Isolation and Analysis of Six *timeless* Alleles That Cause Short- or Long-Period Circadian Rhythms in Drosophila

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ABSTRACT

In genetic screens for Drosophila mutations affecting circadian locomotion rhythms, we have isolated six new alleles of the *timeless* (*tim*) gene. Two of these mutations cause short-period rhythms of 21–22 hr in constant darkness, and four result in long-period cycles of 26–28 hr. All alleles are semidominant. Studies of the genetic interactions of some of the *tim* alleles with period-altering *period* (*per*) mutations indicate that these interactions are close to multiplicative; a given allele changes the period length of the genetic background by a fixed percentage, rather than by a fixed number of hours. The tim^{L1} allele was studied in molecular detail. The long behavioral period of tim^{L1} is reflected in a lengthened molecular oscillation of *per* and *tim* RNA and protein levels. The lengthened period is partly caused by delayed nuclear translocation of TIM^{L1} protein, shown directly by immunocytochemistry and indirectly by an analysis of the phase response curve of tim^{L1} flies.

CIRCADIAN rhythms have been studied genetically in species ranging from prokaryotes to mammals. The organisms in which mutations have led to significant insights into the working of the daily clock include Synechococcus, Neurospora, Drosophila, and mouse (reviewed in Dunlap 1999). All such clocks share a common fundamental mechanism of negative autoregulation, and even the genes involved in the fly and mouse clock are conserved.

The first mutations of a single gene affecting the daily locomotion rhythm were found in the fruit fly *Drosophila melanogaster*. Three alleles of the *period* (*per*) gene were isolated that resulted in short- and long-period rhythms of locomotion and eclosion, or complete arrhythmia (Konopka and Benzer 1971). Since then, other alleles of *per* have been isolated that include ultrashort- and other long-period rhythms (Hamblen *et al.* 1998). The second central clock gene isolated in flies was called *timeless* (*tim*). As with *per*, a null mutation at *tim* results in arrhythmic individuals, indicating that *tim* is necessary to generate rhythmic behavior (Sehgal *et al.* 1994). Another allele of *tim* was found as a specific suppressor of the per^{L} mutation, but by itself, tim^{SL} has a very subtle phenotype (Rutila *et al.* 1996). A third allele, tim^{ri}, from a natural population, shows long-period phenotypes that deteriorate into arrhythmicity at 30° (Matsumoto *et al.* 1999).

Three more clock genes were found in forward genetic fly screens: *dClock* (*dClk*) and *cycle* (*cyc*) mutations both result in arrhythmia (Allada *et al.* 1998; Rutila *et al.* 1998). The *dClk* gene is the fly homologue of the mouse *Clock* gene, which was found by a mutation leading to long-period and arrhythmic mice (Vitaterna *et al.* 1994). The *double-time* (*dbt*) gene was found via period-altering alleles and was shown to be required for molecular rhythms by studying a pupal lethal allele (Kloss *et al.* 1998; Price *et al.* 1998).

The continued investigation of these genes has led to the following model of the fly clock (reviewed in Hardin 1998): PER and TIM are negative autoregulators, and their RNA levels cycle, peaking at dusk. The protein levels also oscillate and PER and TIM proteins accumulate from dusk onwards in the cytoplasm of pacemaker cells to form a heterodimer that translocates into the nucleus later at night. There, the PER/TIM complex interferes with the transcriptional activators dCLK and CYC and represses transcription of *per* and *tim*. After the turnover of PER and TIM, the cycle restarts with *per* and *tim* RNA accumulation in the morning. Proper turnover of PER requires phosphorylation and the activity of DBT, a casein kinase I family member. Additional genes, like *vrille* (Blau and Young 1999), modulate this central oscillation.

The TIM protein also functions as an important sensor for light-induced phase shifts and entrainment (Young 1998). Light causes TIM degradation via the proteasome pathway (Naidoo *et al.* 1999). However, little is known

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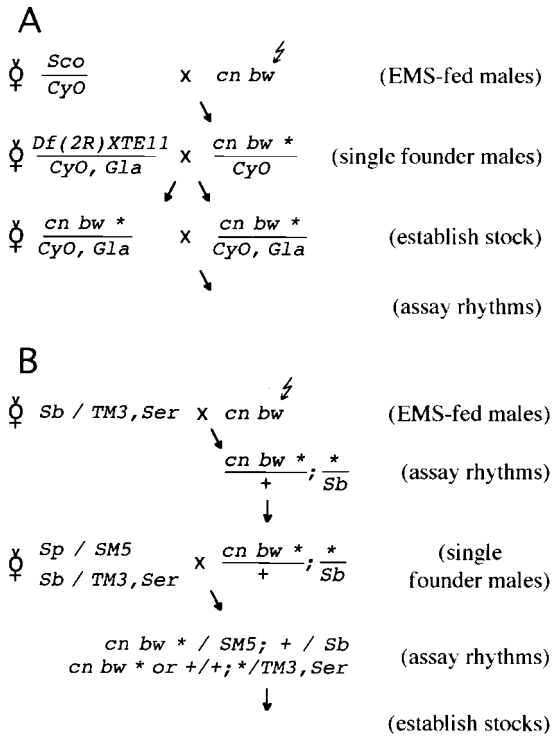

FIGURE 1.—Crossing schemes followed to screen for novel mutations affecting circadian locomotion rhythms (see MATERIALS AND METHODS for details).

about the domains in TIM important for degradation or repression, and TIM has no homology to other known proteins. The only defined domain in TIM is the cytoplasmic localization domain (CLD), deletion of which allows monomeric TIM to enter the nucleus without PER in cultured cells (SAEZ and YOUNG 1996). However, there is normally a mutual requirement between PER and TIM for nuclear localization, and two binding domains have been defined with respect to TIM (SAEZ and YOUNG 1996).

One common property of period-altering mutations is that they show semidominant phenotypes (see DUNLAP 1993, 1996; HALL 1998). We made use of this fact in a screen for mutations that would either dominantly or semidominantly alter circadian locomotion rhythms and report the isolation of four new alleles of *tim* from this screen. Two further mutations of *tim* were isolated in a prior screen for homozygous mutations on the second chromosome affecting rhythmic behavior. These alleles show phenotypes ranging from 21- to 28-hr rhythms and may define important portions of TIM for the maintenance of a 24-hr day.

MATERIALS AND METHODS

Fly methods and mutational screens: Flies were raised on standard cornmeal/agar medium. EMS mutagenesis was done according to LEWIS and BACHER (1968). The crossing scheme depicted in Figure 1A was followed for the locomotion screen for homozygous mutations on the second chromosome. Four to six homozygous males per stock (if viable, otherwise heterozygotes) were screened for their locomotion rhythms. [Note: *Df(2R)XTE11* was included to find lethal genes in the interval 51D3-52A10, notably a mutation in the *igloo* gene].

For the screen for dominant mutations on the second or third chromosome the scheme in Figure 1B was employed. Primary males (each unique in their genotype) were assayed for their rhythms and were crossed to two balancer strains only if they showed an interesting phenotype. Depending on whether a reproducible phenotype was observed segregating with a mutant second or third chromosome, a balanced stock was then established with that chromosome.

Behavioral assays: For eclosion, flies were entrained for at least 3 days in cycles of 12 hr light followed by 12 hr dark (LD) and then released into constant darkness (DD). Every 2 hr, the freshly emerged adults were collected under a Kodak GBX-2 red safe light and then counted. Locomotion assays (at 25° unless otherwise noted) and chi-square periodogram analysis were performed as described in PRICE *et al.* (1998). Anchored phase response curve: flies were entrained to LD for ≥3 days. Groups of flies (12–16) were exposed to 10-min light pulses (~3000 lux) at various times after the last lights off, and their locomotion activity was assayed for 5–7 days. Their average activity offset was determined and compared to the average offset of unpulsed flies to calculate a phase shift. The offset was determined as the point where the activity fell below the mean activity.

Molecular biology and biochemistry: RNase protection assays and Western blotting were performed as described (PRICE *et al.* 1998). Sectioning and immunocytochemistry was done according to MYERS *et al.* (1996), using a 1:1500 dilution of rat anti-TIM antibody. For sequencing mutant and parental strains, total RNA was isolated and reverse-transcribed with random hexamers, or genomic DNA was isolated. Gene-specific primers for *tim* were used for PCR, and DNA sequencing was performed using a cycle-sequencing kit (Perkin Elmer, Branchburg, NJ).

RESULTS

Isolation of novel *tim* alleles: In two genetic screens for mutations changing circadian locomotion rhythms (see MATERIALS AND METHODS), we isolated new alleles of the *timeless* gene. The tim^{L1} and tim^{L2} alleles (Figure 2) were found as homozygous mutants, while tim^{L3}, tim^{L4}, tim^{S1}, and tim^{S2} (Figure 3) were isolated due to their semidominant phenotypes. The tim^{L1} and tim^{L2} mutations showed no recombination with the original tim^{01} allele (415 and 143 potentially recombinant chromosomes were assayed, respectively; data not shown), indicating very tight linkage of these mutations. Sequencing of the *tim* loci from tim^{L1} and tim^{L2} mutant strains revealed the presence of single missense mutations that mapped to the PER-TIM interaction domains (Figure 4), confirming the genetically derived conclusion that they are alleles of *tim*. The tim^{S2} mutation was also genetically mapped to the *tim* genomic region, with 114 recombinants using *Sp* and *dp* markers (data not shown). The other mutations were determined to be alleles of *tim* due to their noncomplementation with amorphic tim^{0} alleles (Table 1). Since the *tim* gene is dosage insensitive (see below), *i.e.*, tim^{0}/tim^{+} has no phenotype itself, com-

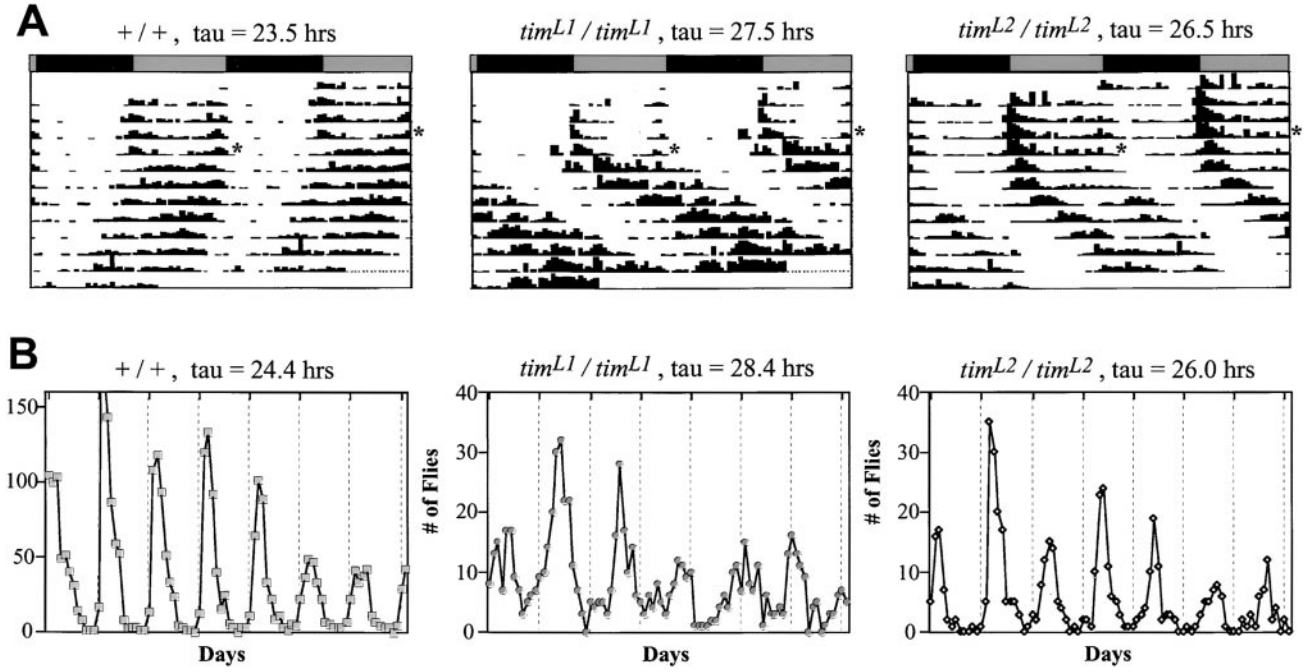

FIGURE 2.—Locomotion and eclosion profiles of wild-type, tim^{L1}, and tim^{L2} flies. (A) Locomotion activity was measured for 4 days in LD (represented by bars on top). The flies were then released into DD (indicated by an asterisk) and assayed for 7.5 days. The genotypes and individual period lengths of representative flies are indicated. (B) Eclosion profiles of flies emerging in DD. Strains were previously entrained to LD cycles (prior lights-on are indicated by stippled lines). The genotype and most likely eclosion period of the fly populations are shown. The periods are ~±0.5 hr for wild type and ±1 hr for tim^{L1} and tim^{L2}.

plementation tests are straightforward (in contrast to some *per* complementation tests; see HALL 1998). Nevertheless, in the absence of sequencing data it is still formally possible that the three unmapped mutations are not *tim* alleles and that they show an uncommon intergenic noncomplementation with tim^{0} alleles.

Five of the six alleles show the same fully penetrant phenotype when homozygous or hemizygous over tim^{0} (while one, tim^{L3}, is on a homozygous lethal chromosome). In addition, all six alleles show a semidominant phenotype where the tim^{mutant}/tim^{+} period length falls between the mutant and wild-type homozygous phenotypes. The two alleles tested, tim^{L1} and tim^{L2}, also showed an eclosion period similar to the periods of their locomotion rhythms (Figure 2B).

The *tim* gene is dosage insensitive: Most of the central clock genes isolated are dosage sensitive. Flies hemizy-

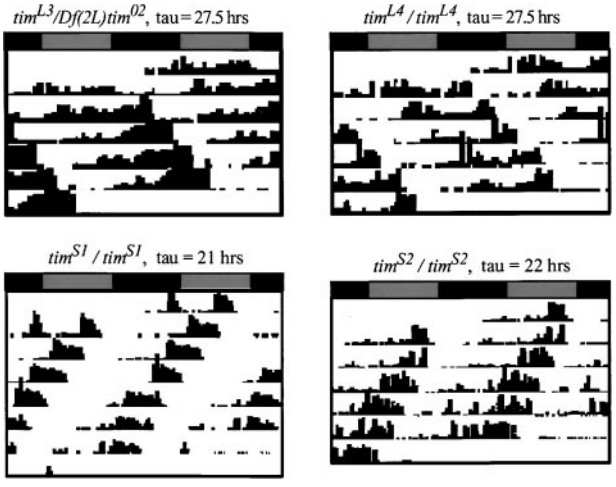

FIGURE 3.—Locomotion actograms of two further tim^{L} alleles and short-period *tim* mutants. Activity was measured in DD after LD entrainment and the genotype and individual period length are indicated. Note that the chromosome carrying tim^{L3} also harbors an unlinked mutation resulting in homozygous lethality.

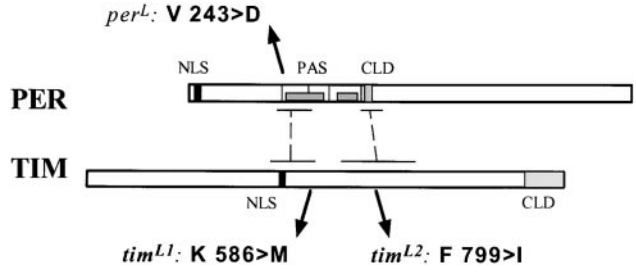

FIGURE 4.—Schematic of PER and TIM proteins. The tim^{L1} and tim^{L2} mutations map to the PER-TIM interaction domains. The interaction domains of the two proteins are connected by dashed lines in the middle: PER 233-365 interacts with TIM 514-587, and PER 452-512 with TIM 724-923 (SAEZ and YOUNG 1996). Mutations that fall into these domains are shown along with their amino acid change and position. They include PER^{L} and the two TIM^{L} variants sequenced. NLS, nuclear localization signal; CLD, cytoplasmic localization domain; and PAS, PER-ARNT-SIM homology.

TABLE 1

Locomotion period length of various *tim* alleles

	Homozygous				Heterozygous				Hemizygous			
Genotype	τ	±SEM	R	AR	τ	±SEM	R	AR	τ	±SEM	R	AR
tim^{L1}	27.8	±0.2	17	2	25.5	±0.1	20	0	27.6	±0.1	12	0
tim^{L2}	26.2	±0.1	13	1	25.1	±0.2	16	0	26.3	±0.2	11	0
tim^{L3}		NA			25.9	±0.2	6	0	27.6	±0.2	6	0
tim^{L4}	28.3	±0.2	7	0	25.7	±0.3	5	0	27.9	±0.1	9	0
tim^{S1}	21.1	±0.2	9	0	22.2	±0.1	15	0	20.7	±0.2	19	0
tim^{S2}	21.9	±0.1	8	0	22.6	±0.1	5	0	21.6	±0.1	14	0

Mutant *tim* alleles (on the left) were assayed as homozygotes, as heterozygotes over tim^{+}, and as hemizygotes over the null point mutation tim^{01}. The hemizygous phenotype was in all cases very similar to the one observed over $Df(2L)tim^{02}$, a deficiency that removes the *tim* locus (Myers *et al.* 1995; data not shown). The periods (τ, in hours) are indicated, with the number of rhythmic flies (R) and the number of arrhythmic flies (AR). Homozygous data for tim^{L3} were not available (NA) due to a secondary lethal mutation on the chromosome.

gous for *per* or *cyc* have ~25-hr period lengths (Konopka and Benzer 1971; Rutila *et al.* 1998). Dosage sensitivity for *dClk* is somewhat tentative, since the point mutant $dClk^{Jrk}$ probably produces a dominant-negative protein rather than a null mutation, and dosage sensitivity is inferred from a large deficiency removing the *dClk* locus, resulting in ~25-hr flies (Allada *et al.* 1998). Amorphic *vri* mutations result in period-shortening of about half an hour in hemizygous flies (Blau and Young 1999). So far, the only Drosophila clock gene isolated that was shown not to be dosage sensitive is *dbt* (Price *et al.* 1998). We tested the dosage sensitivity of the *tim* gene by assaying hemizygous tim^{-} flies and flies carrying a duplication of tim^{+}, *Dp(2;3)DTD33*. Flies harboring one to three copies of tim^{+} all showed indistinguishable locomotion period lengths (Table 2), showing that *tim*, in contrast to its molecular partner *per*, is dosage insensitive. The molecular basis for this difference in dosage sensitivity may be related to the fact that PER is destabilized by the activity of the kinase DOUBLE-TIME, but stabilized by physical interaction with TIM. Such a relationship tends to promote higher levels of TIM than PER protein throughout most of the circadian cycle (Price *et al.* 1998). Indeed, higher levels of TIM than PER have been measured directly in heads from wild-type Drosophila (Zeng *et al.* 1996; Suri *et al.* 1999).

TABLE 2

Dosage insensitivity of the *tim* locus

Genotype	τ	±SEM	R	AR
$+/tim^{01}$	23.6	±0.1	20	0
+/+	23.6	±0.1	20	0
$+/Df(2L)tim^{02}$	23.9	±0.1	22	0
+/+	23.8	±0.1	23	0
+/+; *Dp(2;3)DTD33*/+	23.8	±0.2	13	1
+/+; +/+	23.6	±0.2	10	4

Locomotion periods (τ, in hours), with number of rhythmic (R) and arrhythmic (AR) flies of various *tim* doses shown. The high background of arrhythmia (4 of 14) in the controls is due to the presence of two balancer chromosomes.

Genetic interactions among *tim* alleles: Since *tim* is dosage insensitive, but all the period-altering *tim* alleles are semidominant, these alleles cannot be hypomorphs. Nor can they be hypermorphs since tim^{mutant}/tim^{0} flies show a more severe phenotype than tim^{mutant}/tim^{+} flies. We characterized some alleles more carefully, utilizing the tim^{+}-carrying duplication *Dp(2;3)DTD33*. We assayed tim^{L1}, tim^{L2}, and tim^{UL} [described more closely in Rothenfluh *et al.* (2000)]. For all three alleles tested, the period length of locomotion rhythms was dependent on the ratio of mutant to wild-type *tim* allele (Table 3).

Tendency toward multiplicative interaction of period-altering alleles: We also tested the genetic interactions of period-altering *tim* alleles with period-altering *per* alleles. Table 4 shows the resulting period length of various double-mutant combinations. The phenotypes generally reflect the action of alleles at both loci in such combinations (see below), and no period-altering mutation is epistatic to another. Thus, these mutant alleles seem to independently affect circadian rhythmicity.

The absolute number of hours a given tim^{mutant} allele changes the period length of the genetic background is rather variable, and higher, the longer the period of the genetic background. However, the percentages by which a tim^{mutant} alters the period length of the different *per* genetic backgrounds are generally similar. The genetics of period-altering alleles is perhaps best described by a multiplicative interaction, where each allele changes the period of the genetic background by a fixed percentage. This would be in contrast to an additive interaction, where each allele changes the period by a fixed number of hours.

Temperature compensation of *tim* alleles: One characteristic of the biological clock is that it runs at the same speed over a broad span of temperatures, *i.e.*,

TABLE 3

Three long-period *tim* alleles are semidominant

Genotype	τ	±SEM	R	AR	L:+
tim^{L1}/tim^{L1}; +/+	27.5	±0.2	13	1	2:0
tim^{L1}/tim^{L1}; *Dp(2;3)DTD33,*tim^{+}/+	26.0	±0.2	15	2	2:1
tim^{L1}/+; +/+	25.6	±0.2	10	0	1:1
tim^{L1}/+; *Dp(2;3)DTD33,*tim^{+}/+	25.0	±0.1	10	1	1:2
+/+; +/+	24.5	±0.7	4	1	0:2
tim^{L2}/tim^{L2}; +/+	26.2	±0.2	14	6	2:0
tim^{L2}/tim^{L2}; *Dp(2;3)DTD33,*tim^{+}/+	25.4	±0.1	15	3	2:1
tim^{L2}/+; +/+	24.5	±0.1	14	4	1:1
tim^{L2}/+; *Dp(2;3)DTD33,*tim^{+}/+	24.4	±0.1	17	0	1:2
+/+; +/+	23.7	±0.1	17	1	0:2
tim^{UL}/tim^{UL}; +/+	32.8	±0.2	15	1	2:0
tim^{UL}/tim^{UL}; *Dp(2;3)DTD33,*tim^{+}/+	28.4	±0.3	18	1	2:1
tim^{UL}/+; +/+	25.6	±0.1	11	5	1:1
tim^{UL}/+; *Dp(2;3)DTD33,*tim^{+}/+	25.1	±0.1	17	0	1:2
+/+; +/+	23.8	±0.1	13	2	0:2

Locomotion periods (τ, in hours), with number of rhythmic (R) and arrhythmic (AR) flies of various tim^{L} alleles in combination with the tim^{+}-containing duplication *Dp(2;3)DTD33*. For all three alleles, the severity of the phenotype (*i.e.*, deviation from 24 hr) is determined by the ratio of tim^{L} to tim^{+} gene doses (indicated as L:+).

it is temperature compensated. Many period-altering mutations are defective in temperature compensation: the per^{S} and per^{L} alleles all show stronger deviations from wild type at higher temperatures (Konopka *et al.* 1989), while the period of per^{SLIH} is closer to wild type at higher temperatures (Hamblen *et al.* 1998). We tested a number of other mutant alleles for their temperature compensation. Table 5 shows that the three long-period alleles of *tim* tested are still temperature compensated. This contrasts with tim^{rit} flies, whose period is progressively longer at higher temperature, and at 30° most flies are arrhythmic (Matsumoto *et al.* 1999; see also Table 5 legend). The period length of tim^{S2} flies increases slightly at higher temperatures, but the effect is subtle, with a deviation of <1 hr over a span of 10°. The period of dbt^{L} flies shows an improvement toward wild type at higher temperatures, indicating a defect in temperature compensation that is similar to the response observed in per^{SLIH} flies (see also Hamblen *et al.* 1998). Taken together, these data show that in Drosophila, a period-altering mutation does not necessarily disrupt temperature compensation or show temperature sensitivity. This is most clearly seen for tim^{UL} flies: even a 33-hr clock can run accurately over a span of 10°. Similarly, in Neurospora, certain period-altering mutations do not affect temperature compensation (*cf.* Loros *et al.* 1986; Dunlap 1993).

Long-period molecular oscillations in tim^{L1}: To test whether a change in the behavioral periods (see Figure 2) is also reflected in a change of the underlying molecular oscillation, we decided to study tim^{L1} mutant flies in molecular detail. Heads from tim^{L1} flies were collected for 1 day in LD and two subsequent days in DD. RNA and protein were isolated and tested for levels of *per* and *tim* (Figure 5). In LD, a phase delay of ~2 hr is observed in the accumulation of *per* and *tim* RNA and protein in tim^{L1}. In DD, the first peak of *per* and *tim* RNA is delayed by 4 hr and while two full RNA cycles are completed in 2 days of DD in wild type, only one and a half cycles are observed over the same time span in tim^{L1}, reflecting the lengthened behavioral period in tim^{L1} flies. A delay can also be seen in the PER protein oscillation in tim^{L1} in DD, especially the shift from hyper- to hypophosphorylated forms of PER, an indication for newly accumulating PER (Edery *et al.* 1994). In wild type these shifts occur between CT 6 and 10 on day 1 (CT, circadian time, reflects time measured in DD with CT 6 = subjective noon and CT 18 = subjective midnight) and then between CT 2 and 6 on day 2. In tim^{L1} they are delayed to around CT 14 of day 1 and then between CT 14 and 18 of day 2 in DD (Figure 5A), again consistent with a long-period cycle.

Delayed nuclear translocation in tim^{L1}: The missense mutations in tim^{L1} and per^{L} fall into the PER-TIM interaction domains (see Figure 4), thus opening the possibility that the similar behavioral phenotypes of these two mutations might result from similar molecular defects. The long-period phenotype of per^{L} was correlated with delayed nuclear translocation of PER^{L} protein by (Curtin *et al.* 1995). The entry of PER^{L} into the nuclei of lateral neurons was further delayed by increasing the assay temperature, thereby correlating the per^{L} behavioral period lengthening at higher temperatures with a molecular phenotype. Since this temperature effect was also

TABLE 4

Nearly multiplicative interaction of period-altering alleles

	per^S						per^+						per^L					
	τ	±SEM	R	AR	Δτ	%τ	τ	±SEM	R	AR	Δτ	%τ	τ	±SEM	R	AR	Δτ	%τ
tim^{S1}/tim^{01}	16.0	±0.1	14	0	−2.6	−14	20.7	±0.2	15	0	−3.1	−13	24.5	±0.2	15	0	−4.1	−14
tim^{L2}	20.9	±0.2	16	1	2.3	12	26.2	±0.1	11	1	2.5	11	30.9	±0.3	12	0	3.0	11
dbt^{L}	21.1	±0.2	14	1	2.5	13	26.8	±0.1	12	0	3.1	13	33.3	±0.2	19	0	5.3	19
tim^{L1}	20.6	±0.1	17	0	2.0	11	27.5	±0.1	16	0	3.8	16	31.6	±0.2	17	1	3.7	13
tim^{UL}	24.5	±0.1	12	0	6.0	32	32.8	±0.1	15	1	9.0	38	41.3	±0.3	22	0	12.7	44

Genetic interaction of period-altering *tim* alleles (and one *dbt* allele, left) with three *per* alleles (top). The period length (τ, in hours) is shown with the number of rhythmic (R) and arrhythmic (AR) flies. The change in the period length of the per^X background observed by introducing the alleles on the left is indicated in absolute hours (Δτ) and in a relative change in percentage (%τ). Comparisons were made between per^X; tim^{mutant} and sibling per^X; tim^+, with latter data not shown.

correlated with a decrease in PER^L/TIM interaction in yeast cells at increased temperatures (Gekakis *et al.* 1995), the following model of the per^L molecular defect has emerged: PER^L is an amino acid substitution in one of the two PER/TIM interaction domains that leads to decreased affinity of the proteins. This delays heterodimer formation in the cytoplasm of clock cells, and thus PER/TIM nuclear translocation occurs later than in wild type.

If a similar molecular mechanism is at least partially responsible for the tim^{L1} phenotype, then a delay in nuclear translocation could also be expected in this genotype. We found that nuclear staining of TIM protein in the photoreceptor cells is delayed in tim^{L1} relative to wild type (Figure 6). At early time points, diffuse cytoplasmic staining with little nuclear TIM was observed (Figure 6, A and D). Then the outer photoreceptors (1–7) showed nuclear staining (solid arrows), while the R8 photoreceptors (open arrows) continued to show little nuclear TIM (Figure 6, B and E). Still later, nuclear TIM staining predominated in both photoreceptor populations (Figure 6, C and F). Note the different phases of nuclear staining in wild type and tim^{L1} (*e.g.*, no R8 staining could be seen at ZT 17 in tim^{L1}, Figure 6D, while in wild type, nuclear staining was observed in all photoreceptors at ZT 17, Figure 6B; ZT, Zeitgeber time, reflects time measured in LD where ZT 0 = lights on and ZT 12 = lights off).

Yet in contrast to per^L, where a delay of ~5 hr in nuclear staining closely reflects the behavioral period lengthening (Curtin *et al.* 1995), nuclear entry in tim^{L1} is only delayed by ~2 hr, while tim^{L1} flies show a behavioral period lengthening of ~4 hr. The delay in nuclear translocation of TIM^{L1} protein presumably contributes to the behavioral phenotype, but does not account for the full 4-hr-longer period.

To test delayed nuclear translocation in tim^{L1} in an indirect way, the phase response curve (PRC) for tim^{L1} was established (Figure 7). Light pulses at different times in DD result in different phase changes, depending on the time of the pulse. Three time domains can be defined in a PRC: (i) a phase delay domain in the early subjective night, when light pulses reset the fly clock to late afternoon; (ii) a phase advance domain, late at night, where light pulses advance flies into the early morning; and (iii) a domain of relative insensitivity to light during the subjective day. Each of these domains has a molecular correlate: the delay domain corresponds to the time of PER and TIM cytoplasmic accumulation, while during the advance domain PER and TIM are in the nucleus. The transition point (where delay changes to advance) occurs at the time of PER/TIM nuclear translocation and is delayed in per^L flies (Rutila *et al.* 1997). Thus the PRC transition point can be regarded as an indirect behavioral means of measuring the timing of nuclear translocation. Figure 7 shows that in wild type the transition occurs shortly after ZT 18,

TABLE 5

Temperature compensation of various *tim* alleles

	19°				25°				29°				
	τ	±SEM	R	AR	τ	±SEM	R	AR	τ	±SEM	R	AR	Q_{10} ±SEM
Wild type	23.6	±0.3	10	0	23.4	±0.1	15	0	23.8	±0.1	15	0	0.99 ±.02
per^S	20.1	±0.1	6	0	18.9	±0.1	15	0	18.5	±0.1	15	0	1.09 ±.01
per^L	26.5	±0.2	13	0	28.3	±0.2	13	0	30.1	±0.5	8	0	0.88 ±.02
per^{SLIH}	29.2	±0.3	8	10	28.1	±0.2	12	1	27.1	±0.2	13	1	1.08 ±.02
dbt^L	27.4	±0.2	12	0	26.8	±0.1	12	0	25.6	±0.2	15	0	1.08 ±.02
tim^{S2}	21.2	±0.2	9	2	21.9	±0.1	8	0	22.1	±0.2	7	0	0.96 ±.02
tim^{L2}	26.9	±0.3	14	0	26.5	±0.3	21	0	26.8	±0.4	13	0	1.00 ±.03
tim^{L1}	28.0	±0.2	10	0	28.1	±0.3	13	0	27.5	±0.1	10	0	1.02 ±.01
tim^{UL}	33.0	±0.2	17	3	32.9	±0.2	16	2	33.6	±0.2	12	2	0.98 ±.01

The locomotion period (τ, in hours) of various mutants (left) at three temperatures (top) is shown with the number of rhythmic (R) and arrhythmic (AR) individuals. For each genotype a Q_{10} was calculated from the periods at 19° and 29°. Q_{10} indicates the measurement of a reaction at a given temperature divided by the measurement at a temperature 10° higher (*i.e.*, $\tau_{19°}/\tau_{29°}$). For comparison, tim^{rit} flies show a Q_{10} of ~0.85 in the range of 17°–27° (Matsumoto *et al.* 1999).

while in tim^{L1} it is delayed to about ZT 19.5. Note that the difference is only ~1.5 hr, again indicating that delayed nuclear translocation contributes to the 4-hr period lengthening observed in tim^{L1} flies, but does not fully explain it.

To ensure that the delayed transition point is not just a reflection of a long period and concomitant phase delay in LD conditions (*cf.* Figure 5), we established a PRC for dbt^L. The long-period phenotype of dbt^L (27 hr; see Table 5) is the result of delayed PER phosphorylation and turnover late at night in the nucleus and includes a phase delay of PER and TIM protein accumulation in LD similar to tim^{L1} (*cf.* Figure 5; Price *et al.* 1998). The transition point of the dbt^L PRC is very close to wild type, at about ZT 18.5, indicating that an ~2-hr phase delay in PER and TIM protein accumulation does not automatically lead to a delay of the PRC transition point. At ZT 19, a light pulse results in an ~1.5-hr phase advance in dbt^L flies, while tim^{L1} flies are still phase delayed by ~3.5 hr at this time point. The late transition point and delayed nuclear translocation of the PER/TIM complex seen in tim^{L1} is therefore a specific phenotype of tim^{L1} flies.

The amplitude of the advance domain in the tim^{L1} PRC is more than double that seen with wild-type flies. This is similar to the phenotype observed in tim^{UL} flies, where it has been correlated with prolonged stability and nuclear localization of the PER/TIM^{UL} complex (Rothenfluh *et al.* 2000). Thus a secondary defect, possibly similar to the one seen in tim^{UL} flies late at night, is likely to occur as well as delayed nuclear translocation, and together these defects lead to the 4-hr period lengthening observed in the tim^{L1} strain.

Since delayed nuclear translocation in per^L was correlated with a decreased interaction between PER^L and TIM, we tested the PER/TIM^{L1} interaction in the yeast two-hybrid system. Our results were inconclusive. While PER 233-685 showed a reproducible decrease in interaction with TIM^{L1} 457-1014 to ~65% PER/TIM^+ interaction, a TIM^{L1} 300-1237 construct showed no difference in interaction with PER 233-685 compared to wild-type TIM (data not shown). Therefore, we do not know whether the delayed nuclear translocation is a result of a diminished interaction between PER and TIM^{L1}, analogous to that seen with PER^L/TIM.

DISCUSSION

New timeless alleles: In this report, we describe the isolation and characterization of six new alleles of the *timeless* gene. All alleles result in period alterations of locomotion rhythms with full penetrance, and the two alleles tested for eclosion, tim^{L1} and tim^{L2}, also show a comparably altered period. These two mutations were isolated in a screen for homozygous mutants affecting circadian rhythms. Since they are both semidominant, and previously isolated period-altering alleles of *per* also show semidominance (Konopka and Benzer 1971), we changed our behavioral screen and assayed unique F_1 males for (semi)dominant locomotion phenotypes (see materials and methods). Using this new strategy, we increased our throughput of mutant chromosomes tested by 8- to 10-fold (and even more per person-hour), and we isolated five additional *tim* alleles, one of which, tim^{UL}, is described in Rothenfluh *et al.* (2000). Two of these alleles, tim^{L3} and tim^{L4}, are similar to tim^{L1}. The other two, tim^{S1} and tim^{S2}, are short-period mutations, the first reported for *tim*. There are now 10 *tim* alleles reported that include arrhythmic, short, long, and ultralong phenotypes (Sehgal *et al.* 1994; Matsumoto *et al.* 1999; Rothenfluh *et al.* 2000) and an allele-specific suppressor of per^L, which by itself has a very small pheno-

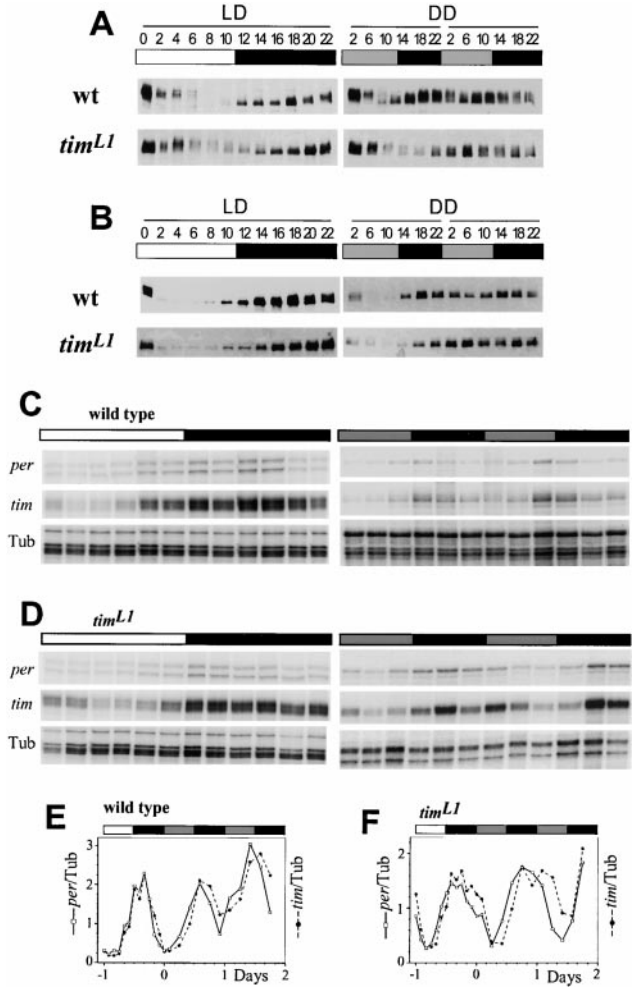

FIGURE 5.—*per* and *tim* protein and RNA oscillations in tim^{L1}. Three days were measured, the first one in LD and two subsequent ones in DD. Anti-PER (A) and anti-TIM (B) Western blots from wild-type (wt) and tim^{L1} head extracts. Equal amounts of protein were loaded (controlled with a nonspecific cross-reacting band on the PER blots, not shown). (C and E) Wild-type and (D and F) tim^{L1} RNase protections and their corresponding quantitations. Quantitations in E and F can be compared directly, since tim^{L1} gels always included wild-type samples as well. Three experiments yielded similar results.

type (RUTILA *et al.* 1996). Clearly, the *tim* gene can be altered in many ways to result in abnormal circadian rhythmicity.

In contrast to most period-altering *per* mutations (HAMBLEN *et al.* 1998), the *tim* alleles we isolated are well temperature compensated. Little is known about the molecular mechanism of temperature compensation, but our data suggest that the *tim* gene plays a lesser role in this mechanism than the *per* gene. That loss of temperature compensation in tim^{ri} can be rescued by introducing an additional dose of the *per* gene (MATSUMOTO *et al.* 1999) supports this notion that *per* is central to the mechanism of temperature compensation.

The genetics of period-altering mutations: As mentioned above, all period-altering *tim* alleles described here are semidominant, and we found that, for those alleles tested, the ratio of mutant to wild-type product determines the severity of the phenotype. KING *et al.* (1997a) found that the mouse *Clock* mutation, which causes an internal deletion of 51 amino acids (KING *et al.* 1997b), is semidominant and behaves as an antimorph, or dominant negative mutation (MULLER 1932). Wild-type CLOCK and BMAL-1 heterodimers can transactivate transcription from m*per-1*-derived E-boxes in mammalian tissue culture (GEKAKIS *et al.* 1998), yet mutant CLOCK and BMAL-1 cannot activate, although they are still capable of binding one another and of binding to target DNA. Thus, mutant CLOCK acts in a dominant-negative fashion, titrating out BMAL-1 protein and CLOCK/BMAL-1 DNA binding sites. This results in reduced activation of m*per-1 in vivo* (JIN *et al.* 1999) and in long-period behavioral rhythms that deteriorate to arrhythmia (VITATERNA *et al.* 1994). Thus in the instance of *Clock*, dominant-negative antimorphic behavior can be explained in a straightforward manner.

Can this dominant-negative interpretation apply for the tim^{L} mutations? In contrast to mutant CLOCK protein, TIM^{L} proteins clearly retain most of their function, since tim^{L} mutants are all highly rhythmic. Thus they are incompletely dominant and not particularly negative. We have previously interpreted the semidominant nature of *dbt* mutations by proposing the formation of distinct pools of stably interacting proteins (KLOSS *et al.* 1998). In the case here of semidominant *tim* alleles this could also apply: in a cell where TIM^{L} and TIM^{+} proteins are present, both proteins bind PER and two distinct pools of PER/TIM^{+} and PER/TIM^{L} are generated. Both pools can perform their function, albeit at different rates, and the two pools may equally contribute at the molecular level. In such a model, since both pools feed back onto the transcriptional regulation of both *tim* alleles present, an integration of the independent reactions results, which ultimately contributes to an intermediate period length.

A nearly multiplicative interaction: Period-altering mutations are generally thought to affect a specific step in the circadian cycle: per^{L} delays cytoplasmic PER^{L}/TIM complex formation (see above), per^{S} increases nuclear turnover of PER^{S} (ZERR *et al.* 1990; MARRUS *et al.* 1996), and tim^{UL} results in a prolonged nuclear PER/TIM^{UL} complex (ROTHENFLUH *et al.* 2000). These changes seem to be achieved independently, and it is therefore not surprising that double-mutant combinations give phenotypes that reflect the contribution of each mutant gene. On further inspection though, the genetic interaction was found not to be additive. Rather, as previously seen with some period-altering mutations in Neurospora (LAKIN-THOMAS and BRODY 1985), interactions among mutant alleles tend to be multiplicative or nearly so. That tim^{UL}, for example, lengthens the period of per^{S} flies by 6 hr, but lengthens the per^{L} rhythm by almost

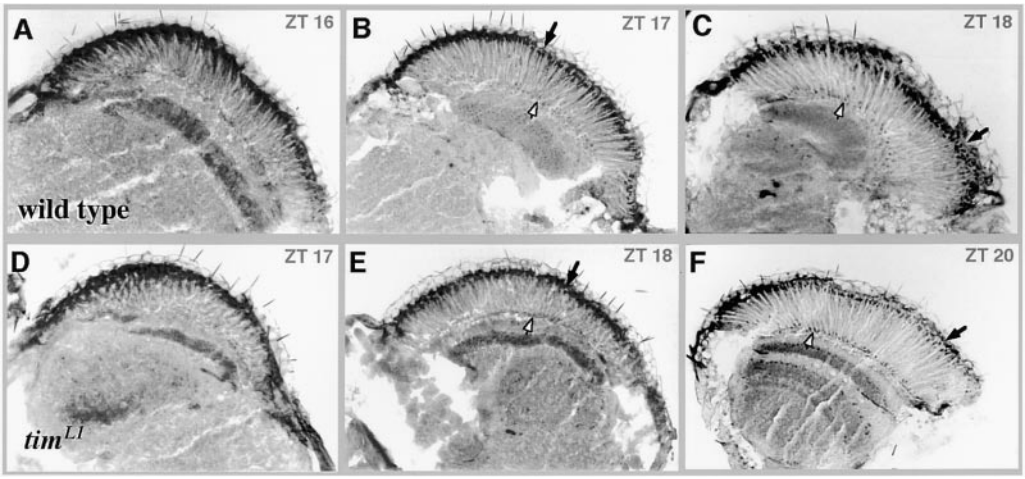

FIGURE 6.—TIM nuclear translocation in wild-type and tim^{L1} photoreceptor cells. Frontal head sections from flies kept in LD were stained with anti-TIM antibody. Wild-type sections are (A) ZT 16, (B) ZT 17, and (C) ZT 18. tim^{L1} sections are (D) ZT 17, (E) ZT 18, and (F) ZT 20. Nuclear staining in R1-7 photoreceptors (black arrows). Nuclear staining of R8 photoreceptors (open arrows). On a subjective scale from 1 (predominantly cytoplasmic staining) to 3 (exclusive nuclear staining) we obtained the following staining intensities for wild-type sections (with standard error and number of sections in parentheses): ZT 14, 1.0 ± 0.0 (4); ZT 15, 1.4 ± 0.3 (5); ZT 16, 1.9 ± 0.2 (8); ZT 17, 2.5 ± 0.2 (10); ZT 18, 2.8 ± 0.2 (8). For tim^{L1} we obtained the following: ZT 15, 1.2 ± 0.2 (6); ZT 16, 1.4 ± 0.2 (9); ZT 17, 1.6 ± 0.3 (5); ZT 18, 1.8 ± 0.2 (10); ZT 19, 2.2 ± 0.2 (10); ZT 20, 2.6 ± 0.2 (9). Note that in photoreceptors nuclear translocation is 2–3 hr advanced compared to the lateral neurons (*cf.* CURTIN *et al.* 1995).

13 hr seems surprising if indeed different steps are affected by these mutations. One explanation is that a mutant phenotype that affects a single step in the cycle has consequences throughout the cycle. tim^{L1} affects nuclear translocation of PER/TIM^{L1}, but the net result of this effect is that both *per* and *tim* RNA oscillations are affected in timing and in amplitude (Figure 5). Similarly, tim^{rit} and tim^{UL} affect both *per* and *tim* RNA oscillations and levels (MATSUMOTO *et al.* 1999; ROTHENFLUH *et al.* 2000). Thus the cause of a mutant phenotype may be one step in the cycle, but the consequences reach over the whole cycle. This altered cycle may now be more (if lengthened) or less (if shortened) susceptible to further perturbations by introducing additional period-altering mutations, which themselves may have a unique cause but affect the whole molecular cycle.

The molecular defect in tim^{L1}: Because the missense mutation in tim^{L1} maps to one of the PER/TIM interaction domains, it was an attractive hypothesis that TIM^{L1} would interact less strongly with PER and result in delayed formation and nuclear translocation of the PER/TIM^{L1} complex, analogous to the model of the per^{L} defect (CURTIN *et al.* 1995; GEKAKIS *et al.* 1995). We were unable to observe a reproducible difference in PER/TIM^{L1} interaction compared to the PER/TIM interaction in the yeast two-hybrid system. However, we did observe delayed nuclear translocation of TIM^{L1} protein in photoreceptor cells. This was also reflected by a delayed transition point from phase delay to phase advance in a tim^{L1} phase response curve. In both cases, the difference between wild type and tim^{L1} was only ~2 hr. The cause for the delayed nuclear translocation we observed may be a subtle PER/TIM^{L1} interaction difference that emerged with some two-hybrid constructs, but not others.

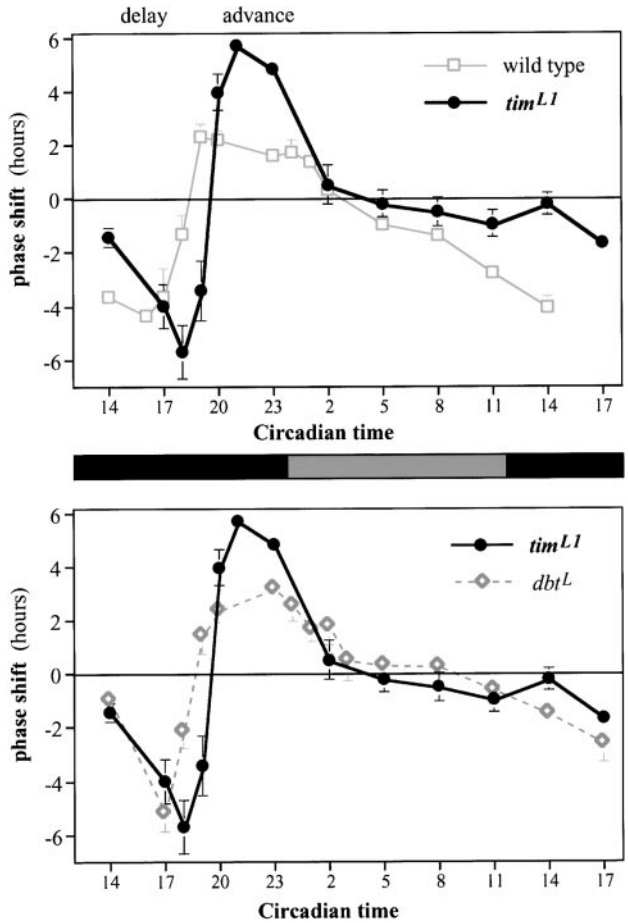

FIGURE 7.—Anchored phase response curve of wild-type, tim^{L1}, and dbt^{L} flies. The phase shift for each time point was assayed two to five times, and standard error bars are shown (unless they are smaller than the plot symbol). At CT 19, there is a significant difference ($P < 0.01$, Student's *t*-test) between tim^{L1} and wild type or dbt^{L}, showing that the transition point is delayed specifically in tim^{L1} flies.

The 2-hr phase delay we detected in PER and TIM^{L1} protein accumulation in an LD cycle could delay complex formation and thus nuclear translocation. The same steady-state phase delay of PER and TIM accumulation is also seen in dbt^{L} flies (Price *et al.* 1998), yet according to their PRCs there is some difference in nuclear entry of PER/TIM in tim^{L1} compared to dbt^{L}, suggesting specificity of delayed nuclear translocation to the tim^{L1} mutation. Even if delayed nuclear entry is a cause for the tim^{L1} period lengthening, the delay observed does not fully reflect the 4-hr behavioral period lengthening seen in tim^{L1} flies. The differences in wild-type and tim^{L1} PRCs lead us to propose that there is an additional defect in tim^{L1} mutant flies when PER/TIM^{L1} complexes are nuclear.

Conclusion: We have isolated six new alleles of the *timeless* gene. They are associated with long- and short-period rhythms, showing that *tim* mutant phenotypes for altered period lengths can be as diverse as those found for *tim*'s partner, *per*. The genetics of these mutations suggests that there is a finely tuned inter- and even intragenetic interplay between clock genes that is easily disturbed by subtle changes in their encoded proteins. Further analysis of these mutants will allow more insight into the various steps that are involved in generating a precise 24-hr internal clock, as well as identifying domains of TIM protein important for its proper function.

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LITERATURE CITED

Allada, R., N. E. White, W. V. So, J. C. Hall and M. Rosbash, 1998 A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell **93:** 791–804.

Blau, J., and M. W. Young, 1999 Cycling *vrille* expression is required for a functional *Drosophila* clock. Cell **99:** 661–671.

Curtin, K. D., Z. J. Huang and M. Rosbash, 1995 Temporally regulated nuclear entry of the *Drosophila period* protein contributes to the circadian clock. Neuron **14:** 365–372.

Dunlap, J. C., 1993 Genetic analysis of circadian clocks. Annu. Rev. Physiol. **55:** 683–728.

Dunlap, J. C., 1996 Genetic and molecular analysis of circadian rhythms. Annu. Rev. Genet. **30:** 579–601.

Dunlap, J. C., 1999 Molecular bases for circadian clocks. Cell **96:** 271–290.

Edery, I., L. J. Zwiebel, M. E. Dembinska and M. Rosbash, 1994 Temporal phosphorylation of the *Drosophila* period protein. Proc. Natl. Acad. Sci. USA **91:** 2260–2264.

Gekakis, N., L. Saez, A.-M. Delahaye-Brown, M. P. Myers, A. Sehgal *et al.*, 1995 Isolation of *timeless* by PER protein interaction: defective interaction between *timeless* protein and long-period mutant PER^{L}. Science **270:** 811–815.

Gekakis, N., D. Staknis, H. B. Nguyen, F. C. Davis, L. D. Wilsbacher *et al.*, 1998 Role of the CLOCK protein in the mammalian circadian mechanism. Science **280:** 1564—1569.

Hall, J. C., 1998 Genetics of biological rhythms in *Drosophila*. Adv. Genet. **38:** 135–184.

Hamblen, M. J., N. E. White, P. T. J. Emery, K. Kaiser and J. C. Hall, 1998 Molecular and behavioral analysis of four *period* mutants in *Drosophila melanogaster* encompassing extreme short, novel long, and unorthodox arrhythmic types. Genetics **149:** 165–178.

Hardin, P., 1998 Activating inhibitors and inhibiting activators: a day in the life of a fly. Curr. Opin. Neurobiol. **8:** 642–647.

Jin, X., L. Shearman, D. Weaver, M. Zylka, G. De Vries *et al.*, 1999 A molecular mechanism regulating output from the suprachiasmatic circadian clock. Cell **96:** 57–68.

King, D. P., M. H. Vitaterna, A. M. Chang, W. F. Dove, L. H. Pinto *et al.*, 1997a The mouse *Clock* mutation behaves as an antimorph and maps within the W19H deletion, distal of *Kit*. Genetics **146:** 1049–1060.

King, D. P., Y. Zhao, A. M. Sangoram, L. D. Wilsbacher, M. Tanaka *et al.*, 1997b Positional cloning of the mouse circadian *Clock* gene. Cell **89:** 641–653.

Kloss, B., J. L. Price, L. Saez, J. Blau, A. Rothenfluh *et al.*, 1998 The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. Cell **94:** 97–107.

Konopka, R., C. Pittendrigh and D. Orr, 1989 Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. J. Neurogenet. **6:** 1–10.

Konopka, R. J., and S. Benzer, 1971 Clock mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **68:** 2112–2116.

Lakin-Thomas, P. L., and S. Brody, 1985 Circadian rhythm mutations in *Neurospora crassa*: interactions between clock mutations. Genetics **109:** 49–66.

Lewis, E. B., and F. Bacher, 1968 Method for feeding ethyl methane-sulfonate (EMS) to *Drosophila* males. Dros. Inf. Serv. **43:** 193.

Loros, J. J., A. Richman and J. F. Feldman, 1986 A recessive circadian clock mutation at the *frq* locus of *Neurospora crassa*. Genetics **114:** 1095–1110.

Marrus, S. B., H. Zeng and M. Rosbash, 1996 Effect of constant light and circadian entrainment of per^{S} flies: evidence for light-mediated delay of the negative feedback loop in *Drosophila*. EMBO J. **15:** 6877–6886.

Matsumoto, A., K. Tomioka, Y. Chiba and T. Tanimura, 1999 tim^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. Mol. Cell. Biol. **19:** 4343–4354.

Muller, H. J., 1932 Further studies on the nature and causes of gene mutations. Proc. Sixth Int. Congr. Genet. **1:** 213.

Myers, M. P., K. Wager-Smith, C. S. Wesley, M. W. Young and A. Sehgal, 1995 Positional cloning and sequence analysis of the *Drosophila* clock gene, *timeless*. Science **270:** 805–808.

Myers, M. P., K. Wager-Smith, A. Rothenfluh-Hilfiker and M. W. Young, 1996 Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. Science **271:** 1736–1740.

Naidoo, N., W. Song, M. Hunter-Ensor and A. Sehgal, 1999 A role for the proteasome in the light response of the Timeless clock protein. Science **285:** 1737–1741.

Price, J. L., J. Blau, A. Rothenfluh, M. Abodeely, B. Kloss *et al.*, 1998 *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell **94:** 83–95.

Rothenfluh, A., M. W. Young and L. Saez, 2000 A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. Neuron **26:** 505–514.

Rutila, J. E., H. Zeng, M. Le, K. D. Curtin, J. C. Hall *et al.*, 1996 The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron **17:** 921–929.

Rutila, J. E., O. Maltseva and M. Rosbash, 1997 The tim^{SL} mutant affects a restricted portion of the *Drosophila melanogaster* circadian cycle. J. Biol. Rhythms **13:** 380–392.

Rutila, J. E., V. Suri, M. Le, W. V. So, M. Rosbash *et al.*, 1998 CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell **93:** 805–814.

Saez, L., and M. W. Young, 1996 Regulation of nuclear entry of the *Drosophila* clock proteins Period and Timeless. Neuron **17:** 911–920.

Sehgal, A., J. L. Price, B. Man and M. W. Young, 1994 Loss of

circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. Science **263:** 1603–1606.

Suri, V., A. Lanjuin and M. Rosbash, 1999 TIMELESS-dependent positive and negative autoregulation in the *Drosophila* circadian clock. EMBO J. **18:** 675–686.

Vitaterna, M. H., D. P. King, A.-M. Chang, J. M. Kornhauser, P. L. Lowrey *et al.*, 1994 Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. Science **264:** 719–725.

Young, M. W., 1998 The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*. Annu. Rev. Biochem. **67:** 135–152.

Zeng, H., Z. Qian, M. P. Myers and M. Rosbash, 1996 A light-entrainment mechanism for the *Drosophila* circadian clock. Nature **380:** 129–135.

Zerr, D. M., J. C. Hall, M. Rosbash and K. K. Siwicki, 1990 Circadian fluctuations of *period* protein immunoreactivity in the CNS and the visual system of *Drosophila*. J. Neurosci. **10:** 2749–2762.

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