Molecular and Behavioral Analysis of Four *period* Mutants in *Drosophila melanogaster* Encompassing Extreme Short, Novel Long, and Unorthodox Arrhythmic Types

Melanie J. Hamblen,* Neal E. White,* Philip T. J. Emery,[†,1] Kim Kaiser[†] and Jeffrey C. Hall*

**Department of Biology, Brandeis University, Waltham, Massachusetts 02254 and [†]Institute of Genetics, Glasgow University, Glasgow G11 5JS, Scotland*

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ABSTRACT

Of the mutationally defined rhythm genes in *Drosophila melanogaster*, *period* (*per*) has been studied the most. We have molecularly characterized three older *per* mutants—per^T, per^{Clk}, and per^{04}—along with a novel long-period one (per^{SLIH}). Each mutant is the result of a single nucleotide change. per^T, per^{Clk}, and per^{SLIH} are accounted for by amino acid substitutions; per^{04} is altered at a splice site acceptor and causes aberrant splicing. per^{SLIH} exhibits a long period of 27 hr in constant darkness and entrains to light/dark (L/D) cycles with a later-than-normal evening peak of locomotion. per^{SLIH} males are more rhythmic than females. per^{SLIH}'s clock runs faster at higher temperatures and slower at lower ones, exhibiting a temperature-compensation defect opposite to that of per^{Long}. The *per*-encoded protein (PER) in the per^T mutant cycles in L/D with an earlier-than-normal peak; this peak in per^{SLIH} is later than normal, and there was a slight difference in the PER timecourse of males *vs.* females. PER in per^{04} was undetectable. Two of these mutations, per^{SLIH} and per^{Clk}, lie within regions of PER that have not been studied previously and may define important functional domains of this clock protein.

BIOLOGICAL rhythms have been studied genetically in organisms ranging from microbes to mammals. Analysis of clock genes and the mutations that define them in Neurospora and Drosophila have led to insights about how two circadian oscillators work (most recently reviewed by Dunlap 1996; Seghal *et al.* 1996; Iwasaki and Thomas 1997; Rosato *et al.* 1997).

Among the many rhythm mutants in *Neurospora crassa* (Dunlap 1996), most map to one locus, *frequency* (*frq*). All *frq* mutants induced *in vivo* turned out to have single nucleotide changes within the protein-coding part of the gene; the circadian period-altered cases have amino-acid substitutions, and the mutation-causing arrhythmicity is a molecularly null allele (Aronson *et al.* 1994; Garceau *et al.* 1997). The latter correlation between genotype and phenotype is readily appreciated; however, it is fair to say that the reason a given missense mutation causes a shorter- or longer-than-normal periodicity is unknown. Nevertheless, it is interesting that the most extreme *frq* mutations are caused by amino acid substitutions within 24 codons of each other, that period-shortening mutations are associated with conservative changes in amino acids, and that the long-period mutants suffered nonconservative substitutions, resulting in charge changes as well as differences in side-chain bulk (Aronson *et al.* 1994). It is also notable that the long-period *frq* mutants exhibit defects in temperature compensation of Neurospora's conidial banding rhythm: the period becomes progressively shorter as the temperature is raised (Gardner and Feldman 1981; Dunlap and Feldman 1988; Aronson *et al.* 1994; also see Coros and Feldman 1986).

In *Drosophila melanogaster*, there are about 10 genes known to cause rhythm alterations when mutated (Hall 1995; Sehgal *et al.* 1996). Of these, *period* (*per*) has been studied the most. A corollary is that *per* has been hit several independent times by *in vivo* mutagenesis, leading to mutations that speed up (per^T, per^S, and per^{Clk}), slow down (per^L), or abolish (per^{01}, per^{04}) the organism's biological rhythms. Many additional *per* mutants have been engineered *in vitro* (Baylies *et al.* 1992; Rutila *et al.* 1992; Curtin *et al.* 1995). These mutants, when tested for locomotor behavior, exhibited a wide range of rhythm alterations (a few of them showed no change; see below). Among the expected instances of period shortenings, lengthenings, or arrhythmicity was a case of temperature sensitivity for circadian periodicities: a deletion of the Thr-Gly repeat—from the central region of the *per*-encoded protein (PER)—resulted in a slower clock pace at high temperature (Ewer *et al.* 1990). This is analogous to the per^L mutant's temperature sensitivity (Konopka *et al.* 1989; Ewer *et al.* 1990) but opposite to that exhibited by the aforementioned *frq* mutants of Neurospora. These temperature dependencies should

Corresponding author: Jeffrey C. Hall, Department of Biology, Mailstop 008, Brandeis University, 415 South Street, Waltham, MA 02254-9110. E-mail: hall@binah.cc.brandeis.edu

[1]*Present address:* Department of Medicine, University of Manchester, Manchester M13 9PT, UK.

be kept in mind in the context of a new *per* mutant first reported here. A similarly relevant category of engineered mutations in this gene involves a set of point mutations deliberately made within a 40-amino-acid span in the vicinity of the per^S-defined site. These *in vitro*-manufactured variants usually resulted in shorter-than-normal locomotor rhythms (Baylies *et al.* 1992), as if this ~3% of the polypeptide's length might be special in this regard (damage it in almost any manner, get a fast clock).

Additional subsets of PER have taken on the status of "domains" (for review see Hall 1998). These regions variously interact with another clock-gene product, the TIMELESS protein (reviewed in general by Sehgal *et al.* 1996), or they are involved in subcellular localization of PER, or both. Only one of these regions, the PAS domain (reviewed by Hall 1998), harbors an *in vivo*-induced *per* mutation (per^L). The other side of this coin is that further subsets of PER may turn out to be functionally significant, and attention could be drawn to them by the molecular mapping of *in vivo* mutants. This is one theme of the current study, involving a different perspective from selecting an intragenic region for *in vitro* mutagenesis. For example, who would choose to effect a change in the C-terminal half of PER? It seems to be featureless, it is poorly conserved evolutionarily (Colot *et al.* 1988), and the C-terminal one-third of the protein is gone altogether in one insect species (Reppert *et al.* 1994). Moreover, one alteration that was made *in vitro* within PER's C-terminal region, which involved a possible cAMP-dependent protein kinase site, led to no change in behavioral rhythmicity (Baylies *et al.* 1992).

Another molecular theme of clock gene investigations is that the products of several such loci exhibit daily cyclings in their abundance (for reviews see Dunlap 1996; Sehgal *et al.* 1996; Hall 1998). The fact that in Drosophila's per^S mutant, which was studied immunochemically in light/dark (L/D) conditions, protein levels rise earlier than in wild type exemplifies that there are also feedback effects on the mRNA cyclings (*e.g.*, Zerr *et al.* 1990). In per^S, PER is phosphorylated earlier as well (Edery *et al.* 1994); temporal dependence of that post-translational modification is a normal feature of the protein's quality. As part of the current study, we ran similar timecourses on two relatively new *per* mutants; like in per^S, these exhibit substantial period changes in D/D conditions, peaks of locomotor activity that are much earlier or later than normal in L/D (Konopka *et al.* 1994; this article), and altered PER protein time courses.

We have also (intragenically) mapped the two *per* mutations just alluded to, along with the other ones resulting from *in vivo* mutagenesis that have not been characterized molecularly (Hamblen-Coyle *et al.* 1989; Dushay *et al.* 1990). Moreover, we have phenotypically analyzed a new long-period mutant; a prominent feature of its properties is a novel kind of temperature sensitivity (*i.e.*, for slow-clock mutants in Drosophila): the flies become more normal when behaviorally monitored at high temperature, hence $per^{SomeLikeitHot}$.

MATERIALS AND METHODS

Strains: Flies were grown in media containing cornmeal, dextrose, agar, sodium potassium tartrate, calcium chloride, and the mold inhibitor Lexgard (Inolex Chemical Co., Philadelphia, PA). The growth chamber was an environmental room maintained at 25° and 70% relative humidity.

The long-period strain originally named *43Y* was tested for complementation with *per* mutations. Stocks carrying a given one of five *X*-chromosomal *per* mutations (see results) or an *X* chromosome from a Canton-S wild-type stock were crossed to *43Y*, resulting in females heterozygous for the new mutation and a given *per* mutation or per^+; these females were collected as <1-day-old imagoes before behavioral testing (see below). *43Y* mutant males were also crossed to females carrying deletions (*Df*s) that remove *per* from the *X* (first) chromosome, *Df(1)TEM-202* and *Df(1)64j4* (*e.g.*, Reddy *et al.* 1984), and to males carrying a w^+Y chromosome in which the w^+ duplication includes the *per* locus (*e.g.*, Hamblen *et al.* 1986). These crosses led to flies in which *43Y* was suspected to be uncovered or covered, respectively. Another kind of "*per* duplication" crossed into a *43Y* background was a 13.2-kb transgene (strain 13.2:2 in Table 2), which covers the effects of arrhythmic-inducing *per* genotypes (*e.g.*, Citri *et al.* 1987; Yu *et al.* 1987b).

To outcross the new mutant, *43Y* and Canton-S females were mated to males hemizygous for *In(1)FM7* (an *X*-chromosome balancer marked with *Bar*); F_1 females expressing *Bar* and carrying either *43Y* or per^+ were crossed to F_1 males carrying the same nonbalancer *X*; the result was a pair of mutant *vs.* control strains with similar genetic backgrounds. Another wild-type strain used (as a source of to-be-sequenced *per* locus DNA, Figure 1B) was Oregon-R.

Rhythm tests: Locomotor behavior of the flies was monitored mostly as described in Hamblen *et al.* (1986). Briefly, the relevant devices involve infrared emitters and detectors that flank arrays of glass tubes, into each of which is placed an individual fly, which breaks the light beam during most of its movements within the tube; such signals are routed from the detector into digital storage. Modifications of these procedures were as follows: (1) Flies were placed in the monitoring tubes immediately after collecting them as <1-day-old imagoes; infrared emitter/detector-flanked tubes were immediately put into an incubator, usually set at 25°, but at 18° or 29° in certain tests (although all flies had been reared at 25°). (2) Each test ran for ~2 wk. First-week conditions: 12-hr/12-hr L/D cycles; second week: constant darkness (D/D). (3) The flies' behavior began to be monitored during the very first L/D cycle. (4) Most of the data were collected using DAM software provided by Trikenetics, Inc. (Waltham, MA; *cf.* Konopka *et al.* 1994), although some were handled via the older Apple IIe interface (Hamblen *et al.* 1986).

Data files from each individual fly's monitoring record were analyzed by periodogram and phase analysis programs (*cf.* Hamblen *et al.* 1986; Hamblen-Coyle *et al.* 1992; Wheeler *et al.* 1993). The Phase program gives estimates for the times of peak activity within the L/D cycles, with reference to the L-to-D or D-to-L transitions (*e.g.*, Hamblen-Coyle *et al.* 1992). Chi-square periodogram analysis determined whether a fly was significantly rhythmic ($\alpha = 0.05$), and if so, gave a best estimate of periodicity. These determinations were augmented by taking into account the so-called "power" of the rhythm and the width of the significance peak (in 0.5-hr bins); the previously used cutoffs (power >20) and width (>2) were

applied as additional demands for tabulating a behavioral record as "rhythmic" (*cf.* Ewer *et al.* 1992; Frisch *et al.* 1994). VAX-based programs led to graphic actogram outputs of an individual fly's locomotor behavior and periodogram result, as well as average activity plots, in which that individual's L/D behavioral record had each day of behavior superposed; for this, each bar in the resulting histogram is a per-day average of the activity event count.

For eclosion tests, the aforementioned pair of per^+ *vs. 43Y*-containing cultures were handled as described in Konopka *et al.* (1994). A modification was to start the cultures at 25° (12 hr:12 hr L/D) but then shift them to 20° for 2–3-days (L/D condition); this allowed for an optimal number of staged pupae. Such animals were glued to the plastic disks of an eclosion monitor (Konopka *et al.* 1994), put into an incubator at 25°, and after one more lights off, they were left to eclose in D/D.

PCR amplification and direct sequencing of *per* mutants: Flies from each mutant strain were frozen in a 15-ml conical tube on dry ice. DNA was extracted by homogenizing 100 flies in a buffer containing 8 mm NaCl, 160 mm sucrose, 50 mm EDTA, 125 mm Tris (pH 8.5), and 0.5% sodium dodecyl sulfate, using a glass homogenizer. The homogenate was transferred to a 1.5-ml Eppendorf tube and put in a 65° water bath for 30 min. 160 ml of 8 m potassium acetate was added, and the tube was placed in ice for 30–60 min. The samples were spun for 10 min at 4°, the supernatant was transferred to a new tube, and 2 ml of 5 mg/ml RNase (Sigma, St. Louis, MO) was added. This mixture was then incubated at 37° for 30 min. After two phenol/chloroform extractions, 2 vol of ethanol were added. These materials were mixed, held at room temperature for 2 min, and spun for 15 min in a microcentrifuge at 14,000 rpm. The DNA pellet was washed with 70% ethanol, allowed to dry, and resuspended in 200 μl dH_2O.

Double-stranded DNA from the per^T, per^{04}, per^{Clk}, and per^{SLIH} (née *43Y*) mutants were used as templates with five pairs of primers. All mutants were sequenced from bp 2000 to 9227 (see open arrows in Figure 1). *per*-specific, 20-mer primers were designed such that the gene could be amplified into five overlapping products, including introns. The primer pairs were as follows: (1)"upper" primer (U): 5′-GTTGGCGGACG GCAGAGGCA-3′ corresponding to bp 1983–2003 of the *per* sequence in Citri *et al.* (1987), with the "lower" primer (L): 5′-CGGCGCCCTTGTTCTTCTTC-3′ corresponding to 3121–3141; (2) U: 5′-AGTCAACCAACTGGGCAAGC-3′ (2800–2820); L: 5′-TCCACGTGCGATATGATCCC-3′ (4155–4175); (3) U: 5′-TG CCCCCCCCAGTTCCCGAC-3′ (4084–4104); L: 5′-AGCCGCT GCTGCCGCTCCTG-3′ (5721–5741); (4) U: 5′-GAGGAGGAT CCGGAACAGGC-3′ (5622–5642); L: 5′-GTGTACGTCGGTC AGCAGCA-3′ (6888–6908); and (5) U: 5′-CAAGACCACGGA CGGATCGG-3′ (6675–6695); L: 5′-AGCCGACATCACTGTTT CAG-3′; this corresponds to bp 9227–9247 of genomic the per sequence from Oregon-R wild type (A. C. Jacquier and M. Rosbash, unpublished results).

PCRs were cycled in 95° for 1 min, 1 min at 60°, and 1.5 min at 72° for 30 cycles using a thermocycler (MJ Research, Watertown, MA). The PCR products were gel purified on a 1.5% agarose gel, followed by purification with Qiaex2 (Qiagen, Chatsworth, CA). The templates were then prepared for PCR sequencing with nested primers and fluorescently labeled dNTPs (the relevant ones included in a kit called ABI Prism; Perkin Elmer Applied Biosystems, Foster City, CA). The sequencing reactions were cycled at 92° for 3 min, 50° for 20 sec, and 60° for 4 min for 25 cycles. These samples were cleaned using a Sephadex column, dried, and run on a sequencer (model 373A; Perkin Elmer Applied Biosystems). Some regions of the large *per* DNA fragments gave mediocre results; in these instances, smaller templates were prepared. Every region from different PCR templates and different DNA samples was sequenced more than once (*i.e.*, different groups of starting flies carrying a given *per* allele).

Sequence analysis: The sequences of the *per* mutations were compared to that of a per^+ allele cloned from wild-type Canton-S, whose genomic sequence was donated to the EMBL database by T. A. Bargiello (id = DMPER; AC = 03636; *cf.* Jackson *et al.* 1986; Baylies *et al.* 1993). Because this sequence does not include the extreme 3′ untranslated region (UTR) region of *per* (encompassed by nt 7400–9400), the extreme 3′ sequences in the mutants were compared to *per* data obtained from sequencing the aforementioned per^+ DNA cloned from Oregon-R. Sequences from the various strains were compared by using "best fit" of the GCG70 sequencing programs. When we compared the two wild-type sequences (nt 2000–7474 of Oregon-R to nt 2004–7487 of Canton-S), we found 20 presumably silent polymorphisms; 10 were within introns and 10 were within exons (the latter did not lead to amino acid substitutions). When comparing the mutant strains to both wild types, 15 unique silent polymorphisms in introns and 12 in exons were found. In comparisons of the different mutant strains to Oregon-R base numbers 7474–9227 (3′ UTR), 12 unique base changes and 14 missing bases (in the mutants) were detected.

Other miscellaneous molecular results are now described in some detail; these interstrain variations may not be significant as far as rhythm phenotypes are concerned (*e.g.*, Hall *et al.* 1992); however, elements of the "repeat region" differences could be (*cf.* Sawyer *et al.* 1997). Moreover, consider that the per^+ strain from which a given *per* mutant is derived can be obscure (*e.g.*, Hamblen-Coyle *et al.* 1989; also per^{SLIH}); thus, elements of the following allows one to guess the source of a given mutant (*cf.* Yu *et al.* 1987b): Analysis of DNA from per^T revealed that its Thr-Gly amino acid repeat region (encoded by *per* exon 5; see Figure 1A) carries only one of the relevant 18-bp direct-repeat cassettes; therefore, this mutant is missing two of the possible three cassettes, the result being 17 Thr-Gly pairs out of 23, the maximum number known (Kyriacou *et al.* 1996). per^{Clk} was found to contain the (empirical) maximum number of repeat cassettes (hence 23 Thr-Gly pairs); intralocus regions for per^{Clk} beyond the ORF in both directions (5′ and 3′) were as in per^+, except that this mutant is missing 11 bases within the first intron of the 0.9-kb transcript-encoding sequence (see Figure 1B for what that sequence is in general terms). per^{04} contains one of the repeat cassettes (thus, 17 Thr-Gly pairs); within the first (and largest) intron, this mutant contains the same two extra base pairs as in the case of per^T. per^{SLIH} contains two repeat cassettes (encoding 20 Thr-Gly pairs); the 5′ region of per^{SLIH} is like per^T and per^{04} (see above), and it is missing 11 bases in the 3′ UTR (the same as in per^{Clk}).

cDNA production: Drosophila heads were separated from their bodies and appendages by sieving in dry ice (as described in Levy and Manning 1981). The heads (mass = 0.2–0.5 g) were homogenized in 500 ml of TRIzol reagent (GIBCO BRL, Gaithersburg, MD), and then spun in a microcentrifuge at 4° for 10 min. The supernatant was moved to a new tube, and 100 ml of chloroform was added to the supernatant. This material was vortexed and spun again for 15 min at 4°; 250 ml of isopropanol was added to the removed supernatant, which was incubated at room temperature for 10 min (after mixing) and spun for 10 min at 4°. The pellet was then washed with 70% ethanol, dried, and resuspended in 40 ml dH_2O.

Reverse Transcriptase-PCR was carried out with a commercial kit (SuperScript Preamplification System for First Strand cDNA Synthesis; GIBCO BRL), using total head RNA (5 μg) and random primers, followed by application (to the cDNA) of the following *per*-specific primer pairs: (1) U: 5′-CACCTTC TGCGTGATGCTGC-3′ (3823–3843); with L: 5′-GCGGCAGCT CCAGCTTGAGA-3′ (4735–4755) and L: 5′-AGCCGCTGCT

GCCGCTCCTG 3′ (5721–5741), and (2) a separate experiment, U: 5′-CAAGCAGGAGGTTTCCCGCC-3′ (4657–4677) with L: 5′-CCTTTGGATGAGCTGGCGGT-3′ (5264–5284) and L: 5′-AG CCGCTGCTGCCGCTCCTG-3′ (5721–5741).

The PCR products were separated by electrophoresis on a 1.5% agarose gel, purified with Qiaex2, and cloned into a pGEM-T vector (Promega, Madison, WI). cDNAs from the clones were sequenced as described above.

Western blotting: Flies were assayed for PER protein abundance, as described in Edery *et al.* (1994), with minor modifications. For time-based collection of flies, ~3–7-day-old flies were placed in either vials or bottles containing fly food. Mixed males and females from per^T, per^{04}, or per^{SLIH} strains were placed in bottles; for certain timecourses, per^{SLIH} males and females were separated before their placement in vials. The flies were placed in incubators set at 25° and were subjected to three cycles of 12 hr:12 hr L/D cycles, after which they were frozen at different Zeitgeber times: per^{04} flies were taken every 4 hr beginning at ZT0 ("0" referring to the beginning of the light period), per^T and per^{SLIH} every 2 hr beginning at ZT0, and wild-type controls were collected at least at ZT8 and ZT20). After the homogenization of separated heads in Hepes-EDTA buffer (100 mm KCl, 20 mm Hepes, 5% glycerol, 10 mm EDTA, 0.1% Triton X-100, 1 mm dithiothreitol, 0.5 mm phenylmethylsulfonyl fluoride, 10 μg aprotinin, 5 μg leupeptin per milliliter, 1 μg pepstatin per milliliter) and centrifugation to extract the supernatant, the relative protein abundance was assayed by combining 1 μl of sample and 1 ml Coomassie Protein Assay Reagent (Pierce Chemicals, Rockford, IL) in a cuvette and quantifying absorbance in the 590-nm range. Samples in a given experiment were equilibrated before electrophoresis using the values obtained by this method. Gels were electroblotted onto nitrocellulose for 40 min at 0.2 A/blot or 0.1 A/mini-blot (Integrated Separation Systems electroblotter; Bio-Rad, Richmond, CA).

The nitrocellulose blot was immersed in Ponceau S stain (Sigma) and checked for equal loading. After destaining with tris-buffered saline with Tween (TBST; 10 mm Tris-HCl, 140 mm NaCl, 0.05% Tween 20, pH 7.5), the membrane was blocked in 1% BSA in TBST for 30 min and then immediately incubated with a polyclonal anti-PER antibody made in a rabbit (Stanewsky *et al.* 1997); here it was diluted at a concentration of 1:10,000 in 5% dry milk in TBST for either 2 hr at room temperature or overnight at 10°. After a washing in TBST, the blot was incubated for 30 min at room temperature with anti-rabbit IgG horseradish peroxidase-conjugated antibody and was diluted 1:5,000 in 5% dry milk in TBST. After another TBST wash, PER signals were visualized using the Enhanced ChemiLuminesence Kit (Amersham, Arlington Heights, IL) followed by autoradiography.

To quantify the signals, membranes were exposed to a chemoluminescence-sensitive screen that was scanned using a Phosphorimager (Bio-Rad). Band intensities obtained by the imager were quantified by creating a boxed-in area around a PER band to be analyzed; an identically sized box was made directly above (but not overlapping) the PER band in a given lane. The exposures in the boxed areas were then quantified, and the "background" value was subtracted from the PER band. The highest value was set equal to one, with all others adjusted in the same manner; these values were then plotted as further detailed in Figure 4.

RESULTS

Sequencing of mutants: per^T is a very fast 16-hr clock mutant (Konopka *et al* 1994). Comparing its *per* sequence to that of Canton-S wild type revealed per^T to have a single nucleotide mutation at bp 4800 (with reference to the *per* gene numbering scheme of Citri *et al.* 1987). This **g**-to-**a** transition (with lowercase and boldface indicating nucleotides as opposed to amino acids) results in a glycine-to-aspartic acid substitution at residue 593; this is four amino acids more C-terminal than the site at which another short-period mutant, per^S, is amino acid substituted (Figure 1A; *cf.* Baylies *et al.* 1987; Yu *et al.* 1987a). The per^T mutation also lies within an intragenic region that has been *in vitro* mutated, usually resulting in short-period mutants (Baylies *et al.* 1992). In fact, these investigators happened to mutate the per^T-defined glycine, changing it to cysteine and to serine; the behavioral results were, respectively, locomotor rhythms with periods 4 hr longer or 5.5 hr shorter than the relevant control values (Baylies *et al.* 1992). Aspartic acid and serine contain polar groups, with aspartic acid having the additional property of being negatively charged (Taylor 1986). Perhaps the latter attribute is related to the extreme period-shortening effects of per^T.

In the 3′ UTR region of the per^T, a total of 3 bp were found to be deleted with respect to the published (and presumably normal) sequence (Lorenz *et al.* 1989). However, we found this to be true of all the other mutant strains sequenced, along with two wild-type strains (Oregon-R and Canton-S). This *per* UTR region overlaps the ORF of the adjacent (centromere-proximal) gene, which encodes a 0.9-kb transcript (Reddy *et al.* 1984; Lorenz *et al.* 1989). With regard to the anomalous sequence in per^T, which creates no coding changes for PER, one of the (noncontiguous) deleted bases creates a frame shift in a seven-amino-acid stretch of the ORF of the 0.9-kb transcript: SGRAGAH (*cf.* Lorenz *et al.* 1989) to VAALEPI (Figure 1B); these changes remove two positively charged amino acids and add three nonpolar amino acids. Two additional bases are deleted such that the translation of this ORF goes back into frame (Figure 1B). We suspect that this additional (apparent) change in the per^T sequence is unrelated to its rhythm phenotype because the corresponding nucleotides in strains that carry a variety of other *per* alleles (see legend to Figure 1) spell the same sequence of amino acids (VAALEPI). It could be that the reported sequence—from a per^+ strain, leading to SGRAGAH (see above)—was in error.

With regard to the 5′ end of the *per* gene in the per^T mutant (and the other three described below), this part of the ORF was sequenced, along with ~1 kb of more upstream material; special attention was paid to the nucleotide sequences corresponding to the N terminus because small insertions in this part of the protein can lead to period changes (so far, meaning shorter than normal; Rutila *et al.* 1992). per^T had a normal (conceptual) nucleotide sequence near the translation start site; there were no extra bases in this region, nor were there any extra amino acids coded for. We found, however,

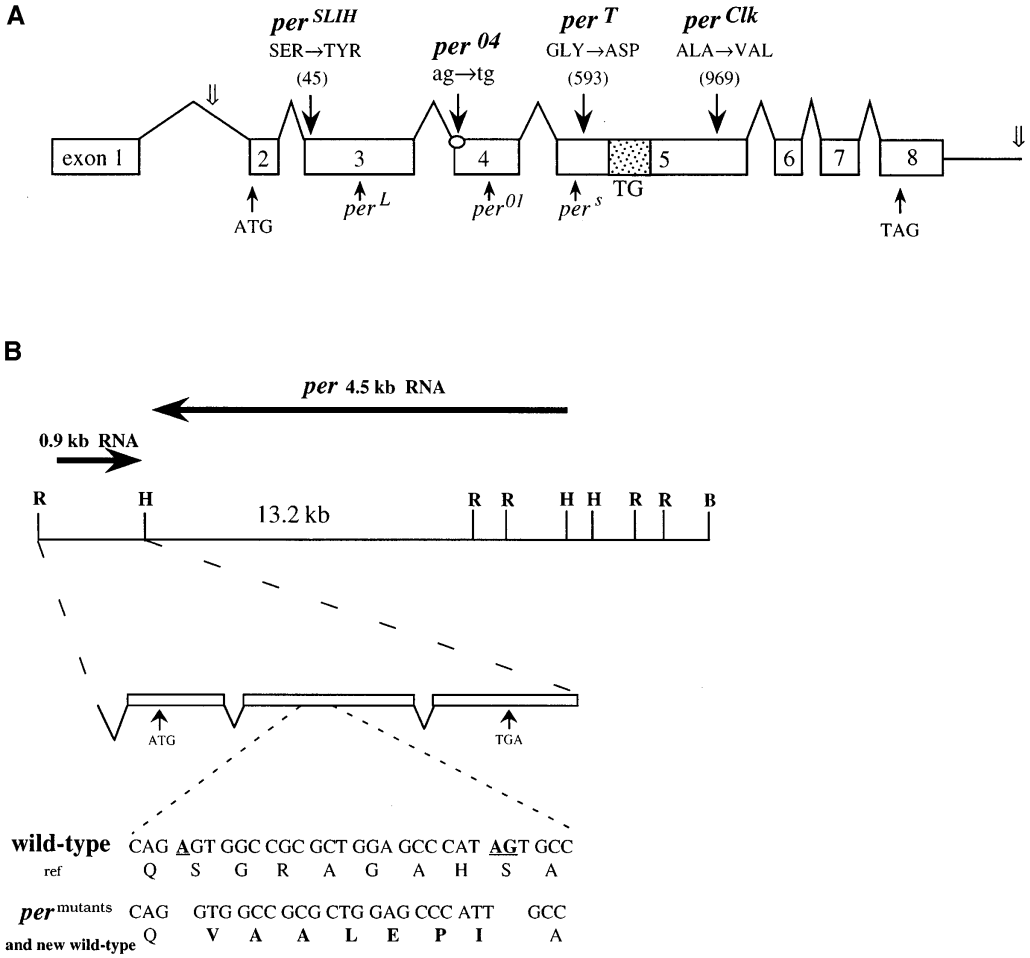

Figure 1.—Genomic map of the *per* gene with locations of mutations. (A) The open numbered boxes are exonic material; angled lines are introns; the straight line is the 3′ UTR, including material from which a 0.9-kb RNA is transcribed (see text and B below); the filled box indicates the threonine-glycine (TG) repeat; the area between open arrows (pointing downward) indicates the material sequenced to compare mutant to wild-type nucleotides; and black arrows (pointing downward) indicate where the newly revealed mutations lie. Accompanying these indications are the names of these four mutants, the amino-acid substitution, and residue number of the protein that is changed (for per^{SLIH}, per^{T}, and per^{Clk}), or the nucleotide change only (per^{04}; see also Figure 5); upward-pointing arrows show the (normal) coding start and stop sites, as well as the positions of previously sequenced *per* mutations, each accounted for by a single base change resulting in an amino-acid substitution or intragenic stop codon (Baylies *et al.* 1987a; Yu *et al.* 1987a). (B) A partial restriction enzyme map of a 13.2-kb *Bam*HI/*Eco*RI genomic DNA fragment that encodes the *per* (4.5-kb) transcript and possibly the adjacent 0.9-kb transcript (Bs = *Bam*HI sites; Rs = *Eco*RI ones); this fragment rescues arrhythmicity in flies whose only other *period* allele is per^{01} (*e.g.*, Yu *et al.* 1987b). The dark, horizontal arrows show where and in what direction the two mRNAs are transcribed. The large, dashed lines show detail from where the 0.9-kb species is transcribed; smaller, dashed lines show sequence detail and thus the anomalous bases detected in this region of the per^{T} mutant (*cf.* Lorenz *et al.* 1989, from which the so-called wild-type sequence shown here is taken). These anomalies, however, were found in DNA from flies expressing the other *per* alleles included in this report; see bold underlined nucleotides, which are missing in all four of the newly sequenced *per* mutants and in the current versions of two wild-type strains (Canton-S and Oregon-R).

two additional bases in the per^{T} sequence, compared to the wild-type one, 800 bases upstream of the translational start site in intron 1.

When comparing the sequence of the 22.5-hr per^{Clk} mutant to per^{+} (Canton-S), the mutant allele was found to be associated with a single base pair change at number 5928: **c** to **t**, which causes an amino acid substitution from alanine to the aliphatic valine at residue 969 (Figure 1A). The molecular alteration of per^{Clk} in this region of the gene correlates with the fact that apparent intragenic meiotic recombinants (Dushay *et al.* 1990) placed this short period mutation to 3′ or C-terminal to the site of the nonsense mutation of per^{01} (which is N-terminal to the mutated sites in per^{S} and per^{T}; Figure 1).

Sequencing the nearly arrhythmic per^{04} mutant (Hamblen-Coyle *et al.* 1989) revealed that it contains a splice-site mutation at the boundary of the third intron and the fourth exon (Figure 1A). An **ag** sequence has been changed to **tg**; the latter is the wrong kind of dinucleotide for a proper splice-acceptor site. The molecular

TABLE 1

Locomotor behavior of *per*^{SLIH} hemi-, homo-, and heterozygote types, including complementation tests with *per* mutations and contemporaneous controls

	L/D			D/D	
Genotype	No. entrained (%)	Morning phase hr ± SEM	Evening phase hr ± SEM	No. rhythmic (%)	Period hr ± SEM
per^{SLIH}/*Y*	49 [98]	+0.30 ± 0.10	+1.50 ± 0.20	36 [80]	27.2 ± 0.1
per^{SLIH}/*per*^{SLIH}	40 [82]	+0.50 ± 0.15	+1.15 ± 0.15	14 [35]	27.3 ± 0.1
per^{SLIH}/*w*$^{+}$*Y*	15 [100]	+0.45 ± 0.30	+0.10 ± 0.25	14 [100]	25.6 ± 0.2
per^{SLIH}/*Y;13.2:2*	12 [100]	+0.50 ± 0.10	+1.20 ± 0.10	11 [92]	26.1 ± 0.1
per^{SLIH}/*Y* OC	23 [100]	−0.45 ± 0.20	+1.50 ± 0.10	19 [100]	27.3 ± 0.1
per^{SLIH}/*per*^{SLIH} OC	52 [94]	+1.10 ± 0.10	+1.10 ± 0.15	33 [70]	27.5 ± 0.1
per^{+}/*Y* OC	28 [100]	−0.70 ± 0.15	−0.55 ± 0.15	26 [100]	23.8 ± 0.1
per^{+}/*per*$^{+}$ OC	35 [100]	+1.20 ± 0.10	−1.30 ± 0.05	27 [97]	24.0 ± 0.1
per^{T}/*per*^{SLIH}	19 [100]	+0.20 ± 0.15	−1.55 ± 0.10	15 [88]	23.2 ± 0.1
per^{S}/*per*^{SLIH}	15 [58]	−0.55 ± 0.25	−0.75 ± 0.60	15 [65]	23.8 ± 0.1
per^{Clk}/*per*^{SLIH}	23 [92]	+0.65 ± 0.25	+0.05 ± 0.25	17 [55]	25.6 ± 0.1
per^{+}/*per*^{SLIH}	41 [93]	+0.20 ± 0.15	+0.80 ± 0.10	14 [61]	26.1 ± 0.1
per^{01}/*per*^{SLIH}	47 [100]	+1.35 ± 0.10	+1.25 ± 0.10	29 [71]	28.4 ± 0.1
per^{04}/*per*^{SLIH}	23 [79]	+1.20 ± 0.20	+0.85 ± 0.20	19 [78]	28.2 ± 0.1
per^{L}/*per*^{SLIH}	26 [84]	+1.35 ± 0.10	+0.55 ± 0.25	17 [55]	27.6 ± 0.2
Df(1)TEM202/per^{SLIH}	20 [77]	+0.70 ± 0.15	+0.25 ± 0.15	16 [64]	27.2 ± 0.1
Df(1)64j4/per^{SLIH}	5 [16]	+1.35 ± 0.20	+0.75 ± 0.30	15 [48]	28.2 ± 0.1
per^{+}/*Y*	32 [97]	−0.55 ± 0.10	−0.80 ± 0.10	29 [96]	24.0 ± 0.1
per^{01}/*Y*	25 [78]			28 [0]	

Genotypes of flies tested are in the first column. Genotypes in the second block from the top are from outcrossings (OC) involving the *FM7* balancer strain (see materials and methods). One genotype designation that bears explanation is 13.2:2, which refers to a 13.2-kb transgene containing *per*$^{+}$ DNA (transgenic strain no. 2, in which the inserted *X*-chromosomally derived DNA is on chromosome *3*; *cf.* Citri *et al.* 1987). Second, third, and fourth columns: data from flies behaving in L/D cycling conditions. Second column: numbers of flies that entrained to the L/D cycles, followed in brackets by the percentages of the total number of flies tested that were in synchrony with the fluctuating environmental condition. Third column: morning phase (hr ± SEM) from the time of D-to-L transition (− means before that time; +, after that time). Fourth column: evening phase (similarly quoted values for behavior near the time of L-to-D transitions); peaks of activity (hence, the phase values) were determined as described in Hamblen-Coyle *et al.* (1992). Fifth and sixth columns: behavioral data from constant dark (D/D) and constant (25°) temperature conditions; fifth: numbers of rhythmic individuals, with percentages of flies that behaved rhythmically in brackets; sixth: average period (± SEM) from these D/D behavioral monitorings. No phase or period values are entered for the *per*^{01} control row because these flies exhibit no clock-regulated rhythmic behavior, neither in L/D (Wheeler *et al.* 1993) nor in D/D (Hamblen *et al.* 1986).

change within *per*^{04} should be considered in light of the Northern blotting data for this mutant. That is, *per*^{04} showed an anomalous RNA species, detected by *per* probes, together with the normal 4.5-kb mRNA (Hamblen-Coyle *et al.* 1989); the former species was considerably shorter than normal and appeared in females heterozygous for *per*^{04} and the normal allele, as well as in extracts of flies taken from a true-breeding, fully mutant stock.

The *per*^{SLIH} mutant (whose biological properties are detailed below) was found to have a single nucleotide change at number 3035: **c** to **a**, which causes an amino acid substitution from serine to tyrosine at residue 45 (Figure 1A).

Locomotor rhythms: An *X*-chromosomal long-period rhythm mutant was detected in conjunction with "GAL4" (transposon) mobilization [see Brand and Dormand (1995) for general information on such transpositions]. The flies exhibited locomotor rhythms of 27–28 hr in preliminary tests. (The transposons carried by the strains in question were inserted in widely different genomic locations, but several of these strains contained slow-clock flies.) A long-period derivative of one of the transposon-bearing strains was generated by repeated outcrossing and reextraction of individuals carrying the *w*$^{+}$-marked transposon in question, which in this case was located in a centromere-distal region of the *X* chromosome (cytogenetic interval 2C-D), somewhat near where *per* is located (3B1-2). One such derivative remained abnormal for its locomotor activity and was

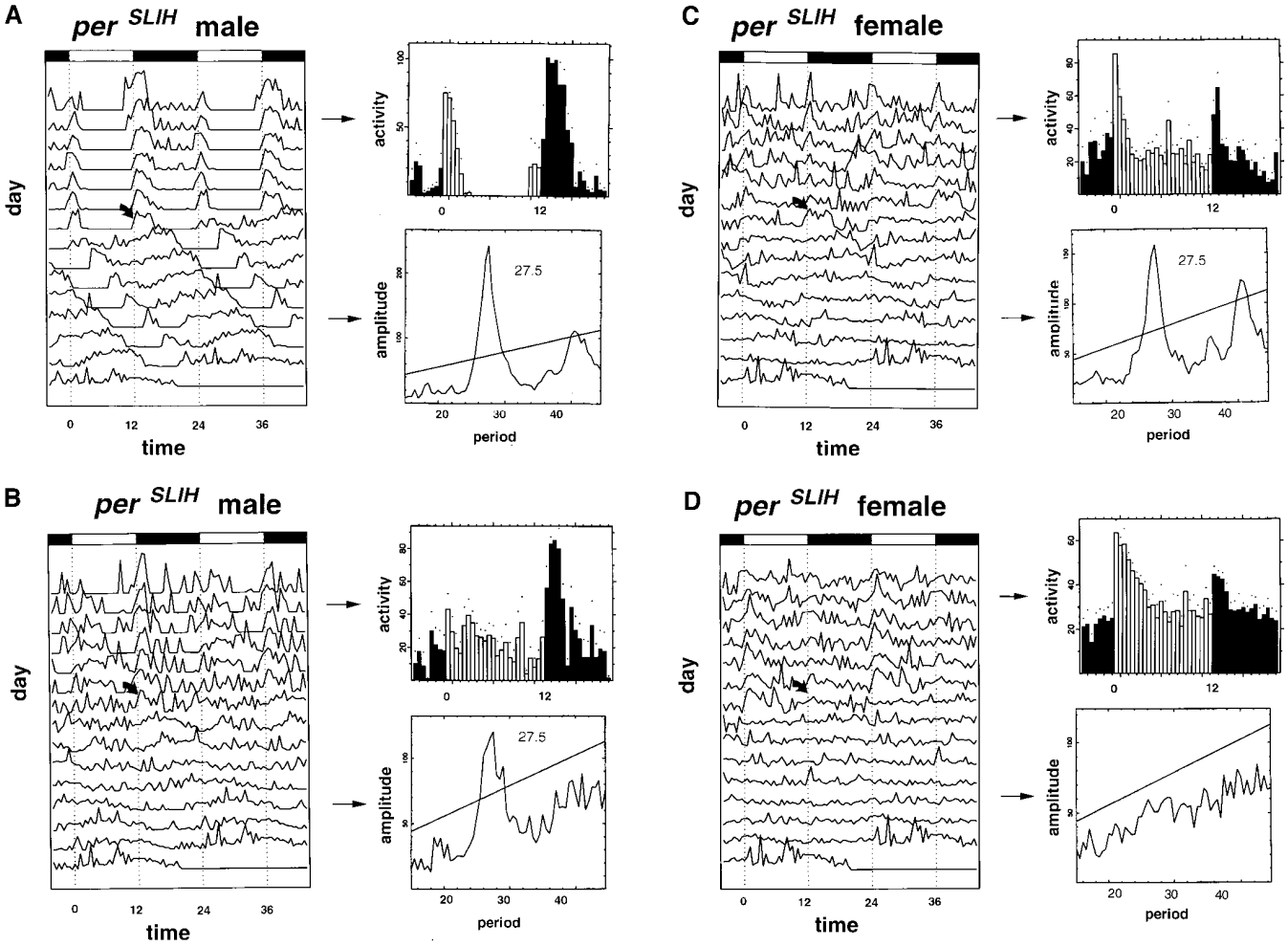

Figure 2.—Locomotor activity of per^{SLIH} males and females in environmentally cycling and constant conditions. Individual activity plots of males (A and B) and females (C and D), showing a range of rhythm strengths (*cf.* power and width values, below, and as explained in materials and methods). Each panel consists of an actogram of an individual fly's activity during L/D cycling conditions followed by constant D/D cycling conditions (the arrow indicates the last lights off before the beginning D/D), an average (per day) activity histogram derived from that individual's L/D behavior, and a periodogram (plot of the chi-square analysis; $\alpha = 0.05$). The period, power, and width values determined from these chi-square periodograms are as follows: (A) 27.5 hr, 170 arbitrary units, 9 time-bins; (B) 27.5, 47, 8; (C) 27.5, 85, 6.

called *43Y*. Long periodicity was clearly linked closely to the transposon's insertion site and hence to the *per* locus. Thus, a small portion of the *per* gene, in and around the site at which the classical per^{L} variant is altered (Baylies *et al.* 1987; Gailey *et al.* 1991), was obtained from *43Y* flies by PCR and sequenced; the sequence was identical to those reported for per^{+}. Yet, in conjunction with finding a *per*-mutated site in this mutant (see above), the *43Y* mutation was shown to be a new *per* allele. The mutation, now called per^{SLIH}, had its period-lengthening effects uncovered by null or near-null *per* variants (per^{01}, per^{04}, or deletions of the *per* locus); in particular, the periods exhibited by the relevant heterozygous females were longer than the ~27-hr rhythms that were measured for per^{SLIH} males or homozygous per^{SLIH} females (Table 1; Figure 2). Note that the majority of the females in the original version of the (*43Y*) stock were arrhythmic (65%); this improved to 30% arrhythmicity of mutant females after outcrossing (see materials and methods). That value, however, is still unusually high and also reflects a greater proportion of arrhythmic individuals than for per^{SLIH} males (Table 1).

per^{SLIH} is appreciably semidominant for its period-lengthening effects (Table 1). This may be a universal feature of period-altered clock mutants (*e.g.*, Hall 1995). In any case, the closer to normal periods of $per^{SLIH}/+$ females, compared to the per^{SLIH}/per^{-} females (see above), validates the apparent lack of complementation in the latter females. Further tests of heterozygous genotypes showed that per^{SLIH} lengthened the effects of short-period *per* mutations and it shortened the effects of per^{L}. In general, these transheterozygotes had periods intermediate between those associated with the two alleles that had been combined (Table 1). Adding a per^{+}

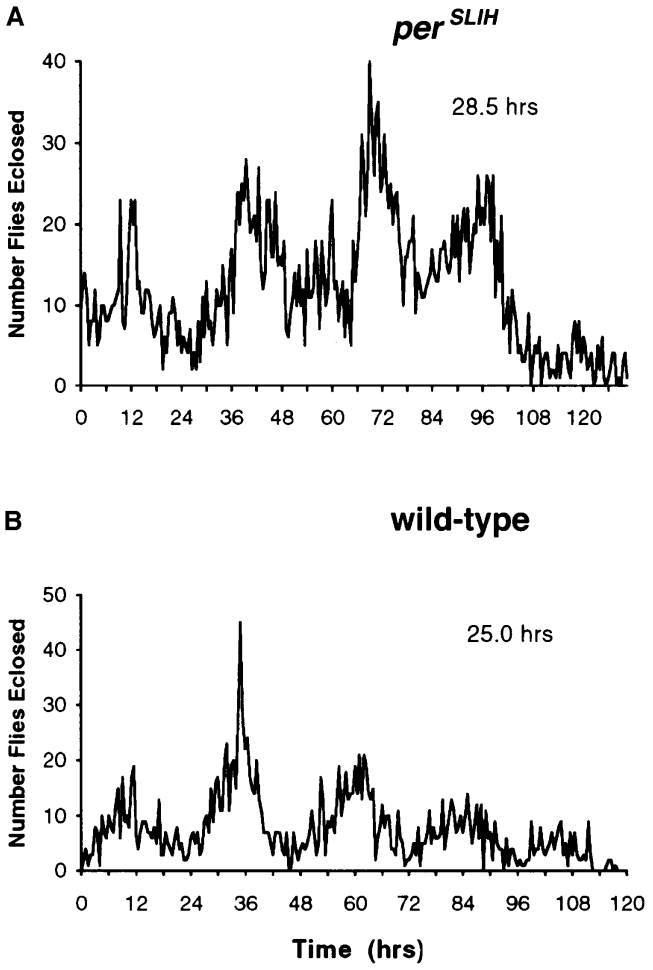

Figure 3.—Eclosion profiles of per^{SLIH} and normal populations. The plots show five days' worth of adult emergence numbers from automated monitorings performed as described in materials and methods. The abscissas represent the numbers of hours after the last lights-off transition, and the ordinates represent the number of newly eclosed adult flies counted every 30 min. (A) This per^{SLIH} plot was rhythmic with the anomalously long period indicated (determined from chi-square periodogram, $\alpha = 0.05$; power: 25, width: 9; the latter two indicators of rhythm strength are defined in materials and methods). The number of adults emerging in two separate runs was 3180 (such results were merged for this plot). (B) Eclosion of a per^{+} (Canton-S-derived) strain was also rhythmic with the closer-to-circadian period indicated ($\alpha = 0.05$; power: 10, width: 11); the number of emerged flies (one run) was 1814.

allele to males hemizygous for per^{SLIH} largely covered the effects of the mutation (Table 1), although its semidominant effects were still discernible. [Note that an extra dose of per^{+} by itself speeds up the clock slightly (Smith and Konopka 1982), but such a duplication (in a per^{+} genetic background) does not cause ~1.5 hr of shortening, as observed in per^{SLIH}-with-per^{+} males, thus implying coverage of this mutation's effect in the current tests.]

In L/D cycles, the per^{SLIH} mutant entrained. The mutant flies' locomotor was in synchrony with the environmental cycles, giving rise to 24-hr periodicity; however, the mutant females did not entrain as well as the males did (Table 1; Figure 2). The phase of the morning peak was just after lights on, as is observed for wild-type adults of this species; the mutant's evening peaks were 1–2 hr after lights off, which is later than normal (Hamblen-Coyle *et al.* 1992). As in previous studies, therefore, the evening peak's phase is more movable under the influence of a period-altered *per* mutation (*cf.* Hamblen-Coyle *et al.* 1992). The evening peak associated with the mutant's L/D behavior proceeded into the major free-running locomotor peak component (Figure 3; *cf.* Hamblen-Coyle *et al.* 1992; Konopka *et al.* 1994), indicating that the daily ~3-hr reset (leading to the aforementioned synchrony) is genuine entrainment.

Flies carrying the per^{SLIH} mutation were tested at different temperatures, given that other *per* mutants can exhibit different kinds of temperature dependence for their free-running periods (Konopka *et al.* 1989, 1994; Ewer *et al.* 1990; Curtin *et al.* 1995; Huang *et al.* 1995). per^{SLIH}'s period did change with temperature, but unlike the case of per^{L}, the new mutant's clock pace sped up as the temperature was raised; the period range, resulting from testing over a >10° temperature range, was >1.5 hr (Table 2). Experimental temperature variations accompanied the tests of heterozygous females carrying per^{SLIH}. The results were that the mutation is at least partly dominant to per^{+} in terms of faulty temperature compensation, although this effect was not observed when per^{SLIH} was heterozygous with per^{Clk} or per^{L} (Table 2), respectively. per^{Clk} does not alter the fly's temperature compensation (Dushay *et al.* 1990) or lead to "over compensation," as does per^{L} in the formal sense. In this regard, per^{L}'s higher temperature/slower clock properties could be counteracting those of per^{SLIH} in the trans-heterozygous females (Table 2). Combining per^{SLIH} with the other mutation that (by itself) causes the fly's clock to be more of a thermometer (per^{T}) led to a period change that was about the same as in the case of hemi- or homozygosity for per^{SLIH} by itself (Table 2).

Eclosion rhythmicity: per^{SLIH} was expected to be abnormal in its eclosion rhythm (*cf.* Hall 1995). Indeed, the periodic adult emergence for the mutant cultures occurred with much longer than normal cycle durations (Figure 3).

PER cycling in *per* mutants: We tested the new mutant per^{SLIH} to determine whether PER cycling was affected. Because of the differences in locomotor behavior observed in males and females, Western blots containing timecourses of males or females were analyzed separately. Two different experiments involving the same timepoints and flies were consistent in showing that, although the peak (*ca.* ZT22) (Figure 4C) and trough (*ca.* ZT10) times were similar (or identical), the accumulation of PER in males was delayed or took longer to reach its peak. The slope of the rise of PER levels is steeper in males than in females (Figure 4D). The rising

TABLE 2

Free-running locomotor behavior of per^{SLIH} types tested at different temperatures

	Temperature					
	18°		25°		29°	
Genotype	Period ± SEM	No. rhy. (%)	Period ± SEM	No. rhy. (%)	Period ± SEM	No. rhy. (%)
per^{SLIH}/Y	28.1 ± 0.1	49 (87)	27.2 ± 0.1	36 (80)	26.4 ± 0.1	15 (93)
per^{SLIH}/per^{SLIH}	27.3 ± 0.1	8 (40)	27.3 ± 0.1	14 (35)	25.5	1 (10)
per^{SLIH}/Y;13.2:2			26.1 ± 0.1	11 (92)	25.2 ± 0.1	14 (93)
per^{T}/per^{SLIH}	23.5 ± 0.1	9 (100)			22.0 ± 0.1	6 (67)
per^{Clk}/per^{SLIH}	24.8 ± 0.1	9 (39)			25.0 ± 0.1	8 (66)
per^{+}/per^{SLIH}	26.0 ± 0.2	7 (77)			24.8 ± 0.2	3 (37)
per^{01}/per^{SLIH}	28.7 ± 0.3	5 (55)			27.7 ± 0.1	6 (75)
per^{04}/per^{SLIH}	28.6 ± 0.3	16 (100)			27.5 ± 0.2	10 (100)
per^{L}/per^{SLIH}	27.3 ± 0.1	13 (56)			27.8 ± 0.2	11 (46)

The first column contains the per^{SLIH}-including genotypes. See the legend to Table 1 for an explanation of the 13.2:2 transgene. All three parts of columns indicate period ± SEM values (in hr) from D/D behavioral monitorings, along with the numbers of rhythmic (No. rhy.) individuals (percent of rhythmically behaving flies in parentheses). Each pair of columns gives data from testing at the three different temperatures indicated. The blank portions of certain rows imply that tests of these genotypes at this temperature were not done.

phase in this case was also steeper when compared to wild-type or another *per* mutant (Marrus *et al.* 1996).

We also tested per^{T} to determine whether PER is altered in its cycling quality. PER in extracts from this fast-clock mutant had a distinct, predictably earlier phase than the wild type (Figure 4A). The trough was much earlier (ZT2 *vs.* ZT6-8) and reached a plateau at ~ZT12–ZT18. This plateau resulted from an average of four different experiments that each gave a different PER^{T} peak time (Figure 4B). The rise time for this mutant was earlier (in L/D cycles) than in the case of the "5-hr-fast" per^{S} mutant (Marrus *et al.* 1996).

To properly gauge the quantity of PER present in wild-type and mutant strains, three peak time points for each genotype [*i.e.*, for wild-type ZTs 16, 18, and 20; for per^{T} ZTs 12, 14, and 16; and for per^{SLIH} males and females (separately) ZTs 20, 22, and 0] were run at one time on a Western blot, and each blot was quantified for PER presence (Figure 4E). In two separate experiments, the wild-type peak amount was greater than those of the other PER-producing genotypes tested (see legend to Figure 4).

Protein extracts of per^{04} were tested in two six-point timecourses and showed no detectable PER (Figure 4, A and E), as is routinely found for per^{01}.

Splicing defects in per^{04}: cDNA from per^{04} RNA was made using *per*-specific primers corresponding to the region in the vicinity of the mutation (Figure 1A). The pieces of cDNA were cloned and sequenced (see materials and methods). From these data, we infer that the ruined splice-acceptor in question can lead to deletion of 23 downstream coding nucleotides because the RNA processing machinery looked for another nearby **ag** 3′ junction. Such a deletion would cause a frameshift in the translation of the protein, leading to a premature stop codon (Figure 5). The novel 3′ splice-acceptor site implied was not at the first **ag** dinucleotide downstream of the mutated site; presumably this is because the first such site is preceded by a **g**—hence **gag**, which has been shown to prevent splicing (Mount 1993). Analysis of the cDNA fragments cloned from per^{04} indicates that the "next" intron (number 4 in Figure 1A) is spliced out correctly. Analysis of another cDNA fragment that was amplified from further downstream—into exon 5, upstream of the Thr-Gly repeat region—revealed another novel splice site (indicated by the diamond in Figure 5).

To see if temperature could have an effect on the splicing mutation (*cf.* Krawczak *et al.* 1992), cDNA was prepared from per^{04} and wild-type (Canton-S) flies that had been kept at different temperatures (18°, 25°, or 29°) for at least 4 days. The same primers were used to compare the cDNA around the mutation. No changes were seen in the products from the cDNAs taken from this mutant at different temperatures.

DISCUSSION

Genotypic defects: We have completed the elementary molecular analysis of *per* mutants that have occurred *in vivo*. Each of these four mutants is caused by a single base change, three of which are within the *per* ORF, and the fourth at a splice site. The previously sequenced *per* mutants ($n = 3$) also result from single base pair substitutions (see legend to Figure 1).

per^{T} is mutated in an evolutionarily conserved region of the PER ORF (Figure 1) called C2 (*cf.* Colot *et al.* 1988; Reppert *et al.* 1994). C2 has been reported to be

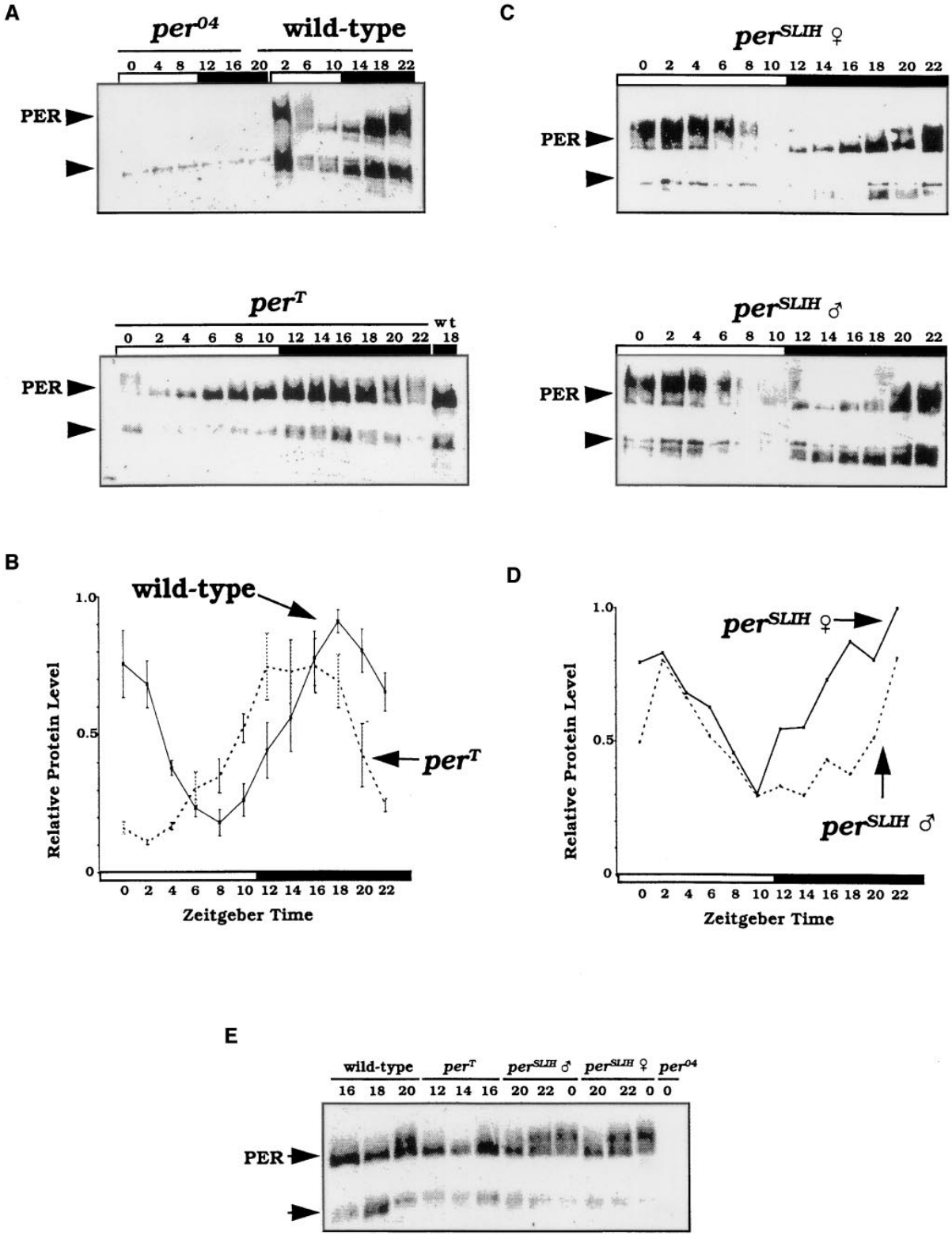

Figure 4.—Western blots of head extracts from wild-type and *per* mutants. (A) Wild-type, per^{04}, and per^{T} flies were entrained and killed at different Zeitgeber times (ZT0 representing the beginning of the light phase of a 12-hr:12-hr L/D cycle); these times are indicated above each lane. The open box below the ZTs indicates when lights were on; the darkened box indicates lights off in A–D; the top arrow indicates where PER runs; the bottom arrow indicates cross-reacting band (owing to its absence from *per*-null controls; see below) in A, C, and E. (B) Quantification and averaging of three or four per^{T} timecourses (hence, *n* is variable among timepoints) and four, five, or six wild-type timecourses for other genotypes (see materials and methods). (C) Western blots of separated per^{SLIH} females and per^{SLIH} males that were entrained and killed at different ZTs; these times are indicated above each lane. (D) Quantification and averaging of two per^{SLIH} experiments. (E) Western blot of wild-type, per^{T}, and per^{SLIH} males and females at their respective high timepoints, allowing side-by-side comparison of PER levels. per^{04} provided the

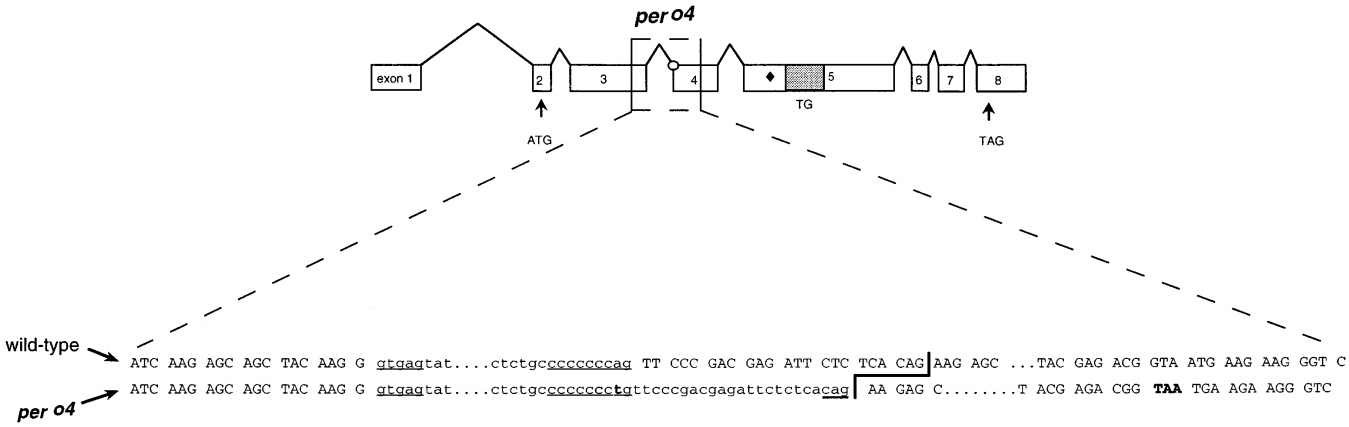

Figure 5.—How the *per*^{04} mutation affects *per* transcript splicing and coding potential. The top figure is the genomic structure of *per* showing the location and nature of the *per*^{04} mutation. Dotted lines circumscribe a region of sequence detail. The sequence at the top is from wild type, and the bottom sequence is from *per*^{04}. Uppercase letters designate coding DNA, and lowercase letters are intron sequences. Underlined small letters are splice site regions. The boldface letters indicate how the *per*^{04} mutation leads to an early translation stop site. The diamond in exon 5 indicates a second novel splice site that was detected in a separate experiment (see text).

involved in intramolecular interactions (Huang *et al.* 1995; however, see the critique by Kay and Millar 1995). The *per*^{Clk} mutant has an amino-acid change (Figure 1) downstream of the Thr-Gly repeat region (Figure 1A) and >300 residues away from the so-called "short region" of Baylies *et al.* (1992); clearly, another distinct region of the polypeptide can give the mild shortening that was associated with many of these *in vitro*-created mutants. More generally, no *in vivo per* mutants or *in vitro* ones with appreciable rhythm defects have previously been localized to the *per*^{Clk}-identified part of PER. The amino-acid substitution in the *per*^{SLIH} mutant (Figure 1) is within the N-terminal, most conserved region (known as C1; see above); this is upstream of PER's putative nuclear localization signal (*cf.* Saez and Young 1996). C1 is perhaps featureless, but it is one of several possible glycogen synthase kinase substrates within PER ($n = 39$ such sites; our MacVector subsequence search).

The locations of two of these mutations, *per*^{Clk} and *per*^{SLIH}, are within regions that have never been mutated before. Both are outside the PER/TIM interaction sites (*cf.* Saez and Young 1996) and are not within any other PER domains known (whether or not associated with *per* mutants). Thus, *per*^{Clk} and *per*^{SLIH} may prove to define functionally important regions within the polypeptide. A corollary to the intragenic uniqueness of these two mutants is that (more generally) the period-altering *per* mutations have occurred over most of the length of the protein; they are not restricted to regions to which attention has been drawn previously, often by having been selected for *in vitro* mutagenesis (Baylies *et al.* 1987, 1992; Yu *et al.* 1987b; Rutila *et al.* 1992; Curtin *et al.* 1995).

per^{04} is a different kettle of fish in this study, being essentially arrhythmic (however, see below). Yet the *per*^{04} phenotypes become comprehensible in the context of the splice junction mutation carried in this mutant. There are previous examples of such mutations in Drosophila (Lee *et al.* 1987; Collier *et al.* 1990; Lichtinghagen *et al.* 1990), as well as mouse (*e.g.*, Tumer *et al.* 1997; Yu *et al.* 1997; see below); and in humans, it is estimated that 15% of point mutations associated with diseased states result in splicing defects (Krawczak *et al.* 1992). The nonrarity of this kind of mutation is further exemplified by the recent case of a molecularly identified rhythm mutant in the mouse; the relevant splice site mutation leads to exon skipping in this case, not (on paper) to a protein null (King *et al.* 1997).

A polypeptide produced from the anomalously spliced RNA in the *per*^{04} mutant (inferred from a section of cDNA obtained by RT-PCR) would be frameshifted in a way that an intra-ORF nonsense codon would soon be encountered (Figure 5). No aspects of this analysis (including characterization of additional cDNA types) allowed us to infer the production of any normal or at least nearly full-length PER in this mutant. But additional kinds of analogous splicing events may occur in *per*^{04} (not detected), along with an aberrant aspect of

null control (more generally, the lower molecular weight bands seen in some of the lanes in this figure are interpreted as cross-reacting material because of their routine presence in extracts from *per*-null flies; with respect to the currently used antibody, see Figure 11 in Stanewsky *et al.* 1997). The relevant times are indicated above each lane (here in E, as in the other parts of the figure). PER peaks for the two rhythmic mutants analyzed in this manner (that is, excluding *per*^{04}) were measured (see materials and methods): PER^{T}'s range was 70–73% of wild type; the values for PER^{SLIH} females and PER^{SLIH} males were 60–74 and 51–73%, respectively.

post-transcriptional processing that would lead to a truncated transcript. The latter would be the ~3-kb RNA species that is produced in per^{04} with the normal 4.5-kb mRNA (Hamblen-Coyle *et al.* 1989). None of the current results, however, specifically explain the appearance of the truncated transcript in the relevant Northern blots. Nevertheless, it seems as if splicing problems, as implied by the sequencing (Figure 1) and detected accordingly (Figure 5), must be connected to the existence of a normal sized (but, as we now infer, intrinsically abnormal) *per* mRNA with a shorter than normal transcript. In this regard, a mutation within the *tottering* locus in mice results in two transcripts—one smaller and one larger than normal; both are predicted to produce abnormal truncated proteins (Fletcher *et al.* 1996).

The effects of per^{04} on behavior imply that the mutation is a severe hypomorph, not quite a null. Mutant adults exhibit near-normal locomotor behavior in L/D cycles, and in D/D, per^{04} is not a thoroughgoing arrhythmic mutant (Hamblen-Coyle *et al.* 1989). Putting these earlier results together with the current ones, we infer that certain splicing events caused by the nucleotide substitution in per^{04} result in PER molecules that are least quasi-functional. No molecular evidence was readily obtainable for such PER molecules, as if they are extremely rare in a per^{04} brain, including that they may not be made at all of the relevant brain cells (*cf.* Frisch *et al.* 1994). It follows that this mutant would seem to be a protein null in Western blottings (Figure 4A), which jibes with a previous immunohistochemical test of the same mutant (Siwicki *et al.* 1988). But the behavioral results provide more powerful evidence for per^{04}'s hypomorphy, as opposed to the seeming amorphy just discussed. A corollary is that a level of (final) product just above the amorphic floor is sufficient for nearly normal locomotor activity in L/D conditions, given the daily environmental boost that would therefore appear to be important for promoting such behavior, whereas the nearly amorphic molecular property is woefully inadequate for free-running rhythmicity.

Newly revealed rhythm-defective phenotypes: Inasmuch as per^{SLIH} is a novel long-period mutant biologically (irrespective of its intriguing molecular etiology, see above), its phenotypes bear comment. Although per^{SLIH}'s free-running periods are at least 3 hr longer than normal, in L/D cycles, mutant individuals entrain to the 24-hr Zeitgeber (Table 1; Figure 2). Indeed, the 3-hr daily advance implied is well within Drosophila's limits of entrainment (Hamblen-Coyle *et al.* 1992; Wheeler *et al.* 1993). There was a slight difference between males and females in relation to per^{SLIH}'s penetrance of rhythmicity (Table 1); mutant males were less likely to give an arrhythmic behavioral result than females. This may correlate with elements of the PER expression phenotype in this mutant (see below). per^{SLIH} exhibits a temperature compensation defect different from that of its tau cousin, per^{L}: per^{SLIH}'s clock runs faster at higher temperatures and slower at lower ones. Whereas per^{SLIH} is semidominant—for period lengthening in a standard test involving per^{+}—its temperature compensation abnormality was dominant or recessive, as the case may be, in tests with other *per* alleles (Table 2). It is not possible molecularly to rationalize any of these per^{SLIH}-including genotypes in terms of the corresponding flies' anomalous responses to temperature changes. Suffice it to say that four separate intrapolypeptide regions of PER are implicated in temperature compensation of circadian rhythms (Ewer *et al.* 1990; Huang *et al.* 1995; Sawyer *et al.* 1997) and implicitly Konopka *et al.* (1994), when considered in conjunction with per^{T}'s mutated site (see the current Figure 1). As this increasingly complicated story unfolds the extreme N-terminal region mutated in per^{SLIH} will have to be taken in account.

Western blotting of per^{SLIH} head extracts showed that PER levels cycle with a peak amplitude at ZT22, whereas wild-type PER peaked at ZT18 (Figure 4). The later protein peak for this mutant corresponds to its later activity maximum in L/D behavior. Males and females differed biochemically and behaviorally: the mutant female protein rose earlier than that of males so that the former followed the rising phase of the wild-type protein. This resulted in a broader PER peak in females; the trough of PER in per^{SLIH} females occurred at the normal time but was broader and later in males (Figure 4). These biochemical results do not *per se* explain the sexually dimorphic behavioral phenotypes. The overall increased levels of PER in mutant females (compared to males and wild type) may, however, account for their increased arrhythmicity by these analogies. In transgenic flies carrying a *heat shock promoter/per* fusion, PER is expressed in many more cells than in wild type, and the flies exhibit a relatively low proportion of rhythmic individuals, as well as long-period behavior (Ewer *et al.* 1990). Also, per^{L} yields a PER timecourse in which it is difficult to pinpoint an accurate (peak) phase (Zerr *et al.* 1990; Rutila *et al.* 1996), although one study yielded a PER^{L} peak at ZT24 (Huang *et al.* 1995). A possible corollary to the biochemical results is that per^{L} is weakly rhythmic, including mediocre penetrance of periodic behavior (Hamblen *et al.* 1986; Dowse and Ringo 1987).

A further feature of the current Western blotting results may correlate with a mutant's behavior. The PER in per^{T} reached peak levels at ZT12 and stayed high until ZT18—again, more of a plateau than a peak. A similar kind of broad temporal maximum (for PER) was reported for the short-period per^{S} mutant (Marrus *et al.* 1996). Compare these biochemical results to the timecourses defined by the flies' rhythmic behavior in the same conditions (L/D): Whereas the width at half height of wild-type Drosophila's evening peak of locomotor activity is 2 hr (Hamblen-Coyle *et al.* 1992), the corresponding values for per^{S} and per^{T} are appreciably wider: 4 hr (Hamblen-Coyle *et al.* 1992; Konopka *et*

al. 1994). per^L exhibits a broad middle-of-the-night peak of activity, with a half-height width of 6–8 hr (Hamblen-Coyle *et al.* 1992). The value for per^{SLIH} males is 2.5 hr (Figure 2, A and B), just slightly wider than wild type, but the female peak is harder to define (see above).

A final point about mutant proteins (from the current data): The amount of PER in the per^T and per^{SLIH} Westerns at their respective peaks (or plateaus) was comparable to wild type levels (~70% of normal levels for these two mutants). This does not conform to the gene dosage analysis by Coté and Brody (1986), which can be taken to predict that per^{SLIH} would express one-fifth of the wild-type amount, and that per^T would express >200 times more than wildtype. This does not mean that the authors who were just cited were mistaken; instead, it means that the PER^T and PER^{SLIH} proteins are intrinsically defective [a point made previously and more generally by Rutila *et al.* (1992)].

Conclusion: We have mapped and characterized four *per* mutants, three that have been previously reported and one that is novel. Each carries a point mutation: three are within PER's ORF, and one carries a splice-acceptor site alteration. Two of these mutants are located within regions of the corresponding protein to which we believe attention should be newly paid. We further believe that these findings will add clues in terms of the eventual connections that should be made between clock factor genotypes and various features of the fly's rhythm phenotypes. Such connections should ultimately contribute to our understanding of how the action of *per* participates in setting the pace of the organism's clocks—whether they are circadian under the influence of certain *per* genotypes or temporally beyond the pale, as in several of the mutant types.

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