The *tim*^{SL} Mutant Affects a Restricted Portion of the *Drosophila melanogaster* Circadian Cycle

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Abstract The circadian rhythm genes *period* (*per*) and *timeless* (*tim*) are central to contemporary studies on *Drosophila* circadian rhythms. Mutations in these genes give rise to arrhythmic or period-altered phenotypes, and *per* and *tim* gene expression is under clock control. *per* and *tim* proteins (PER and TIM) also undergo circadian changes in level and phosphorylation state. The authors previously described a period-altering *tim* mutation, *tim*^{SL}, with allele-specific effects in different *per* backgrounds. This mutation also affected the TIM phosphorylation profile during the mid-late night. The authors show here that the single amino acid alteration in TIM-SL is indeed responsible for the phenotype, as a *tim*^{SL} transgene recapitulates the original mutant phenotype and shortens the period of *per*^{L} flies by 3 h. The authors also show that this mutation has comparable effects in a light-dark cycle, as *tim*^{SL} also accelerates the activity offset during the mid-late night of *per*^{L} flies. Importantly, *tim*^{SL} advances predominantly the mid-late night region of the *per*^{L} phase response curve, consistent with the notion that this portion of the cycle is governed by unique rate-limiting steps. The authors propose that TIM and PER phosphorylation are normally rate determining during the mid-late night region of the circadian cycle.

Key words Drosophila, circadian rhythms, behavior, timeless

INTRODUCTION

In *Drosophila melanogaster*, circadian rhythms were originally studied behaviorally by analyzing how an endogenous clock affects both the locomotor activity of individual flies and the eclosion rhythm of a population (Konopka and Benzer, 1971). Using this model system, recent studies have analyzed how primary zeitgeibers such as light and heat can both entrain and reset the fly circadian clock (Hamblen-Coyle et al., 1992; Sehgal et al., 1992; Wheeler et al., 1993; Edery et al., 1994a; Saunders et al., 1994; Qiu and Hardin, 1996; Marrus et al., 1996). The use of mutants that are either blind or altered in their endogenous periodicity (*per* mutants) helped to describe how photic inputs to the clock are perceived and how the circadian clock functions in constant conditions (Konopka and Benzer, 1971; Helfrich and Engelmann, 1983; Dushay et al., 1989; Rutila et al., 1992; Baylies et al., 1992; Wheeler et al., 1993). *per* mutant effects were manifest on a wide range of clock phenomena, including temperature compensation—the fact that the endogenous period normally varies minimally with temperature (Konopka et al., 1989, 1994; Ewer et al., 1990; Huang et al., 1995; Sawyer et al., 1997).

These phenotypic studies have been complemented by molecular studies of how circadian clocks function, with the 2 genes *period* (*per*) and *timeless* (*tim*) as the cornerstones of the *Drosophila* clock model (Rosbash et al., 1996). Both of these genes were initially identified as mutants that either altered or abolished fly circadian rhythms (Konopka and Benzer, 1971;

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Sehgal et al., 1994). Transcription initiation of the *per* gene is under clock control, which contributes to the cycling of the *per* mRNA abundance as a function of circadian time (Hardin et al., 1992; So and Rosbash, 1997). The transcription of this gene is not only under clock control but also under feedback control, since missense mutations of the *per* gene product (PER) affect temporal regulation of its own gene transcription (Hardin et al., 1990). The current view is that this is a negative feedback loop, in which the *per* protein (PER) inhibits its own transcription (Zeng et al., 1994; Marrus et al., 1996; Darlington et al., 1998). PER levels then also cycle due to this transcriptional regulation as well as to posttranscriptional regulation. These posttranscriptional events include PER phosphorylation (Edery et al., 1994b) and temporal gating of nuclear entry in pacemaker neurons (Vosshall et al., 1994; Curtin et al., 1995), which may be relevant to the transcriptional negative feedback loop. Recently, 3 mammalian homologues for *per* have been identified. They undergo circadian cycling and are present in brain pacemaker cells, suggesting that aspects of the fly paradigm might extend to mammalian systems (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Sun et al., 1997; Tei et al., 1997; Zylka et al., 1998).

per-analogous cycling of *tim* RNA levels and transcription rates has been described, and *tim* protein (TIM) cycles both in abundance and in the phosphorylation state (Zeng et al., 1996; Hunter-Ensor et al., 1996; Myers et al., 1996; Lee et al., 1996; So and Rosbash, 1997). PER and TIM dimerize, and the dimer is almost certainly relevant to fly clock function (Gekakis et al., 1995; Zeng et al., 1996; Lee et al., 1996; Saez and Young, 1996). Since PER-TIM dimerization is under circadian control and since dimers are most prevalent at times when phosphorylation of PER and TIM is minimal, PER and TIM phosphorylation (or dephosphorylation) is also likely to be important for pacemaker function. Phosphorylation could affect PER-TIM dimerization and therefore monomer as well as dimer levels, or phosphorylation could have a more direct impact on activities of the individual proteins. Interpretation of how phosphorylation and dimerization are related is further complicated by the circular nature of the clock. For example, the per^{L} mutation affects multiple aspects of clock function, including free-running period, temperature compensation, the strength of the behavioral rhythms, the timing of PER nuclear entry, and the timing and extent of PER phosphorylation (Konopka and Benzer, 1971; Smith and Konopka, 1981; Baylies et al., 1987; Konopka et al., 1989; Curtin et al., 1995; Rutila et al., 1996). It is unknown whether the changes in PER-L phosphorylation act through the PER-TIM dimer, and it is even unclear whether they are the cause or the effect of some of the other phenotypes. In this context, it has been proposed that time-dependent effects on PER phosphorylation are not the cause of the timing changes in nuclear entry but a consequence of the subcellular compartment within which the protein resides (cytoplasm in the early night, nucleus in the late night) (Lee et al., 1996).

Temporal changes in TIM phosphorylation have also been observed, and a role of phosphorylation in timing was reinforced by studies of a period-altering allele of *tim*, tim^{SL} (Rutila et al., 1996). This mutation, which was identified as a suppressor of per^{L} in a behavioral mutagenesis screen, shortens per^{L} rhythms from 29 to 26 h. tim^{SL} has a much milder effect on the per^{+} (24 to 23 h) and per^{s} (19 to 20 h) genotypes. The period of the per^{L}:tim^{SL} double-mutant combination was also no longer temperature sensitive: the tim^{SL} mutation also suppressed the temperature compensation defect of per^{L} flies. Since the cycling of PER and TIM is altered in *per* mutants and is reflective of the endogenous mutant periods, it was expected that the cycling pattern of tim^{SL} mutants would be similarly altered. Surprisingly, however, the pattern of TIM as well as PER cycling was indicative of the *per* genotype present rather than of the *tim* genotype or of the behavioral rhythm (Rutila et al., 1996). The only change in TIM-SL observed by Western analysis was an alteration in the TIM phosphorylation state in the late night, with more intense and more prominently phosphorylated forms of TIM-SL seen from ZT21 to CT1 (Rutila et al., 1996). It was suggested that this change in phosphorylation might alter the nature of the PER-TIM dimer interaction, which might be relevant to the allele-specific effects as well as the timing changes.

TIM also features prominently in the current view of how light affects the *Drosophila* circadian clock. TIM levels decrease rapidly in response to illumination, and a comparable effect on PER is not detectable (Zeng et al., 1996; Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Suri et al., 1998; Yang et al., 1998). Light also causes a TIM decrease in arrhythmic, per^{0} (PER null mutant) flies. Although these flies have no discernable rhythms, TIM protein levels still oscillate during light-dark cycles: there are higher levels of TIM at night than during the day (Zeng et al., 1996; Myers et al., 1996). Therefore, TIM is probably an important light-sensitive clock component, and it is possible that

the phosphorylation state of TIM contributes to features of this light response (Zeng et al., 1996).

The effect of light on TIM levels has been used to explain results obtained from a behavioral paradigm long used in circadian biology, where flies are exposed to brief pulses of light at different times during their circadian cycle. The behavioral assay then determines if the fly's activity peak is phase advanced or phase delayed relative to nonpulsed flies, and the magnitude and direction of the phase shift are plotted versus circadian time. This plot is the phase response curve (PRC) (Pittendrigh, 1981). It is a characteristic feature of species and genotype and can be used to define the state of the clock: a particular phase shift is indicative of a particular location within the cycle. Under favorable circumstances, PRC effects can be interpreted to indicate altered light sensitivity or an altered clock, one that runs too fast or too slow. It is known, for example, that *per* mutants alter both the phase and the amplitude of the PRC (Saunders et al., 1994).

A similar effect of the tim^{SL} mutant on the PRC might help to elucidate the roles of TIM phosphorylation and the PER / TIM dimer in clock entrainment and resetting. We have therefore undertaken a set of analyses to see how these mutant flies behave in light-dark cycling conditions and how they respond to light pulses. For this second purpose, we have constructed tim^{SL} mutant PRCs in 2 *per* genetic backgrounds, using a slightly different protocol that was first described by Aschoff (1965; see also Levine et al., 1994; Suri et al., 1998) and that we refer to as an anchored phase response curve (APRC). Our results indicate that the increased phosphorylation of TIM seen in tim^{SL} mutants in the late night appears to affect how rapidly the clock progresses at this time. During these studies, we have also verified that the *thr* to *ile* change at *tim* amino acid 494 is in fact responsible for the mutant behavioral phenotype initially described.

MATERIALS AND METHODS

Fly Strains

Two *tim* cDNA sequences, generated by PCR with hemaglutinin tags at their 3' ends (as described in Zeng et al., 1996; Rutila et al., 1996), were cloned into CasPeR4. These cDNAs are identical except for the C to T transition at nucleotide 1724 in the tim^{SL} cDNA. A genomic clone that contains both *tim* coding and 5' noncoding sequences, as well as the first intron, was identified from a CosPeR genomic library (Tamkun et al., 1991). From this clone, a 6 kb BamHI / SalI fragment whose 3' end is at the *tim* translation start was isolated and cloned upstream of the *tim* cDNA in CasPeR4. At the 3' end of each cDNA, a 2.1 kb HindIII / EcoRI fragment obtained from the 3' end of *per* was cloned in the appropriate orientation. These constructs were used to generate germ-line transformants by injecting *y w*; tim^{0}; *Ki* p^{p} P[ry^{+} Δ2-3] / +. Three independent lines were generated for each construct.

Wild-type Canton-S, per^{L}, and per^{L};tim^{SL} flies were used for average activity and APRC analyses (see below) and as controls for the locomotor activity analyses.

Behavioral Analyses

When flies were assayed for locomotor activity, they were entrained for two 12-h light:12-h dark cycles before being assayed in constant dark conditions at 25ºC for 5 days (Hamblen et al., 1986). Activity periods were determined by χ^2 periodogram analysis, $\alpha = 0.01$ (Hamblen-Coyle et al., 1992).

The analysis of flies' activity patterns in light-dark cycling conditions has been described previously (Hamblen-Coyle et al., 1992). Briefly, a fly's activity is assayed in 12-h light:12-h dark (12:12 L:D) cycling conditions for 5 days, and each day's data are superimposed on top of one another. A single plot that includes the average daily activity profile for an entire genotype can be generated from the individual fly averages. This average activity plot is possible since, regardless of the flies' endogenous period, they nearly always entrain to a 24-h cycle if given constant entraining light cues. The phase of the activity peaks was analyzed as described previously (Hamblen-Coyle et al., 1992).

In the APRC protocol, the flies were first entrained to a 12:12 L:D cycle for 5 days. During the sixth dark phase of the cycle, flies were given a 10-min saturating white light pulse (2000 lux) at 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 h after the last lights on. A separate control group of flies was not given a light pulse. Flies were then put into constant darkness for another 5 days. The phase of locomotor activity peaks after the light pulse was determined similar to what was described previously (Edery et al., 1994a), using activity offset at 50% of the peak as the phase reference point. To calculate the phase shift of these activity peaks, the average phase value 2 days after the pulse was compared to the average phase value of the relative activ-

ity peaks of nonpulsed controls. The standard error of the mean was calculated from the standard deviations of the average phase values of the pulsed flies and the standard deviations of the average phase values of the nonpulsed control flies. The data were plotted using the "smooth" function of KaleidaGraph (Abelbeck Software) to generate the curves.

RESULTS

One of the notable aspects of the tim^{SL} mutation is its apparent allele-specific interaction with per^{L}, as it has more modest effects in either a wild-type or per^{s} background (Rutila et al., 1996). Before trying to explain this phenomenon, we sought to verify an underlying assumption—namely, that the tim^{SL} mutant phenotype is really due to a change at amino acid 494 in the TIM gene, which is just upstream of the PER-interaction domain (Saez and Young, 1996). Definitive proof requires that this specific mutation be shown to result in the defined behavioral phenotype. To accomplish this, we created a biologically active *tim* gene following the general strategy previously used to obtain rescue with *per* DNA (Citri et al., 1987); ca. 4.5 kb of upstream *tim* DNA, including the first intron, was followed first by a *tim* cDNA sequence and then by ca. 2.5 kb of 3' flanking DNA derived from 3' *per* genomic sequences (Figure 1). Introduction of the tim^{+} transgene rescues arrhythmicity in a tim^{0} null mutant in both a wild-type *per* and mutant per^{L} background (Table 1). A second *tim* transgene was constructed that is identical to the first, except for a single C to T transition at nucleotide 1724, leading to the *thr* to *ile* amino acid change at residue 494. One copy of this tim^{SL} transgene also restores rhythmicity to tim^{0} flies, albeit with the same 1-h period shortening seen with the endogenous tim^{SL} mutation (Table 1). When the fly harbors the per^{L} mutation, the tim^{SL} transgene causes a dramatic period shortening, as is observed in per^{L};tim^{SL} flies (Table 1). The results show that both genes function as predicted from their strains of origin. We conclude that the single missense mutation at position 494 is indeed responsible for the tim^{SL} phenotype and causes the per^{L} clock to run 3 h fast.

In an attempt to clarify how the tim^{SL}; per^{L} interaction was altering the clock, we asked 2 questions: first, does the tim^{SL}; per^{L} clock run ca. 10% faster than the per^{L} clock under conditions other than constant darkness? Second, is the 3-h advance restricted to one or more defined subsets of the cycle, or does the clock run a uniform 10% faster around the whole cycle? One way of addressing the first question is to examine how flies behave in entraining light-dark (LD) cycles. *per* mutant flies can entrain to 12-h light:12-h dark (12:12 LD) cycles, but their patterns of locomotor activity show substantial differences from wild-type flies. These differences are manifest as displacement of the evening activity peak during the imposed 24-h LD cycle (Hamblen-Coyle et al., 1992). We therefore examined the timing of the evening activity peak of various genotypic combinations in LD cycling conditions.

Table 1. A tim^{SL} transgene rescues the tim^{SL} behavior.

Genotype	*Period* ± SEM[a]	N[b]
wild type	24.2 ± 0.1	15
tim^{SL} / +	23.7 ± 0.2	24
per^{L}	29.2 ± 0.2	19
per^{L}; tim^{SL} / +	26.7 ± 0.2	17
y w	23.6 ± 0.1	32
y w; tim^{0}	AR	10
y w; tim^{0}; P(tim^{+}) / +[c]	24.5 ± 0.1	10
y w; tim^{0}; P(tim^{SL}) / +[d]	23.4 ± 0.1	24
per^{L} *w sn*; tim^{0}; P(tim^{+}) / +	30.7 ± 0.6	6
per^{L} *w sn*; tim^{0}; P(tim^{SL}) / +	25.7 ± 0.2	15

NOTE: AR = arrhythmic.
a. Period of activity in constant darkness, given in hours ± the standard error of the mean.
b. Number of animals tested.
c. 37% (6/16) of the flies tested were arrhythmic.
d. 25% (8/32) of the flies tested were arrhythmic.

As expected, and consistent with its small effect on period in a wild-type background and per^{s} background, tim^{SL} has little or no effect on the location of the evening activity peak on these 2 *per* genotypes (Table 2). However, tim^{SL} also has a surprisingly small effect on the timing of the evening activity peak in a per^{L} background. The advance was 1 h rather than the 3-h period shortening observed in constant darkness. But other flies, containing mutant transgenes, have endogenous periods similar to per^{L};tim^{SL} and exhibit average activity peaks that are even closer to the 12.5-h evening peak value characteristic of wild-type flies (see Hamblen-Coyle et al., 1992).

To examine these LD patterns in more detail, we evaluated the average activity profiles from which the peak locations are calculated. As expected, tim^{SL} has little or no effect on an otherwise wild-type pattern; the timing of both morning and evening activity peaks is essentially identical in a per^{+} background (Figure 2A).

As previously described, the per^{L} evening activity peak is profoundly delayed into the middle of the night, consistent with the slow-running per^{L} clock ob-

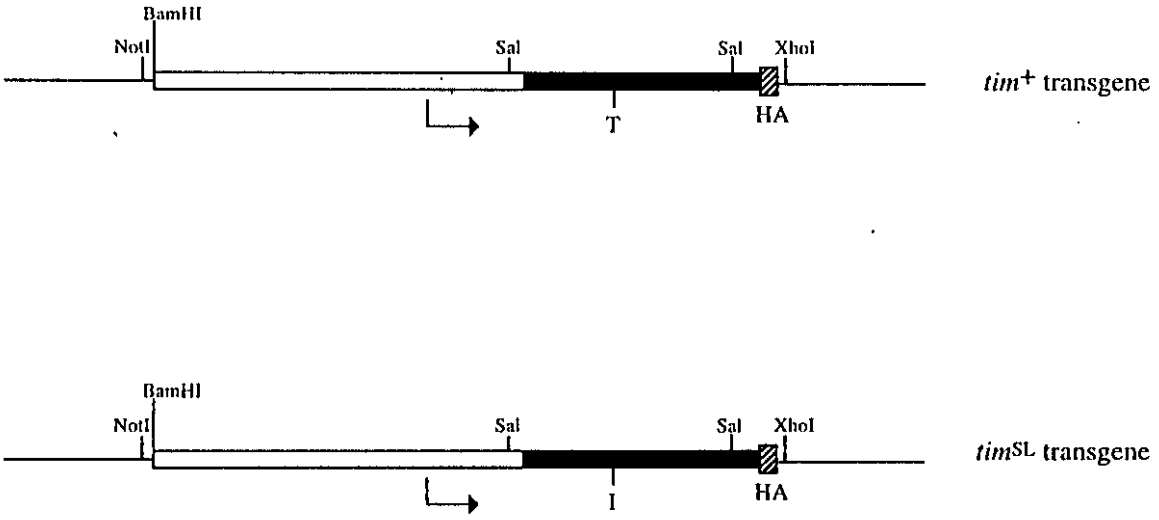

Figure 1. Diagram of *tim* transgenes. The open bars denote 5' genomic sequences, and the closed bars are cDNA coding sequences. The hatched box shows where the hemagglutinin tag is located. Material 3' of the tag is derived from *per* 3' sequences. The transcription start site is shown by the arrow, and the *thr* (T) to *ile* (I) change seen in tim^{SL} at amino acid 494 is shown beneath each transgene.

Table 2. Activity peaks in light-dark cycling conditions.

Genotype	*Peak (ZT)*[a]	SEM[b]	N[c]
wild type	12.5	0.1	29
tim^{SL} / tim^{SL}	12.5	0.1	6
per^{L}	15.6	0.3	28
per^{L}; tim^{SL} / tim^{SL}	14.6	0.3	26

a. The peak of activity is given as zeitgeiber time (where ZT0 is lights on, and ZT12 is lights off).
b. Standard error of the mean.
c. Number of animals.

served in constant darkness (Figure 2B) (Hamblen-Coyle et al., 1992). It is also noticeably less acute and more spread out than the wild-type or per^{s} evening peaks, consistent perhaps with the less robust rhythms characteristic of per^{L}. At the time when the wild-type evening activity peak normally occurs, at ZT12, per^{L} flies only exhibit a "startle" response. Unlike the wild-type evening peak, this per^{L} evening peaklet occurs in response to rather than in anticipation of the LD transition (Hamblen-Coyle et al., 1992). Addition of tim^{SL} to the per^{L} genotype does not cause a dramatic change in the average activity profile, as the evening peak is comparably delayed in both per^{L} and per^{L};tim^{SL} genotypes. It appears, however, that the activity shutoff of the evening activity peak occurs several hours earlier in the double-mutant background. This advance in the mid-late night would be consistent with the previously observed altered accumulation and phosphorylation profiles of *tim* protein seen in per^{L};tim^{SL} flies from ZT21 to ZT24 during a light-dark cycle (Rutila et al., 1996).

This behavioral result suggests a response to the second question—namely, that tim^{SL} is having its period-shortening effect in per^{L} flies during a restricted portion of the circadian cycle. To confirm this conclusion, we generated a PRC. In this assay, flies are exposed to a light pulse at various points in their dark cycle, and one then determines when their next activity peak occurs compared to a control set of flies kept in constant conditions. The resulting phase shifts are plotted as a function of the time light was administered. Traditionally, the light pulse is given after a light-dark entrainment phase followed by a sufficient period in constant darkness to ensure that the fly's circadian clock is free-running. PRCs can be difficult to compare, however, because it is not obvious how to align or compare the curves from genotypes with different endogenous periods. To obviate this problem, we exploited a simple modification of the standard PRC method, which we have called APRC (Aschoff, 1965; Levine et al., 1994; Suri et al., 1998). Rather than subjecting animals to light pulses at different times of a free-running cycle in constant darkness as in a standard PRC, the APRC protocol subjects flies to a light pulse during the dark phase of an entraining LD cycle, between ZT12 and ZT24, and during the subsequent 12 h of the first cycle in constant

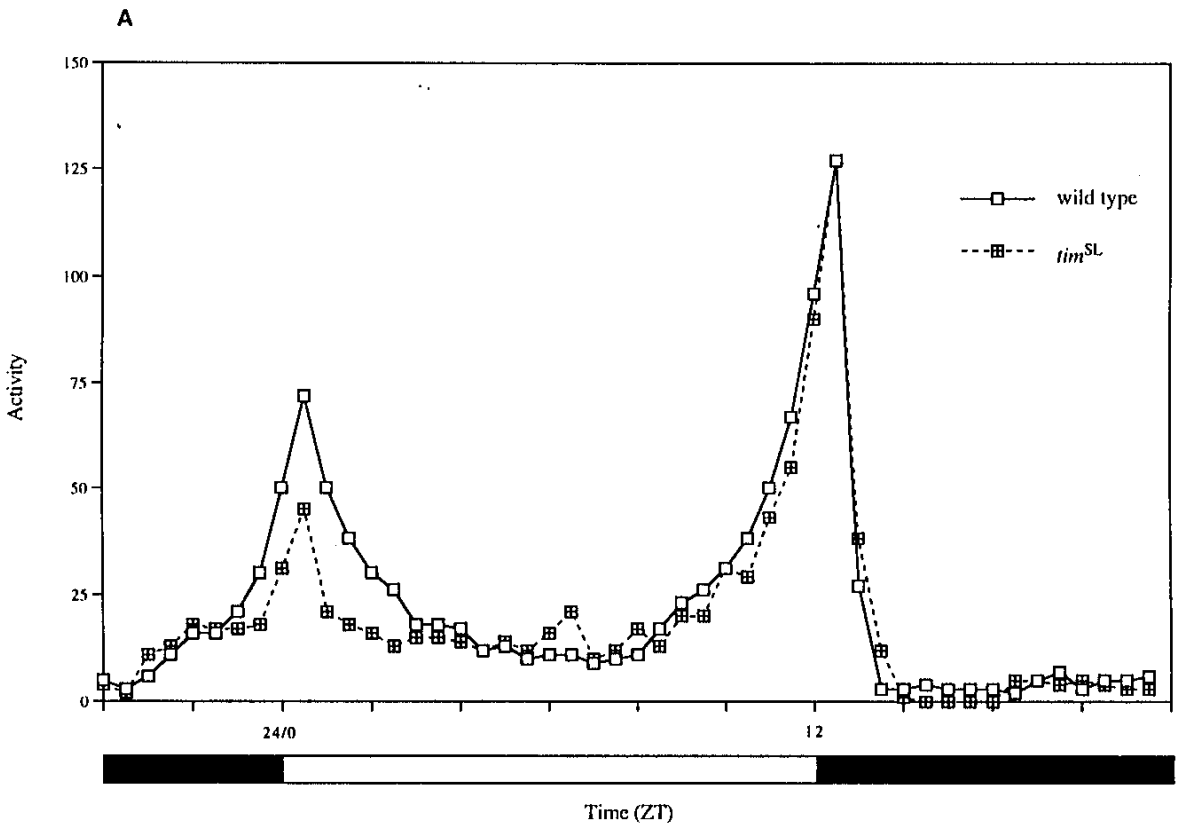

Figure 2a. Pattern of locomotor activity in 12-h light:12-h dark (12:12 L:D) cycling conditions. The timing of the light-dark cycle is shown by the bars below the graph. Figure 2A plots activity amounts for wild-type CS (*N* = 29) and tim^{SL} flies (*N* = 10), while Figure 2B replots data for wild type with per^{L} (*N* = 28) and per^{L};tim^{SL} flies (*N* = 26).

darkness, between CT0 (same as ZT24, the end of the LD cycle) and CT12. Since the *per* mutants entrain to a 24-h LD cycle (Hamblen-Coyle et al., 1992), the cycles of different genotypes are anchored to a specific time point defined by the lights-off transition at ZT12 (Qiu and Hardin, 1996; Marrus et al., 1996). This should allow a more direct comparison of PRCs from genotypes with different endogenous periods and might provide an indication of when during the circadian cycle one genotype progresses more rapidly or more slowly than another.

The APRC of wild-type flies is virtually indistinguishable from a standard PRC (Figure 3A) (Saunders et al., 1994; Levine et al., 1994). This is due in part to the 24-h endogenous period of *D. melanogaster*, which matches the 24-h period of the 12:12 LD cycle used in the APRC; that is, wild-type animals manifest 24-h periods under both LD and DD (constant dark-dark) conditions. The APRCs of wild-type and tim^{SL} flies are also essentially indistinguishable, as expected from the small differences in the endogenous period (Figure 3B). When one compares the APRCs of wild-type versus per^{L}, however, it is apparent that there is a shift in the per^{L} APRC since there are substantial differences for per^{L} already apparent immediately after the light-dark transition (at the earliest times that one can apply a light pulse; see Figure 3A). This suggests that the per^{L} clock is already well behind the wild-type oscillator by ZT12.

On the other hand, the per^{L} and per^{L};tim^{SL} APRCs are very similar, and a detailed comparison is therefore appropriate (Figure 3C). The 2 APRCs track very closely until ZT20, after which the double-mutant flies are clearly advanced by a few hours relative to the single-mutant per^{L} flies. The data are broadly consistent with the average activity comparisons shown above as well as with the nighttime changes in TIM-SL accumulation and phosphorylation (Rutila et al., 1996). This indicates that much and perhaps most of the tim^{SL}-mediated advance does indeed occur during the late night. This indicates further that the 3-h period shortening imparted by tim^{SL} does not represent a uniform speeding up of the per^{L} clock. It also recalls the original isolation of tim^{SL} as a suppressor of the per^{L}

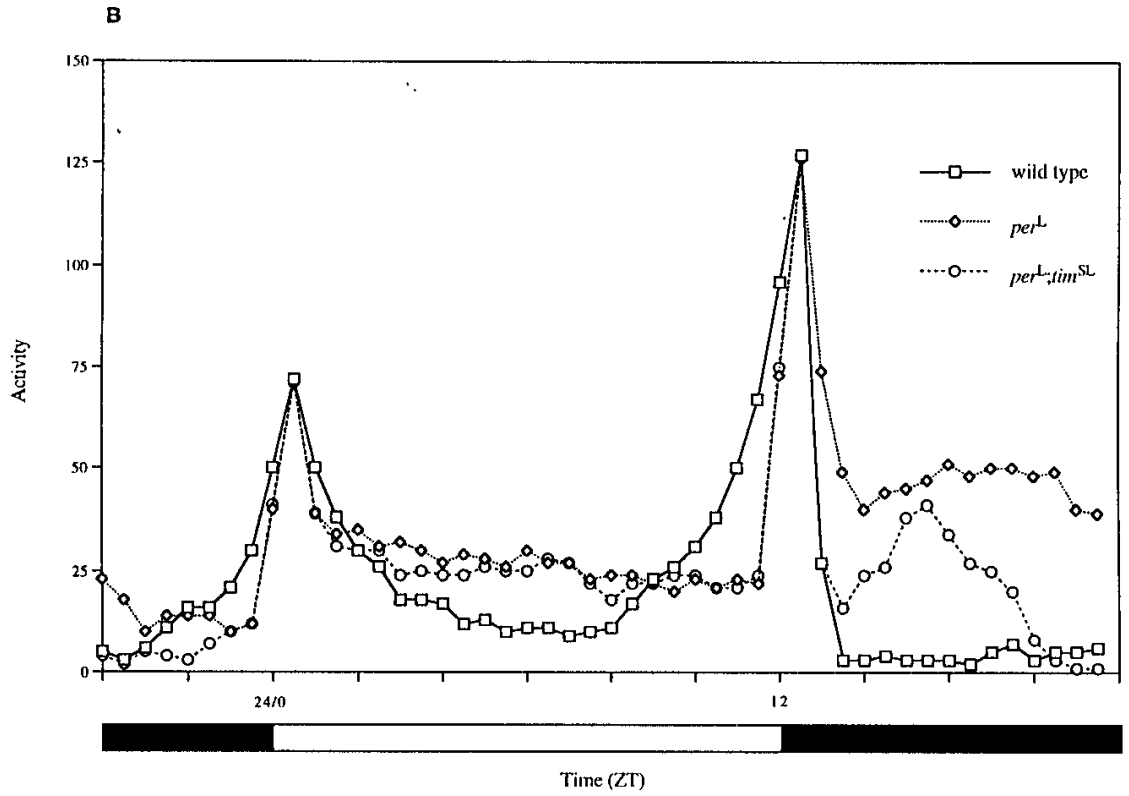

Figure 2b.

long-period and temperature compensation phenotypes, suggesting that these 2 aspects of tim^{SL} may be mechanistically linked.

DISCUSSION

In this article, we have exploited the tim^{SL} mutant to learn more about the relationship between *tim* gene expression and the circadian pacemaker. Despite the extensive evidence that *tim* has been correctly assigned, transgenic rescue with *tim* cDNA was not initially reported. Our results indicate that this cDNA does indeed rescue the arrhythmic tim^{0} mutation (Figure 1 and Table 1) and is therefore the correct reading frame of the gene responsible for the arrhythmic tim^{0} phenotype. We have not explored the transgenic phenotype in detail, and there could be small differences between the behavioral phenotypes of the rescued flies and those of wild-type flies. Therefore, we cannot conclude that the 4.2 kb of *tim* promoter used in the construct contains all relevant upstream regulatory information. We also cannot be certain that there is no *tim*-specific information downstream of the stop codon, as the construct used *per*-derived DNA on this side of the ORF. But rescue is robust, so much relevant *tim* coding and regulatory information is present in the construct. Similar results have been reported by Ousley et al. (1998).

The same conclusion can be drawn for the tim^{SL} construct, which generates a set of behaviors very similar to that of the original mutation. This makes it likely, if not certain, that the single amino acid change associated with that mutant gene is responsible for the tim^{SL} behavioral phenotypes—in particular, allele-specific suppression of the per^{L} phenotypic characteristics. Although we can only speculate about the direct molecular consequences of this *thr* → *ile* change at position 484, the previous characterization of this genotype provided 3 additional pieces of information (Rutila et al., 1996). The first suggested that this mutation does not directly alter the nature or stability of the TIM / PER-L interaction, at least in vitro. The second indicated that the mutation may directly affect TIM phosphorylation since hyperphosphorylation of TIM-SL during the late night was observed by Western

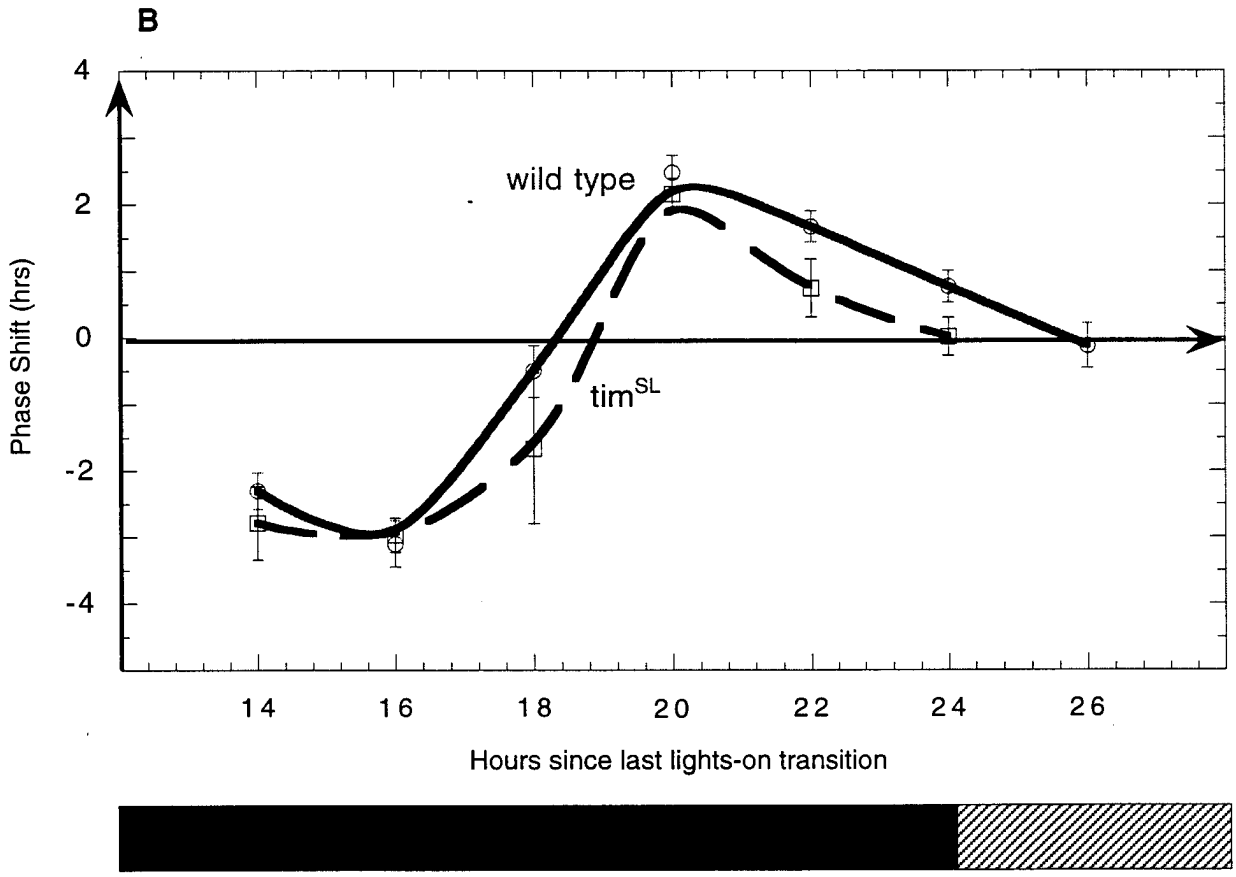

Figure 3a. Anchored phase response curves. The change in the activity phase of light-pulsed versus nonpulsed flies is plotted in hours (where positive values are phase advances and negative values are phase delays) against the time after the last lights on when the light pulse was administered. The bar below the graph indicates if the flies were in the dark phase of an LD cycle (solid bar) or in the next subjective day (hatched bar) when they were pulsed. Figure 3A looks at the phase response of wild type and per^{L}, while 3B plots wild type versus tim^{SL}, and 3C plots per^{L} versus per^{L};tim^{SL}. The number of flies tested (*N*) varied from 11 to 48 for each time point tested.

blotting. The third showed that when the tim^{SL} mutation is present, PER-L protein enters the nucleus ca. 2 h earlier, again in the mid-late night at ZT19. Given that the wild-type amino acid at position 484 is a threonine, the normal temporal program may require threonine phosphorylation at this position. In its absence, other phosphorylation events may be aberrant, perhaps more extensive and more rapid than what occurs on the wild-type protein. This could affect protein turnover and even lead to an aberrant TIM-PER interaction or to a PER nuclear entry phenotype as secondary consequences, especially since *thr*-484 is adjacent to a region in TIM that contains both a nuclear localization signal and a PER interacting domain (Saez and Young, 1996). Of course, it is not certain that altered phosphorylation causes these other phenotypes: for example, a direct effect on the timing of nuclear entry could cause a secondary effect on TIM phosphorylation (cf. Lee et al., 1996).

It is curious that the TIM-SL hyperphosphorylation is only apparent during the last half of the night, between approximately ZT18 and ZT24; the phosphorylation pattern during the first half of the night was essentially indistinguishable from that of the wild-type protein. The most interesting possibility was that the double-mutant flies are affected primarily during this restricted phase of the cycle and move 3 h more rapidly through the late night by comparison to single-mutant per^{L} flies (Figure 4). This is consistent with visualization of PER-L entering the nucleus ca. 2 h earlier at ZT21 in a TIM-SL-containing genotype. A number of alternative explanations are possible as well. These include the fact that the 3-h period change may reflect a more uniform advance during all phases

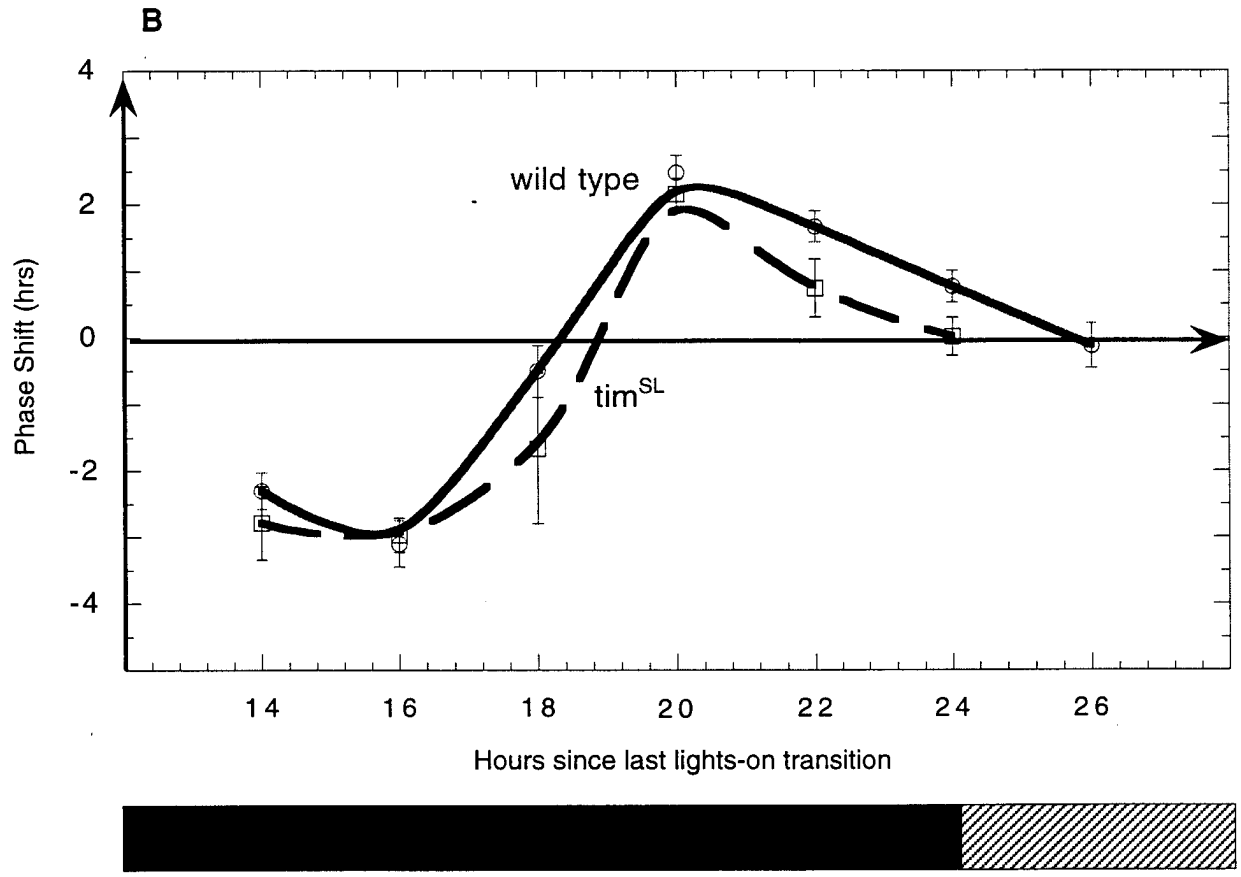

Figure 3b.

of the cycle, which is assumed when a mutant PRC is plotted on an expanded or contracted circadian time scale. In the case of a more uniform advance, the phosphorylation analysis by one-dimensional denaturing gels may fail to detect comparable phosphorylation changes at other times in the cycle that result in less dramatic mobility changes. Of course, it is also possible that the phosphorylation phenotype is unrelated to the 3-h shortened cycle of the double-mutant flies.

The biochemical alterations had been visualized during 12:12 LD cycles, which provide substantial temporal synchrony among different genotypes. This reflects the fact that many mutant *Drosophila* genotypes manifest 24-h periods under these environmental conditions (Saunders, 1982; Helfrich and Engelmann, 1987; Petersen et al., 1988; Hamblen-Coyle et al., 1989, 1992; Dushay et al., 1989, 1990). Therefore, our strategy was to address tim^{SL} clock progression with other assays based on a 12:12 LD cycle. To this end, we used the APRC protocol (Aschoff, 1965; Levine et al., 1994; Suri et al., 1998). This procedure anchors the flies to the light-dark cycle of the previous day and provides a tighter population distribution than seen with a more traditional PRC generated after several days in constant darkness (Saunders et al., 1994).

The wild-type APRC is very similar to what has been published previously (Saunders et al., 1994; Levine et al., 1994; Myers et al., 1996). As various rhythm mutant flies also manifest 24-h periods under these conditions, it was hoped that curves from 2 genotypes could be superimposed to provide some idea of when during the cycle one genotype progresses more rapidly or more slowly than another. Indeed, this type of analysis is useful for the wild-type versus tim^{SL} APRC's (Figure 3B). A careful comparison of these 2 curves shows that they are almost identical, although the trailing edge of the tim^{SL} advance zone does appear to return to baseline faster in the late night than that of wild-type flies, consistent with the late-night advance hypothesis. A wild-type versus per^{L} compari-

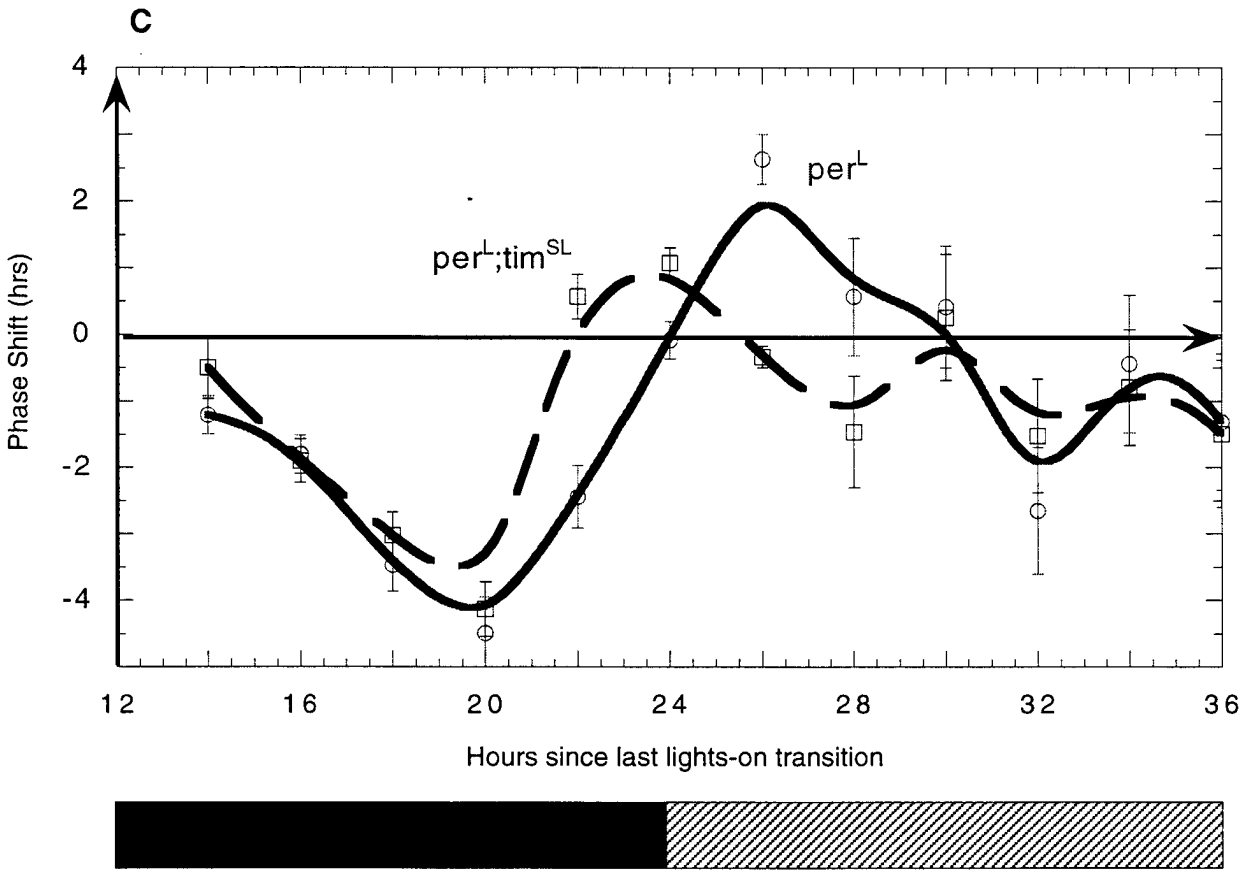

Figure 3c.

son, however, is more difficult. Since these 2 APRCs differ immediately after lights off at ZT12, *per*^{L} is already substantially delayed when the analysis begins.

The same problem does not pertain to the *per*^{L} versus *per*^{L};*tim*^{SL} APRC comparison. Although the free-running periods of these 2 genotypes differ by 3 h, their APRCs track very closely until ZT20, after which the double-mutant flies are clearly advanced relative to the single-mutant *per*^{L} flies. The advance appears to be 2 to 3 h in magnitude, which would localize much and perhaps all of the period shortening to this zone. These APRCs, and the previously mentioned change in the *tim*^{SL} versus wild-type curves, are consistent with the notion that *tim*^{SL} predominantly accelerates a restricted mid-late-night portion of the temporal program (Figure 4).

An enigmatic feature of *tim*^{SL} is that it also suppresses the temperature compensation defect of *per*^{L}. If *tim*^{SL} only affects a restricted portion of the cycle, then *per*^{L} may also have a nonuniform effect on the temporal program; much of the period increase versus temperature effect may also occur during the mid-late night. As *tim*^{SL} does not completely suppress the *per*^{L} period phenotype (double-mutant flies have a 25.5-h period, which is essentially unaffected by temperature) (Rutila et al., 1996), it suggests that the *per*^{L} phenotype is unexpectedly complex. There appears to be a 1.5-h component, which is temperature compensated, and a temperature-sensitive variable component, which becomes larger with increasing temperature. These 2 components could reflect different biochemical consequences of the *per*^{L} mutation, and the current data are insufficient to predict when during the circadian cycle the first component manifests an effect. Only the second component is predicted to occur in the mid-late night and to be suppressed by *tim*^{SL}. Unfortunately, it is not yet possible to address the temperature compensation issue in a more coherent fashion (cf. Huang et al., 1995; Gekakis et al., 1995).

This bipartite view of *per*^{L} and its interaction with *tim*^{SL} is also consistent with a casual inspection of the PRC amplitudes (the amplitude of the PRC is the magnitude of the phase shift that takes place in response to a light pulse). In the delay zone, the *per*^{L} PRC

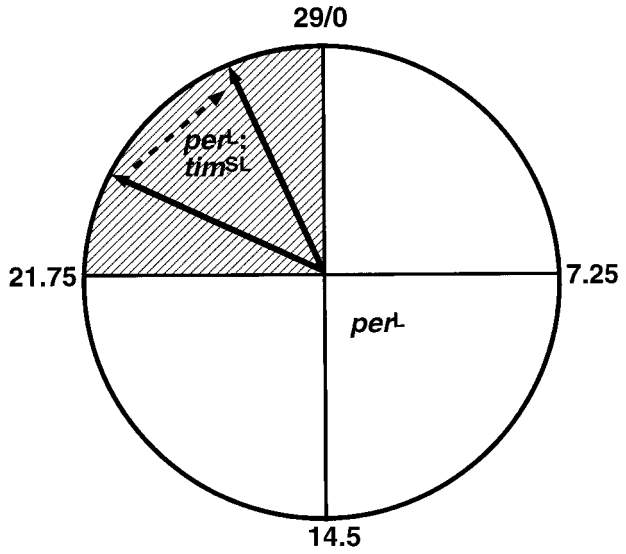

Figure 4. TIM-SL phosphorylation predominantly affects a portion of the cycle. In this model, the circadian cycle consists of subdomains with biochemically distinct rate-limiting steps. Either TIM or PER can be rate limiting at any point during the cycle. Here, the hyperphosphorylation of TIM-SL is resulting in clock advances of the *per*^{L} clock during a restricted portion of the cycle.

amplitude is enhanced relative to wild-type flies (Figure 3A) (Saunders et al., 1994). This value is not dramatically altered in the *per*^{L};*tim*^{SL} double-mutant flies (Figure 3C). But the magnitude of the phase shifts in the *per*^{L} advance zone are clearly suppressed in the *per*^{L};*tim*^{SL} double-mutant genotype. As the advance zone occurs in the mid-late night, this consideration of PRC amplitude complements the clock progression and period phenotypes. It strengthens the bipartite view of *per*^{L} and the late-night view of *tim*^{SL}. We note that *tim*^{SL} has additional effects on the circadian response to light (Suri et al., 1998).

Remarkably, the *tim*^{SL}-mediated late-night acceleration of *per*^{L} also fits with the other type of LD behavioral analysis, the average LD activity plots (Figure 2B). Only in the mid-late night, after ZT16, do the 2 curves diverge substantially. At these times, it is apparent that the evening activity peak is more short-lived in the case of the double-mutant flies: the activity offset occurs more rapidly in this genotype. This complicated evening activity profile explains why the phase calculations less clearly reflect the period differences between genotypes (Table 2). The timing of the activity change in the night roughly parallels the timing in the change of TIM-SL phosphorylation states previously seen in Western blots (Rutila et al., 1996). The activity decline is somewhat earlier than the PRC change or the phosphorylation change, which may simply reflect a more sensitive assay. We suggest that the TIM-SL late-night phase advance leads to a premature triggering of the activity offset, although we cannot exclude a more direct effect of TIM on activity offset. In any case, the observations suggest that aspects of the LD activity profile—in particular, the evening activity peak offset—is not only programmed by the clock but is also closely tied to specific features of the PER-TIM cycle.

These TIM-SL biochemical changes seen with *per*^{L} occur similarly in a *per* wild-type background, raising the question of why the period changes in this background are so modest compared to the *per*^{L} background (cf. Table 1). As described above, we cannot exclude the possibility that the period changes are unrelated to the TIM-SL phosphorylation and abundance changes and are in fact caused by some other aspect of TIM activity. But we prefer the interpretation that the very modest period effects in other genetic backgrounds reflect different biochemical steps that normally contribute to rate-limiting processes for clock movement (Figure 4). For example, if wild-type PER phosphorylation is normally an important rate-limiting event for clock progression during the mid-late night, it would reduce the magnitude of a TIM-SL-mediated advance in a *per* wild-type background. In a *per*^{L} background, however, the normally robust PER phosphorylation program has largely disappeared (Rutila et al., 1996), which may leave TIM phosphorylation as the major determinant of clock progression, at least during this phase of the cycle. A semidominant role of TIM-SL phosphorylation during this portion of the circadian cycle could also affect the *per*^{s} clock, resulting in the lengthened period seen in this background (Rutila et al., 1996).

We have previously proposed that *tim*^{SL} acts as a bypass suppressor of *per*^{L} (Rutila et al., 1996). The genetic formalism does not require a direct PER-TIM interaction, nor are PER and TIM required to participate directly in the events that determine the rate of clock movement. For example, they may affect the expression of other genes that are more directly engaged in the rate of clock movement. However, PER and TIM undergo robust oscillations in abundance and phosphorylation states, and the 2 proteins interact strongly. This suggests that phosphorylation may occur predominantly or exclusively within the heterodimer and that the rate and extent of phosphorylation may determine the rate of clock progression, at least during certain phases of the cycle. Moreover, it is attractive to ascribe the allele-specific effects to detailed aspects of protein-protein interactions (Rutila

et al., 1996). We therefore suggest that proper phosphorylation of the heterodimer is critical to detailed features of the timing program, especially in the mid-late night. The effects on PRC amplitude suggest that the heterodimer may also participate in aspects of the light response (Suri et al., 1998). Verification of either possibility will require a more detailed characterization of the mutant effects on phosphorylation.

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