity in DD do the same for the new mutation (Table III). In addition, the “strengths” of the rhythmic phenotypes—in terms of periodicities and proportions of tested flies exhibiting overt and significant rhythms—paralleled the data of this kind obtained previously for the other per-nulls (cf. Zehring et al., 1984; Hamblen et al., 1986; Citri et al., 1987; Yu et al., 1987a, b). Therefore, per\(^{04}\) appears to be an orthodox mutation that has been induced squarely at the period locus.

There were, however, anomalies accompanying these results. Rescue of the hyperactivity described above (cf. Table II) was not straightforward, in that there was no particular correlation between these transformed flies’ rhythmicity and normal levels of activity. This means that some of these transformed individuals were not hyperactive whether or not their arrhythmicity was rescued; also more activity could occur in the rescued individuals (within a transformant type) than in the still-arrhythmic records. [Note that this lack of full “penetrance” is a routine feature of per transformants in which the transduced DNA does not encompass the entirety of this genetic locus (cf. Zehring et al., 1984; Hamblen et al., 1986; Baylies et al., 1987).] These results are difficult to interpret, because (1) there is, in general, considerable variability in activity levels exhibited by flies expressing per\(^{04}\) (see the large standard errors in Table II); (2) the genetic backgrounds among transformant types are rather different (see Materials and Methods), and (3) the numbers of transformed flies tested is not large. A possible exception to the latter problem concerns transformant type “14.6:21,” for which rather more data were collected; in this case there was appreciably less hyperactivity exhibited by the rhythmic individuals (Table III). Yet, it was not possible to conclude from these experiments that the genetic etiology of per\(^{04}\)’s aberrant rhythmicity (Table I) is the self-same factor as that which erratically leads to hyperactivity.

**Molecular Biology of per\(^{04}\)**

The primary lesion in per\(^{04}\) is a nucleotide substitution that changes a CAG glutamine codon (corresponding to amino acid 464 in the conceptual per protein) to a TAG stop codon (Yu et al., 1987a; Baylies et al., 1987). This base change also creates on paper (cf. Zain & Roberts, 1977) a novel Xba I restriction site in the gene, providing a nicely
FIGURE 5  Southern blot analysis of per^

A. Results of blotting Drosophila genomic DNA that had been digested with Xba I (exception, lane 1: phage λ marker DNA digested with Hind III). Lane 2: wild-type (Canton-S) DNA. Lane 3: per^

4. Lane 4: per^

2. Lane 5: per^

. Lane 6: per^

The filled arrow denotes the 8.1 kb DNA fragment resulting from digestion at sites flanking the per coding region; the open arrow designates the 1.9 kb fragment resulting from digestion of the Xba I site associated with per^

4.3. B. Restriction map of the per locus (after Yu et al., 1987a, b). The position of the 8.0 kb fragment, which was used as a probe for the blot shown in A, is denoted by the bold line beneath the map. Restriction sites are represented as follows: "B" = BamH I, "Bg" = Bgl II, "E" = EcoR I, "H" = Hind III, "X" = Xba I. The position of the novel Xba I site created by all the per^

mutations except for per^

is indicated by the circled ♦.

diagnostic molecular change for the per^

lesion (originally mentioned by Yu et al., 1987a). Unexpectedly, this extra Xba I site has also been found in DNA from the per^

2 and per^

3 mutants (Fig. 5). Southern blotting was carried out using genomic DNA, which was obtained from flies expressing a variety of per alleles and then digested with Xba I. The pattern of hybridizing fragments indicated that DNA surrounding the per locus, from each of the first three per^

mutants, contains the same Xba I restriction
sites, including the unique one known from the sequencing data for per01. In contrast, both wild-type and per04 DNA lack this additional Xba I site (Fig. 5), i.e., the digests yielded only a ca. 8.1 kb fragment resulting from complete digestion at Xba I sites flanking the per coding region. All other per0 mutant DNAs were internally cut, resulting in hybridization to both 6.2 kb and 1.9 kb fragments (Fig. 5), which is diagnostic for the nonsense mutation responsible for per04. Restriction digests using two other enzymes, Bgl II and Sst I, yielded (data not shown) identical hybridization patterns for all of the genotypes indicated in Fig. 5, suggesting that no gross changes at the DNA level are associated with these four mutants. Also, DNA from flies expressing two other “rhythmic genotypes,” per0 and per(L) (cf. Konopka & Benzer, 1971; Hamblen et al., 1986; Yu et al., 1987b; Baylies et al., 1987), resulted in Southern-blotted bands that exhibited no anomalies, including no novel Xba I sites (data not shown).

At the time of its isolation as an apparent arrhythmic mutant, per04 was hypothesized to be a null variant. Thus, we considered that it produces an inactive (or truncated) protein (cf. per01). Alternatively, the new mutation might lead to an absence of mRNA from this gene, because of a “regulatory” mutation, or possibly a deletion of all or part of per (though the Southern blotting results belie this explanation). Given that per04 has turned out not to be an entirely null factor on phenotypic grounds (e.g., Table I, Fig. 3), we assume that it must be transcriptionally active at some level. In fact, Northern blotting experiments showed that “the” 4.5 kb per RNA (which is in reality a small family of similarly sized, alternatively spliced transcripts; Citri et al., 1987) is produced in what appears to be normal amounts by per04 adults (Fig. 6). Transcript levels have been briefly noted to be normal, as well, in per+ vs. per0, per(L), and per04 (Bargiello & Young, 1984; Reddy et al., 1984; Young et al., 1985; Hamblen et al., 1986).

The Northern blotting data for per04 revealed, however, a striking anomaly. DNA probes that are specific to this gene (Fig. 6A) or that cover a portion of per itself (and a neighboring, more proximally located subsegment of the X chromosome, Fig. 6B) detected an RNA species that is only ca. 3 kb. There are a variety of possible explanations for this smaller-than-normal transcript (see Discussion). We believe, in any event, that it is not an artifact, because (1) the 3 kb band has failed to appear in previous studies of this type (see citations at end of the previous paragraph); (2) it was not present in RNA extracted from flies expressing other per-mutant or normal genotypes [Fig. 6; and another Northern blotting experiment involving RNA from Oregon-R and Hikone adults (data not shown)]; and (3) it was found in three experiments involving not only separate blotting and probing operations, but also independently grown flies and different RNA extraction protocols [see legend to Fig. 6 for description of two of these experiments; in the third (data not shown), the anomalous band was found in extracts of per04/ per0 flies]. Finally, the appearance of the 3 kb band was found to be dominant to per+, in the same Northern blot as that which analyzed RNA from the two wild-type strains (see above).

**DISCUSSION**

**Basic Properties of the New period Mutant**

The elementary aspects of per04 as a newly isolated “clock mutant” are straightforward. First, this new allele was recovered only after screening a large, but not vast, number of mutant “candidates” (see Materials and Methods), as opposed to this new
FIGURE 6 Northern blot analysis of transcripts from per". Flies of the indicated genotypes (see below) were grown as usual (see first section of Materials and Methods); young adults, emerging from completely separate groups of cultures for blot A vs. B (see below), were collected (A) during the daytime or (B) during the middle of the light and middle of the dark phases of a 12h:12h LD cycle. p(A)+ RNAs were loaded, nominally at 10 μg/lane for blot A (though see below) and 5 μg/lane for B; these materials were fractionated on 1% agarose formaldehyde gels, transferred, and probed for per-locus transcripts (see last section of Materials and Methods for details). A. The probe was homologous to only the 4.5 kb per transcript. B. The pairs of lanes for each genotype contained RNA from daytime (left) vs. nighttime (right) collections (see above); the probe was homologous to both the 4.5 kb mRNA (bands arrayed across the top of the figure) and a 0.9 kb RNA encoded at this locus (intense bands across the bottom). A novel ca. 3 kb transcript, detected in both experiments in the RNA from per" (04) adults, is indicated by the arrows. RNAs from wild-type (+) or from per" (01) did not yield anomalous bands. In blot A, the abundance of the 4.5 kb RNA in the wild-type appears to be less than in the two mutants, but application of a control probe—i.e., a portion of the gene encoding ribosomal protein #49 (cf. Al-Atia et al., 1985; James et al., 1986)—indicated lower loading for this (left-most) lane (data not shown). When the blot in A was rehybridized with probes for 2.7 and 1.7 kb transcripts (Reddy et al., 1984), whose genomic sources are X-chromosomal regions just proximal to those for the 4.5 and 0.9 kb species, the bands indicated approximately equal levels of the former two RNAs from all three genotypes and helped to determine the size of the novel transcript (data not shown).

variant being ridiculously easy or extremely difficult to induce. The same sort of frequencies for finding new rhythm mutants have accompanied several earlier screens (reviewed by Hall & Rosbash, 1987, 1988). It is notable that about 80% of the rhythm-altering mutations induced on D. melanogaster's X chromosome have occurred at the per locus (Konopka, 1987a, b; and this report).

Since per" was chemically induced, it could be a point mutation, consistent with certain elements of its phenotypic effects and inferred molecular expression. For example, the new arrhythmic mutation seems to be neither a real zero allele nor a per deletion on a variety of criteria: (1) Usually hidden, but bone fide, long-period activity
rhythms are readily extractable from, and occasionally observable in, the behavioral data collected by monitoring per\(^{14}\)'s locomotor activity in constant darkness (Table I, Fig. 3B); that there are also cryptic ultradian rhythms associated with these data records does not suggest that per\(^{14}\) is (or is not) a null mutant, since even per\(^{1}\) flies exhibit these kinds of periodicities (Dowse et al., 1987). (2) Activity records stemming from LD experiments brought out the still-functional nature of peper\(^{14}\)'s (implied) gene product, because these data (Fig. 3) resemble those from wild-type flies more than per\(^{-}\)-type actograms; there is a proviso, however, in that the new mutant exhibits the same relatively weak phase lead as does the original per\(^{1}\) mutant (Fig. 4).

Such LD experiments also served to describe the basic characteristics of Drosophila rhythms more extensively than has been reported in previous studies, most of which have involved periodic eclosion or activity monitoring in constant darkness only (i.e., after unmonitored LD entrainment). Thus, it is useful to specify that a “clock function” underlies wild-type behavior in LD, given that the flies tend to become rather active well in advance of a given dark:light transition (Fig. 4). That is, the wild-type’s cyclical patterns of activity vs. inactivity are not merely “forced” responses to the environmental cycles (also see Petersen et al., 1988; and Dushay et al., 1989). In contrast, the anticipation of lights-on seems much less than normal in per-null flies and or in those expressing per\(^{14}\) (Figs. 3, 4). Yet, Helfrich & Engelmann (1987, their Fig. 2) show an example of per\(^{-}\) behavior in LD with a fair amount of lights-on anticipatory activity. However, the phases of “arrhythmic” per mutants’ activities against a background of LD cycling, have not been determined from formal analyses of behavioral records. Thus it is not known whether per\(^{14}\) and possibly per\(^{1}\) as well are able to “entrain” to 12:12 LD. If not, these mutants’ periodic behavior in such conditions could in fact be forced responses to the lights coming on and going off.

An organism whose internal pacemaker is not running at exactly 24 h entrains to 12:12 LD by resetting its clock each day to give a periodicity of 24.0 h, accompanied by a manifestation, in the relevant behavioral records, of an oscillator’s function, e.g., a phase lead (see Fuchs, 1983, for an example of this phenomenon from another organism). If a given per\(^{14}\) fly has no functioning circadian pacemaker it might behave like per\(^{1}\) in LD (see Fig. 4C). If another fly expressing per\(^{14}\) is having its activity governed by a very long-period clock (Table I, Fig. 3B), it might not be able to reset itself enough to entrain, and so this animal might exhibit morning and evening “bursts” of activity only at lights-on and lights-off. An inspection of Fig. 4B, however, does not support this notion very well: These per\(^{14}\) flies’ behaviors seem, overall, to be more than startle responses to the environmental changes. It would be instructive, in any case, to examine the behavior in LD of per\(^{14}\) and per\(^{1}\), to determine whether the patterns resemble those summarized in Fig. 4B: Do these data in fact reflect a low-frequency (i.e., much greater than circadian) oscillator for per\(^{14}\) individuals? Are these flies, and the straight long-period mutants, entrainable?

We suggest from these kinds of results and inferences that per\(^{14}\) is a severely hypomorphic mutant, i.e., one whose level of actual or effective gene product is quite subnormal. A common interpretation of per’s expression, in terms of correlating such gene action with activity rhythm periods, is that the lower the level of the gene product (experimentally determined, e.g., Baylies et al., 1987; or inferred, e.g. Smith & Konopka, 1982) the longer the periodicity (also see Bargiello et al., 1984, and Coté & Brody, 1986). In fact, another per variant, caused by a chromosomal breakpoint within the locus [in a strain called T(1; 4)JC43], is somewhat like per\(^{14}\), in that it (1) causes adults to be either (apparently) arrhythmic or long-period (i.e., depending on the individual fly tested), and (2) results in a low level of anomalous (\(\approx 4.5\) kb) per mRNA (Bargiello & Young, 1984; Reddy et al., 1984). The array of (DD-based) phenotypes
associated with per^{ab} (Table I) is rather similar to that just described for the effects of this chromosome aberration.

**Molecular Properties and Effects of per^{ab}**

Given the behavioral differences between per^{ab} and the previously isolated or constructed per-null variants, we guessed that the new mutant resulted from a missense mutation, which would hypothetically cause the gene’s protein product(s) to be present at reduced levels, to function poorly on a per-molecule basis, or both. This notion was based on (1) the fact that per^{ab} is not knowingly explained by a nonsense mutation (Fig. 5; but see below); and (2) the inverse relationship between levels of per expression and length of inactivity rhythm periods discussed above; in this regard, then, the inferred missense mutant would be hypomorphic, which is not an unreasonable assumption.

The novel, smaller than normal transcript associated with the new mutant’s expression (Fig. 6) muddles the “missense mutant hypothesis.” If per^{ab} is caused by a nucleotide substitution in a coding region, which under this hypothesis has led to an amino acid substitution, then it would seem also to have caused: (1) an anomalously spliced product from the gene to be processed from the primary transcript; or (2) a sometimes-used transcription start-site, which would be far downstream of the normal one; or (3) a premature transcription termination site. [The latter conjecture is dubious, given the “3’ untranslated” probe that, in Fig. 6B, detected the anomalous band.] In principle, it is possible that one nucleotide substitution could lead, in one of these ways, to a qualitatively abnormal transcript as well as to a missense mutant, e.g., if a reasonable (though not obligatory) splice-junction could be mutationally created at a site within the gene that is also part of a codon. One alternative would be that per^{ab} is a double mutant; for example, two base substitutions could have been induced within the gene, one of which changes the coding information within the 4.5 kb RNA and the other leading to a smaller than normal form of this transcript.

If we assume for the moment that a coding-region alteration is responsible for the biologically mutant phenotypes of per^{ab} (Fig. 1, Table I), this leads to a question about the fate of the novel transcript. Perhaps it is inconsequential, e.g., simply degraded, whereby it would have no effects on rhythm-related or other biological phenotypes. Alternatively, the 3 kb RNA might be translated to produce a per protein fragment. This could either have no biological consequences (see above), in which case the “basic” (hypothetical) missense mutant would be solely responsible for the rhythm defects; or, the partial protein product might interfere with the function of the polypeptide encoded by the 4.5 kb mRNA. Under this hypothesis, it might even be that this normal-size transcript (Fig. 6) is also intrinsically wild-type, in which case the nucleotide substitution surmised for per^{ab} would not even have to be within a coding region of the gene. The implications of this idea would be, in general terms, similar to those presented by Coté & Brody (1986); they inferred from an analysis of rhythm periods associated with various genotypes involving per (cf. Smith & Konopka, 1982) that the qualitatively altered protein produced under the instruction of this allele (cf. Yu et al., 1987a; Baylies et al., 1987) can interact with a normal polypeptide, i.e., in the relevant heterozygous flies, such that the very short periodicities connected with this mutation’s effects are “dragged down” towards the normal range. Yet, a simple prediction of the damaging-protein-fragment hypothesis for per^{ab} is that this mutation would be a “dominant arrhythmic” mutation, i.e., when heterozygous with a normal allele; this is not the case (Table I).
Clearly, it will be important eventually to determine the exact nature of the genomic change that occurred when per\textsuperscript{04} was induced (cf. Yu et al., 1987a; Baylies et al., 1987). In the meantime, the new mutant can be viewed formally as a hypomorphic per variant, in spite of the complexities and puzzles just discussed. Thus, we now discuss per\textsuperscript{04} in the context of the protein product of this gene. Immunohistochemical experiments, using an antibody reagent specific for the per polypeptide, showed no staining in tissue sections of per\textsuperscript{04} adults; the same results were obtained in parallel experiments on per\textsuperscript{01} and per\textsuperscript{-} animals (Siwicki et al., 1988). Such findings do not necessarily mean that per\textsuperscript{04} is a “protein null” variant, in that (1) the antigenicity per se—as opposed to the level—of the pertinent protein may be altered by per\textsuperscript{04} (if indeed an amino acid substitution is involved; see above); or (2) a low level of the protein could have been present in the sectioned material, but below some threshold of detectability. If the second of these possibilities describes per\textsuperscript{04}’s protein product, then it is likely that the polypeptide is “unstable” (degraded so readily that its steady-state levels are relatively low), probably because of an intrinsic abnormality (i.e., if per\textsuperscript{04} is indeed a missense mutation). Such a suggestion would be consistent with the fact that the level of per mRNA was not detectably reduced in per\textsuperscript{04} flies—although the possibility of a poorly translatable 4.5 kb mRNA (Fig. 6) from the mutated gene is not ruled out.

At all events, the new period mutation is probably unique at the molecular level in addition to having a rather special spectrum of phenotypic consequences. If an amino acid substitution is involved, what kind of change like this would render the product nearly nonfunctional as well as non-antigenic or, alternatively, badly unstable? Might per\textsuperscript{04}’s hypothetical missense mutation designate a “new” region of the polypeptide, which will turn out to be significant in terms of this molecule’s structure and function? The sites altered in the two molecularly mapped missense mutants, per\textsuperscript{01} and per\textsuperscript{41} (Yu et al., 1987a; Baylies et al., 1987), may have defined regions of the protein that are interesting in this regard (see discussions in Hall & Rosbash, 1987, 1988). The putative amino acid substitution in the new mutant could have occurred in a part of the gene completely separate from these two previously determined sites.

Perhaps, though, there is no such substitution in per\textsuperscript{04}’s polypeptide. This mutation might, therefore, have led to a stop codon (which may or may not have also caused the anomalous per transcript to be produced; see above). If per\textsuperscript{04} involves a nonsense mutation, it would presumably have been induced in a codon different—and probably downstream—from that where the original three per\textsuperscript{01}’s occurred (Fig. 5), given the several different phenotypic defects between the new mutant and the others. This supposition implies that protein “fragments,” as translated in certain per nonsense mutants, could retain some biological activity, allowing for patterns of rhythmicity appreciably stronger (Table I, Fig. 3) than in a completely non-expressible per variant. This possibility has been raised before (Yu et al., 1987a), in conjunction with per\textsuperscript{01}’s and per\textsuperscript{-}’s behavioral non-equivalence (cf. Hamblen et al., 1986; and Table II of the current report).

A final comment in this section focuses on the striking non-uniqueness of the original three per\textsuperscript{01} alleles. In addition to all three of these genetic variants seeming to be similar in their basic properties and phenotypic effects (Konopka & Benzer, 1971; Smith & Konopka, 1982; Hamblen et al., 1986, and Table II of this report), they have turned out to be identical nonsense mutants (Fig. 5 of this report). How? Unfortunately, the pedigree histories for these three mutants cannot be thoroughly reconstructed (including the matter of which specific wild-type X chromosome was, when mutated, the source of per\textsuperscript{01}; see Materials and Methods). Thus, it is possible that per\textsuperscript{01} itself was merely “reisolated” as a consequence of mistakes made in the handling of animals involved in mutant screens carried out subsequent to the original one (Kon-
opka & Benzer, 1971). Yet, if memory serves, the per⁰'s isolated subsequently to #01 may have been genuine for the following reasons: Approximately 10 years after the original arrhythmic allele was recovered, per⁰² was isolated (by one of us, R.J.K.) at the California Institute of Technology (Smith & Konopka, 1982), and per⁰³ (Hamblen et al., 1986) as well as per⁰⁴ (these two also found by R.J.K.) at Clarkson University. Since care was taken to avoid contamination of cultures with stray (e.g., per⁰⁴) flies, and since mistakes of this kind would have had to occur at widely separate times and places, it is argued that the identity of the first three per⁰'s might not have resulted from mishandling. A more intriguing possibility is that the nucleotide in question (cf. Fig. 5) is a "hot spot" for induction of mutations at this locus. This possibility could be assessed by searching for further null or near-null per mutants. Such screens are warranted in any case, given that there are only two unique mutations of this type and that more molecular data on important domains of this clock gene's product would be welcome.

Behavioral Anomalies and per⁰⁴'s Effects on Locomotor Activity

The ultradian, and other, rhythms that were readily extractable from the behavioral records for per⁰⁴-expressing adults (Table I, Fig. 2) provide further evidence to support the hypothesis that Drosophila's circadian clock involves communication among high-frequency oscillators. In some formalistic manner (e.g., not necessarily intercellularly) these individual oscillating components would need to be well coupled in order that the animal's circadian clock be "constructed" (cf. Dowse et al., 1987; Dowse & Ringo, 1987).

The array of periodicities found for the new mutant, by the relevant high-resolution analyses (Dowse & Ringo, 1989b), is not the same as in the case of per mutants that are known to be null variants at the level of gene action (cf. Fig. 5, and see below). There were four "oscillatory modes" describable for the pattern of period values determined, by MESA and VESTAL, for per⁰⁴ (Table I), i.e., essentially all known modes for this organism except for wild-type- and per⁰²-like rhythms (which have very strong periodicities in the 24 h and 19 h ranges, respectively, cf. Konopka & Benzer, 1971; Smith & Konopka, 1982; Hamblen et al., 1986; Dowse & Ringo, 1987). The results from similarly analyzing per⁰⁴ activity records fell into these same categories (Table I), but there were considerably more long-period cases in the per⁰⁴ records, and the ultradian rhythms exhibited by the original arrhythmic per mutant tended not to be as sharply discernible (Figs. 1, 2; compare to Dowse et al., 1987).

Several of the high resolution spectral analyses performed on the new mutant's behavior revealed multiple modes within a given fly (Table I, legend: these dual-period spectra were obtained for some of the per⁰⁴'s as well, but essentially only by VESTAL). These kinds of results have been found before: (1) in the behavioral records from per⁰¹ and per⁰² adults, which not infrequently have quite-short (ultradian) components accompanying their expected long-period MESA peaks (Dowse & Ringo, 1987); (2) in wild-type D. melanogaster that were reared then monitored for activity (i.e., always maintained) in constant darkness; these have been termed per-mutant phenocopies (Dowse & Ringo, 1989a), in that they are frequently "arrhythmic" but actually exhibit the wide array of oscillatory modes revealed in the current report as well (Table I, especially for per⁰⁴, i.e., by both VESTAL and MESA); (3) in adults expressing the disconnected visual system mutation, LD-reared then DD-tested flies are also most always arrhythmic by inspection of elementary periodogram analysis (Dushay et al., 1989), but in reality these mutants are (roughly) genocopies
of the dark-reared/tested cases; hence, they are similar to per^mutant in their overall patterns of periodicities (Dowse et al., 1989).

Thus, the new period mutant we report here is not unique in the global sense, but it is a special kind of mutation among those that have occurred in this particular clock gene. Unlike the case of the per^-s, per^mutant is usually not overtly rhythmic. But, like these long-period mutants, per^mutant can be thought of as lying on a continuum between the "null state"—with chaotically functioning, i.e., almost completely "uncoupled" ultradian oscillators (cf. Dowse & Ringo, 1987)—and the "long state," for which a nascent (i.e., somewhat well coupled) circadian clock is coalescing out of the chaos. So the new per variant may be just at the point where this clock is beginning to be "formed," not necessarily in the neural developmental sense, but—which is equally likely (cf. Ewer et al., 1988)—in terms of physiologically mediated interactions among the relevant component oscillators in adult flies, whose circadian pacemaker is trying to function in part via this kind of communication in the pertinent part of the brain (cf. Siwicki et al., 1988).

The new mutation also causes adults to be hyperactive when being monitored in DD for circadian rhythmicity (Table II)—with certain caveats (e.g., Fig. 1 vs. Fig. 3; and the inconsistent aspects of this phenotype in the results from testing transformants, Table III). Hyperactivity for this mutant may occur only in certain circumstances, i.e., during relatively long-term behavioral testing that occurs in constant darkness (Fig. 1, Table II). In the short-term activity tests, per^mutant flies could not be said to be hyperactive (see text in Results); and this in a sense fits with the fact that no abnormalities were detected during short-run assessments of this specific mutant’s phototactic response and optomotor behavior (Dushay et al., 1989).

Nevertheless, the "DD hyperactivity" exhibited by per^mutant seems intriguing, in part because this kind of behavior has been noticed before, i.e., in the actograms resulting from monitoring the locomotion of per^-adults (Hamblen et al., 1986; now quantified in the current report, Table II). The similar behaviors shown by flies expressing these two different per variants would not apparently involve equivalent “nullness” of per^- and per^mutant, since the latter seems not to have this property and because the other pep mutants, which are null (Fig. 5), do not lead to appreciable hyperactivity (Hamblen et al., 1986; and this report, Table II). We considered that per^mutant somehow affects the two other transcripts whose genomic sources are missing from per^- However, data from Northern blotting analysis of p(A)+ RNA, extracted from per^mutant and hybridized to a DNA probe from a chromosomally more proximal region of the locus than those used in Fig. 6, indicated that these other mRNAs were normal in sizes and amounts (see legend to the figure just mentioned). Therefore, it may be that the change within the per gene—which is probably responsible for the abnormal circadian behaviors associated with per^mutant—also causes hyperactivity.

It is impossible to speculate more specifically about how a per^mutant-altered protein might lead to these excess levels of locomotor behavior. It is, however, worth conjecturing that the control exerted by this gene on behavioral rhythms need not be limited to some kind of “utterly central” oscillator function—one that would be far removed in its effects on the “output” phenotype, in this case, locomotion. The levels of such behavior obviously change in conjunction with per-influenced activity rhythms, so it is not farfetched to imagine that this gene’s action has a rather direct effect on the actual amount of behavior occurring on a given day. In this regard, it should be mentioned that the original per^- mutations’ effects on activity rhythms, in their elimination of overtly rhythmic locomotor behavior, do not seem to have been a case of inappropriately boosting the subjective-night levels of activity (in DD) up to those
measured during the subjective day. This would have meant that these mutants tend to be active all the time ("day and night"). Instead, an inspection of the activity scores for per$^{Dn-03}$ (Table II) reveals them to be similar to that of wild-type (i.e., on a crude per 0.5 h basis). Thus, it is possible to describe the effects of the original per$^{Dn}$'s as causing the normal overall level of the fly's activity to be spread more or less evenly through a given day (during DD). In contrast, per$^{Dn+}$ and per$^{-}$ can be thought of as insomniacs, who tend to continue being as active during a given subjective night as they did during the previous subjective day.

So the per gene, in its various forms, seems to contribute to more than rhythmicity per se. Similar conclusions—about this clock gene's influence on the quality of circadian rhythms, as opposed to their mere presence and periodicities—have recently been arrived at by studying the locomotor activity of D. pseudoobscura flies and the effects of this species' per gene when transduced into D. melanogaster (Petersen et al., 1988).

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Note added in proof: The cytogenetic localization of the per$^{+}$ DNA transduced onto chromosome 2 in transformant strain “8.0:4” (see Table III) has recently been corrected from “26F-27A” (cf. Hamblen et al., 1986) to 27E (N. Bonini, personal communication).

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