Conserved Regions of the *timeless* (*tim*) Clock Gene in Drosophila Analyzed Through Phylogenetic and Functional Studies

Andrea Ousley, Khaja Zafarullah, Yifeng Chen, Mark Emerson, Lesley Hickman and Amita Sehgal*

*Howard Hughes Medical Institute, Department of Neuroscience, and *Center for Sleep and Respiratory Neurobiology, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104*

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ABSTRACT

Circadian (~24-hr) rhythms in *Drosophila melanogaster* depend upon cyclic expression of the *period* (*per*) and *timeless* (*tim*) genes, which encode interacting components of the endogenous clock. The *per* gene has been isolated from other insects and, more recently, a *per* ortholog was found in mammals where its expression oscillates in a circadian fashion. We report here the complete sequence of a *tim* gene from another species, *Drosophila virilis*. TIM is better conserved than the PER protein is between these two species (76 *vs.* 54% overall amino acid identity), and putative functional domains, such as the PER interaction domains and the nuclear localization signal, are highly conserved. The acidic domain and the cytoplasmic localization domain, however, are within the least conserved regions. In addition, the initiating methionine in the *D. virilis* gene lies downstream of the proposed translation start for the original *D. melanogaster tim* cDNA and corresponds to the one used by *D. simulans* and *D. yakuba*. Among the most conserved parts of TIM is a region of unknown function near the N terminus. We show here that deletion of a 32 amino acid segment within this region affects rescue of rhythms in arrhythmic tim^{01} flies. Flies carrying a full-length *tim* transgene displayed rhythms with ~24-hr periods, indicating that a fully functional clock can be restored in tim^{01} flies through expression of a *tim* transgene. Deletion of the segment mentioned above resulted in very long activity rhythms with periods ranging from 30.5 to 48 hr.

CIRCADIAN (~24-hr) rhythms are displayed by virtually all organisms, ranging from cyanobacteria to mammals (Dunlap *et al.* 1995; Hall 1995). Circadian rhythms, as distinguished from phenomena that are driven solely by cues in the environment, are generated by an internal time-keeping mechanism called the circadian clock. Because the circadian clock is normally synchronized with the environment, models of a circadian system include an input pathway that conveys information about the environment to the clock (Eskin 1979). Cues in the environment, such as light, reset the phase of the clock, thereby synchronizing the ~24-hr endogenous rhythm with the environmental cycle. The mechanism of resetting has been investigated in at least two systems, and in both, the level of a clock component changes in response to light, producing a predictable phase shift (Crosthwaite *et al.* 1995; Hunter-Ensor *et al.* 1996; Lee *et al.* 1996; Myers *et al.* 1996; Zeng *et al.* 1996). A circadian system also includes an output pathway that relays temporal information from the clock to other sites, effecting the circadian regulation of different physiological processes and behavioral activities (Eskin 1979).

Genetic analysis of circadian rhythms has identified four genes that encode clock components. These are the *timeless* (*tim*) and *period* (*per*) genes in Drosophila, and the *frequency* and *white collar-2* (a transcriptional activator) genes in Neurospora (Dunlap *et al.* 1995; Sehgal *et al.* 1996; Crosthwaite *et al.* 1997). Recently, two mammalian genes that function in the circadian system were isolated. The first, *Clock*, is the gene affected in a circadian mutant mouse, and it encodes a novel member of the bHLH-PAS family of transcription factors (King *et al.* 1997; Antoch *et al.* 1997). The second is a mammalian ortholog of the *per* gene (Sun *et al.* 1997; Tei *et al.* 1997).

Where characterized, the clock mechanism involves a molecular feedback loop. In Drosophila, RNA and protein levels of the *per* and *tim* genes cycle with an ~24-hr period, and the TIM and PER proteins affect expression of their own mRNAs through a feedback mechanism that requires nuclear entry of TIM and PER (Siwicki *et al.* 1988; Hardin *et al.* 1990; Zeng *et al.* 1994, 1996; Vosshall *et al.* 1994; Sehgal *et al.* 1995; Hunter-Ensor *et al.* 1996; Myers *et al.* 1996). Nuclear localization of TIM and PER is mediated, at least in part, by association of the two proteins with each other through known interaction domains (Vosshall *et al.* 1994; Gekakis *et al.* 1995; Saez and Young 1996). The Neurospora *frequency* gene operates in a similar autoregulatory feedback loop whereby both RNA and protein cycle and the protein inhibits synthesis of its own RNA (Aronson *et al.* 1994). Finally, while the role of

Corresponding author: Amita Sehgal, Department of Neuroscience, 233A Stemmler Hall, University of Pennsylvania, Philadelphia, PA 19104-6074. E-mail: amita@mail.med.upenn.edu

the mammalian *per* gene in the circadian system has not yet been established, it is known that levels of its RNA cycle in the suprachiasmatic nuclei and retina. Both these tissues contain endogenous oscillators (Tosini and Menaker 1996; Turek 1996).

A phylogenetic analysis carried out for *per* from a number of Drosophila species revealed that much of the coding region (approximately one-third) is not conserved (Colot *et al.* 1988; Thackeray and Kyriacou 1990). The *per* gene was also isolated from a non-Dipteran species, *Antheraea pernyi*, the giant silkmoth (Reppert *et al.* 1994). Although the giant silkmoth *per* gene is only 39% identical at the amino acid level to the most closely related Drosophila gene, it was able to rescue circadian rhythms in the arrhythmic per^{01} Drosophila mutant (Levine *et al.* 1995). Sequences conserved between the insect *per* genes, particularly those corresponding to the PAS domain, were used to isolate a mammalian *per* homolog (Tei *et al.* 1997). The same gene was isolated fortuitously by a group studying transcripts that mapped to human chromosome 17 (Sun *et al.* 1997). Surprisingly, given the low level of homology between the insect *per* genes, mammalian *per* is ~44% homologous to Drosophila *per* (including identical amino acids as well as conserved and neutral substitutions; Sun *et al.* 1997). Conserved regions of PER include all known functional domains, including the PAS domain and the cytoplasmic localization domain, both of which also mediate the interaction with TIM (Gekakis *et al.* 1995; Saez and Young 1996; Huang *et al.* 1993, 1995).

Since the cloning of the *tim* gene (Myers *et al.* 1995), no one has reported the sequence of a *tim* homolog. We undertook a phylogenetic analysis of *tim* by isolating and characterizing a *tim* homolog from *D. virilis*, and also by sequencing selected regions of a *tim* homolog from *D. hydei*. In this report, we present the intron/exon organization of the gene and the conservation profile of the TIM protein. We found that the amino acid sequence of TIM is more highly conserved overall than that of PER from different Drosophila species, and that most functional domains of TIM are conserved. In addition to known functional domains, such as the PER interaction domains and the nuclear localization signal (NLS), the conserved parts of TIM include an additional region that we show here to be important for function. *tim* transgenes lacking a 32 amino acid sequence from this region showed aberrant rescue of behavioral rhythms in tim^{01} flies, while a wild-type *tim* transgene restored completely normal behavior.

MATERIALS AND METHODS

Fly strains: The *D. hydei* flies were obtained from Carolina Biological Supply Co. (Burlington, NC), and the homozygous *yw*; *Ki* Δ*2-3* Drosophila strain was kindly provided by Paul Hardin (Texas A&M University, College Station, TX).

Library screening: The genomic library of *D. virilis* made in λ phage EMBL3 was kindly provided by R. K. Blackman (University of Illinois, Urbana, IL). This genomic library was screened with a 1.5-kb *Eco*RI fragment, which corresponds to nucleotides 1974–3492 of the *D. melanogaster tim* cDNA (Myers *et al.* 1995; EMBL/GenBank accession number U37018). Hybridization was performed under low stringency conditions (37°, 40% formamide, 6× SSPE, 0.5% SDS, 5× Denhardt's solution, and 0.1 mg/ml salmon sperm DNA). Washes were repeated twice at 50° in a 5× SSPE, 0.1% SDS solution. The library screen yielded two recombinant phage clones that were subjected to Southern blot analysis to identify hybridizing restriction fragments. An ~4-kb *Bam*HI fragment that showed strong hybridization to the *tim* probe was subcloned and sequenced in both directions. To extend this sequence in the 5′ and 3′ directions, we synthesized primers based on the sequence obtained and used these to sequence phage DNA isolated from our λ phage clones.

A recombinant phage clone containing the upstream sequence of the *D. melanogaster tim* gene was isolated from a genomic library made in EMBL3A (Spradling and Mahowald 1981) as described above, except that more stringent conditions were used for the hybridization.

RT-PCR experiments of *D. melanogaster tim* RNA: For the RT-PCR experiment, total RNA was isolated from fly heads by homogenizing them in 0.5 ml of buffer (150 mM sodium acetate, 50 mM Tris, pH 9.0, 5 mM EDTA, pH 8.0, 1% SDS containing 1/100th volume diethyl pyrocarbonate) followed by two phenol/chloroform (1:1) extractions and ethanol precipitation. RNA (in 10 mM dNTP mixture, 20 mM DTT, 1× reverse transciptase buffer containing 10 μM random hexamers and 2 units/μl RNasin) was heated at 65° for 5 min and then cooled to 42°. The reaction mixture was incubated with 1 μl of AMV-reverse transcriptase (10 units/μl, Promega, Madison, WI) for 1 hr at 42°, and the enzyme was then inactivated at 65°. Reverse-transcribed cDNA was amplified by PCR (in 2.5 mM $MgCl_2$, 1× PCR buffer, 2 mM dNTP mixture containing 5 pmol of each primer and 1 μl of Promega Taq polymerase) under the following conditions: 95°, 3 min; 30 cycles of 95°, 30 sec, 55°, 30 sec, 72°, 1 min; 72°, 10 min. A pair of specific primers, which amplifies a fragment corresponding to nucleotides 839–1501 of the original *tim* cDNA sequence (Myers *et al.* 1995; GenBank accession number U37018) and spans the 32 amino acid sequence (GenBank accession number AF038501; see results), was used to amplify this region from the reverse-transcribed cDNA and from the original 5′ *tim* cDNA clone.

3′ RACE: For the 3′ RACE experiment, total RNA was isolated and the reverse transcription was done as described above, except that an oligo-dT primer was used (5′-$[C]_{13}$ AAGC$[T]_{17}$-3′) and the reaction was carried out at 37°. Reverse-transcribed cDNA was amplified by PCR as described above, with some modifications. During the first round of amplification, a *D. virilis*-specific primer (5′-TTGGCTGCAGT TGGTCAT-3′; *D. virilis tim* sequence, GenBank accession numbers AF038502 and AF040096) and a shorter oligo-dT primer (5′-$[C]_8$ AAGC$[T]_8$-3′) were used, and the annealing temperature was 42° for the initial three cycles and 52° for the remaining 47 cycles. An aliquot of this PCR reaction was then reamplified by PCR using a second *D. virilis*-specific primer internal to the first (5′-ATGCGCAGCAAATGCAGCA-3′; *D. virilis tim* sequence, GenBank accession numbers AF038502 and AF040096) and the short oligo-dT primer. The conditions were the same as for the first round of amplification, except that the annealing temperature was 52° for all 40 cycles. The amplified fragments were cloned into the pCR2.1 Vector (Invitrogen, San Diego, CA) and sequenced as below.

Isolation of *D. hydei tim* sequences: Pairs of degenerate

primers were used to amplify, by PCR, regions of the *tim* gene from *D. hydei*. The primers were designed based on sequences within regions of the *tim* gene that were highly conserved between *D. melanogaster* and *D. virilis*. The primers were 1S (sense primer) (5′-AA(G/A) CCI CA(A/G) CA(T/C) CA(G/A) AA(G/A/) CC-3′), 4S (5′-GA(T/C) CA(G/A) AT(C/T/A) AA(C/T) AA(T/C) TG(T/C) CT-3′), 13S (5′-GA(C/T) ATG GA(A/G) CA(C/T/) AT(C/T/A/) GA(T/C/) AC-3′), 1A (antisense primer; the exact complement of 1S), and 12A (5′-TC(A/G) TA(G/A) TCI GC(T/C) TCC CA(A/G/T/) AT-3′). Advantage KlenTaq polymerase (Clontech Laboratories, Palo Alto, CA) was used to amplify the following *D. hydei* fragments. The primer pairs (13S, 12A) and 4S, 1A) amplified a fragment corresponding to nucleotides 2127–2749 and 74–1804 of the *D. melanogaster tim* cDNA (Myers *et al.* 1995; GenBank accession number U37018), respectively. The primer pair (1S, 12A) amplified an overlapping fragment corresponding to nucleotides 1785–2749 of the *D. melanogaster tim* cDNA. A pair of specific primers were made based on the *D. hydei* sequence to amplify a fragment spanning the 1S/1A primer region. PCR amplification using degenerate primers was done (in 1.5 mM $MgCl_2$, 1× PCR buffer, 0.4 mM dNTP mixture containing 2 pmol of each primer and 1 μl of Taq polymerase) under the following conditions: 95°, 3 min; 3 cycles of 95°, 1 min, 42°, 1 min, 72°, 2 min; 50 cycles of 95°, 1 min, 50°, 1 min, 72°, 2 min; 72°, 10 min. PCR amplification using specific primers was done as described above for 30 cycles, except for omitting cycles 2–4 at a lower annealing temperature. Each fragment was cloned into the pCR 2.1 Vector (Invitrogen) and sequenced as described below.

Sequencing and analysis: Sequencing was done at the University of Pennsylvania Sequencing Facility on an automated sequencer (Applied Biosystems, Foster City, CA) using a dye-terminator and thermal cycling method. The Sequencher program (version 3.0; Gene Codes Corporation, Ann Arbor, MI) was used to create the contigs of independent sequencing reactions. The intron/exon structure of the *D. virilis* gene was predicted based on its consensus 5′ and 3′ splice sites and by comparison with the *D. melanogaster tim* cDNA sequence (Myers *et al.* 1995). We used the DSPL, FGENED, and FEXD programs found on the Baylor College of Medicine Gene-finder site (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html) for prediction of intron/exon junctions, and the Gap and BestFit programs in the Genetics Computer Group (GCG) Wisconsin Software package (version 8.0.1-UNIX) was used for pairwise alignments. The PileUp program (GCG) was used to make multiple sequence alignments, and the amino acid sequence similarity plot was generated by the Plot-Similarity program (GCG).

Construction of plasmids: Plasmids for *P*-element-mediated DNA transformation were generated by fusing ~4.3 kb of sequence upstream of the transcription initiation site of the *D. melanogaster tim* gene to the *D. melanogaster tim* cDNA (Myers *et al.* 1995; see Figure 4 in this paper). Upstream sequences were isolated by screening a genomic library made in EMBL3A (Spradling and Mahowald 1981) with the 5′ region of genomic clone Ec1 (Myers *et al.* 1995). An ~7 kb *Sac*I-*Sac*I DNA fragment, which contained a *Sac*I-*Swa*I fragment extending from approximately −4300 to +180 relative to the transcription initiation site, was isolated from the phage clone.

A full-length *tim* cDNA was generated by piecing together fragments from partial *D. melanogaster* cDNA clones (35c and 35g; see Myers *et al.* 1995). Individual fragments were successively cloned into pBluescript: first, a *Bam*HI-*Apa*I fragment from the 3′ end, then a *Sac*I-*Bam*HI from the 5′ end, followed by the internal *Bam*HI-*Bam*HI fragment. The orientation of the *Bam*HI-*Bam*HI fragment was verified. The resulting cDNA insert extended from nucleotide positions 1–4955 of the original cDNA sequence and had a 3′ alternatively retained intron inserted after position 3555 (Myers *et al.* 1995; GenBank accession number U37018), but it did not include the additional 32 amino acid sequence reported in this paper (see results). This full-length cDNA was then excised using *Sac*I and *Kpn*I (polylinker sites) and cloned into the pet-17b vector to introduce a *Spe*I site at the 5′ end.

The ~4.5-kb *Sac*I-*Swa*I fragment containing the upstream sequence (see above) was substituted for the 180-bp *Sac*I-*Swa*I fragment in the *tim* cDNA pet-17b construct. A *Spe*I-*Kpn*I fragment derived from the resulting construct, which contained the upstream sequence fused to the *tim* cDNA, was then cloned into the *P* element vector pCaSper4, which contains a miniwhite gene p(*white*), thus enabling selection of transgenic flies by eye color. The resulting pCaSper4 construct was called Tim 1. Another pCaSper4 construct (Tim 4) was made, which was identical to Tim 1, except that it also contained the additional 32 amino acid sequence (see Figure 4). Since the 32 amino acid sequence was contained within a unique *Sph*I fragment in the *tim* gene, the *Sph*I fragment in the Tim 1 construct was replaced with the corresponding fragment from the genomic clone Ec1 (Myers *et al.* 1995). The resulting construct was called Tim 4.

Production of transgenic strains: These two constructs were introduced into the Drosophila genome by *P*-element-mediated DNA. Embryo injections were performed using a *yw/yw; Ki Δ2-3* strain (Robertson *et al.* 1988), and multiple

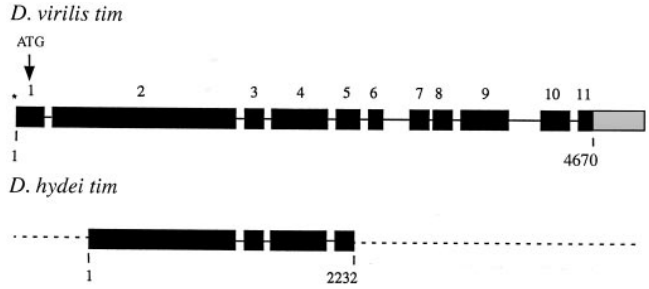
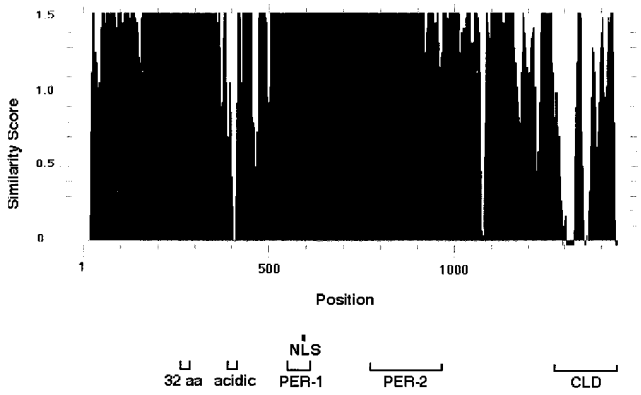


Figure 1.—Genomic organization of the *tim* gene. The intron–exon structure of the *D. virilis tim* gene and part of the *D. hydei tim* gene is shown schematically. The *D. virilis* genomic *tim* homolog (4682 bp) begins at a consensus cap site (asterisk), extends through a codon corresponding to amino acid 1194 of the *D. virilis* TIM sequence, and contains 11 exons (black boxes) and 10 introns (lines between boxes). The translation initiation site (ATG) is at nucleotide position 68. The shaded box represents the sequence obtained from the 3′ RACE experiment (see materials and methods), which codes for the C-terminal 149 amino acids missing from our genomic *tim* homolog. The *D. hydei* sequence (2232 bp) begins at a position corresponding to the middle of exon 2 and extends through most of exon 5. Introns 1–11 of the *D. virilis* gene occur immediately downstream of the following nucleotide positions, and their respective sizes (in base pairs) are shown in parentheses: intron 1, 164 (60); intron 2, 1722 (71); intron 3, 1935 (59); intron 4, 2503 (65); intron 5, 2767 (64); intron 6, 2959 (242); intron 7, 3355 (55); intron 8, 3563 (68); intron 9, 3992 (288); intron 10, 4521 (59). Introns in the *D. hydei* sequence corresponding to introns 2–4 of the *D. virilis* gene occur immediately downstream of the following nucleotide positions, and their respective sizes (in base pairs) are shown in parentheses: intron 2, 1208 (75); intron 3, 1425 (67); intron 4, 2000 (60). The *D. virilis* and *D. hydei tim* sequences have been deposited in GenBank under accession numbers AF038502 and AF040096, and AF038579, respectively.

A

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D. virilis          642 DLRPYLRRMH LVVTAIREFL QAIEAYNKVT HLSEDDRYRL RQLQLQISAT 691
D. melanogaster     698 ..K..... .......... ...DT..... ..N...KAH. ........EM 747
D. hydei            513 ..Q..... .......... .T..T....S .......L.. H......G.. 562
                                                                              [-

D. virilis          692 TDLRCLFVLL LRRFNPSIHS KQYLQDLVVT NHILLLILDN AAKLEGGQTI 741
D. melanogaster     748 S..... .......... .......... .........S S...G.C... 797
D. hydei            563 .......... ......R... .......... ....M....S .......... 612
                        ----------------------------------PER-2----------------------------------

D. virilis          742 GLSEHISQFA TLEVMHYYGI LLEDFSNNGE YVNDCIFTMM HHIGGDLDQV 791
D. melanogaster     798 R.....T... .......... .....N.... F......... .......G.I 847
D. hydei            613 .......... .......... .....D.... F......... .......G.I 662
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D. virilis          792 GVLYQPIILK AYSRIWEADY DICDDWSDLI EYVIHKFLNT PPKSPMAIPT 841
D. melanogaster     848 ...F...... T......... EL........ .......M.. .....LT... 897
D. hydei            663 ...F...... T...                                        676
                        ----------

D. virilis          842 ASLTELTKEH NQEHTACPWS QEDMDSLCWY FVQSKRQNDV IGNIAKLFSN 891
D. melanogaster     898 T....M.... .....V.S.. ..E..T.Y.. Y....KN..I V.K.V..... 947
                        ----------]

D. virilis          892 NGNKIKTRIS IIQQLLQQDI ITLLEYDDLM KFEDAEYQRT LLATPTSLTT 941
D. melanogaster     948 ....L..... .......... .......... .......... ..T....A.. 997

D. virilis          942 DSGIELKESA YGKPSDDVQV LLDLIRKENK SQHLVWLQKL LLECCYVKMM 991
D. melanogaster     989 E....I..C. .........I .....I.... A...L...RI .I...F..LT 1047

D. virilis          992 IKCGSCQTDV EPIMEPVVYH CMFKQKPIPV VQWNNEQSST MLYQPFMLLL 1041
D. melanogaster    1048 LRS.LKVPEG DH.....A.. .IC...S... ........T. ......V... 1097

D. virilis         1042 HKLGIQLPAD AGLIFARIPD FWTPETMYGL AKKLGPLDKL TLKFDPRDLE 1091
D. melanogaster    1098 .......... ..S....... Y......... .......... N....ASE.. 1147

D. virilis         1092 DA----PPSR HHTGARNSLS SISSLEADFG DSEGLALIPE VDPAVEKAAA 1137
D. melanogaster    1148 ..TASS.SRY ....P..... .V...DV.L. .T.E...... ..A.....H. 1197

D. virilis         1138 AASAIPNNEI FALPRVKHCN SIIRYTPDPT PPVPSWLQLV MRSKCSKRRS 1187
D. melanogaster    1198 M..TPSPS.. ..V.KT.... .......... ....N..... .....NH.TG 1247

D. virilis         1188 PATDASDCTS TSTMIADDEI KSYASMAQAS HTKLNAGYPT STLVCMNKLS 1237
D. melanogaster    1248 .SG.P...VG S.STTV...G FGKSIS.AT. QAASTSMSTV NPTTTLSLNM 1297
                                [---------------------------------- CLD ----------

D. virilis         1238 NCSFAAPPNE NSSSSGCGGT ASSMSMPNMP D-------GN S-------DA 1273
D. melanogaster    1298 LNT.MGSH.. .......... V..L..VALM STGAAGGG.. TSGLEMDV.. 1347
                        ----------

D. virilis         1274 LMKTSFERLA VTGARYLRPS NTDQDYSALV ASVYENE-FA NSDNVSVASD 1322
D. melanogaster    1348 S..S.....E .N.SHFS.AN .L..E...M. .....K.KEL ......L... 1397
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D. virilis         1323 LTRMYVSDED EKHELQLQQVE     1343
D. melanogaster    1398 .......... DRL.RTEIR.PHYH 1421
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B

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D. virilis          1  MDWLLATPQL QSVFSSLGSL VGGTYVVSPN ALAILEEINH KLTYEDQTLR  50
D. melanogaster    24  .......... Y.A.....C. E.D....N.. .........Y ..........  73

D. virilis         51  TFRRAIGFGQ NVRVDLIPLL ENAKDDAVLE SVIRILVNLT VPVECLFSVD 100
D. melanogaster    74  .......... ...S...... .......... .......... .......... 123

D. virilis        101  LMYRTEVGRH TIFELNKLLY NSKEAFTDPK STKSVVEYMK HILESDPKLS 150
D. melanogaster   124  V....D.... .......... T......EAR .......... .......... 173

D. virilis        151  PHKCDQINNC LLLLRNILHI PETHAHFLMP RLQPGSGHQV SMQNTILWNL 200
D. melanogaster   174  .......... .......... ......CV.. MM.-SMP.GI .......... 222
D. hydei.           1             ......... .......... V..SSG.... .......... 39

D. virilis        201  FIQSIDKLLL YLMTCPQRSL WGVTMVQLIA LIYKDQHVST LQKLLNLWFE 250
D. melanogaster   223  .......... ........AF .......... .......... .....S.... 272
D. hydei.          40  .......... ........A. .......... M.......N. .......... 89
                                                                  [----32 aa region------

D. virilis        251  ASLSESSDDN ESNTTPPKQA SGDSSPMLTS DPTSDSSDN- ----GSGGKK 295
D. melanogaster   273  .......E.. ....S....G .......... .........G SNGR.M..-- 320
D. hydei          90   .......E.. ....S...KG .......... .........G SNGG..S... 139
                       ------------------------------------]

D. virilis        296  ESCEERRQAL REGTDATLHE VSRKGHEYQN AMAS------ --SNAANYIL 337
D. melanogaster   321  --------GM ....A...Q. .....Q.... ...RVPADKP DG.EE.SDMT 362
D. hydei          140  .G.D...... ....E...Q. ......D... ...RVTADKP DI.EV.SDSF 189

D. virilis        338  ----EGPCSA QQPWS----- ---------- ---------- --DCEMQE-- 354
D. melanogaster   363  GNDS.Q.G.P E.SQPAGESM DDGDYEDQRH RQLNEHGEED EDED.VE.EE 412
D. hydei          190  ----.V...P ..HLNTEEAM DDIDY----- ---------- --EEQV..-- 216
                                              [--------------------acidic domain-------------------]

D. virilis        355  YKQMTAVI-S EPLNLS-QPA DNVNYTTNAN YARTTSTDIL TKT-----TS 397
D. melanogaster   413  .L.LGPA--. .....TQ... .K..N...-- ---------- -P.SSAPQGC 447
D. hydei          217  .E.EA.AVS. ......-... N........V ..S..A.ETQ .TSSLCAM.. 265

D. virilis        398  LKHEGFKPPA P---RRNTLS AILSDNY--- APLSFISAVK LGQKSPHAGQ 441
D. melanogaster   448  .GN.P....P .LPV.AS.SA HAQMQKFNES SYA.HV.... .......... 497
D. hydei          266  .CY.P..... .LPT...... EM.....--- TSH.HV.... .....S.... 312

D. virilis        442  LQLIKGKCCP QKRECPSSQS EHSDCGYGTQ MENPESISTS SNDDDGPQGK 491
D. melanogaster   498  ...T...... .......... .L........ V..Q...... .......... 547
D. hydei          313  ...T...... .......... .L.....A.. V..........  .......... 362
                                                    I (tim^SL)      [----------PER-1----

D. virilis        492  PQHQKPPCSS KHRSKQRIFA VPQDTKDLRR KKLVKRSKSS LINMKGLVLH 541
D. melanogaster   548  ........NT .P.N.P.TIM S.M.K.E... .......... ........Q. 597
D. hydei          363  ........NT .P.N...TLM S...K.E... .......... ........Q. 412
                       ----------------------------------------------------[.....NLS.....]-------------------------

D. virilis        542  TPNDDDISNL LKEFTVDFLL KGYNYLVEEL HSQLLSNAKM PIDTSHFFWL 591
D. melanogaster   598  ..T....... .......... ...S...... .M.......V .......... 647
D. hydei          413  ..TNY..... .......... .......... LK...TS..V L.......... 462
                       --------------------------]

D. virilis        592  VTFFLKFAAQ LELDMEHIDT ILTFDVLSFL TYEGVSLCEQ LELNARQEGA 641
D. melanogaster   648  ..Y....... .......... ...Y....Y. .......... .........S 697
D. hydei          463  ......L... .........S ...Y....Y. .......... .....Q...S 512
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Figure 2.—Profile of amino acid similarity between the *tim* homologs. (A) The amino acid similarity plot was generated from the multiple sequence alignment (shown in B) of the entire coding region of the *D. virilis* and *D. melanogaster* homologs, as well as specific regions of the *D. hydei tim* gene (see Figure 1), using the PlotSimilarity program (GCG). The total number of positions indicated on the x axis is extended to accommodate gaps in each of the three homologs. (B) The sequence of the *D. virilis tim* gene is shown. *D. melanogaster* and *D. hydei* residues are indicated only where they are different from *D. virilis*. Dots represent identical residues, and dashes correspond to gaps. The amino acid changed by the tim^{SL} mutation is indicated. The sequences were aligned using the PileUp program (GCG). The locations of the PER interaction domains (PER-1 and PER-2), the NLS, the CLD, and acidic region found in *D. melanogaster* are shown in both panels. Also indicated is a 32 amino acid sequence (32 aa) within the N-terminal conserved region, which we demonstrate to be important for function (see Figure 4). Note that the acidic region and the CLD are within the least conserved regions.

independent lines carrying each of the two transgenes were derived from the progeny of surviving G_0 adults. Each transgene was then introduced into a *yw tim*^{01} background.

Behavioral assays: Behavioral analysis was done on transgenic flies carrying only the *tim* transgene [p(*white*)*tim*^{01}/*tim*^{01}], on siblings that carried a wild-type copy of the *tim* gene (*CyO*/*tim*^{01}), on siblings that carried neither the endogenous *tim* gene nor the transgene (*tim*^{01}/*tim*^{01}), and on wild-type flies (*yw* strain). Locomotor activity rhythms were monitored using the Trikinetics system, and analysis was done exactly as described previously (Sehgal *et al.* 1994).

RESULTS

Cloning of a *tim* homolog from *D. virilis*: We screened a *D. virilis* genomic library under low stringency conditions using a *D. melanogaster tim* cDNA probe that corresponds to nucleotides 1974–3492 (Myers *et al.* 1995). This probe includes the PER interaction domains of the *tim* gene, which we assumed would be well-conserved because the TIM interaction domains of the per gene are well conserved between *D. melanogaster* and *D. virilis* (Colot *et al.* 1988). Two recombinant phage clones were isolated. An ~4-kb *Bam*HI fragment that strongly hybridized with the *tim* cDNA probe on Southern blots was subcloned and sequenced. To extend the sequence in the 5′ and 3′ directions, we synthesized primers based on the sequence obtained and used these to sequence phage DNA prepared from our clones.

Both genomic clones lacked sequences corresponding to the 3′ end of the *D. melanogaster tim* gene, which includes the cytoplasmic localization domain (CLD) of TIM (Saez and Young 1996). Since this was a known functional domain that was reported to be conserved in TIM (Saez and Young 1996), we used a 3′ RACE method to obtain sequences from the 3′ end of the *D. virilis tim* cDNA. By using a *D. virilis*-specific primer that overlapped the coding region in the 3′ end of our genomic clone and an oligo-dT primer, we amplified from *D. virilis* cDNA a fragment that included nucleotides homologous to the C-terminal 149 amino acids of TIM.

Genomic organization of *tim* homologs: We characterized >4 kb of *D. virilis* genomic sequence corresponding to the *tim* gene. The schematic in Figure 1 represents ~4.7 kb of genomic sequence from the *D. virilis* homolog beginning at a consensus cap site (Cherbas and Cherbas 1993) just upstream of a methionine codon and continuing through a codon corresponding to amino acid 1194 of the *D. virilis* TIM sequence. The C-terminal, 149 amino acid stretch obtained through the 3′ RACE experiment is also indicated. The total length of the coding region is 4029 bp, from which we predict a protein of 1343 amino acids.

The genomic organization in Figure 1 shows the intron exon structure of *tim*. The figure shows this organization for the *D. virilis tim* gene and for part of the *D. hydei tim* gene, which was subsequently isolated for comparison of specific sequences (see below). Our limited data on the organization of the *tim* gene in *D. melanogaster* and *D. hydei* indicate that, where examined, the position of introns within the gene is conserved. We found that the positions of introns corresponding to introns 2–4 of the *D. virilis* gene are conserved in *D. hydei* (see Figure 1). Also, the position of an intron corresponding to intron 1 in the *D. virilis* gene is conserved in *D. melanogaster*. The DNA sequence of introns was not conserved (data not shown).

The predicted translation start site in the *D. virilis* gene corresponds to a methionine downstream of the proposed start site in the *D. melanogaster* gene (Myers *et al.* 1995). This finding is consistent with a recent report that this methionine may, in fact, also be the start site in some strains of *D. melanogaster* as well as in other Drosophila species (Rosato *et al.* 1997). A polymorphism identified in the *tim* gene from certain *D. melanogaster* strains, as well as from *D. simulans* and *D. yakuba*, generates a premature stop codon shortly after the originally proposed site (Rosato *et al.* 1997). Use of the downstream start site would shorten the *D. melanogaster* TIM protein by 23 amino acids.

Conservation profile of the TIM protein: The amino acid similarity plot in Figure 2A shows the level of conservation across the TIM protein. The predicted amino acid sequences of the *tim* gene from different Drosophila species are aligned in Figure 2B, with domains relevant to our analysis of TIM indicated. We found that TIM is more highly conserved between *D. virilis* and *D. melanogaster* than PER is conserved between these two species, with TIM having a 76% overall amino acid identity compared with 54% overall identity for PER. In contrast to the *per* gene, which has five nonconserved domains interspersed within the relatively conserved portion of its coding region (Colot *et al.* 1988), the coding region of the *tim* gene has only two regions of low homology. The stretch of acidic amino acids found in *D. melanogaster* TIM (Myers *et al.* 1995) falls within one such region (Figure 2, A and B).

Most of the known functional domains in TIM are highly conserved. The NLS in *D. virilis* is identical to that in *D. melanogaster*, except for the conservative change of a glutamate to an aspartate. Relative to *D. melanogaster*, the PER interaction domains in *D. virilis* are 80–85% identical at the amino acid level, a level of conservation that is similar to that for the TIM interaction domains of *per* (Colot *et al.* 1988; Thackeray and Kyriacou 1990; Saez and Young 1996). The N-terminal region of *D. virilis tim* also shows a high degree of homology (90% identical) to the *D. melanogaster* gene although no functional domain of *tim* has been mapped to this region. Incidentally, a 32 amino acid sequence within this region was lacking in the original *tim* cDNA (see below). The CLD recently mapped to the C-terminal end of TIM (Saez and Young 1996), while retained, is one of the least conserved regions,

having only 56% amino acid identity between *D. virilis* and *D. melanogaster.*

Of note is that the single amino acid mutated in the tim^{SL} allele of *D. melanogaster* (Rutila *et al.* 1996), which occurs just upstream of the first PER interaction domain, is conserved in *D. virilis.* This allele contains a missense mutation that converts threonine 494 to an isoleucine. The mutation does not produce a dramatic phenotype by itself (the wild-type rhythm is shortened by ~0.5 hr in homozygous tim^{SL} lines), but it suppresses the per^{l} (long period) mutation, shortening the period by 4 hr and also restoring temperature compensation (Rutila *et al.* 1996).

Isolation of homologous sequences of the *tim* gene from *D. hydei*: Analysis of *D. virilis tim* provided some interesting revelations about *tim* structure. Most importantly, perhaps, it revealed that the acidic region in *D. melanogaster,* which was thought to be similar to activation domains of transcription factors (Myers *et al.* 1995), was not conserved, and that an additional 32 amino acid sequence was present within a highly conserved region. To determine the extent to which these findings were universally applicable, we amplified specific sequences of the *tim* gene from yet another Drosophila species *D. hydei*. The genomic *D. hydei* sequence analyzed here begins in the middle of exon 2 in the *D. virilis* gene and extends through most of exon 5 (see Figure 1). Protein-coding sequences within this region are represented in Figure 2B and were included in the profile analysis shown in Figure 2A. The following facts were confirmed by our analysis of the *D. hydei* sequence: (1) the acidic region is poorly conserved, (2) the region N-terminal to this is very well-conserved and includes the additional 32 amino acids, (3) the PER interaction domains are conserved, and (4) the amino acid mutated in the tim^{SL} allele is conserved.

The *tim* RNA in *D. melanogaster* contains an additional sequence: The *D. virilis* TIM sequence contained an extra 32 amino acids within the highly conserved N-terminal region of the protein (see Figure 2, A and B). Note that the *D. hydei tim* gene also contains the additional 32 amino acids; however, the original *D. melanogaster tim* cDNA that was characterized lacked this sequence (Myers *et al.* 1995). To determine whether this sequence was included in a differentially spliced form of the *tim* RNA, we carried out RT-PCR experiments. We used a pair of specific primers to amplify a fragment spanning the 32 amino acid region from head RNA of *D. melanogaster* (*yw* strain) and from the original 5′ *tim* cDNA clone (Myers *et al.* 1995). In Figure 3, lanes 1 and 2 (*yw* 14 and *yw* 16) show the fragment amplified by RT-PCR from head RNA collected at Zeitgeber times 14 and 16, when *tim* RNA is expressed at high levels (Sehgal *et al.* 1995). The RT-PCR product is of the size predicted (758 bp) if the fragment includes the additional 32 amino acids. The PCR product amplified from the *tim* cDNA clone (Figure 3, lane 3) is ~100 bp smaller than the RT-PCR product, as expected, since this cDNA clone lacks the 32 amino acid sequence. Other experiments in which control genomic fragments were also amplified confirmed that endogenous tim RNA was ~100 bp longer than the *tim* cDNA (data not shown). Since we failed to amplify a smaller band from head RNA in all experiments, it is unlikely that the 32 amino acid sequence is part of a differentially spliced message. The other possibility is that the shorter version represents a polymorphic form of the *tim* RNA that occurs in other strains. However, given that the 32 amino acid sequence is found in both *D. virilis* and *D. hydei* homologs of *tim* and that it is important for *tim* function (see below), we believe that this is un-

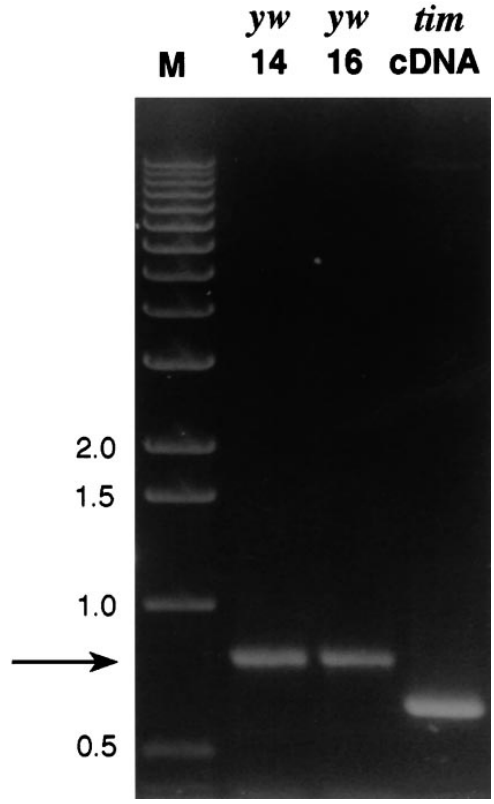

Figure 3.—Presence of an additional sequence in the *D. melanogaster tim* RNA. We amplified a region of the *tim* gene thought to include coding sequences that were lacking in the original *D. melanogaster tim* cDNA, but which were present in both the *D. virilis* and *D. hydei tim* genes (see Figure 2). Amplified products were resolved on a 1% agarose gel and stained with ethidium bromide. Locations of DNA size markers are indicated (far left). The same primer pair was used to amplify a fragment from *D. melanogaster* wild-type fly head RNA collected at Zeitgeber times 14 and 16 (lanes 1 and 2), and from the original *D. melanogaster tim* cDNA clone (lane 3; Myers *et al.* 1995). *yw* (*yellow white*) refers to the genotype of the Drosophila line used, which is wild type for rhythms. Note that the fragment amplified from fly head RNA is larger than the one amplified from the *tim* cDNA by ~100 bp, a size difference that corresponds to the additional 32 amino acids reported in Figure 2.

likely. Thus, we conclude that it is an integral part of the *D. melanogaster tim* RNA.

A protein of 1389 amino acids was predicted from the original sequence of the Drosophila *tim* gene (Myers *et al.* 1995). The predicted size becomes 1421 after including the additional 32 amino acids. Use of the methionine in *D. melanogaster* corresponding to the translation start we report here for *D. virilis* would shorten TIM by 23 amino acids (see also Rosato *et al.* 1997).

A *tim* transgene lacking a sequence in the N-terminal conserved region produces aberrant rhythms in *tim^{01}* flies: The functional significance of the conserved region containing the extra 32 amino acid sequence was determined through behavioral rescue experiments. Arrhythmic *tim^{01}* flies were transformed with a *tim* transgene that either contained (Tim 4) or lacked (Tim 1) the 32 amino acid sequence. Each transgene construct consisted of a *tim* cDNA from *D. melanogaster*, which included a 3′ alternatively retained intron (Myers *et al.* 1995) placed under the control of ~4.3 kb of sequence upstream of the transcription initiation site in the *D. melanogaster tim* gene (see materials and methods). Flies carrying either transgene were examined in constant darkness after entrainment in 12 hr: 12 hr light/dark cycles for several days. We evaluated two independent lines of flies carrying the Tim 4 transgene (see Table 1). In each of these lines, a high percentage of individual flies were rhythmic (72%), and the rhythmic individuals displayed wild-type periods. For the Tim 4-1 line, the average period was 23.7 ± 0.58, and for the other line, Tim 4-6, the average period was 23.4 ± 0.74. These periods are comparable to those seen in wild-type flies (see Table 1). As expected, *tim^{01}* siblings that lacked the transgene were arrhythmic. In Figure 4B, we show the locomotor activity plot and periodogram for a representative fly from one of these lines, Tim 4-1.

To determine whether the Tim 1 construct could rescue the arrhythmic phenotype, we evaluated four independent lines of *tim^{01}* flies carrying this transgene. In three of these lines, 27–40% of individual flies showed rescue of behavioral rhythms, but the periods were long, ranging from 30.5 to 48 hr; individual lines had average periods of 36.8–38.0 hr (see Table 1). In Figure 4B, we show the locomotor activity plot and periodogram for a representative fly from one of these lines, Tim 1-1. In a fourth line (Tim 1-6), only one of the individuals tested was rhythmic (Table 1). As before, flies carrying a wild-type copy of the *tim* gene (*CyO/tim^{01}* siblings or yw flies) were rhythmic with an average period of 23.6 ± 0.52 (see Table 1). The behavioral analysis of the Tim 1 and Tim 4 flies demonstrates that the 32 amino acid sequence in TIM is necessary for restoring wild-type rhythms to *tim^{01}* flies.

DISCUSSION

We report here the characterization of *tim* homologues from *D. virilis* and *D. hydei*. Our data show that the overall conservation of TIM is higher than that of PER. All known functional domains of TIM are highly conserved (>80% amino acid identity), with the exception of the CLD (Saez and Young 1996), which shows only 56% amino acid identity between *D. virilis* and *D. melanogaster*. The PER interaction domains in TIM show the same level of conservation as the TIM interaction domains in PER (Colot *et al.* 1988). We propose that since interaction between the PER and TIM proteins is an integral part of the clock mechanism, there is genetic selection to conserve the interacting domains. While the CLD, as currently defined, is less well conserved, there are regions within this domain that are identical between *D. virilis* and *D. melanogaster* (see

TABLE 1

Period Estimates and the Percentage of Rhythmic Flies for Different *tim* Transgenic Lines

Transgenic line	Number rhythmic	Number arrhythmic	Percent rhythmic	Average period	Range
Tim 1-1	50	74	40.3	37.9 ± 3.2	30.5–45.0
Tim 1-7	20	54	27.0	36.8 ± 3.1	33.5–47.0
Tim 1-13	21	44	37.5	38.0 ± 3.7	32.5–48.0
Tim 1-6	1	23		36.5	
Tim 4-1	30	11	73.2	23.7 ± 0.58	23.0–24.5
Tim 4-6	26	10	72.2	23.4 ± 0.74	22.5–25.5
Wild type[a]	61	13	78.7	23.6 ± 0.52	23.0–25.0

Locomotor activity data were obtained in constant darkness for flies from each Tim 1 and Tim 4 line (see text and Figure 4). Period analysis was done as described in Figure 4B. The number of flies that were rhythmic (and arrhythmic) for each line is shown, as well as the percentage of flies that were rhythmic. The average period (±SD) for rhythmic flies and the range in period are given in hours.

[a] Wild type indicates flies carrying a wild-type copy of the *tim* gene (*CyO/tim^{01}* siblings or *yw* flies; see materials and methods).

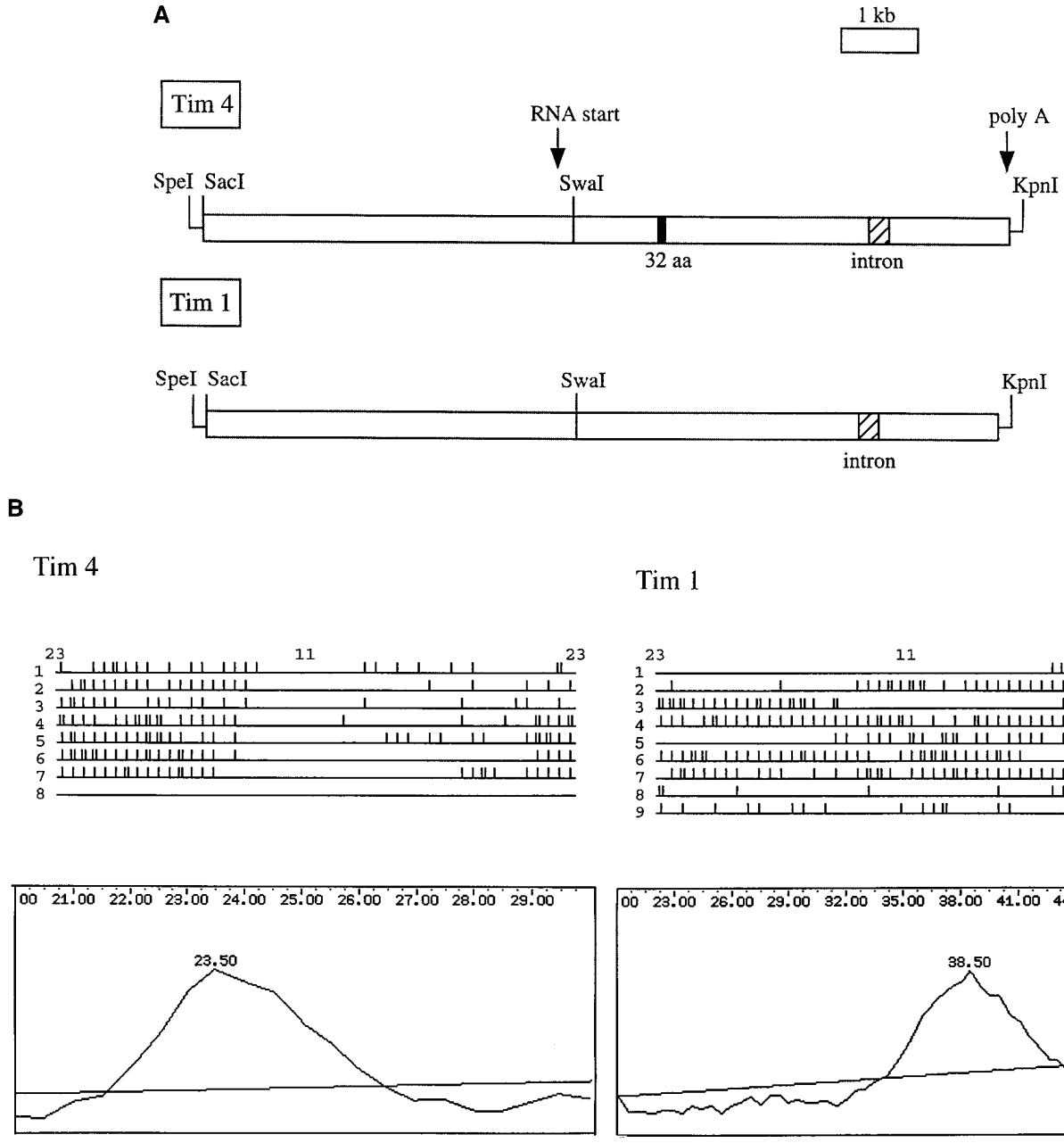

Figure 4.—Rescue of activity rhythms in *tim*^{01} flies by *tim* transgenes. (A) Schematic representation of the *tim* transgenes. The *tim* transgene constructs (Tim 4 and Tim 1) were made by ligating an upstream ~4.5-kb SacI-SwaI genomic fragment extending from approximately −4300 to + 180 relative to the *tim* transcription start site to a *tim* cDNA (Myers *et al.* 1995). The *tim* cDNA either included the additional 32 amino acid sequence reported in this paper (in Tim 4) or did not contain the sequence (in Tim 1). The *tim* cDNA also contained a 3′ alternatively retained intron (Myers *et al.* 1995). The *Spe*I-*Kpn*I (polylinker sites) fragments shown were cloned into pCaSper4. (B) Locomotor activity plots of *tim*^{01} flies carrying either of the two *tim* transgenes were obtained as follows. Flies were maintained in light/dark cycles (12 hr:12 hr) for 3 days and were subsequently monitored for 7–11 days in constant darkness. Activity records shown on the top are for representative flies from the Tim 1-1 and Tim 4-1 transgenic lines (see Table 1). The horizontal lines indicate successive 24-hr intervals (shown top to bottom for each record). The vertical lines indicate activity above a threshold value. The time of day is indicated at the top of each record in hours. Note that 11 days of data were obtained for the Tim 1-1 fly although the activity record only plots 9 days. The activity data were subjected to χ square periodogram analysis, and the corresponding periodogram for each activity record is shown at the bottom. For each periodogram, the lower limit of statistical significance ($P < 0.05$) is indicated by a sloping line. The Tim 1-1 fly showed rescue of rhythms with a long period (38.5 hr), while the Tim 4-1 fly showed wild-type rescue with a period of ~24 hr). See Table 1 for the average period for each individual line and the percentage of flies in each line that were rhythmic.

Figure 2B). We also report an additional highly conserved region close to the N terminus. Deletion of a 32 amino acid sequence within this region affects the function of a *tim* transgene.

This is the first report of rescue of the arrhythmic tim^{01} mutant phenotype using a *tim* transgene. The wild-type *tim* transgene, Tim 4, gave wild-type rescue (average period of ~24 hr) in a high percentage of individual flies. We also obtained full rescue using a *tim* construct in which almost all cDNA sequences (up to amino acid 1228) were replaced with corresponding genomic sequences. Full rescue of the tim^{01} phenotype contrasts with that often reported for the arrhythmic per^{01} phenotype. Rescue of per^{01} flies by genomic *per* constructs has usually resulted in periods that are somewhat longer than wild type. Best results are produced with a 13.2-kb *per* construct that includes ~4 kb of upstream sequences and generates periods of ~24.7 hr (Citri *et al.* 1987). However, fusion of a *per* cDNA to the same upstream sequence generated wild-type periods (average 23.8 in one line), suggesting inefficient processing of the genomic constructs (Citri *et al.* 1987). As mentioned above, we didn't see any difference in the behavioral rescues effected by a *tim* cDNA versus a *tim* genomic construct.

Behavioral analysis of arrhythmic tim^{01} flies carrying a *tim* transgene that either included (Tim 4) or excluded (Tim 1) the additional 32 amino acid sequence reported here demonstrated that this sequence is necessary for restoring wild-type rhythms to tim^{01} flies, and thus is likely to be part of an important functional domain. Deletion of this domain does not prevent rescue, as might occur if the protein were rendered nonfunctional through instability or incorrect folding, but it lengthens circadian period to levels rarely observed before. A high percentage of arrhythmia accompanies the long-period phenotype, which is also true, although to a lesser extent, of the per^{l} phenotype (unpublished observations). It may be the case that long periods are associated with arrhythmia, perhaps because of variable expressivity or low penetrance. Interestingly, when *Clock* mutant mice are monitored under freerunning conditions, they display long periods that eventually degenerate into arrhythmicity (Vitaterna *et al.* 1994).

Since *tim* was only recently isolated, previous structure-function studies focused on *per*, and it should be noted that mutagenesis of *per* does not always produce a phenotype. Deletion of the Gly-Thr repeat in PER has no observable effect on activity rhythms (Yu *et al.* 1987). Other mutations in *per*, such as those in the per^{s} domain (Baylies *et al.* 1992), or a 177-bp deletion within another conserved part of *per* generate short periods (A. Sehgal, unpublished results). Deletion of PER's NLS, on the other hand, lengthens circadian period, but not to the extent reported here for the Tim 1 construct (Baylies *et al.* 1992). As we learn more about the biochemical activities of these proteins, the function of specific domains and the effects of mutations in these domains will become clear.

Since *tim* is only the second Drosophila clock gene characterized, the phylogenetic conservation of this gene is encouraging. The *per* gene was found to be poorly conserved among Drosophila species, and yet the information gleaned from this analysis facilitated the isolation of additional homologs and will likely help the characterization of these homologs. Likewise, we believe that our analysis of *tim* will be useful for addressing clock mechanisms in other species. The Neurospora *frequency* gene has also been isolated from other species (Merrow and Dunlap 1994), and the mouse *Clock* gene appears to be conserved among vertebrates (King *et al.* 1997). Mutations affecting circadian rhythms have now been identified in several other organisms (Ralph and Menaker 1988; Kondo *et al.* 1994; Millar *et al.* 1995; Hicks *et al.* 1996), and the near future will likely see the isolation of new clock genes or homologs of those already known.

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