Molecular mapping of point mutations in the period gene that stop or speed up biological clocks in *Drosophila melanogaster*

(circadian rhythms/arrhythmic mutant/short-period mutant/P-element transformation/DNA sequencing)

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ABSTRACT The *per^{ol}* and the *per^s* mutations in *Drosophila melanogaster*, which seem to eliminate or speed up, respectively, the clocks underlying biological rhythmicity, were mapped to single nucleotides. Chimeric DNA fragments consisting of well-defined wild-type plus mutant DNA subsegments were constructed, introduced into flies by germ-line transformation, and assayed for biological activity. These experiments localized both *per^{ol}* and *per^s* to a 1.7-kilobase DNA fragment that is mostly coding DNA. Sequencing of this subsegment from each mutant showed that *per^{ol}* is completely accounted for by a nonsense mutation in the third coding exon of a 4.5-kilobase RNA transcribed from this locus. The *per^s* mutation is also a single nucleotide substitution, in the fourth coding exon, which results in a serine-to-asparagine substitution in the *per* gene protein product. The functional significance of these changes is discussed with reference to the phenotypes of the two mutations.

The period gene of *Drosophila melanogaster* is defined by some of the most interesting behavioral mutations isolated to date. These mutations affect biological rhythms either by eliminating periodic eclosion or behavior or by causing dramatic changes in rhythmic phenotypes (for review see refs. 1 and 2). The original *per* mutations were induced by Konopka and Benzer (3). Two of the mutants were *per^s* and *per^L*, isolated with respect to circadian eclosion rhythms whose periods are about 5 hr shorter or longer, respectively, than the normal 24 hr. The *per^o* mutant was also recovered (3), based on aperiodic eclosion. This mutation is now called *per^{ol}*, because there are currently four independently isolated “arrhythmic” mutations at this locus (refs. 4 and 5; R. J. Konopka and M.H., unpublished results).

Detailed genetic and phenotypic analyses of these “clock” mutants suggested that *per^{ol}* may be a null mutation, behaving like a deletion of this apparently nonvital locus within segment 3B1-2 of the X chromosome (6, 7). Manipulations of the dosage of the wild-type allele and the *per^s* allele suggested that the increased *per* activity seemingly associated with *per^s* is caused by a qualitative change in the activity of the gene product (4, 8).

A 6-kilobase (kb) subset of the *per* region (9, 10) appears to be necessary for *per⁺* activity—i.e., rescues *per^o*-induced arrhythmicity of (i) circadian locomotor activity (5, 11, 12), (ii) eclosion (12), and (iii) variations in the male courtship “singing” behavior (5, 12). This portion of the *per* region corresponds to a 4.5-kb RNA species (9, 10), allowing one to focus on its informational content (13–15).

The results we present here include a determination of the precise sites of the mutations in the original arrhythmic and short-period variants. These experiments were undertaken to

address some of the issues raised above and the role of the gene in the development (e.g., refs. 16 and 17) and the function (e.g., refs. 15 and 18) of biological clocks in *Drosophila*. Thus, we set out to determine if *per^{ol}* is in fact a null mutant and to identify the nature of the *per^s* mutation. A combination of molecular and behavioral analyses shows convincingly that *per^{ol}* is a stop codon and that *per^s* is a missense mutation.

MATERIALS AND METHODS

Cloning of *per^{ol}* DNA. *per^{ol}* fly DNA was partially digested with *Sau3A* and ligated into *Bam*HI-digested EMBL4 (19) phage arms. Then, 2×10^5 plaques were screened with a 32 P-labeled 8-kb *Eco*RI–*Eco*RI *per* region fragment (5, 11). Three positive clones were isolated, and their identity was verified by restriction mapping. Since no phage insert included the entire 8 kb, a mutant 8-kb fragment was constructed from DNA excised from two such clones. The synthesized fragment was then cloned into the vector pEMBL18.2, constructed by inserting an *Xho*I linker into the polylinker of pEMBL18+ in place of all of the restriction sites between (but not including) the *Eco*RI and *Xba*I sites [pEMBL18+ is pEMBL8+ (20) with its polylinker replaced by that of pUC18 (21)].

Cloning of *per^s* DNA. Genomic DNA from 40 *per^s* flies was digested to completion with *Eco*RI and *Bam*HI. The 4.5- and 3.5-kb fragments (corresponding to *per* locus material) were isolated from a 0.9% low-melting agarose gel and ligated separately into pUC9 (22) that had been digested with *Eco*RI and *Bam*HI. The ligations were then transformed into bacterial strain JM83. About 500 colonies from each transformation were screened by colony hybridization with the 8-kb probe from *per⁺* (see above), labeled with 32 P. Two positive colonies, one for the 4.5-kb and the other for the 3.5-kb segment, were identified and verified by restriction mapping. The two *Eco*RI–*Bam*HI DNA fragments, which sum up to the 8-kb *Eco*RI–*Eco*RI *per⁺* fragment described above, were then ligated together and cloned into the pEMBL18.2 plasmid.

DNA Sequencing. Sequencing was, in general, performed as described (15). The data in Fig. 5 were generated by separately subcloning a 1.7-kb *Sal*I–*Sst*I fragment from *per^{ol}* and *per^s* into pEMBL18+ and pEMBL19+ (20, 21); in addition, a 0.71-kb *Pst*I–*Sst*I fragment (the downstream 40% of the region just noted) was cloned from each mutant into pEMBL18+. The ends of all these subclones were sequenced using standard M13 primers. The more centrally located regions were sequenced using a series of synthetic oligonucleotide primers (from the DNA Synthesis Facility at the University of Massachusetts Medical School), homologous to seven sites along this 1.7 kb of *per* DNA.

FIG. 1. Structure and expression of the period gene. (Top) A restriction map of 13 kb of DNA cloned from the *per* locus (11) is shown. The thick part of the map designates the genomic material analyzed (29), from which the amino acid sequence (Fig. 2) was derived. Abbreviations for the restriction sites are as follows: B, *Bam*HI; R, *Eco*RI; H, *Hind*III; X, *Xba* I; SII, *Sst* II; SI, *Sst* I. The double lines below R and SII indicate a pair of closely spaced *Eco*RI and *Sst* II restriction sites, respectively. (Middle) Two subsets of this region, which give rise to a 4.5- and a 0.9-kb RNA, are depicted. (Bottom) Detailed view of the precursor for the 4.5-kb transcript is shown. The exons are numbered 1-8 in the 5' → 3' direction; the arrows pointing to exons 2 and 8 indicate the positions of the translation initiating and terminating codons, respectively; the corners above exons 3 and 5 frame the portion of this gene (*Sal* I-*Sst* I fragment) for which DNA from the *per^{ol}* and *per^s* mutants was sequenced (Fig. 5); this fragment is almost identical to the *Sst* II-*Sst* I fragment used in Fig. 3. The scale bar for 1 kb refers to only the Bottom of the figure.

Construction of Plasmids Carrying Chimeric *per* DNA. All of the plasmids were constructed by three-way and four-way ligations. *per* DNA derived from the mutants (this report) or from Oregon-R wild-type flies (10) was digested with *Xho* I, *Xba* I, *Sst* I, and *Sst* II; the relevant DNA fragments were isolated from 0.9% low-melting agarose gels. The P-element sequence-containing plasmid vector cp20.1 (e.g., ref. 23) was double digested with *Sal* I and *Xba* I. Separate *per* DNA fragments (e.g., one purified from gels-containing *per*^{ol} DNA

and the other from gels containing *per*⁺ DNA, see Fig. 3B; or one from *per*^s and two from *per*⁺, see Fig. 3H) were then ligated into cp20.1. HB101 cells were used for bacterial transformation. Correct plasmid constructions were selected on the basis of size and verified by restriction mapping.

Generation and Analysis of Transformants. Germ-line transformants were produced by methods reported previously (5; see ref. 24 for general strategies). The source of embryos for microinjection was a *per^{ol}*; *ry⁵⁰⁶* stock. After establishment of the various transformed lines individual males expressing the vector-tagging marker (*ry*⁺, cf. ref. 24) were monitored for general locomotor activity as in ref. 5.

RESULTS

Organization of the Period Gene. Biologically active *per* DNA has been localized to a region of cloned DNA from 3B1-2 on the X chromosome (5, 11, 12). These transformation experiments, in conjunction with transcript mapping, indicated that the 4.5-kb RNA from this region, rather than the neighboring 0.9-kb RNA [the level of which cycles in a circadian manner (10)], is the biologically active *per* transcript. A restriction map of this region as well as the location of these two transcripts is shown in Fig. 1. The distal end of this map is a *Bam*HI restriction site; it defines a border for the DNA most distal to the X chromosome centromere that has been used in our transformation experiments (5, 11). This *Bam*HI site is upstream of the transcription start site of the 4.5-kb RNA (Fig. 1). The 3' border is an *Eco*RI site that lies to the right of the flanking 0.9-kb transcript and is the most proximal (downstream) DNA we have used. Based on RNA gel blots (10), the 4.5-kb transcript appeared to be contained largely within the borders of a 7.2-kb *Hind*III-*Hind*III fragment (Fig. 1), although it is now known that the 3' end of the transcript extends beyond the right-hand *Hind*III site, as indicated in Fig. 1 (cf. ref. 14).

Most of this 13.2 kb of *per* region information has been subjected to DNA sequence analysis in our laboratories. Also, the entire 4.5-kb transcript region has been covered by cDNA clones. Putative splice junctions, suggested by RNase mapping, were defined by sequencing of cDNA clones across such junctions (29). The data indicate that the seven translated exons (exons 2–8) code for a 1218-amino acid protein

FIG. 2. The predicted *per* gene protein sequence from Oregon-R flies. The *per* gene protein sequence of 1218 amino acids, deduced from the DNA sequence of genomic and cDNA clones (29), is presented. Exon-exon joining sites are indicated by arrowheads above the sequence, and exons are numbered beside the arrowheads. An arrowhead directly above an amino acid indicates that its codon is divided by an intron; an arrowhead between two amino acids indicates that the intron lies between two codons. The positions of the *per*^{ol} mutation (residue 464) and the *per*^s mutation (residue 589) are indicated (*). The complete Gly-Thr amino acid repeat (40 amino acids) is underlined. The length of the Gly-Thr repeat reported here (for Oregon-R flies) is 6 amino acids (3 pairs) shorter than that from Canton-S flies (14). This is a bona fide strain polymorphism.

(Fig. 2). The predicted molecular weight of the protein is 127,000, in the absence of any post-translational modification (cf. ref. 15). The amino acid sequence presented here agrees largely with that published by Jackson *et al.* (14), but our exon 2 is missing in their report. The presence of this additional exon adds 62 amino acids to the N terminus of the predicted protein. Our exon 5 is a long contiguous coding exon, but is interrupted by an intron of 102 nucleotides in the description by Jackson *et al.* (14). The absence of this intron adds another 35 amino acids to the predicted protein sequence. These 35 amino acids (Fig. 2, amino acids 727–761) are inserted between amino acids 670 and 671 in the sequence presented by Jackson *et al.* (14).

Mapping of *per* Mutations in Transformants. Genetic studies carried out by Smith and Konopka (7) have suggested that *per^{ol}* is a null allele. Cytogenetic analysis of the *per^s* allele (4) suggested that it leads in some way to hyperactivity of the gene or its product. Molecular studies (5, 9, 10) have revealed no gross changes at the DNA level or in the abundance of the 4.5-kb RNA in *per^{ol}* or *per^s* flies. Therefore, we considered it probable that these mutations affect biological activity at the protein level. We set out to verify this prediction and attempted to localize these two mutations within the coding region of the 4.5-kb RNA by using an 8-kb genomic DNA fragment from the *per* region in transformation experiments.

This *EcoRI*–*EcoRI* fragment, when cloned from wild-type flies, can readily confer rhythmicity on *per^o*-containing transformants (5, 12). It is effective for somewhat mysterious reasons—since this segment is missing half of the large first intron (Fig. 2), the first exon (which is noncoding, see above), and all of the putative regulatory material is located upstream of the transcription start site. It is not known how transcription of the *per* coding information is promoted from the 8-kb fragment (or, more likely from a given insert region containing it); but it is, as determined by the behavioral bioassays and by RNA gel blots (5).

To test whether the original arrhythmic mutation is indeed located within the 8-kb subsegment of the *per* gene, this material was cloned from *per^{ol}* DNA, inserted into *P*-element vector cp20.1 (23), and transduced into *per^{ol}*, arrhythmic flies. We found that all the flies carrying this mutant fragment were arrhythmic (Fig. 3D), whereas transformants carrying the corresponding *per* locus information cloned from wild-type flies were frequently, but not always, rhythmic (cf. refs. 5 and 12) and exhibited circadian periods slightly longer than the normal range (Fig. 3C). To narrow the localization of *per^{ol}*, two chimeric genes were made using DNA from *per⁺* ligated to that of *per^{ol}*. When the biological activities of such segments were tested in transformants, the results showed that they could be separated into two distinct behavioral categories. Those containing the 1.7-kb *Sst* II–*Sst* I restriction fragment from wild-type DNA gave about 24.5-hr periods (when rhythmic, Fig. 3A). When the portion of the gene on the 5' side of the *Sst* I site was from *per^{ol}*, all individuals were arrhythmic (Fig. 3B).

Transformation mapping of *per^s* by a similar strategy led to similar kinds of clear distinctions between behaviors associated with *per⁺* or *per^s* fragments. Examples of the behavioral data and analyses thereof are shown in Fig. 4. When the *Sst* II–*Sst* I-defined region was from *per^s*, the periods for rhythmic flies were 19–20 hr (Fig. 3F and H). If this same subsegment of *per^s* was included in the portion of the chimeric gene derived from wild-type DNA, the periods were again “normal” (Fig. 3E and G), like those of control transformants (Fig. 3C). As in the *per^{ol}* mapping experiment, substantially fewer than 100% of the individuals from the short/normal chimeric transformant types were rhythmic (Fig. 3). However, this impenetrance did not interfere with measurements of the periods of rhythmic individuals. It should be noted that the penetrance of rhythmicity in our tests of *per^s* (i.e., straightfor-

Fig. 3. Germ-line transformants involving mutant *per* DNA and analysis of circadian behavioral rhythms. Transformants containing 8-kb fragments cloned from the *per* locus were generated and analyzed. The sources of *per*-derived information, transduced into the genome of these strains, are depicted schematically by the horizontal bars. +, DNA from wild-type flies; 0, DNA from *per^{ol}* flies; S, DNA from *per^s* flies. For each of the newly created transformant types (types A, B, D–H), several independently isolated strains, all of which carry autosomal inserts, were recovered. For the new transformant types that in general exhibited rhythmicity (types A, E–H), the results from individual strains that included at least one rhythmic individual are pooled and summarized here. Type A: 13 strains, 5 of which included at least one rhythmic fly. Type B: 6 strains, 0 rhythmic. Type C: summary of data and periodogram analyses (refs. 5 and 11 and Fig. 4), augmented by a few more tests of behavior expressed by five independently isolated strains. These carry either the 8-kb fragment or a 7.2-kb subset of it (5), different from the former only with respect to 800 base pairs of intronic material (see vertical line near left-hand end of horizontal bar in type C). Such control transformants of the 8-kb type involved a *P*-element vector (11) different from that used in the present study and in the 7.2-kb types. As the behavioral data from these several separate strains were essentially homogeneous, all the individual rhythm periods were pooled. Type D: 3 strains, 0 rhythmic. Type E: 12 strains, 9 rhythmic. Type F: 15 strains, 9 rhythmic. Type G: 10 strains, 6 rhythmic. Type H: 15 strains, 7 rhythmic.

wardly mutant adults) was very high (94%) and that the mean period was 19.4 ± 0.1 hr ($n = 33$).

Determination of *per* Mutated Sites. Both the *per^{ol}* and *per^s* mutations map to the same *Sst* II–*Sst* I fragment, which is a 1.7-kb subset of this gene. The two mutant segments (actually a region slightly longer than that defined by the *Sst* II–*Sst* I fragment, see Fig. 1) were sequenced and compared to each other and to the corresponding wild-type information. Only one single-base change was found in each mutated DNA sequence. A portion of the sequencing gels, showing the two mutant sites, is in Fig. 5. The *per^{ol}* mutation site is a cytosine to thymine change and that for *per^s* is a guanine to adenine change. According to the predicted protein sequence (Fig. 2), the *per^{ol}* mutation changes a glutamine codon (CAG) to a translational stop codon (TAG) at position 464 in exon 4 (as shown in Fig. 2); this results in a truncated protein containing 463 amino acids. It would not be surprising if such a polypeptide has no *per⁺*-like function. Indeed, this interpretation is in agreement with genetic predictions (e.g., ref. 7), and the fact that a transformant carrying a DNA fragment whose 3' end is well downstream of the *per^{ol}* position is

FIG. 4. Circadian rhythms of transformants. The actograms depicted (for one fly in A, two in B) are activity/rest cycles of adults monitored for their locomotor activity, as in ref. 5. The actograms are "double plotted" (days 1 and 2 on the first horizontal line, days 2 and 3 on the second, etc.). The results of χ^2 periodogram analyses (cf. ref. 5) for the two genotypes here are also plotted in A and B; the several plots for a genotype are superimposed. Values above the sloping line refer to periodicities that are significant at the 5% level. (A) Near-normal-period transformants—i.e., type E in Fig. 3; note that, in the actogram, the ends of the "main active phases" (regions of densest markings, displayed horizontally) are at slightly later times on successive days, and the periodogram peaks are dispersed in the vicinity of 25 hr. (B) Short-period transformants—i.e., type F in Fig. 3; for these actograms, the ends of the active phases are noticeably earlier on successive days, and the periodogram peaks range from 18 to 21 hr.

completely arrhythmic (5, 11). The *per^s* mutation is a serine-to-asparagine missense mutation (AGC to AAC) at position 589 (Fig. 2). The positions of these two mutated sites (Fig. 2) support the hypothesis that the 4.5-kb transcript (Fig. 1) is the *per* mRNA.

DISCUSSION

The amino acid sequence of the period gene product of wild-type *D. melanogaster* (Fig. 2) forms a basis for begin-

ning to understand how mutations at this locus affect behavioral rhythms and, therefore, how the fly builds and "runs" its biological clocks. Other than the homology to proteoglycans (14, 15), analysis of the period gene protein sequence has yielded few insights as to its structure or function. No signal sequence is visible at the 5' end, and only one region (within exon 3) is a reasonable candidate for a transmembrane segment. The absence of well-defined membrane-seeking or membrane-spanning domains in the predicted protein sequence is not without precedent for proteoglycans (25). Presumably, the high degree of modification (i.e., glycosylation) predicted for this protein (cf. refs. 14 and 15) is related in some way to the absence of diagnostic secondary sequence features. The N-terminal region is particularly serine-rich. The protein contains a high proportion of glycine (12.0%), serine (12.7%), threonine (9.0%), and alanine (7.6%) residues, consistent with the proposals that the protein is a proteoglycan (ref. 26 and see discussion in refs. 14 and 15).

To minimize potential problems that would be created by finding more than one difference between a mutant vs. wild-type sequence, we first defined a subset of the 8.0-kb *per* DNA by transformation bioassays that contains these chemically induced changes. Since both *per* mutants studied here were derived contemporaneously from mutagenesis of a Canto-S stock (3), it was likely that they would be isogenic with each other (except at the sites of the mutations), which was the case. It was not, however, predictable that all the rest of this 1.7-kb region from the mutants would match the corresponding information contained in Canton-S (14) and Oregon-R (29) *per⁺* DNA perfectly. Since the rest of the

FIG. 5. DNA sequencing gels of DNA fragments cloned from the *per^{ol}* and *per^s* mutants. The *Sal*I-*Sst*I fragment of the *per* gene (see Fig. 1) was sequenced using a series of complementary primers. In addition, a *Pst*I-*Sst*I fragment (from a *Pst*I site, at about the position of the *per^o* mutation, to the *Sst*I site) was subcloned so as to allow sequencing from the *Pst*I site. The bases are as indicated at the bottom; 5' → 3' direction is from bottom to top. Lanes are labeled at the top as to whether the DNA was from *per^s* (S) or *per^{ol}* (O). The arrows indicate the positions at which sequence differences are apparent between *per^{ol}* and *per^s*.

sequences were identical, these two wild-type strains exhibit no polymorphic differences in this part of the gene.

From sequencing the relevant subsets of *per* DNA from the two mutant strains, we conclude that both mutations are single base-pair substitutions. The former mutation is a stop codon at amino acid 464 (Figs. 1 and 5). This arrhythmic mutation creates a new *Xba* I restriction site at this position; this site is present in genomic DNA from *per^{ol}* flies but not in that from any rhythmic strain we have examined (data not shown). It is also present, somewhat unexpectedly, in genomic DNA from two other *per* mutant strains, *per^{o2}* and *per^{o3}* (data not shown, cf. refs. 4 and 5).

The behavioral phenotypes associated with *per^{ol}* are not identical to those induced by a homozygous *per⁻* deletion of the locus (18). Also, the latter leads to anomalously hyperactive behavior (5). Perhaps the *per^{ol}* nonsense fragment retains some weak or aberrant biological activity.

The *per^s* variant is a serine-to-asparagine missense mutation at amino acid 589 (Figs. 1 and 5). To the best of our knowledge, this is the first time that a behavioral mutation has been identified at the amino acid sequence level, although we note that this mutation and *per^{ol}* have been independently localized in another laboratory (M. W. Young, personal communication). We, however, cannot more definitively interpret the biochemical change that accounts for the *per^s* phenotype, as it could be due to the absence of the wild-type serine or the presence of the mutant asparagine. At present, we favor the former possibility; the serine may be normally modified, and the absence of this modification in the *per^s* protein may cause the clock to "run too fast." Although many of the abundant serines in the *per* protein are probably glycosylated (cf. ref. 15), there is an arginine located two amino acids upstream of this serine. Such a configuration resembles a diagnostic feature of at least two protein kinase target sites (27, 28). As appropriate upstream arginines are associated with relatively few of the serines encoded within *per* (Fig. 2 and ref. 14), we suggest that the serine at position 589 could be a phosphorylation site that is an especially important component of normal gene function.

Although the data establish quite firmly that the sequenced region codes for the *per* gene product, they do not define the nature of the biologically active *per* protein. For example, the indicated primary translation product may be subjected to proteolytic cleavage, and only a portion of the protein sequence may contribute to rhythmic behaviors. Also, differential splicing, now firmly established for this gene (29), further complicates the identification of the protein or proteins that influence a given rhythm affected by *per*. In any case, the localization of the *per^s* mutation indicates that this region of protein sequence is probably important for all of its rhythm-related activities, inasmuch as the minor *per* gene transcripts identified to date differ from the major one (Fig. 2) only in regions downstream of the *per^s* mutated site (29). Thus, the *per^s* serine-to-asparagine change would be present in several if not all of the *per* gene products.

Experiments similar to those reported here should be applicable to the other extant *per* alleles, the most interesting of which might be the two *per^L* variants (1, 3). Also, we anticipate that *in vitro* mutagenesis experiments will provide further insight into the importance of the region of this gene defined by *per^s*. Further analyses of this kind may identify

additional subsets of the locus that are important elements of its contributions to biological clocks of *Drosophila*.

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