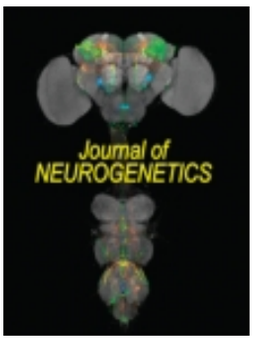

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THE ANALYSIS OF NEW SHORT-PERIOD CIRCADIAN RHYTHM MUTANTS SUGGESTS FEATURES OF *D. MELANOGASTER PERIOD* GENE FUNCTION

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A number of new *period* gene (*per*) mutants were generated by *in vitro* mutagenesis and germ line transformation. Missense mutations were made at amino acid 589, which is altered in the 19 h short-period (*per*^{s}) mutant, and insertion mutations were generated with peptides commonly used for epitope tagging. Most of these new *per* mutants had short behavioral rhythms. Flies with heteroallelic combinations of these new mutant *per* genes were found to have "hybrid" periods, i.e., they had values that were usually in between those of the individual alleles. These findings suggest that short-period *per* mutants are not unusual gain-of-function mutants but rather more traditional loss-of-function mutants that are unable to influence the circadian pacemaker in a proper manner. The data also suggest that the *per* protein may engage in important intermolecular interactions.

Keywords: Clock mutants, circadian rhythms, in vitro *mutagenesis, locomotor activity, epitope tagging, P-element transformation.*

INTRODUCTION

For species where rhythm mutants exist, studies of these genes and their mutant phenotypes have led to insight into time-keeping mechanisms (Rosbash and Hall, 1989; Young et al., 1989; Dunlap, 1990). Mutants in the X-linked *period* gene of *D. melanogaster* (*per*) affect the circadian rhythms of eclosion and locomotor activity as well as ultradian rhythms (very short periods = ca. 1 min) of the male courtship song (Konopka and Benzer, 1971; Hall and Kyriacou, 1990). This gene and its products are apparently important components of a fly pacemaker (Rosbash and Hall, 1989).

The three original mutations that defined the *per* locus lead to three very different circadian rhythm phenotypes. *per*^{01} flies are essentially arrhythmic, whereas *per*^{s} flies have short (ca. 19 hr) periods and *per*^{L1} flies have long (ca. 29 hr) periods (Konopka and Benzer, 1971). The generally accepted interpretation of these three mutant phenotypes is that they reflect the quantitative effects of the mutations on *per* "activity" (Smith and Konopka, 1982). For example, the *per*^{01} allele eliminates *per* activity so that circadian rhythms are undetectable. The short periods of the *per*^{s} strain are due to an increase in *per* activity relative to the wild-type *per*$^{+}$ allele, and the long periods of the *per*^{L1} allele are due to a decrease in activity relative to *per*$^{+}$ (Coté and Brody, 1986). In favor of these interpretations are experiments that ma-

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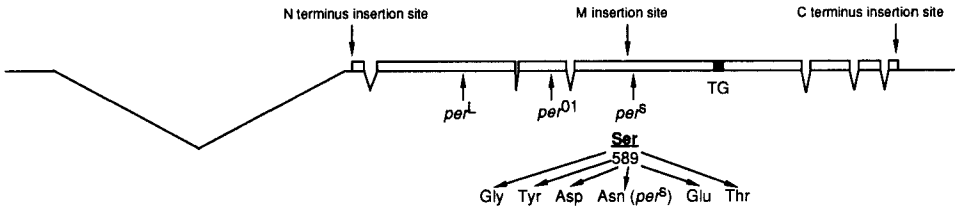

FIGURE 1 Map locations of *in vitro* generated *per* mutations. The molecular locations of the three *per* mutations per^L, per^{01} and per^s, are shown along with the *in vitro*-generated mutations described here. Five amino acids, Gly, Tyr, Asp, Glu and Thr, were introduced at amino acid position 589, which is normally a serine in wild-type *per*. Either the HA or myc peptide was introduced at the three locations, N terminus, M (middle) and C terminus, diagrammed above the map. The boxes denote coding region, the narrow horizontal lines show nontranslated exon material, and the diagonal lines refer to introns. The Thr-Gly (TG) repeat region (Reddy et al., 1986; Jackson et al., 1986) is denoted by the shaded box.

nipulated, by classical genetic means, the number of copies of the *per* gene and measured the fly's circadian period as a function of *per* gene dose (Smith and Konopka, 1982). An increased *per* copy number led to shorter periods whereas a decreased dose (such as a single copy in a female that normally has two copies of this X-linked gene) led to longer periods. More recent studies that used recombinant DNA procedures to alter the levels of *per* gene expression gave rise to similar conclusions (Baylies et al., 1987). Also consistent with this view was the fact that the rhythms of per^L flies are generally weak, whereas the rhythms of per^s flies are very strong (Dowse and Ringo, 1987).

While the per^{01} mutation is due to a stop codon, consistent with its putative null, arrhythmic phenotype (Yu et al., 1987a; Baylies et al., 1987), the per^L and per^s alleles are more enigmatic. They are due to single amino acid replacements: per^{L1} has a valine to aspartic acid change in exon 3, whereas per^s contains a serine to asparagine substitution at amino acid 589 in exon 5 (Yu et al., 1987a; Baylies et al., 1987; Figure 1). Presumably these mutations affect *per* protein level or activity because *per* mRNA levels are roughly comparable in all three genotypes (Hardin et al., 1990).

per protein levels are indeed affected by some *per* mutations. For example, no *per* protein was detected in per^{01} flies, as expected (Liu et al., 1992). The per^{L1} phenotype might also be due, at least in part, to an effect on *per* protein levels. In adult heads from this genotype, *per* protein levels appeared much lower than those observed in per^+ flies (Zerr et al., 1990; Liu et al., 1992). Although the protein assay was histochemical (and therefore not necessarily a direct reflection of *per* protein levels), the observation was consistent with the hypomorphic features of the per^{L1} mutant phenotype and with the fact that the head is the focus for the *per* gene's effect on circadian rhythms (Konopka et al., 1983; Ewer et al., submitted). The missenese mutation might decrease the stability of the *per* protein, thereby leading to reduced protein activity and long rhythms.

In contrast, no change in *per* protein levels was apparent in per^s flies (Zerr et al., 1990). Because missense mutations that increase gene activity should be rare, we generated other amino acid replacements at position 589 and assayed the circadian periods of flies transformed with these novel *per* mutations. Most of these strains had short periods. We were surprised to discover that two other *in vitro*-generated *per* mutations in other locations within the molecule also gave rise to short-period phenotypes. Taken together with the analysis of several novel mutant combinations,

the results suggest that the per^s protein is defective in some aspect of *per*'s normal function. A reconsideration of data from Konopka and Benzer (1971) and from Smith and Konopka (1982) is also consistent with this view.

Circadian oscillators are likely to contain negative feedback loops, and the phenotype of the various short-period mutants and mutant combinations can be explained by proposing that they give rise to a pacemaker with elements that fail to respond properly to these negative cues. As a consequence, these short-period mutants may mimic the effects of increased *per* protein activity normally associated with a hypermorphic phenotype.

MATERIALS AND METHODS

in vitro *Mutagenesis*

The cloned DNA used as the template for *in vitro* mutagenesis at amino acid position 589 was a subclone of the per^+ gene. The cloned fragment extends from the *Sal*I site in exon 3 to the *Sst*I site in exon 5, and is inserted into pTZ19U. Single stranded DNA was mutagenized by the oligonucleotide-directed *in vitro* mutagenesis system (Amersham), using synthesized 21-mers from nucleotide 4778–4798. (All nucleotide and amino acid numbering as in Citri et al., 1987). Each oligo changed nucleotides 4787–4789 from the wild-type AGC sequence to GGC (Gly), ACC (Thr), GAC (Asp), GAG (Glu), or TAC (Tyr). Mutagenized clones were verified by dideoxy sequencing. Mutations from the pTZ19U-*Sal*I/*Sst*I construction were cloned into the transformation vector cp20.1 (Rubin, 1985) containing a 13.2 kb per^+ insert (Citri et al., 1987; Yu et al., 1987b) via four-way ligations. The resulting plasmids are identical to cp20.1–13.2(per^+), except for the altered nucleotides at positions 4787–4789.

The insertion of sequences that code for the HA (Field et al., 1988) or myc (Munro and Pelham, 1986) peptides into *per* coding sequences employed various synthetic oligonucleotides and PCR to construct the indicated *per* variants. All constructs were verified by DNA sequencing. The three insertions gave rise to the following modified coding sequences. At the N terminus (HA/N), the 11 amino acid HA peptide is preceded by 2 additional amino acids and is inserted before the *per* coding sequence, giving the sequence Met, Glu, [Tyr, Pro, Tyr, Asp, Val, Pro, Asp, Tyr, Ala, Ser, Leu (HA peptide)], Met(1), Glu(2) followed by the remainder of the *per* gene. Note that the original initiating methionine is in the same sequence context as in wild-type, and that all *in vitro* synthesized protein contains the HA epitope (I. Edery, unpublished observations). The relative amounts and location of this HA/N protein in transformant flies is indistinguishable from wild-type *per* 13.2 transformants by histochemical analyses (Xin Liu, unpublished data). The myc insertion near the per^s site (myc/M) occurs at position 4729 of the genomic DNA (Citri et al., 1987), resulting in the amino acid sequence Ser(568), Arg(569), [Glu, Gln, Lys, Leu, Ile, Ser, Glu, Glu, Asp, Leu, Asn (myc peptide)], Ala(570), Asp(571), etc. Both of the C terminal constructions (myc/C and HA/C) place the myc or HA peptide sequences directly before the TAG stop codon used in type A and B cDNAs (Citri et al., 1987). The type C cDNA is not tagged in these constructions, due to its shorter reading frame. Since this message only contributes at most 10% of the total *per* message (Citri et al., 1987), its effect on the final period is most likely minimal. All of these insertions were reconstructed into the 13.2 kb clone in cp20.1 by standard procedures.

Germ Line Transformation and Behavioral Analysis

per^{01}; ry^{506} flies were transformed with each of the mutant *per* P-element constructions as previously described (Rubin, 1985). Two independent lines were obtained for 13.2(per^{Gly589}), 12 for 13.2(per^{Thr589}), 7 for 13.2(per^{Asp589}), 1 for 13.2(per^{Glu589}), 2 for 13.2(per^{Tyr589}), 5 for 13.2(HA/N), 5 for 13.2(myc/M), 2 for 13.2(HA/C) and 1 for 13.2 (myc/C). The lines that were tested for activity rhythms are listed in Tables I and II.

Flies were entrained in a 12 h light:12 h dark regime for 2–3 days before being assayed in constant darkness at 25° C as described previously (Hamblen et al., 1986). Data were collected under these "free-run" conditions for 5 to 10 days and activity periods determined by Chi-square periodogram analysis (Hamblen et al., 1986), $\alpha = 0.05$.

RESULTS

One approach to understanding the significance of the short-period phenotype of the original per^{s} mutation is to generate other *per* mutations and analyze their phenotypic consequences. To this end, we made and analyzed two classes of new *per* mutations (Figure 1). The first consisted of other single amino acid changes at position 589, the location of the serine to asparagine change responsible for the original per^{s} phenotype (Yu et al., 1987a; Baylies et al., 1987). The second consisted of the insertion of two different 33 basepair (bp) sequences coding for two different 11 amino acid peptides. This class of mutations was originally designed for other purposes and made use of peptide sequences widely used for epitope tagging (Munro and Pelham, 1986; Field et al., 1988). They were inserted at three different locations within the *per* coding sequence (Figure 1). Both classes of mutants were generated by standard *in vitro* mutagenesis procedures, confirmed by DNA sequencing, reconstructed into a complete (13.2 kb) *per* gene, and introduced into the germ line by P-element mediated transformation. Locomotor activity rhythms of individual flies harboring these mutant constructs were then assayed and compared with flies that carried a complete wild-type 13.2 kb *per* gene. This 13.2 kb *per* gene had been previously introduced into arrhythmic per^{01} flies and shown to effect nearly complete rescue of the rhythmic deficits of the host flies (Citri et al., 1987).

The first class consisted of 5 mutants. The codon at position 589 (normally serine) was changed to glycine, threonine, aspartic acid, glutamic acid, or tyrosine. At least two independent transformed lines were generated for each mutant, except glutamic acid 589, for which only one line was recovered. Together with the wild-type serine and the asparagine from the per^{s} strain, the five mutants provided us with 7 different *per* genes differing only at amino acid 589.

All five of the new mutant genes rescued well the arrhythmic phenotype of the per^{01} host, i.e., the fraction of rhythmic flies (penetrance) was high (Table I). Examples of single-fly actograms show the activity events for individual flies in the standard double plot format, and the periodograms graph the Chi-square analysis which finds the best period for a given data set (Figure 2). Most of the observed rhythms were strong and reproducible within a line.

Table I shows that most of the transformed lines had periods shorter than the 24.8 h observed for the control wild-type 13.2 kb per^{+} transformants. [Note that the 13.2 kb fragment gave rise to rhythms in two transformant lines that are somewhat long compared to a true wild-type strain (Table I; Citri et al., 1987).] A glycine

TABLE I
Activity averages of *per* point mutant transformants

Genotype	CHRM Location of Insert[a]	#RHY[b]	Period Avg[c]	#AR[d]
CONTROLS				
per^{+}		13	24.1 ± 0.1	0
$per^{+};ry^{506}$		17	24.0 ± 0.1	0
per^{s}		31	19.4 ± 0.1	0
$per^{s};ry^{506}$		18	19.8 ± 0.1	0
$per^{01};ry^{506}$		0	—	10
TRANSFORMANTS[e]				
per^{01};13.2(per^{+})-2	3	30	24.9 ± 0.1	0
per^{01};13.2(per^{+})-34	2	14	24.7 ± 0.1	0
Avg Group Period[f]			24.8	
per^{01};13.2(per^{Gly589})-5	2	26	19.4 ± 0.1	7
per^{01};13.2(per^{Gly589})-45	2	12	20.3 ± 0.1	2
Avg Group Period[f]			19.7	
per^{01};13.2(per^{Thr589})-3	3	7	25.5 ± 0.3	0
per^{01};13.2(per^{Thr589})-6a	3	9	25.9 ± 0.2	1
per^{01};13.2(per^{Thr589})-6b	2	4	25.6 ± 0.2	0
per^{01};13.2(per^{Thr589})-8a	2	3	26.2 ± 0.2	0
per^{01};13.2(per^{Thr589})-8b	3	4	25.3 ± 0.1	0
per^{01};13.2(per^{Thr589})-9	3	3	25.8 ± 0.2	0
per^{01};13.2(per^{Thr589})-11	3	2	25.8 ± 0.8	1
per^{01};13.2(per^{Thr589})-15	3	13	25.3 ± 0.2	1
per^{01};13.2(per^{Thr589})-22	2	10	25.5 ± 0.1	0
Avg Group Period[f]			25.6	
per^{01};13.2(per^{Asp589})-7	2	7	22.5 ± 0.1	4
per^{01};13.2(per^{Asp589})-18	3	21	22.6 ± 0.1	13
per^{01};13.2(per^{Asp589})-19	2	9	22.0 ± 0.2	1
per^{01};13.2(per^{Asp589})-20	2	14	23.2 ± 0.1	0
per^{01};13.2(per^{Asp589})-34	2	13	22.7 ± 0.1	0
Avg Group Period[f]			22.7	
per^{01};13.2(per^{Tyr589})-1	2	17	23.0 ± 0.1	0
per^{01};13.2(per^{Tyr589})-7	2	19	23.5 ± 0.1	0
Avg Group Period[f]			23.3	
per^{01};13.2(per^{Glu589})-39	1	13	22.0 ± 0.1	6

[a]The chromosome (chrm) where each transformed insert is located was identified by balancing crosses.
[b]#rhythmic flies.
[c]All flies tested were single dose males and were monitored in constant darkness. The period is the average period, in h ± the SEM, of the rhythmic flies.
[d]#arrhythmic flies.
[e]Every transformed line contains a 13.2 kb *per* insert in a $per^{01};ry^{506}$ background. The amino acid at position 589 is identified within the parentheses, and the independent transformed line name is denoted after the dash.
[f]The average value given is an average of periods from genotypically identical transformed lines. It is presented to denote an approximate value for a given genotype, and is not meant to ignore the variability seen within lines.

at position 589 resulted in an average period (19.7) very similar to that of the original per^{s} strain (asparagine at position 589) and to previously tested per^{s} transformant lines (Yu et al., 1987a). Glutamate, aspartate, and tyrosine gave rise to periods of 22.0, 22.7, and 23.3 h, respectively, intermediate between the per^{s} asparagine and the wild-type serine. Only the threonine transformants gave rise to a slightly longer than normal period of 25.6 h. Serine and threonine are structurally related, whereas

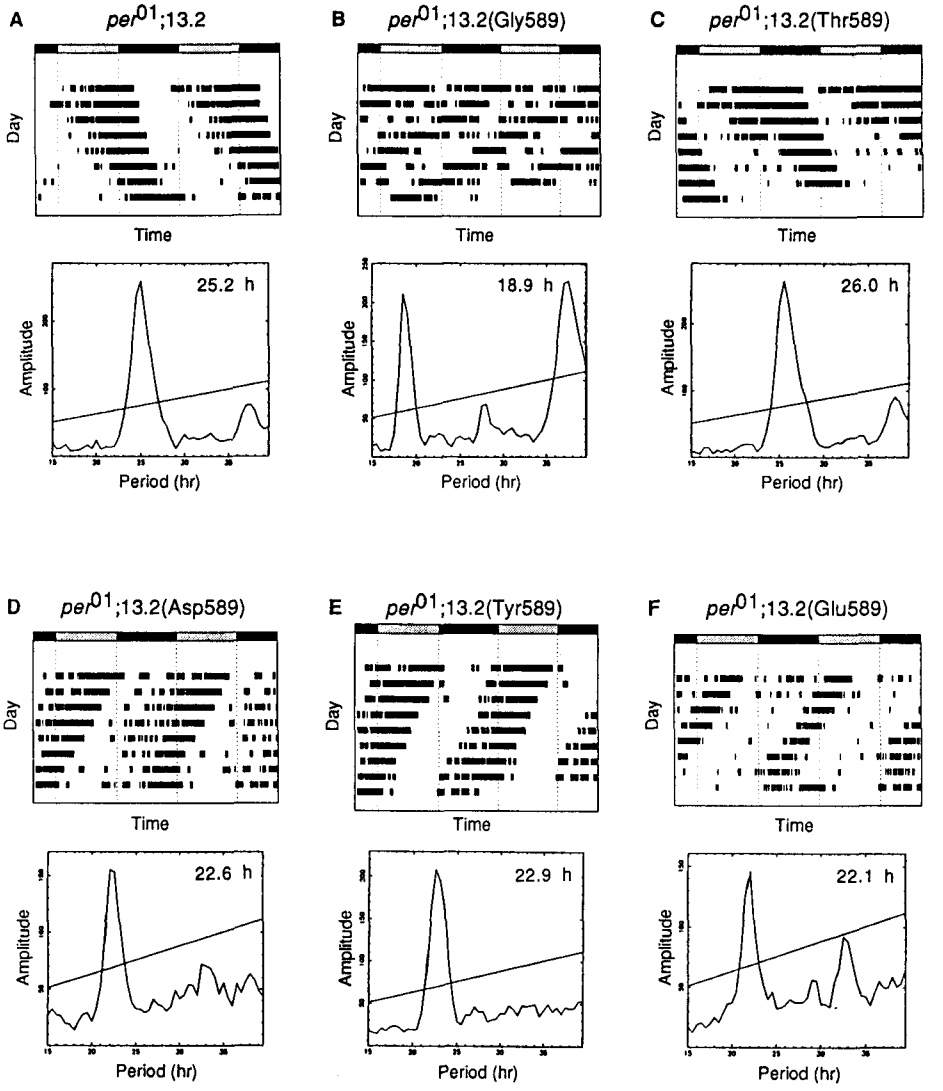

FIGURE 2 Free-running locomotor activity rhythms of *per* point mutant transformants. After individual flies were entrained to 12 h light (L):12 h dark (D) cycles, their locomotor activity patterns were monitored under free-running (constant darkness) conditions (Hamblen et al., 1986). The behavioral records (actograms) are double plotted (days 1–2 of free run on the first line, days 2–3 on the second, etc.). The bars above the activity patterns refer to the subjective day (shaded) or subjective night (black) of a 12:12 L:D cycle. The best estimates of circadian periods were determined by Chi-square periodogram analyses (Hamblen et al., 1986). The values above the sloping lines are significant periodicities (α = 0.05), and the deduced periods are printed in each periodogram. The second peak seen in the per^{01}; 13.2(Gly589) periodogram is a harmonic of the first one (37.8 h vs. 18.9 h), a frequent result of periodogram analysis. The individual flies analyzed were from the transformant lines: **A)** per^{01}; 13.2(per^{+})-34, **B)** per^{01}; 13.2(per^{Gly589})-5, **C)** per^{01}; 13.2(per^{Thr589})-6, **D)** per^{01}; 13.2(per^{Asp589})-34, **E)** per^{01}; 13.2(per^{Tyr589})-1, and **F)** per^{01} 13.2(per^{Glu589})-39.

TABLE II
Activity averages of *per* insertion mutant transformants[a]

Genotype	CHRM Location of Insert	#RHY	Period Average	#AR
CONTROLS				
$per^{+(b)}$		13	24.1 ± 0.1	0
$per^{s(b)}$		31	19.4 ± 0.1	0
per^{01};$ry^{506(b)}$		0	—	10
per^{01};13.2(per^{+})-2[b]	3	30	24.9 ± 0.1	0
TRANSFORMANTS				
per^{01};13.2(myc/C)-18a	2	20	24.8 ± 0.1	5
per^{01};13.2(myc/C)-18b	2	10	25.1 ± 0.1	4
Avg Group Period			24.9	
per^{01};13.2(HA/N)-3	2	19	22.0 ± 0.1	1
per^{01};13.2(HA/N)-21	2	19	22.2 ± 0.1	1
per^{01};13.2(HA/N)-23	2	3	21.1 ± 0.3	1
per^{01};13.2(HA/N)-27	3	4	22.4 ± 0.1	0
per^{01};13.2(HA/N)-36	2	15	22.4 ± 0.1	1
per^{01};13.2(HA/N)-43	2	14	23.0 ± 0.1	6
Avg Group Period			22.5	
per^{01};13.2(HA/C)-16	3	20	24.4 ± 0.1	0
per^{01};13.2(myc/M)-39	1	8	23.3 ± 0.1	3

[a]See notes from Table I.
[b]These controls are repeated from Table I.

the other amino acids have no obvious features in common. The results suggest that a short period is the default phenotype and that only the presence of serine and its structural relative threonine at position 589 can give rise to ca. 24 h periods. Wild-type *per* function may require a modification event at Ser-589 such as phosphorylation or glycosylation. Alternatively, this region may be important independent of any modification at Ser-589 or Thr-589.

The second class consisted of four peptide insertion mutants. We used two different highly antigenic peptide sequences, one derived from the c-myc proto-oncogene and the other from the influenza virus hemagglutinin (HA) protein (Munro and Pelham, 1986; Field et al., 1988). The myc peptide was placed at the C terminus (myc/C) or in the middle of the gene (myc/M) near position 589, i.e., at nucleotide 4729 of the genomic DNA which is 60 bp upstream from the triplet encoding amino acid 589. The HA peptide was placed either at the N terminus (HA/N) or at the C terminus of type A cDNA (HA/C). With the exception of myc/M, at least two transformed lines were recovered for each mutant gene.

In all cases, the insertion mutants rescued the arrhythmic phenotype of the per^{01} hosts with strong rhythms and good penetrance (Figure 3 and Table II). Two of these transformant lines (HA/C and myc/C) had periods very similar to that of the per^{+} 13.2 control strains. The other two (HA/N and myc/M), however, had short periods. The single myc/M line had an average period approximately one and one half hours shorter than the control strains, whereas every HA/N transformed line resulted in rhythms at least 2 hours shorter than the controls. Although only one myc/M line was obtained, the 23.3 h period is likely to be significantly shorter than wild-type, since we have never obtained such a short period strain from a wild-type-derived or a wild-type-like-derived 13.2 *per* gene (see Table I, the myc/C and HA/C lines in Table II, and the 5 ΔTG lines in Yu et al., 1987b). As the peptide sequences prob-

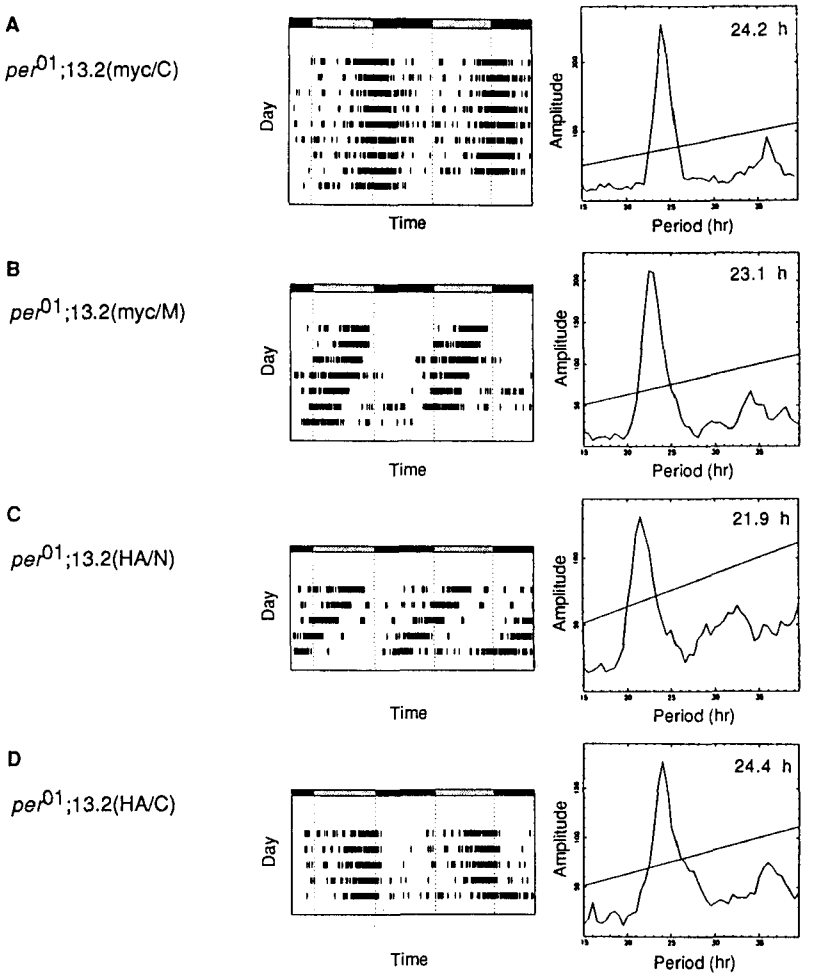

FIGURE 3 Free-running locomotor activity rhythms of *per* insertion mutant transformants. Individual fly activity patterns were analyzed as described in Figure 2. Flies analyzed were from the transformant lines: **A)** per^{01}; 13.2(myc/C)-18a, **B)** per^{01} 13.2(myc/M)-39, **C)** per^{01}; 13.2(HA/N)-3, and **D)** per^{01}; 13.2(HA/C)-16.

ably do not contribute to *per* activity (Note the near normal rhythms of myc/C and HA/C), it would appear that insertions at the N terminus as well as in the middle of the protein can produce a short-period phenotype.

To gain insight into why both classes of mutants gave rise to short-period phenotypes with such a high frequency, we assayed the rhythms of strains that carried *per* mutant gene combinations (e.g., Smith and Konopka, 1982). Analyses of this nature are complicated by the fact that *per* gene activity, as well as *per* expression from the 13.2 kb construct, is subject to dosage compensation (Smith and Konopka, 1982). Yet we were able to compare flies that carried different *per* gene combinations by restricting our analyses to male flies, thereby normalizing for any effect of dosage compensation on period. Initially, we constructed and analyzed strains that carried one or two copies of the wild-type 13.2 kb gene (Ser-589) or of the mutant threonine

TABLE III
Effect of wild-type *per* and per^{Thr589} copies on activity rhythms

Transformed Insert[a]	*per* Background	
	$per^{01};ry^{506}$	$per^{+};ry^{506}$
no insert	AR[b]	24.0[b] (17)
Ser589-2[c]	24.8[b,d] (10)[e]	23.4 (14)
Ser589-2{2}	23.9 (10)	23.7 (6)
Thr589-22	25.5[b] (10)	23.9 (15)
Thr589-22{2}	25.0 (17)	23.8 (3)
Thr589-15	25.3[b] (13)	
Thr589-15{2}	25.0 (8)	

[a]Each insert is a 13.2 kb fragment with the designated amino acid at position 589. Numbers after the dashes refer to the transformant lines tested. The number of copies of the transformed insert is given in brackets. All SEMs are less than 0.3.
[b]These values are repeated from Table I for comparative purposes.
[c]This is the 13.2 transformant line, per^{01};13.2(per^{+})-2, where serine is the amino acid at position 589 in wild-type *per*.
[d]Average period, in h.
[e]Number of flies tested that were rhythmic.

589 *per* gene. Both constructs were assayed in per^{+} and per^{0} backgrounds (Table III).

The results show that the period was shortened by the addition of either the 13.2 kb *per* gene to a per^{+} wild-type fly or by the addition of a second copy to a per^{0} fly that already carried a single insert. In all cases, the presence of another *per* gene copy lowered the period between 0.3 and 0.9 h. There was, however, no further decrease by adding a second copy of the Ser-589 to the wild-type (per^{+}) background (giving a total of three gene copies), consistent with previous observations that the extent of period shortening decreases with increasing *per* gene dose (Smith and Konopka, 1982; Baylies et al., 1987).

Similar dosage effects were observed with the synthetic (Gly-589) short-period gene (Table IV). Addition of a second copy of this gene to a per^{01} background containing a single copy, or to a per^{s} background (containing a single copy of the

TABLE IV
Effect of per^{+} and per^{Gly589} copies on activity rhythms[a]

Transformed Insert	*per* Background				
	per^{s}/per^{+}	per^{s}/per^{01}	$per^{01};ry^{506}$	$per^{s};ry^{506}$	$per^{+};ry^{506}$
No Insert	21.5[b]	19.2–19.9[b]	AR[c]	19.8[c] (18)	24.0[c] (17)
Gly589-5			19.4[c] (26)	19.6 (29)	21.3 (22)
Gly589-5{2}			19.4 (11)	19.0 (12)	20.6 (4)
Ser589-2			24.8[c] (10)	20.4 (23)	
Thr589-22			25.5[c] (10)	20.6 (8)	
Gly589-5;Thr589-15			22.1 (14)		

[a]See notes from Table III. All SEMs are less than 0.3, except for Gly589-5; Thr589-15 (SEM = 0.5).
[b]Data from Konopka and Benger, 1971, Smith and Konopka, 1982 and Dushay et al., 1990. Tested flies were females.
[c]These values are repeated from Table I for comparative purposes.

TABLE V
Effect of combinations of *per* point mutant copies on activity rhythms[a]

Transformed Insert	*per* Background		
	$per^{01};ry^{506}$	$per^{+};ry^{506}$	$per^{s};ry^{506}$
Asp589-20	23.2[b] (14)	22.8 (16)	20.1 (15)
Asp589-20{2}	22.2 (15)	22.3 (8)	21.0 (7)
Asp589-18	22.6[b] (21)		
Glu589-39	22.0[b] (13)		
Tyr589-1	23.0[b] (17)	23.0 (12)	19.9 (16)
Tyr589-1{2}	23.3 (18)		
Thr589-15	25.3[b] (13)		
Thr589-22	25.5[b] (10)		
Gly589-5	19.4[b] (26)		
Asp589-20;Glu589-39	22.2 (11)		
Asp589-20{2};Glu589-39	22.5 (12)		
Asp589-18;Tyr589-1	22.3 (9)		
Asp589-18;Gly589-5	19.6 (8)		
Asp589-20;Thr589-15	23.2 (14)		
Glu589-39;Tyr589-1	22.1 (17)		
Glu589-39;Gly589-5	20.3 (9)		
Glu589-39;Thr589-22	23.2 (13)		
Tyr589-1;Thr589-15	24.3 (15)		

[a]See notes from Table III. All SEMs are less than 0.3.
[b]These values are repeated from Table I for comparative purposes.

Asn-589 gene), had a slight and variable effect on the circadian period (19.4 h vs. 19.4 h, or 19.0 h vs. 19.6 h, respectively). Also shown are combinations of the wild-type gene (Ser-589), or the wild-type-like Thr-589 gene, with a short-period gene (either in a per^{s}; ry^{506} background or a per^{01} background containing a Gly-589 insert). In all cases, the flies exhibited periods that were intermediate between the 19 h periods characteristic of the background strain and the 24–25 h periods characteristic of the wild-type or wild-type-like gene. These results are further examples of the codominance that is characteristic of *per* heteroallelic combinations (Konopka and Benzer 1971; Smith and Konopka, 1982).

The periods resulting from combinations of the remaining position 589 short-period mutants (Tyr-589, Glu-589, and Asp-589) with either per^{+}, per^{s} or themselves are shown in Table V. Only one of two genotypes tested for dosage effects had shorter periods with two copies vs. one copy; the two-dose Asp-589 fly had an average period one hour shorter than its one-dose counterpart, whereas there was no difference between the periods of the one-copy and two-copy Tyr-589 flies. Also, addition of a second copy of the Asp-589 gene to a [Glu-589 + Asp-589] fly (i.e., addition of a third dose of the gene) had no effect on the average period. As described above (Table IV), a "mixing" of periods was observed for many combinations of two different alleles. This effect was only observed, however, if the phenotypes of the individual alleles differed by more than one hour. Also, there were combinations that were unusual, i.e., [Glu-589 + Tyr-589] or [Glu-589 + Asp-589] combinations gave periods characteristic of the "shorter" of the two alleles.

Taken together, the results indicate that increasing the number of *per* genes of any type has less additional effect on the period than the first gene copy. Also, the combination of two different *per* alleles generally leads to a hybrid period, although the shorter allele often has a larger influence on the resulting rhythm.

DISCUSSION

The experiments described in this report were motivated by a desire to understand the nature of the short-period rhythms exhibited by the per^s mutant. As described above, the canonical interpretation is that this mutation is hypermorphic, i.e., the mutant gene gives rise to more *per* "activity" than the wild-type gene. This view is based in part on the observations that increasing the dose of the wild-type gene leads to period decreases whereas decreasing the dose or level of *per* expression leads to period increases. The ca. 19 h period associated with the per^s gene could be due to an increase in per^s protein levels or due to a more active but otherwise normal *per* protein. At least three lines of evidence argue against the former possibility. First, the single nucleotide change has been mapped to the coding region, making it unlikely that it affects *per* mRNA synthesis or turnover (Yu et al., 1987a; Baylies et al., 1987). Second, per^s mRNA levels have been measured, and they are indistinguishable at the peak level from those of wild-type strains (Hardin et al., 1990). Third, per^s protein levels, as estimated by immunohistochemical assays, are also indistinguishable from wild-type levels (Zerr et al., 1990). As a result, a more likely explanation would be that the Ser to Asn change at position 589 gives rise to a *per* gene product that is more active, with a greater specific activity, than the wild-type protein.

This hypermorphic interpretation leads to the expectation that short-period *per* mutants should be rare. Yet most of the mutants generated in this study had short-period rhythms. Although the set was biased because most mutations were made at amino acid 589, the results indicate that it might be worthwhile to reconsider other facts that do not fit easily with the canonical hypermorphic interpretation of the per^s short-period phenotype.

First, additional doses of the per^+ gene do not shorten the period much below ca. 23 h (Table III; Smith and Konopka, 1982). Also, there is little or no decrease in period with more than two copies of a per^s gene (Table IV; Smith and Konopka, 1982). Coté and Brody (1986) have proposed that the circadian period is a very steep function of the *per* dose; this idea would explain, at least qualitatively, the relative insensitivity of period to dose at high levels of *per* activity. Yet this postulate suggests that the per^s protein has an activity equivalent to that from 35 copies of the wild-type gene. Although a more detailed consideration of this issue is hampered by the fact that the biochemical function of the *per* product is not known, it seems unlikely that most amino acids at position 589 as well as two peptide inserts at other locations can give rise to mutant *per* genes with substantial increases in activity. An alternative interpretation for the apparent "buffering" of rhythm periods near ca. 24 h could suggest that another component of the clock becomes limiting in the presence of multiple copies of the *per* gene.

Second, short-period mutants are not particularly unusual in other systems where circadian rhythm mutants have been isolated. In *Neurospora*, four of the eight *frequency* alleles and one of the *prd* mutant alleles lead to short-period rhythms (Dunlap, 1990), while the sole rhythm mutant in hamsters, *tau*, also has a short-period phenotype (Ralph and Menaker, 1988).

Third, the putative hypermorphic character of per^s does not explain the intermediate periods characteristic of flies with heteroallelic combinations, e.g. per^+/per^s. The classic definition of a hypermorph is that it has a more extreme phenotype over a wild-type allele than over a deficiency or a null allele. This means that if per^s/per^{01} flies have 30–35 equivalents of *per* activity and a circadian period of 19–20 h (Coté and Brody, 1986), then per^s/per^+ flies should have an additional unit of ac-

tivity and a period shorter than that of per^{s}/per^{01} flies. This is not the case, however, since per^{s}/per^{+} flies have periods that are considerably longer, i.e., 21.5 (Konopka and Benzer, 1971; Smith and Konopka, 1982). It has also been observed that per^{L} can have an effect on per^{+} rhythms, since per^{L}/per^{+} females have slightly longer rhythms than per^{0}/per^{+} flies (Gailey et al., 1992). A likely explanation for these intermediate phenotypes is that the *per* protein engages in important intermolecular interactions. For example, the *per* product may form homomultimers with intermediate properties in the case of allele mixtures. Alternatively, the *per* product might interact with other proteins or molecules, in which case the intermediate phenotypes of allele mixtures might reflect competition for, or titration of, a limiting component.

Relevant to these considerations are recent observations that indicate that the levels of both *per* mRNA and *per* protein are under circadian clock control and fluctuate with a 24 h periodicity (Hardin et al., 1990; Zerr et al., 1990). The periods of these fluctuations are shortened by the per^{s} mutation, suggesting that there is a feedback loop where the nature of the *per* product influences the periodic fluctuations of its own mRNA and protein. Given recent evidence that the *per* product is predominantly nuclear and can associate with the chromosomes of third instar larval salivary glands (Liu et al., 1992; L.J. Zwiebel, unpublished observations), our current view is that this loop includes a negative autoregulatory circuit, i.e., at an appropriate protein concentration, the *per* product negatively regulates its own mRNA synthesis. This leads to periodic fluctuations in both *per* mRNA and protein levels, which may also contribute to the behavioral fluctuations characteristic of the wild-type fly. The intermolecular interactions referred to above might then be protein-protein interactions necessary for DNA binding or for the repression of *per* mRNA synthesis, or they might consist of direct *per* protein-DNA interactions.

There is also evidence for post-transcriptional regulation of *per* protein levels (Zwiebel et al., 1991). Although the precise type of regulation is not yet established, some *per* protein sequence is necessary for the circadian cycling of a *per*-β-galactosidase fusion protein. Therefore, there are probably other important interactions that also contribute to the circadian cycling of the *per* protein.

Based on these considerations, we suggest that the short-period mutants are more traditional loss-of-function mutations that are defective in some aspect of this autoregulatory loop or that respond poorly to other important aspects of circadian circuitry. Defects of this nature are not incompatible with a hypermorphic character, but they can explain all of the observations described in this and in previous reports (Konopka and Benzer, 1971; Smith and Konopka, 1982), including short-period phenotypes that mimic the effects of putative hyperactive alleles. A complete explanation for all of the *per* mutant phenotypes must await a definition of the biochemical function of the *per* protein.

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