

- 1.2 mM NaH₂PO₄, 10 mM glucose, 20 mM Hepes and 0.5% fatty acid-free BSA, pH 7.4) and were permeabilized for 6 min by addition of 20 μM digitonin in potassium glutamate (139 mM), ATP (2 mM), MgCl₂ (2 mM) EGTA (5 mM), 10.5% fatty acid-free BSA, Pipes (20 mM), pH 6.5 in the presence or absence of the analogs GTP(γ)S (100 μM) or GDP(β)S (750 mM). The cells were then washed twice in HBSS buffer (pH 7.4) containing 0.2% BSA and incubated with or without calcitonin for 30 min before measuring ouabain binding as described (13).
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Molecular Transfer of a Species-Specific Behavior from *Drosophila simulans* to *Drosophila melanogaster*

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***Drosophila* males modulate the interpulse intervals produced during their courtship songs. These song cycles, which are altered by mutations in the clock gene *period*, exhibit a species-specific variation that facilitates mating. We have used chimeric *period* gene constructs from *Drosophila melanogaster* and *Drosophila simulans* in germline transformation experiments to map the genetic control of their song rhythm difference to a small segment of the amino acid encoding information within this gene.**

THE MOLECULAR AND GENETIC analysis of species-specific behavior may enhance the understanding of the evolution of closely related species. Species-specific acoustic components of the courtship behavior of drosophilids provide an excellent model system for the study of molecular ethology.

The courtship song of *Drosophila* is generated by extension and vibration of the males' wings. The most prominent feature of these sounds in the *D. melanogaster* subgroup is intermittent bursts of tone pulses and hums (1–4). The pulses carry information that enhances the females' mating behavior (2, 3). Species-specific pulse information can be conveyed to the female in at least two ways. One way involves the interpulse interval (IPI), which averages about 30 to 40 ms in *D. melanogaster* and 45 to 55 ms in *D. simulans* (1, 4). The second way is the modulation of the mean IPI, which occurs with periods ranging from 50 to 65 s in *D.*

melanogaster and 30 to 40 s in *D. simulans* (3, 5).

The *period* gene (*per*) in *D. melanogaster* influences several temporally dependent phenotypes (6). The gene was first defined by the effects of *per* mutations on circadian rhythms. Mutant alleles shorten (*per^S*), lengthen (*per^{L1}*), or abolish (*per⁰¹*) the circadian rhythms of eclosion and locomotor activity (7). These mutations were shown to have parallel effects on the IPI modulation rhythm in the courtship song: *per^S* males sing with 35- to 45-s periods, *per^{L1}*, 75- to 95-s periods, and *per⁰¹* males appear to be arrhythmic (3, 5, 8–10).

Behavioral studies of *D. simulans*–*D. melanogaster* interspecies hybrids have mapped the species difference in song rhythm periodicity to the X chromosome (3), on which *per* is located (7). Furthermore, deletion of the repeated Thr-Gly-encoding region within *per* by in vitro mutagenesis (11, 12) results in a *D. simulans*-like shortening of the song rhythm in *D. melanogaster* transformants (13). These findings suggest circumstantially that *per* may be responsible for the species-specific components of the courtship song rhythms.

We tested the hypothesis that *per* contains species-specific song instructions by introducing a cloned copy of *D. simulans per* into the genome of a *D. melanogaster per⁰¹* mutant. DNA from the *per* locus of *D. simulans* (Kenscoff strain) was cloned (14) (see Fig.

1A), and a P-element construct was generated (15) that included a *D. simulans*-derived genomic fragment corresponding to the 13.2-kb *D. melanogaster per* DNA used in the restoration of rhythms to *D. melanogaster* flies carrying *per⁰¹* (12, 13, 16, 17).

Because the Thr-Gly repeat of *per* in *D. melanogaster* is implicated in control of song rhythmicity, and because this region of the gene is notable for its interspecific variability (18, 19), further transformation experiments focused on the vicinity of the Thr-Gly repeat in *D. simulans* and *D. melanogaster*. Thus, the transformations involved four different *per* constructs (15): the 13.2-kb fragment from *D. melanogaster* [13.2m-TGm (12, 13)], the corresponding *D. simulans* fragment (13.2s-TGs), the chimeric construct with the *D. melanogaster* Sst I–Bam HI fragment [carrying the Thr-Gly repeat (Fig. 1A)] substituted into *D. simulans per* (13.2s-TGm), and the reciprocal chimeric construct with the *D. simulans* Sst I–Bam HI material inserted within the flanking regions of *D. melanogaster per* (13.2m-TGs).

The biological activity of the transduced *per* constructs was confirmed by testing the locomotor-activity circadian rhythms of the recipient flies. The 13.2s-TGs *D. simulans per* and the two chimeric *per* genes efficiently rescued *per⁰¹*-associated arrhythmicity (20). Resultant circadian periods (about 24.5 to 25 hours) were comparable to those obtained by transduction of *per⁰¹* with the *D. melanogaster* 13.2m-TGm gene (12, 13, 16, 17). Therefore, the 13.2s-TGs fragment and the two chimeric constructs can provide basic *per* function. In this context we also noted that *D. simulans* wild-type adults showed activity rhythms indistinguishable from those of wild-type *D. melanogaster* (20).

Courtship songs were recorded and analyzed blind (21, 22) for males of each of the four transformant types. The mean song cycle of the 13.2m-TGm transformant males (Table 1, Experiment 1, and Fig. 2), gave the expected *D. melanogaster* periods of about 55 s (3, 5, 9, 10). A dramatic effect

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was observed in the *D. melanogaster* (*per*⁰¹) males carrying a transduced 13.2s-TGs (*D. simulans* *per*). The song cycles of these flies, measured by any of the three time series methods used (21), gave mean period lengths of approximately 35 s, characteristic of *D. simulans* (3, 5). [For further comparisons, the song cycles of some recently analyzed wild-type (nontransformed) strains of *D. melanogaster* and *D. simulans* are listed in Table 1, Experiment 2.] Males carrying the chimeric construct with the *D. simulans* Thr-Gly region (13.2m-TGs) gave song rhythms with about 35-s cycles, whereas the reciprocal chimerics (13.2s-TGm) had song periods in the 55- to 58-s range (Table 1, Experiment 1).

The 13.2s-TGs and 13.2m-TGs transformants produced a high proportion of songs with 20- to 30-s periods (Fig. 2). Computer-simulated random songs (9, 10) and songs recorded from *per*⁰¹ males (3, 5, 6, 8-10) also have a preponderance of spectral frequencies in the 20- to 30-s range (6, 9). However, the periodicities from the TGs transformants can be distinguished by analysis of their signal-to-noise (S/N) ratios (6). In a recent study involving the songs of 23 *per*⁰¹ males (10), only 4 gave significant S/N ratios. In contrast, of the 41 13.2s-TGs and 13.2m-TGs songs analyzed (Table 1), 29 gave significant S/N values.

The species-specific period of the courtship song cycle has therefore been mapped to the 700-bp Sst I-Bam HI fragment in the largest exon of *per* (Fig. 1A). The Thr-Gly encoding repeat divides this region into three segments: 60 amino acids upstream of the Thr-Gly repeat, the Thr-Gly motif, and the 122 amino acids downstream. The region upstream of the Thr-Gly repeat is entirely conserved at the nucleotide level (20) between the three *D. melanogaster* and the two *D. simulans* strains shown in Fig. 1B.

The Thr-Gly region itself is polymorphic in length, both within *D. melanogaster* (13) and *D. simulans*. Polymerase chain reaction (PCR) amplification of DNA sequences from seven *D. simulans* and ten *D. melanogaster* strains (Fig. 1C), with oligo primers flanking the Thr-Gly repeat, generated DNA products consistent in size with repeat lengths ranging from about 17 to 24 Thr-Gly pairs (Fig. 1C). The *D. melanogaster* amino acid sequence (Fig. 1B) varies between certain strains in increments of three Thr-Gly-pairs (18 bp) (13): Canton-S (23 pairs), Oregon-R (20 pairs), and Chieti-V (17 pairs). But intermediate Thr-Gly repeat lengths have also evolved in this species (compare, in Fig. 1C, lane 2 with lanes 7 to 9). Two of the *D. simulans* sequences are also illustrated (Fig. 1B), one from the

Kenscoff strain (24 Thr-Gly pairs) used in the transformation experiments, and one from the Australia strain (23 Thr-Gly pairs).

To investigate whether Thr-Gly repeat length variation affects the species-specific song cycle length, we examined the songs

from *D. melanogaster* strains carrying 17, 20, and 23 pairs of Thr-Glys and from three *D. simulans* strains (Australia, Kenscoff, and Georgetown), the *per* alleles of which encode either 23 or 24 Thr-Gly pairs. No significant differences were observed among

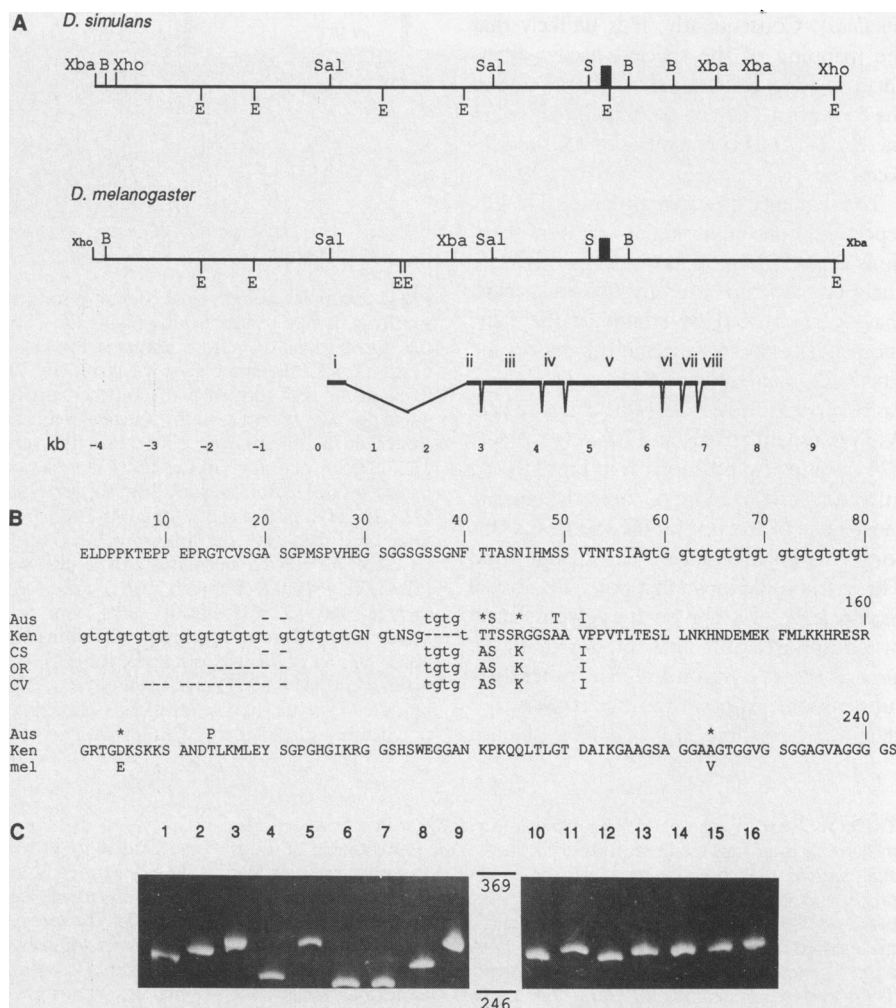


Fig 1. Comparison of *per* cloned from *D. simulans* and *D. melanogaster*. **(A)** The genomic restriction enzyme map of the portion of the *per* locus used to rescue the behavioral abnormalities associated with *D. melanogaster per*⁰¹ mutants. The restriction sites are B, Bam HI; E, Eco RI; S, Sst I; Xho, Xho I; Xba, Xba I; and Sal, Sal I. The black bar indicates the location of the Thr-Gly repeat. The genomic position of the *per* mRNA is indicated below the *D. melanogaster* sequence, with exons numbered in lower-case Roman numerals. Terminal Xho I and Xba I sites at the ends of the *melanogaster* sequence are cloning sites derived from a modified pUC18 vector developed for constructing the P-element vector used in the transformations involving this *per* DNA (12). The scale at the bottom is in kilobases; 0 is the beginning of a published *D. melanogaster* sequence (12). **(B)** Amino acid sequence encoded in the Sst I to Bam HI interval. The Thr-Gly repeat sequence is in lower case. [Aus, Australia; Ken, Kenscoff strains of *D. simulans*; CS, Canton-S; OR, Oregon-R; CV, Chieti-V strains of *D. melanogaster* (13)] (Deletions, -; species-specific amino acid changes, *). Amino acids 1 to 80 were identical in all five strains (amino acid 1 corresponds to position 637 of accession number A26427, PIR data base, National Biomedical Research Foundation). Positions 161 to 242 were identical among the *D. melanogaster* strains (mel is *D. melanogaster*). Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. **(C)** Size variation of DNA from *per* coding for the Thr-Gly repeat. PCR-amplified sequences from several strains of *D. melanogaster* and *D. simulans* were displayed on an ethidium bromide-stained 5% polyacrylamide gel. The primers were 5'-CCGCCAGTAACATACATGAGCAGTG-3' and 5'-GCAGGGATTCCGGTCAGCGTGACTGGCG-3'. Horizontal lines were 246- and 369-bp size markers. *Drosophila melanogaster* strains were: lane 1, Vietnam, and lanes 2 to 4, Raleigh, North Carolina (obtained from C. Aquadro); lane 5, Catonsville, Maryland, and lane 6, Hudson, Massachusetts (wild-trapped, D.A.W.); lane 7, Ithaca, New York; lane 8, Oregon-R, and lane 9, Canton-S (12). *Drosophila simulans* strains were: lane 10, Kenscoff, Haiti; lane 11, Georgetown, Guyana; lane 12, Leticia, Colombia; lane 13, Zamorano, Honduras; lane 14, Australia; lane 15, Lima, Peru; and lane 16, Nueva, California (National Drosophila Species Resource Center, Bowling Green, Ohio).

the *D. melanogaster* or among the *D. simulans* strains, irrespective of the time series analysis used to obtain the individual song periods (Table 1, Experiment 2). All six of these wild-type strains gave song cycles in the usual ranges for these species (50 to 65 s for *D. melanogaster* and 30 to 40 s for *D. simulans*). Consequently, it is unlikely that the mapping of the species-specific differences in song cycle could be simply due to the four extra Thr-Gly pairs encoded within the Sst I-Bam HI fragment of *D. simulans* Kenscoff.

The sequence downstream of the Thr-Gly repeat harbors eight amino acid substitutions (Fig. 1B) among the strains that were analyzed, of which four are species-specific, that is, are shared by strains of the same species. The species-specific differences are Thr in *D. simulans* versus Ala in *D. melanogaster* at position 121, Arg versus Lys at 125, Asp versus Glu at 165, and Ala versus Val at 224 (amino acid positions as in Fig. 1B). By inference, one or more of these changes are most likely to account for the species-specific song rhythm differences. The change from Thr to Ala could mean that posttranslational modification of a Thr, such as glycosylation or phosphorylation, may be absent in *D. melanogaster* at position 121. The other three substitutions appear to be conservative changes, but there are examples of such

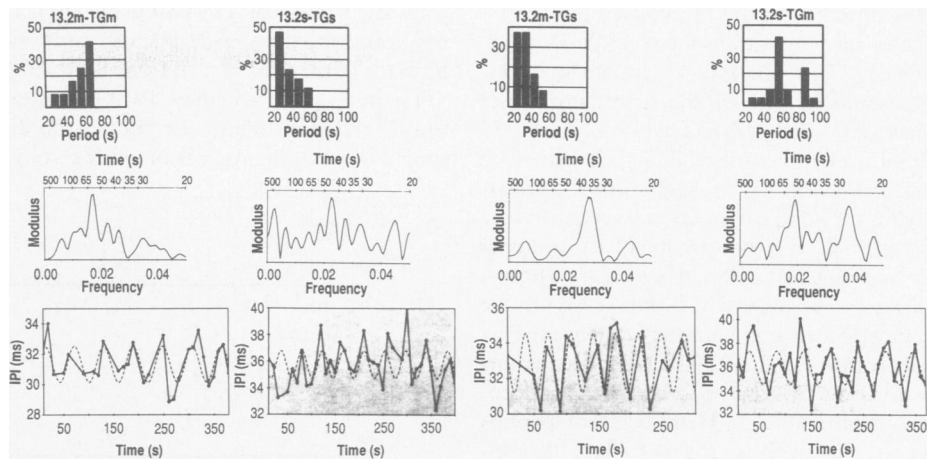


Fig 2. Examples of individual courtship song IPI rhythms of transformants and their corresponding spectrally derived results, from applying the CLEAN algorithm (21, 24). Top row, histograms showing the distributions of periods observed for each group of transformants (averaged in Table 1). The bottom row in the figure gives the mean IPI versus time plots (solid lines) and the best sinusoidal fits through the data (dotted lines), on the basis of nonlinear regression analysis (21). These sine-wave based periods are similar to the principal frequency components observed in the CLEAN spectrograms, shown in the middle row, above each IPI versus time plot (Table 1). (Middle row, from the left: 13.2m-TGm, period = 59.4 s; 13.2s-TGs, period = 44.7 s; 13.2m-TGs, period = 35.7 s; 13.2s-TGm, period = 51.9 s. Bottom row, from the left: 13.2m-TGm, period = 59.3 s; 13.2s-TGs, period = 43.5 s; 13.2m-TGs, period = 35.0 s; 13.2s-TGm, period = 52.4 s.) Analysis of variance (ANOVA) of these song cycle data gave the following F-ratios: 21.0 for the spectrally derived period estimates from CLEAN, 8.7 for the analogous vdB results, and 11.3 for the BMDP3R-derived curve-fitting results (df=3,75, $P < 0.001$ for each such F-ratio). A posteriori analysis of the means showed that males carrying the 13.2m-TGm or 13.2s-TGm constructs gave song rhythm periods that were not significantly different from each other but were each much longer than those obtained from the 13.2s-TGs and 13.2m-TGs transformants ($P < 0.001$); the mean periods from the latter two types of males were not different from each other. ANOVA involving only those songs with significant S/N ratios led to results almost identical to those from the analyses using all the data (Table 1), irrespective of whether we used the CLEAN-, vdB-, or BMDP3R-derived periods.

Table 1. Courtship song cycle of *per*-transformed and wild-type males. The mean cycle durations are in seconds \pm SEM. *N* is the total number of songs analyzed for a given genotype, *n+* is the number that gave significant S/N ratios, and *n-* is the number that did not (Fig. 2 legend). Mean periods based on *N* are given for the CLEAN, vdB, and BMDP3R analyses; a second mean based on *n+* is given. Experiment 1: *D. melanogaster* transformants in a *per*⁰¹ genetic background (15). The *per* construct types included: 13.2m-TGm (TG repeat length, 20 pairs), one of two transformed strains, the behavioral rhythmicity of which was studied (12, 13), though new courtship songs were recorded and analyzed for the present study; 13.2s-TGs (TG repeat length, 24 pairs), four strains; 13.2m-TGs, two strains; 13.2s-TGm, two strains. Within a 13.2-TG type, the interstrain song rhythm results were shown to be homogeneous by nested ANOVA. This also confirmed the significance of the intertype differences (Fig. 2). Experiment 2: Song cycle periods of three polymorphic *D. melanogaster* (*Dm*) strains (Chieti-V, Oxford,

and Canton-S), each carrying within their *per* gene 17, 20, or 23 contiguous Thr-Gly pairs (13), and three *D. simulans* (*Ds*) strains (Australia, Kenscoff, and Georgetown) carrying 23 or 24 Thr-Gly pairs (the bracketed repeat length for the Georgetown strain was estimated by PCR) (Fig. 1C). *D. melanogaster* strains with 17, 20, or 23 Thr-Gly pairs were derived, respectively, from Chieti-V, Oxford, and Canton-S wild-type stocks, made coisogenic except in the vicinity of the *per* locus by repeated backcrossing to a wild-type strain carrying the flanking markers *yellow* (*y*) and *white* (*w*). The final *y*⁺, *w*⁺ strains had retained the Thr-Gly repeat lengths of the starting stocks (R. Costa, personal communication). Because *D. simulans* males tend to sing less vigorously than *D. melanogaster*, a mean IPI for these songs was computed if there were ten or more individual IPIs in a 10-s time bin (Fig. 2). Within each species there were no interstrain song period differences by ANOVA, on the basis of the CLEAN-derived song period estimates.

Strain	Thr-Gly repeat length (no. of pairs)	Number of songs analyzed			Mean song periods \pm SEM(s)					
					CLEAN		vdB		BMDP3R	
		<i>N</i>	<i>n+</i>	<i>n-</i>	<i>N</i>	<i>n+</i>	<i>N</i>	<i>n+</i>	<i>N</i>	<i>n+</i>
<i>Experiment 1: Transformants</i>										
13.2m-TGm	20	12	10	2	53.8 \pm 4.0	58.6 \pm 2.6	52.1 \pm 4.8	56.5 \pm 4.4	53.9 \pm 4.0	58.7 \pm 2.5
13.2s-TGs	24	17	12	5	33.8 \pm 2.4	37.0 \pm 3.0	38.3 \pm 3.7	43.3 \pm 4.4	38.6 \pm 3.6	43.3 \pm 4.4
13.2m-TGs	24	24	17	7	35.0 \pm 2.0	34.7 \pm 2.3	35.6 \pm 2.3	34.6 \pm 2.4	35.7 \pm 2.1	35.1 \pm 2.2
13.2s-TGm	20	21	12	9	58.7 \pm 2.6	58.2 \pm 5.0	55.1 \pm 3.6	58.6 \pm 5.2	56.5 \pm 3.6	58.4 \pm 5.2
<i>Experiment 2: Wild types</i>										
Chieti-V (<i>Dm</i>)	17	13	9	2	49.8 \pm 5.3	52.7 \pm 3.7	51.3 \pm 5.1	53.2 \pm 4.7	51.0 \pm 5.1	52.7 \pm 4.6
Oxford (<i>Dm</i>)	20	13	6	7	51.0 \pm 3.7	54.5 \pm 2.6	53.5 \pm 4.3	54.6 \pm 2.4	58.1 \pm 3.4	54.5 \pm 2.4
Canton-S (<i>Dm</i>)	23	11	9	2	49.8 \pm 5.5	53.6 \pm 5.9	56.5 \pm 4.6	59.0 \pm 4.1	60.6 \pm 3.5	59.4 \pm 4.1
Australia (<i>Ds</i>)	23	5	5	0	39.4 \pm 2.9	39.4 \pm 2.9	34.9 \pm 2.5	34.9 \pm 2.5	35.3 \pm 2.0	35.2 \pm 2.0
Kenscoff (<i>Ds</i>)	24	5	3	2	36.2 \pm 3.0	33.8 \pm 2.6	36.1 \pm 2.9	33.9 \pm 2.6	36.1 \pm 2.9	33.9 \pm 4.6
Georgetown (<i>Ds</i>)	[23]	5	3	2	34.9 \pm 3.6	39.1 \pm 4.7	34.8 \pm 3.8	39.2 \pm 4.6	34.6 \pm 3.7	38.9 \pm 4.6

“mild” differences being functionally important (23).

This region downstream of the Thr-Gly repeat is variable between the species and among strains within species (Fig. 1B), in contrast to the conserved region upstream of the Thr-Gly encoding region. This pattern is also observed when the *per* sequence of another *D. melanogaster* “subgroup” relative, *D. yakuba*, is compared (19).

These experiments suggest that for *D. simulans* and *D. melanogaster* song cycles, perhaps four or fewer amino acid substitutions in the less conserved region of the *per* locus are responsible for the species-specific courtship behavior instructions encoded within this clock gene. Behavioral bioassays of in vitro mutagenized site changes within *per* of either or both species should clarify this situation.

Other transformation experiments implicate *per* in the control of the different circadian rhythm patterns of locomotor activity exhibited by *D. pseudoobscura* versus *D. melanogaster* adults (16). Further scrutiny of the evolutionary constraints on this gene, with respect to both courtship song and locomotor behavior, may eventually determine whether *per* plays a role in drosophilid speciation.

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14. *Drosophila simulans* DNA (Kenscoff strain) equivalent to the 13.2-kb segment of *D. melanogaster* (12–14) was cloned from an EMBL 4 phage library with a *D. melanogaster* 8.0-kb Eco RI probe. The homology of this clone to *D. melanogaster per* was verified by sequencing of the Sal I to Bam HI interval (D. A. Wheeler and A. Jacquier, unpublished data) (Fig. 1A); by detection on Northern blots of a 4.6-kb transcript, similar in size to the *per* transcript of *D. melanogaster* (11, 12); and by in situ hybridization of the 2.7-kb Sal I to Bam HI *D. simulans* (Kenscoff) subclone to the distal portion (3B) of the *D. simulans* X chromosome, where *per* maps in *D. melanogaster* (7). We obtained the *D. simulans* Australia strain Sst I to Bam HI clone from low-melting-point agarose and cloned it in pX1-Blue (Stratagene) by probing with the Sal I to Bam HI fragment subclone. DNA sequencing was by the dideoxy chain termination method [F. S. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)].
15. Construction of the 13.2-kb vector consisting of *D. melanogaster per* (13.2m-TGm) was described (12). An equivalent *D. simulans* 13.2-kb vector (13.2s-TGs) was constructed with genomic DNA extending from the Xba I to the Xho I site (Fig. 1A). The 13.2-kb *D. melanogaster* gene with the *D. simulans* Thr-Gly repeat (13.2m-TGs) was constructed analogously to 13.2ΔTG, a *per* clone with the Thr-Gly repeat deleted (13), except that the Bam HI to Sst I fragment was derived, intact, from *D. simulans*. The 13.2-kb *D. simulans* gene with the *D. melanogaster* Thr-Gly repeat (13.2s-TGm) was constructed in a four-way ligation by means of a cp20.1 vector digested with Sal I–Xba I, the Xho I to Sst I and the Bam HI to Xba I fragments from phage SE2-1, and the Sst I to Bam HI fragment from a previously constructed *D. melanogaster* subclone (13). P-element-mediated germline transformation of *per*⁰¹; *ry*⁵⁰⁶ hosts by *per* and marker DNA (cp20.1 carries the *ry*⁺ marker) was performed with the above vectors as described (8, 12, 13, 16). By in situ hybridization, each transformed line was shown to have acquired one X-chromosomal or autosomal copy of insert DNA (20). From each strain, males carrying one dose of a given insert were monitored for circadian rhythms; and a subset of these transformed lines was tested for song rhythms (Fig. 2 and Table 1).
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21. The procedures to uncover periodicity have been described (5, 6, 8–10). Because subjective elements in the procedure could potentially bias the results (6, 10), each transgenic line used was coded before song recording and all subsequent song analysis was performed blind. The mean IPI was calculated for each 10-s bin of recorded time. Any bin with fewer than 15 individual IPIs was considered empty, and treated as if the male had not sung at all, with no mean IPI value calculated for that time point (6). The time series of mean IPIs (30 to 40 data points) was subjected to Fourier analysis by the CLEAN (9, 24) and vdB (10) algorithms. These two methods deal with gaps in a data record (for example, empty 10-s bins) in different ways (10, 24). The significance of the principal spectral component was tested by nonlinear regression with BMDP program “3R” (BMDP Statistical Software, Berkeley, CA). After all results were obtained, the code was broken and averages were computed for each genotype. Recent experiments using substantial behavioral and analytical modifications of our methods [S. A. Crossley, *Anim. Behav.* **36**, 1098 (1988); A. W. Ewing, *ibid.*, p. 1091] failed to detect rhythmicity in courtship songs; but see (6, 9, 10) for further results confirming the validity of these rhythms.
22. Significant rhythmicity in an individual *Drosophila* male’s courtship song can be difficult to demonstrate statistically [I. G. Logan and J. Rosenberg, *Anim. Behav.* **37**, 860 (1989)]. This stems from the brevity of these data records (which at most have 30 to 40 data points), the existence of gaps in the time series, arising from intervals in the courtship during which the male does not sing, and noise in the data. As in analogous investigations [D. M. Raup and J. J. Sepkoski, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **81**, 801 (1984); *Science* **241**, 94 (1988)], the significance of a spectral peak was confirmed by an independent statistical method; in these experiments, nonlinear regression (6, 10). Only when both methods agreed was a song considered to be periodic. The difficulties in assessing the significance of an individual song rhythm notwithstanding, confidence in the results of this study rests ultimately in the reproducibility of results in multiple trials on each *per* genotype (10).
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