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New Short Period Mutations of the Drosophila Clock Gene *per*

Mary K. Baylies,* Leslie B. Vosshall, Amita Sehgal, and Michael W. Young
Howard Hughes Medical Institute
and Laboratory of Genetics
The Rockefeller University
New York, New York 10021

Summary

Earlier work has indicated that the period length of Drosophila circadian behavioral rhythms is dependent on the abundance of the *period* (*per*) gene product. Increased expression of this gene has been associated with period shortening for both the circadian eclosion (pupal hatching) rhythm and circadian locomotor activity rhythms of adult Drosophila. In this study it is shown that a wide variety of missense mutations, affecting a region of the *per* protein consisting of ~20 aa, predominantly generate short period phenotypes. The prevalence of such mutations suggests that short period phenotypes may result from loss or depression of function in this domain of the *per* protein. Possibly mutations in the region eliminate a regulatory function provided by this segment, or substantially increase stability of the mutant protein.

Introduction

The *period* (*per*) locus of Drosophila melanogaster plays a fundamental role in the construction and/or maintenance of biological clocks (Konopka and Benzer, 1971). Mutations of the gene alter circadian (~24 hr) behavioral rhythms and the rhythmicity of male courtship song, which has a periodicity of ~55 s in wild-type flies (Konopka and Benzer, 1971; Kyriacou and Hall, 1980). The gene is expressed in a variety of neural and nonneural tissues throughout development (James et al., 1986; Bargiello et al., 1987; Liu et al., 1988; Saez and Young, 1988; Siwicki et al., 1988), but only function in the central nervous system appears to be required for rhythmic behavior (Konopka et al., 1983; Hall, 1984).

The *per* locus encodes a protein of 1224 aa in the Canton-S strain of D. melanogaster (Jackson et al., 1986; Citri et al., 1987; Baylies et al., 1993). Three mutations of *per* were isolated and characterized in earlier work (Konopka and Benzer, 1971; Baylies et al., 1987; Yu et al., 1987a), and molecular studies have shown that each affects the structure of the *per* protein. The per^s and per^l mutations are each single amino acid substitutions (Baylies et al., 1987; Yu et al., 1987a). The former shortens the period of circadian behavioral rhythms to 19 hr, while the latter lengthens the period of these rhythms to 28 hr (Konopka and Benzer, 1971). per^o mutants have no detectable circadian rhythms and produce a truncated, apparently functionless protein (Baylies et al., 1987; Yu et al., 1987a). Thus, the gene's control of rhythmicity depends on action of the encoded protein.

Gene dosage studies, including investigations in transgenic Drosophila, indicate that period length is determined by abundance of the *per* protein (Smith and Konopka, 1982; Bargiello et al., 1984; Baylies et al., 1987). For example, flies expressing lower levels of *per* RNA produce circadian rhythms with correspondingly longer periods (Baylies et al., 1987). As per^l and per^s mutants express wild-type levels of *per* RNA (Hardin et al., 1990; T. Bargiello, unpublished data), it has been suggested that the mutants produce hypoactive and hyperactive *per* proteins, respectively (Cote and Brody, 1986; Baylies et al., 1987). To understand how a change in *per* protein structure might increase the activity level of the protein, genes carrying new *per* mutations were produced by in vitro mutagenesis and returned to Drosophila by P element–mediated transformation. In this study it is shown that mutations altering the structure of an ~20 aa segment of the protein, surrounding the site of the original per^s mutation, predominantly confer short period phenotypes. These findings suggest that loss or lowered function in this protein region may dramatically increase the measured level of *per* protein activity.

Results and Discussion

Construction of Transgenic Drosophila Expressing *per* Proteins with New Amino Acid Changes in the per^s Region

A segment of the *per* locus coding for ~40 aa (amino acids 568–608, numbers from Baylies et al., 1993) was modified in vitro by oligonucleotide-directed mutagenesis (Figure 1; Experimental Procedures). The region was chosen for mutagenesis because it includes the site of the per^s mutation (serine 589), which, from earlier work, may produce a protein with greater than wild-type activity (Smith and Konopka, 1982; Cote and Brody, 1986; Baylies et al., 1987); sequence comparisons indicate that the region is unusually well conserved in distantly related Drosophila species (Colot et al., 1988); antibodies directed against this protein region detect cross-reacting material in pacemaker tissues from mollusks and rodents (Siwicki et al., 1989, 1992); and this region encodes possible sites for phosphorylation by protein kinase C and tyrosine kinase.

Sixteen DNA constructs, each encoding one or more amino acid substitutions in the protein region, were independently linked to a vector for P element–mediated transformation of per^o Drosophila (Figure 1

* Present address: Department of Zoology, Cambridge University, Downing Street, Cambridge CB2 3EJ, UK.

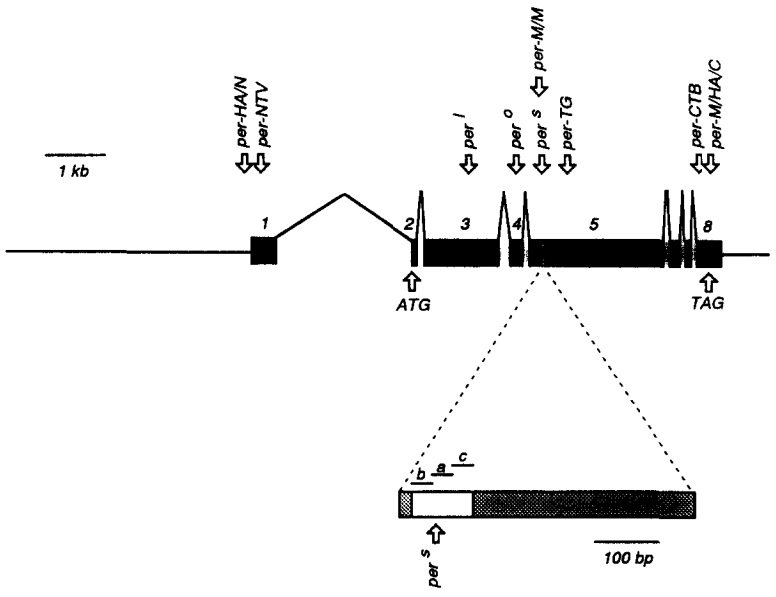

Figure 1. Scheme for Mutagenesis of *per*^{s} Region

(Top) 12.3 kb SphI-XhoI *per* DNA fragment used in all transformation experiments of this study. Fragment contains approximately 4.0 kb of upstream and promoter sequences (horizontal line to left of transcription unit), the *per* transcription unit (closed boxes, exons 1-8), and approximately 0.8 kb of downstream sequences (horizontal line to right of transcription unit; see also Experimental Procedures). Transcription start site and poly(A) addition site correspond to left border of exon 1 and right boundary of exon 8, respectively (Jackson et al., 1986; Citri et al., 1987; Baylies et al., 1993). ATG and TAG indicate the positions of the translation start and stop codons, respectively (Baylies et al., 1993). Locations of the single amino acid changes in the mutants *per*^{l}, *per*^{o}, and *per*^{s} are indicated (Baylies et al., 1987; Yu et al., 1987; Baylies et al., 1993), and positions of mutations affecting other regions of the protein from this and other studies are shown: *per*-HA/N, -M/M, and -M/HA/C are taken from Rutila et al. (1992) (see also text). *per*-TG is from Yu et al. (1987b). *per*-NTV and -CTB are from this study (see text, Table 2, and Experimental Procedures). Stippled portion of exon 5 represents the 531 bp SmaI-XmnI fragment subcloned for mutagenesis of the *per*^{s} region in this study. (Bottom) Expanded map of SmaI-XmnI fragment showing locations of *per*^{s} and oligonucleotides (a-c, see Experimental Procedures) used for mutagenesis.

Table 1. Characteristics of Amino Acid Substitutions in the *per*^{s} Region

Mutant	Side Chain Size	Charge	Predicted Secondary Structure[a]	Other Changes[b]	Period Length ± SE
S589N[c]	=	=	No change	Lose potential phosphorylation site	22.1 ± 0.7[c]
L574M	=	=	No change	–	25.8 ± 0.4
E575G	=	−1 to 0	α helix disrupted	–	25.9 ± 0.8
P577R, H578Q	> <	= +1 to 0	No change	Double mutation, 577 nonpolar > polar	26.2 ± 0.6
E579A	=	−1 to 0	No change	Polar > nonpolar	25.8 ± 0.8
S585A	=	=	No change	Lose potential phosphorylation site, polar > nonpolar	24.0 ± 0.5
E586V, V590D	> <	−1 to 0 0 to −1	α helix disrupted	Double mutation, polar > nonpolar and nonpolar > polar	22.7 ± 0.6
E586G, D588A, M591L	= = =	−1 to 0 −1 to 0 =	α helix disrupted	Triple mutation, 588 polar > nonpolar	24.6 ± 0.5
D588V	>	−1 to 0	α helix disrupted	Polar > nonpolar	22.5 ± 0.4
D588F	>	−1 to 0	No change	Polar > nonpolar	24.3 ± 0.6
S589L	>	=	No change	Lose potential phosphorylation site, polar > nonpolar	22.1 ± 0.5
G593S	=	=	No change	–	21.3 ± 0.8
G593C	>	=	α helix extended	Possible disulfide bond formed	30.7 ± 1.0
P597A	>	=	α helix extended	–	22.2 ± 0.4
P597T, H599Q, Y601F	= < =	= +1 to 0 =	α helix extended	Disrupt potential phosphorylation site, 601 polar > nonpolar	20.6 ± 0.6
D600V	>	−1 to 0	Turn disrupted	Disrupt potential phosphorylation site, polar > nonpolar	24.7 ± 0.7
D600E, S604G	= =	= =	Turn disrupted	Double mutation	25.4 ± 0.7

Size and charge indicate new amino acid in the mutant as compared with wild-type amino acid replaced. For side chain size, (<) indicates replacement is smaller than wild-type amino acid, (>) replacement is larger, and (=) indicates no significant change in size. For charge, (−1) indicates acidic amino acid, (+1) basic amino acid, and (0) uncharged amino acid. Period lengths (from Table 2) are mean periodicities for heterozygous, transgenic Drosophila producing *per* proteins with the indicated amino acid substitution.

[a] Secondary structure as predicted by Chou and Fasman (1974) and Garnier et al. (1978). In the wild-type protein, a predicted α helix extends from approximately amino acids 551-592. A turn region is predicted to follow (amino acids 596-605).

[b] Possible phosphorylation sites from comparisons of consensus sequences (Kemp and Pearson, 1990). Polar and nonpolar refer to interconversion of hydrophilic and hydrophobic amino acids.

[c] Denotes site of original *per*^{s} mutation. Period shown for *per*^{s} is that measured in *per*^{o} lines transformed with *per*^{s} DNA (see also Figure 1 legend).

Table 2. Period Lengths of Transformed Lines

Genotype	Period length	SE	Range (Period Length ± SD)	N	n
p[+]	26.9	0.8	26.7 ± 0.9–27.2 ± 0.5	6	38
p[s]	22.1	0.7	21.4 ± 0.5–22.9 ± 0.6	6	30
p[l]	32.1	0.9	31.1 ± 0.7–32.6 ± 0.6	7	40
p[L574M]	25.8	0.4	25.4 ± 0.7–26.3 ± 0.8	4	99
p[E575G]	25.9	0.8	24.9 ± 0.5–27.7 ± 1.0	5	61
p[P577R, H578Q]	26.2	0.6	24.8 ± 0.4–26.9 ± 1.0	5	55
p[E579A]	25.8	0.8	24.7 ± 0.9–26.6 ± 0.8	7	50
p[S585A]	24.0	0.5	23.3 ± 0.6–26.2 ± 0.2	6	87
p[E586G, D588A, M591L]	24.6	0.5	23.4 ± 0.8–25.7 ± 0.5	5	95
p[E586V, V590D]	22.7	0.6	21.5 ± 0.7–23.6 ± 0.4	9	78
p[D588V]	22.5	0.4	21.2 ± 0.7–23.2 ± 0.7	4	48
p[D588F]	24.3	0.6	23.9 ± 1.1–24.8 ± 1.6	2	30
p[S589L]	22.1	0.5	21.3 ± 0.4–23.9 ± 0.5	7	72
p[G593S]	21.3	0.8	20.3 ± 0.6–22.1 ± 0.6	8	58
p[G593C]	30.7	1.0	29.7 ± 1.0–31.7 ± 0.8	10	80
p[P597A]	22.2	0.4	20.6 ± 0.5–22.9 ± 0.8	4	57
p[P597T, H599Q, Y601F]	20.6	0.6	19.9 ± 0.5–22.1 ± 0.6	8	79
p[D600V]	24.7	0.7	23.5 ± 0.9–25.3 ± 0.8	5	81
p[D600E,S604G]	25.4	0.7	24.2 ± 0.7–25.9 ± 0.9	5	67
p[Def 65-93; A94R, S95A]	Arrhythmic		NA	5	29
p[Def 65-93;A94R, S95A]	32.2 (39%[a])	1.0	30.1 ± 0.3–36.1 ± 3.1	6	61
p[RKKKK73-76 LNNNN]	32.1 (74%[a])	1.7	NA	1	182
p[KKKKNKG74-80 PKKKRKV]	33.7 (7%[a])	0.3	33.5 ± 2.3–33.9 ± 3.2	2	180
p[SMSTS1202-1206VLDR]	26.9	0.8	26.0 ± 0.4–27.5 ± 1.4	6	27

The genetic background for all transgenic flies was *per*^{o}. p[+], p[s], and p[l] are transgenic Drosophila carrying *per*$^{+}$, *per*^{s}, and *per*^{l} transforming DNA. These served as controls (see text). Remaining *per* (p) transformants carry amino acid changes at positions indicated: First letter indicates the wild-type amino acid; the number following indicates its position in the *per* protein; and the subsequent letter shows the amino acid substituted by mutagenesis. Certain lines carry *per* DNA modified so as to produce multiple amino acid substitutions (e.g., p[P577R, H578Q]) or a deletion/substitution (p[Def 65-93; A94R, S95A]). Such lines produce only *per* proteins carrying all indicated changes. Period length shows average period for all lines carrying a particular *per* construct. SE is standard error for that average. Range shows average period length obtained for individual transformed lines showing shortest and longest period lengths, respectively, and the standard deviations (SD) in period length for those individual lines. N, number of independent lines (each with a unique site of P element insertion) tested. n, total number of flies tested. All period length estimates were derived from analysis of locomotor activity as described (Experimental Procedures). NA, not applicable.

[a] Denotes the percentage of flies that were rhythmic.

legend; Experimental Procedures). Table 1 shows the characteristics of the different substitutions. Single amino acid substitutions were chosen so as to affect frequently amino acid size, charge, hydrophobicity, or predicted secondary structure of the *per* protein. Mutants having multiple changes (e.g., mutant E586V, V590D and mutant P597T, H599Q, Y601F) were also produced (Table 1). The phenotypic effects of multiple substitutions were of interest, as they would cause greater divergence from the wild-type protein sequence and possibly further hinder protein function.

For each modified segment of *per* DNA presented in Table 1, multiple strains of transgenic Drosophila were created as described in the legend to Figure 1 and Experimental Procedures. These are also listed in Table 2. As somewhat lower than wild-type levels of *per* RNA expression and slightly longer period lengths were generally observed for our transgenic flies in earlier studies (Bargiello et al., 1984; Baylies et al., 1987), additional transgenic Drosophila strains were generated using wild-type (p[+]), *per*^{s} (p[s]), and *per*^{l} (p[l]) DNA (see Table 2 and legend) to serve as controls. These strains indicate period lengths to be expected from expression of wild-type, *per*^{s}, and *per*^{l} proteins, respectively, in transformed flies. The control and experimental DNAs used in all transformations differed only by the indicated amino acid substitutions (Figure 1 legend). Data provided in Table 1 and Table 2 indicate period lengths of restored rhythms in the transgenic flies.

A "Short Period Mutable" Domain of the *per* Protein

Earlier work has shown that *per*^{s} mutants produce wild-type levels of *per* mRNA (Hardin et al., 1990; T. Bargiello, unpublished data), so short period behavior is linked to altered protein in the mutant (Baylies et al., 1987; Yu et al., 1987). As only one short period mutant was identified in earlier genetic screens (Konopka and Benzer, 1971; Konopka, 1986, 1987), the amino acid substitution in *per*^{s} might uniquely confer a short period phenotype. However, we found that most of the new mutant sequences tested (10/16, Table 1; Table 2) produced short period phenotypes in transgenic Drosophila. Moreover, the changes that

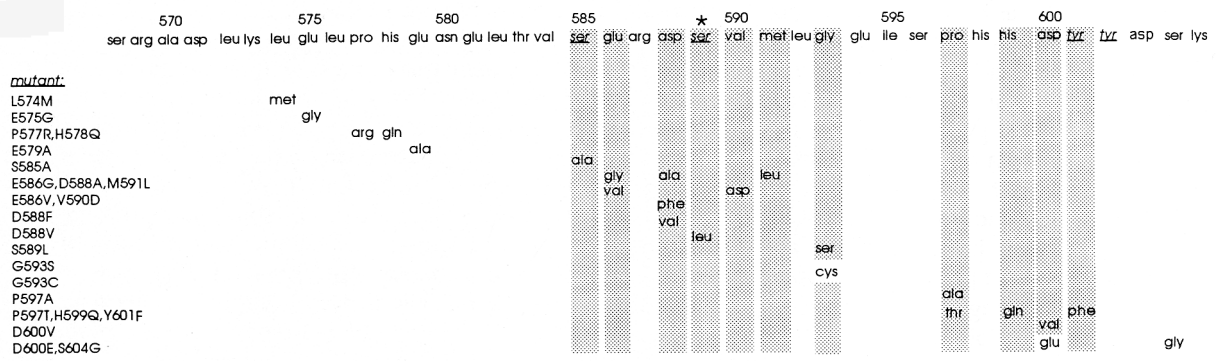

Figure 2. Locations of New Short Period Mutations in the *per*^{s} Region

The wild-type *per* amino acid sequence appears at the top (residues 568–605). The amino terminus is to the left, and the carboxyl terminus is to the right. Asterisk marks the serine to asparagine substitution in *per*^{s}. Underlined residues indicate possible sites for phosphorylation (see text and Table 1). Shading indicates amino acid substitutions associated with short period phenotypes. The substitute amino acid is shown in the shaded region directly beneath the affected wild-type amino acid. Designations of the new mutants are shown in the column to the left of the figure. For each mutant, the first letter indicates the wild-type amino acid, the number following indicates its position in the *per* protein, and the subsequent letter shows the amino acid substituted by mutagenesis.

gave rise to short period phenotypes were clustered: 10/12 mutant sequences carrying substitutions in the interval spanning amino acids 585–601 generated short period phenotypes. None of 4 mutants, which together alter 5 aa in the interval upstream of amino acid position 585, significantly shortened period from the wild type. A remaining mutant with two amino acid substitutions at positions 600 and 604 (D600E, S604G) also gave essentially wild-type rhythms. As indicated in Figure 2, a single, alternative amino acid substitution at position 600 generates a short period rhythm. Thus, the D600E, S604G double mutant marks the downstream boundary of the interval tested and possibly the C-terminus of a region in which short period changes commonly result from mutagenesis. In any event, sequence conservation among different Drosophila species ends in this region approximately 29 aa downstream of our last tested substitution (Colot et al., 1988), and deletion of a threonine–glycine repeat sequence mapping ~90 aa downstream gives wild-type or long period circadian rhythms (Yu et al., 1987b), so this "short-mutable" region of the protein might not be expected to extend much farther than the interval studied.

An exceptional, long period mutant resulted from a glycine to cysteine change at amino acid position 593 (G593C; Table 1; Table 2; Figure 2). If the same glycine is changed to serine, short period rhythms are produced in transgenic flies (G593S; Table 1; Table 2). Thus, it may be the specific change to cysteine that is responsible for the long period phenotype.

This restricted mutational analysis of the *per* protein has shown that a domain of function exists in which short period mutant phenotypes predominate. Rutila et al. (1992) have recently reached a similar conclusion by studying the effects of several substitutions at amino acid position 589 of *per*, the site of the original *per*^{s} mutation. Four out of five amino acid 589 substitutions they tested shortened period length (Rutila et al., 1992). The results of our study indicate that over a region of ~20 aa, a number of amino acid positions can be changed, and a variety of amino acid substitutions can be produced, and still short period rhythms result. Recurrence of the same mutant phenotype strongly suggests that shortened period length stems from loss or lowered function in the protein region. Prior work has shown that increasing gene dosage at *per* shortens period length (Smith and Konopka, 1982), and decreasing *per* gene dosage or RNA (and presumably encoded protein) levels lengthens period (Smith and Konopka, 1982; Baylies et al., 1987). While the mechanism by which *per* protein influences period length is unknown, these data could be reconciled if it is assumed that loss of function in the short-mutable domain studied increases overall activity of the protein. Alternatively, as abundance of the *per* product dictates period length, mutations in this short-mutable region of the protein might dramatically increase protein stability. Any effect on stability must be substantial, as earlier work (Cote and Brody, 1986; Baylies et al., 1987) indicates that an approximately 50-fold increase in abundance of the wild-type product should be required to give period lengths in the same range as the shortest period rhythms achieved in this study.

For the short-mutable interval studied, while amino acid position is an important factor in determining whether a mutation will produce a short period phenotype (Table 1; Figure 2), there is no clear evidence for dependence on the class of amino acid substitution. An exception is the long period mutation that resulted from a glycine to cysteine change at amino acid position 593. As described above, a change to serine at the same location produced short period rhythms (Figure 2). This is the only mutant in our study that involved a substitution to cysteine, and there are

no cysteine residues in or near the short-mutable domain in wild-type *per* protein (closest cysteine residues are at amino acid positions 553 and 652 [Jackson et al., 1986; Citri et al., 1987; Baylies et al., 1993]). The new cysteine might generate a form of the protein that is less active than wild type. For example, lowered activity might result if the new cysteine promoted novel inter- or intramolecular disulfide bonds. As earlier dosage studies indicate, a less active protein should confer a long period phenotype.

We do detect a range of short period phenotypes. Some mutants exhibit rhythms with periods only moderately shorter than wild type (e.g., S585A, D588F, and D600V; Table 2), while several of the new mutants produce short period phenotypes as short or shorter than per^s itself (e.g., D588V, S589L, G593S, and P597A). Of all short period mutants, the most extreme was generated by a triple amino acid substitution (mutant P597T, H599Q, Y601F). This mutant produces rhythms with a period 1.5 hr shorter than transgenic flies expressing per^s protein (Table 2). Although the severe phenotype of this triple mutant is consistent with the suggestion that shorter period lengths may be associated with loss or lowered function of the protein region, the number of substitutions alone is not a simple predictor of the degree of period shortening. For example, the only other triple substitution studied (mutant E586G, D588A, M591L) only shortens period length by about 2 hr from the wild type, and the double substitution (mutant E586V, V590D) produces a period comparable to several of the new short period mutants derived from single amino acid substitutions.

Just outside the short-mutable domain defined by these studies, mutants with substantial changes in amino acid charge (e.g., E579A), size (e.g., P577R, H578Q), or predicted secondary structure (e.g., E575G) still produce wild-type rhythms. The double mutant P577R, H578Q likewise generates rhythms with a wild-type period. Together, the behavior of these mutants indicates that the affected amino acids, although well conserved (Colot et al., 1988), do not have a pivotal role in setting period length.

The short-mutable region we have described may not be the only such domain of the *per* protein. Insertional mutagenesis involving addition of a short (11 aa) segment of the HA protein to the absolute N-terminus of *per* has produced period length shortening, and insertion of an 11 aa myc peptide following amino acid position 569 of the *per* protein also can confer short period rhythms (Rutila et al., 1992; shown in Figure 1 as *per*-HA/N and *per*-M/M, respectively). For the latter insertion it seems likely that the effect is due to an influence on the closely associated short-mutable domain studied here (Figure 1, see also amino acid numbering of Figure 2), but the N-terminal insertion mutant could mark a new short-mutable region. Further work should determine whether these short period rhythms result from lowered activity in the N-terminal domain of the *per* protein.

In vitro deletion, insertion, and point mutageneses of other regions of the *per* locus have been limited, but have not thus far produced any additional short period mutants in transgenic Drosophila. Insertion mutagenesis involving addition of either myc or HA sequences (again 11 aa insertions) directly preceding the *per* termination codon has given transgenic flies with wild-type periods (Rutila et al., 1992; represented as *per*-M/HA/C in Figure 1). Table 2 shows that we have produced a quadruple amino acid substitution and amino acid deletion (amino acids 1202–1206, SMSTS to VLDR) that eliminates a potential cAMP-dependent protein kinase site in the C-terminal region of the protein (Figure 1, *per*-CTB). Transgenic flies producing only this *per* protein have wild-type rhythms (Table 2). We also find that in the N-terminal region of the protein, a deletion of 30 aa (amino acids 65–95; p[Def 65–93; A94R, S95A], Table 2) produces flies that variably generate long period rhythms or are arrhythmic: of 11 lines formed, 5 have given only arrhythmic flies. For the remaining 6 lines, 39% of the flies tested were rhythmic with an average period length of 32.2 ± 1.0 hr (Table 2). In the same region, less extreme changes (mutants RKKKK73-76LNNNN and KKKKNKG74-80PKKKRKV) also produce long period and arrhythmic phenotypes (Table 2; all three classes of sequence changes are represented as *per*-NTV in Figure 1). Yu et al. (1987b) have studied the effects of a deletion of 47 aa approximately 90 aa downstream of the short-mutable domain described in this paper (shown as *per*-TG in Figure 1). The deletion eliminates most of a repeating threonine–glycine coding sequence. Transgenic flies expressing this *per* protein have wild-type or long period circadian rhythms. Finally, Drosophila producing wild-type *per* proteins, but deficient for untranslated 5′ and/or 3′ sequences, so far generate only wild-type or long period rhythms (Hamblen et al., 1986; Jackson et al., 1986; Baylies et al., 1987). Because it is not feasible to study all regions of *per* in this fashion, it is only possible at this time to conclude that mutations in some regions of *per* can produce no change in rhythmicity or arrhythmic and long period phenotypes as would be expected from earlier dosage studies, while decreased rather than increased function in at least one region of the *per* protein produces short period rhythms.

Any one of several mechanisms can be imagined to account for apparent modulation of *per* activity by the short-mutable domain we have studied. The protein region might permit association with a factor that regulates activity of *per* protein or that controls *per* protein stability. Interactions of either sort would then be a critical step in setting period length. Another possibility would be that this domain of the protein forms a substrate for an inhibitory modification, or modification controlling stability. Some of these possibilities are being investigated. In particular it will be important to determine whether this short-mutable domain will regulate period length from novel positions within the *per* protein, whether long and short period rhythms can result from duplicating and delet-

ing the protein region, and whether mutation of unlinked genes can suppress or enhance short period mutations at *per*. It will also be important to determine, in vivo, the stability of the various *per* proteins conferring short period phenotypes.

One third of the D. melanogaster *per* protein sequence is not at all conserved in the distantly related Drosophila species D. virilis and D. pseudoobscura (Colot et al., 1988), yet a D. pseudoobscura *per* protein will restore rhythmicity in *per*^{o} D. melanogaster (Peterson et al., 1988). The region mutated to give short period phenotypes in this study is most highly conserved; no amino acid substitutions distinguish the species in this segment of the protein, while an average substitution rate of about 20% applies to most regions of the *per* protein that are shared in the three species (Colot et al., 1988). Antibodies directed against this protein region react with pacemaker tissues in Aplysia, Bulla, and the rat (Siwicki et al., 1989, 1992). Such a high degree of conservation may indicate the importance of this short-mutable segment of the protein for a particular aspect of *per* function or regulation that extends to the action of quite diverse biological clocks.

Experimental Procedures

Mutagenesis

per^{s} Region

A 531 bp SmaI–XmnI fragment (Figure 1) was subcloned from wild-type (Canton-S) *per* genomic DNA into pGEM-7Zf(+) (Promega). The latter was modified by removing the XmnI site from its ampicillin resistance (β-lactamase) gene by in vitro mutagenesis, and an XmnI site was added in the polylinker. Oligonucleotides for mutagenesis were prepared by the Rockefeller University protein sequencing facility. Three oligonucleotides were created to cover a continuous, 40 aa region: a 29-mer (oligo b), which spanned nucleotides 4783–4812 of the *per* mRNA coding sequence (see Baylies et al., 1993, for complete DNA sequence; and oligonucleotide b in Figure 1), and two 32-mers, which spanned nucleotides 4751–4783 (oligonucleotide a, Figure 1) and 4813–4845 (oligonucleotide c, Figure 1). These were prepared according to the random mutagenesis scheme of Hermes et al. (1989). The level of contamination at each position of the oligomer was set at 9.37%. The procedure followed for mutagenesis was that supplied by Amersham (Oligonucleotide-Directed In Vitro Mutagenesis System RPN.1523). Analysis of each of the mutant progeny was accomplished by sequencing DNA comprising the entire 531 bp SmaI–XmnI fragment depicted in Figure 1 and the legend. Of the 40 new mutants created and sequenced as described, 16 were selected for reinsertion into the *per* 12.3 kb SphI–XhoI construct shown in Figure 1. The resulting 12.3 kb SphI–XhoI mutant *per* constructs were subsequently cloned into the Carnegie 20 vector for P element–mediated transformation (Rubin and Spradling, 1983). Multiple lines of transgenic Drosophila were produced as previously described (Baylies et al., 1987). Constructs (12.3 kb) containing, alternatively, the wild-type SmaI–XmnI fragment, the previously mapped per^{s} mutation, or the per^{l} mutation also were used to form transgenic Drosophila lines (see text). Flies were maintained as heterozygotes with respect to the transforming DNA in a *y per*^{o}; *ry*^{42} background (Baylies et al., 1987).

N-Terminal Region

For p[Def 65–93;A94R, S95A] (Table 2), a 2281 bp XbaI–SmaI fragment was subcloned from wild-type (Canton-S) *per* genomic DNA into pGEM-7Zf(+) (Promega). The latter was altered to remove the BamHI site in the polylinker by cutting with BamHI, end filling with PolIK, and recircularizing. This *per* DNA-carrying plasmid was linearized at nucleotide 3183 (Baylies et. al., 1993) using AccI. After end filling with PolIK, a BamHI linker (CGCGGATCCGCG from New England Biolabs) was inserted, and the plasmid was recircularized. The DNA was transformed into dam^{-} E. coli (K1108) and cut at nucleotide 3089 with BclI and at the linker with BamHI. A 94 bp fragment, corresponding to *per* amino acids 65–95, was removed and the plasmid religated via the cohesive ends of the BclI and BamHI sites. This procedure led to deletion of the indicated amino acids and addition of 2 new amino acids (RA) due to translation of the linker. Before the deletion-bearing XbaI–SmaI fragment was inserted into the *per* 12.3 kb construct (described above), a 354 bp PstI fragment (nucleotides 2910–3264) was subcloned into pEMBL20 and sequenced. The 12.3 kb construct was cloned into the Carnegie 20 vector (as above). Transgenic flies were tested as homozygotes in a *y per*^{o}; *ry*42 background. For p[RKKKK73–76LNNNN] and p[KKKKNKG74–80PKKKRKV] a 341 bp PstI–BglII fragment (nucleotides 2910–3251) was subcloned from Canton-S *per* genomic DNA into pGEM-3Zf(+) (Promega), in which the HindIII site in the polylinker was destroyed and replaced with a BglII linker. Oligonucleotides were prepared by the Rockefeller University protein sequencing facility. According to the method described above for per^{s} region mutagenesis, the coding sequence for amino acids 73–77 was altered with an oligonucleotide (27-mer) spanning nucleotides 3115–3141 (TCGCTCAACAACAACAACAACAAGGGC). Codons for amino acids 74–80 were mutagenized with a second oligonucleotide (27-mer) covering nucleotides 3118–3144 (CGCCCGAAGAAGAAGCGCAAGGTCGCC). Mutant plasmids were analyzed by sequencing the entire 341 bp fragment and were reinserted into the *per* 12.3 kb construct. The resulting DNA fragments were cloned into the Casper vector for P element–mediated transformation of *y per*^{o} *w* Drosophila. Flies were maintained and tested as homozygotes.

C-Terminal Region

For p[SMSTS1202-1206VLDR], a 1585 bp BamHI–HindIII fragment (*per* nucleotides 5650–7235; Baylies et al., 1993) was subcloned from Canton-S genomic DNA into pGEM-7Zf(+) (Promega). This plasmid was partially digested with DdeI, and the ends were filled with PolIK. The fragment corresponding to the plasmid linearized at *per* genomic nucleotide 6836 was isolated. A SalI linker (CGGTCGACCG from New England Biolabs) was inserted, and the plasmid was recircularized. The DNA was further digested with SnaBI at nucleotide 6766 and at the linker with SalI. After end filling with PolIK, the DNA was recircularized. This resulted in a 68 bp deletion within the original Bam–HindIII fragment without changing the *per* reading frame. The intron between amino acid residues 1201 and 1202 is deleted, and codons for amino acids 1202–1206 are deleted. The first 4 bp of the intron and linker would be translated, resulting in the net loss of 1 aa and substitution of 4 adjoining amino acids (see also Table 2 and text). The modified BamHI–HindIII fragment was sequenced, reinserted into the *per* 12.3 kb construct, and cloned into Carnegie 20 for P element–mediated transformation (as above). Flies were maintained as heterozygotes with respect to the transforming DNA in a *y per*^{o}; *ry*42 background (Baylies et al., 1987).

Locomotor Activity Assays

Flies (1- to 2-day-old) were placed individually in cylindrical glass tubes containing a small aliquot of Drosophila cornmeal medium (Bargiello et al., 1984; Young et al., 1985). Generally, male flies were used in this assay. In instances in which the behaviors of female flies were also examined, no significant differences were found in the period lengths of male and female flies from the same transgenic strain. Each glass tube was placed in a monitor flanked by a photodiode (emitting infrared light) and by an infrared detector (Bargiello et al., 1984; Young et al., 1985). Movement of the fly deflects the beam and generates a signal that is picked up by the detector and transmitted to a computer. Activity was generally followed for 7 days using an Apple IIe data collection program (Sulzman, 1982). For each activity record, period length was determined by chi-square periodogram analysis (Hamblen et al., 1986; Sokolove and Bushell, 1978).

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