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A Mutant *Drosophila* Homolog of Mammalian *Clock* Disrupts Circadian Rhythms and Transcription of *period* and *timeless*

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Summary

We report the identification, characterization, and cloning of a novel *Drosophila* circadian rhythm gene, *dClock*. The mutant, initially called *Jrk*, manifests dominant effects: heterozygous flies have a period alteration and half are arrhythmic, while homozygous flies are uniformly arrhythmic. Furthermore, these flies express low levels of the two clock proteins, PERIOD (PER) and TIMELESS (TIM), due to low *per* and *tim* transcription. Mapping and cloning of the *Jrk* gene indicates that it encodes the *Drosophila* homolog of mouse *Clock*. The mutant phenotype results from a premature stop codon that eliminates much of the putative activation domain of this bHLH-PAS transcription factor, thus explaining the dominant features of *Jrk*. The remarkable sequence conservation strongly supports common clock components present in the common ancestor of *Drosophila* and mammals.

Introduction

Circadian (~24 hr) rhythms are characteristic features of diverse life forms, from prokaryotes to humans. These rhythms are manifest in biochemical, physiological, or behavioral oscillations and are driven by self-sustaining pacemakers, as they persist in the absence of environmental time cues. To maintain synchrony with the 24 hr environmental cycle, organisms respond to environmental cues, notably light, to reset or entrain their circadian rhythms. The presence of robust rhythms in individual eukaryotic cells and in unicellular organisms suggests that most circadian pacemakers are fundamentally intracellular machines (Robertson and Takshashi, 1988; Feldman and Dunlap, 1989; Kondo et al., 1993; Welsh et al., 1995). The molecular underpinnings of these oscillators have been revealed primarily by genetic analysis in *Drosophila* (Konopka and Benzer, 1971), *Neurospora* (Feldman and Hoyle, 1973), photosynthetic bacteria (Kondo et al., 1994), and most recently mice (Vitaterna et al., 1994). In all four systems, oscillations in the transcription of specific clock genes appear to play a central role in the generation and maintenance of circadian rhythms (Hardin et al., 1990, 1992; Kondo et al., 1993; Aronson et al., 1994; Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997).

In *Drosophila*, there are two well-characterized clock genes: *period* (*per*) and *timeless* (*tim*). Protein levels, RNA levels, and transcription rates of these two genes undergo robust circadian oscillations (Zerr et al., 1990; Hardin et al., 1990, 1992; Hardin, 1994; Sehgal et al., 1995; So and Rosbash, 1997). In addition, mutations in the two proteins (PER and TIM) alter or abolish the periodicity and phase of these rhythms, demonstrating that both proteins regulate their own transcription (Hardin et al., 1990; Sehgal et al., 1995; Marrus et al., 1996). Although there is no evidence indicating that the effects on transcription are direct, PER contains a PAS domain, which has been shown to mediate interactions between transcription factors (Huang et al., 1993; Lindebro et al., 1995). Most of these PAS-containing transcription factors also contain the well-characterized basic helix-loop-helix (bHLH) DNA-binding domains (Crews, 1998). In contrast, PER lacks any known DNA-binding domain, and there is no evidence that PER interacts directly with DNA. It was therefore proposed that PER regulates transcription by interacting with DNA-binding transcription factors of the bHLH-PAS family.

Recent data have extended this model. First, Hardin and colleagues identified an enhancer in the *per* promoter capable of driving cycling transcription of a reporter gene (Hao et al., 1997). Notably, the activity of this 69–base pair element requires an E box (CACGTG), a known binding site for some bHLH transcription factors including bHLH-PAS transcription factors (Murre et al., 1989; Swanson et al., 1995; Crews, 1998). Second, the cloning of the mouse circadian rhythm gene, *mClock*, revealed that it encodes a bHLH-PAS transcription factor. Recently, mouse *per* genes have been identified and found to undergo circadian oscillation in mammalian clock tissues (Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997). Thus, mCLOCK may drive the cycling transcription of *mper* genes through evolutionarily conserved E box elements in *mper* promoters. If so, one might expect to find *Drosophila* orthologs of *mClock*, which drive cycling of the *Drosophila per* gene.

We identified a novel arrhythmic *Drosophila* mutant, *Jrk*, which severely disrupts cycling transcription of the *per* and *tim* genes. The cloning and identification of the *Jrk* gene revealed that it is the apparent homolog of the mouse *Clock* gene; we therefore named the fly gene *dClock* (*Clk*). The mutation in *Clk* results in a premature stop codon that truncates the protein, deleting most of the putative C-terminal activation domain. This truncation is consistent with the semidominant mutant phenotype, similar to the original *mClock* mutant. Further characterization of the *Clk* mutant phenotype and recent data from elsewhere (Hogenesch et al., 1998) suggest that the wild-type *Drosophila* protein (CLK) interacts directly with the *per* and *tim* E boxes and makes a major contribution to the circadian transcription of clock genes. The similar mouse mutant phenotype and the remarkable sequence conservation strongly support the presence of similar clock mechanisms and components

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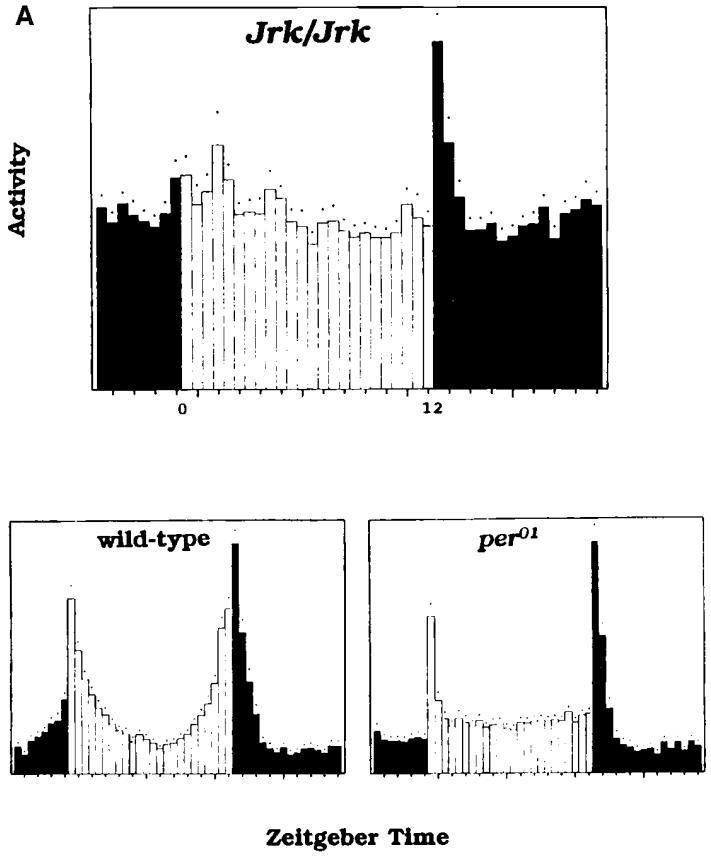


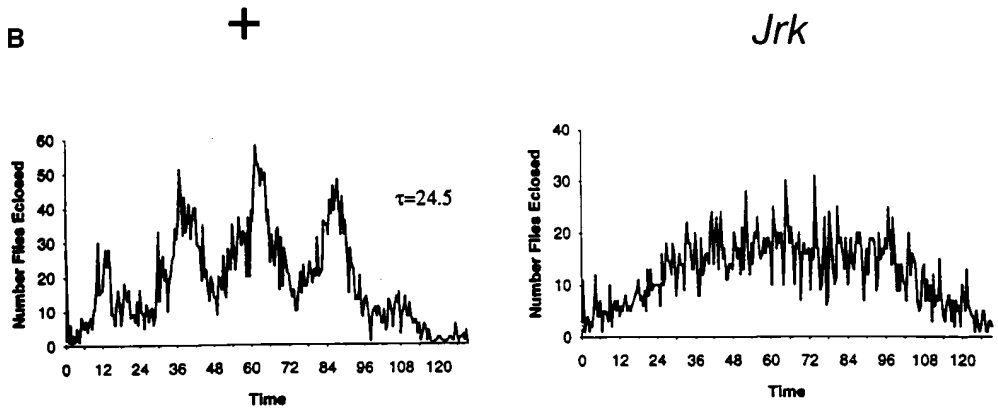


Figure 1. *Jrk* Abolishes the Lights-On Activity Response (A) and *Jrk* Is Arrhythmic for Eclosion (B)

(A) On the Y-axis are plotted internally normalized activity averages of a group of individual flies counted over 30 min intervals (cf. Hamblen-Coyle et al., 1989). On the X-axis is plotted Zeitgeber time in a 12 hr light:12 hr dark (LD) cycle. The Y-axis is dimensionless, owing to the inter-fly normalizations. Closed dark bars indicate locomotor activity levels when lights were off. Open white bars indicate behavior when lights were on. Wild-type indicates flies from a Canton-S (CS) wild-type stock. Data represent averages of 36, 39, and 65 flies for CS, per^{01}, and *Jrk* flies. Though *Jrk* flies on average increase activity in response to lights-off, more than half of individuals do not respond at all.

(B) Data from either four (wild-type) or three (*Jrk*) eclosion runs, performed at 25°C under constant DD conditions, were combined and analyzed using the same parameters mentioned above for locomotor testing. The total number of wild-type flies eclosed was 4695; total number of *Jrk*, 3199. The criteria used in considering a strain rhythmic were different from those used during analysis for locomotor rhythms. Specifically,

Table 1. Behavior of *Jrk* in LD and DD

Genotype	Period ± SEM[a]	% AR	N[b]
LD			
+	24.3 ± 0.0	0	28
Jrk/+	24.3 ± 0.1	36	22
Jrk/*Jrk*	24.2 ± 0.1	86	29
DD			
+	24.0 ± 0.0	7	62
Jrk/+	24.8 ± 0.1	47	55
Jrk/*Jrk*	AR	99[c]	118

[a] Period of activity in constant darkness, given in hours ± SEM.
[b] Number of animals tested.
[c] Experimental Procedures.
AR, arrhythmic.

in the common ancestor of *Drosophila* and mammals more than 500 million years ago.

Results

Jrk Is a Novel Arrhythmic Mutant, Abolishes PER and TIM Cycling, and Markedly Reduces Protein Levels

To identify novel genes involved in *Drosophila* circadian rhythms, we screened chemically mutagenized flies for mutants that alter or abolish circadian locomotor activity rhythms. Specifically, treated sibs were mated to homozygose third chromosomes, permitting the identification of recessive mutations on this chromosome (see Experimental Procedures). More than 6000 lines were screened, approximately 50% of which were free of recessive lethal mutations on the third chromosome; the remainder of the lines were tested as heterozygotes.

In this paper, we describe one novel semidominant third chromosome arrhythmic mutant, initially called *Jrk*. *Jrk* was identified as a homozygous mutant with completely arrhythmic locomotor behavior in constant darkness (DD; Table 1). As heterozygotes, approximately half of all *Jrk* flies were arrhythmic, and those flies that did manifest a rhythm had a slightly longer period than wild-type controls (Table 1). In addition to abolishing rhythmicity under free running conditions, homozygous *Jrk* flies manifested unusual locomotor activity even under 12 hr light:12 hr dark (LD) conditions (Figure 1). First, the percentage of flies with a measurable rhythm in LD fell from 64% for heterozygotes to only 14% for *Jrk* homozygotes (Table 1). Although examination of raw activity counts indicates that wild-type, per^{01}, and *Jrk* flies have similar levels of activity (data not shown), their diurnal activity patterns (Figure 1) differ. Under these standard LD conditions, wild-type flies anticipate the lights on and lights off transitions by slowly increasing activity (Wheeler et al., 1993), reflecting internal clock function. Thus, it is not surprising that arrhythmic *Jrk* flies do not anticipate the L-to-D or D-to-L transitions. But wild-type flies as well as per^{01} and tim^{01} (V. Suri, unpublished data) also react to the light transitions with an acute activity burst that quickly dissipates (Figure 1A). Like wild-type flies, some *Jrk* flies still react to the lights-off transition, but they all fail to react to the lights-on transition (Figure 1A). Optomotor testing of *Jrk* flies revealed no abnormality of the conventional visual system (data not shown, see Experimental Procedures), suggesting that the mutation may also impair some feature of the circadian visual response.

Although *Jrk* flies displayed no obvious morphologic or general behavioral defects, the absence of rhythms could be due to effects on the locomotor output pathway rather than on the clock itself. To address this question, we first assayed an independent manifestation of circadian output, adult emergence, and found that homozygous *Jrk* flies were also arrhythmic for this eclosion phenotype (Figure 1B). To measure more directly clock function, we assayed the fluctuations of the clock proteins, PERIOD (PER) and TIMELESS (TIM) in wild-type, heterozygous, and homozygous *Jrk* flies under LD conditions (Figures 2A and 2B). We observed that both PER and TIM levels are extremely low and noncycling in homozygous *Jrk* flies, approximately equivalent to trough levels of wild-type flies. PER and TIM cycle well in *Jrk* heterozygotes (Figures 2A and 2B), but the amplitude is reduced approximately 50%, consistent with the clear effects on behavioral rhythmicity in these flies.

Low PER and TIM Levels Are Due to Low Transcription Rates

The low PER and TIM levels in *Jrk* flies could be due to reduced protein stability or to reduced protein synthesis in the mutant strains. To distinguish between these possibilities, we measured *per* and *tim* RNA levels. The RNase protection assays revealed low and noncycling RNA levels, suggesting reduced synthesis rather than stability (Figure 3A). Consistent with this notion, *Jrk* heterozygotes had a low amplitude of RNA cycling, which parallels the reduced amplitude of the protein rhythms and semidominance of the behavioral rhythm defect. To measure transcription rates directly, we performed nuclear run-on assays in homozygous *Jrk* flies. We found that *per* and *tim* transcription rates were temporally constant and approximately equal to the very low trough levels of wild-type flies (Figure 3B). We conclude that the behavioral arrhythmicity of *Jrk* is largely due to a defect in the transcription of clock genes, including *per* and *tim*.

To identify specific sequence elements mediating the mutant effects on transcription, we assayed the activity of a *per* promoter-luciferase fusion in transgenic flies (Figure 3C). Promoter activity was consistent with nuclear run-on assays, namely, it was low and noncycling in the homozygous *Jrk* flies and cycled with a reduced amplitude in heterozygous *Jrk* flies. This intermediate

lines were considered rhythmic if chi-square periodogram ($\alpha = 0.01$) gave a result with a power greater than 10 (cf. Ewer et al., 1992) and a periodogram peak spanning three 30 min bins at the 1% significance line (cf. Liu et al., 1991). Wild-type flies manifest a period ($\alpha = 0.01$) of 24.5 hr, with a power of 23.5 and a width of 8 (cf. Ewer et al., 1992). *Jrk* did not show any rhythms judged significant by chi-square periodogram analysis.

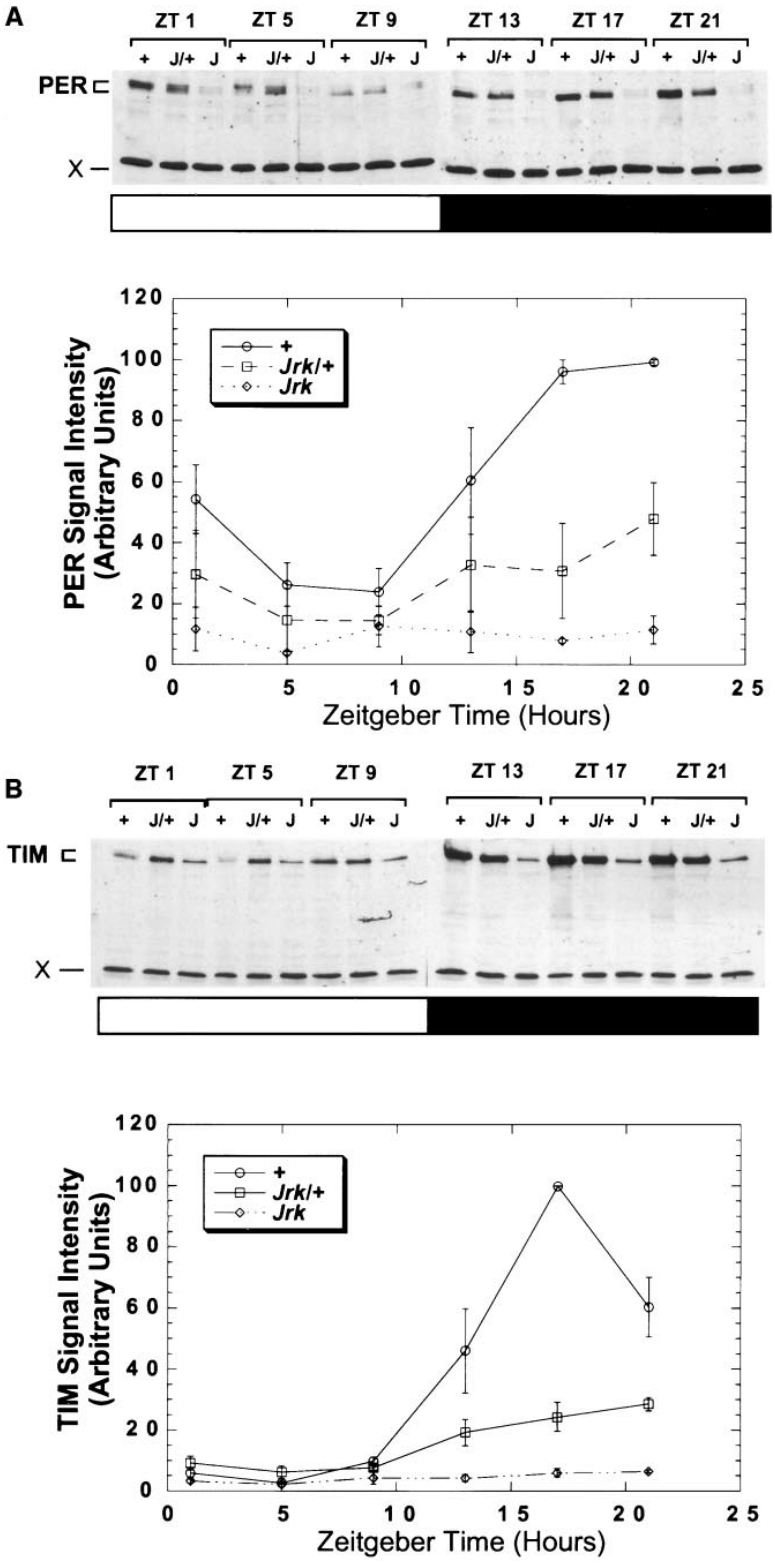


Figure 2. PERIOD and TIMELESS Protein Expressions Are Low in *Jrk*

ZT indicates Zeitgeber time for which lights-on occurs at ZT 0 and lights-off occurs at ZT 12. +, Jrk^{+} flies; J/+, *Jrk* heterozygotes; and J, *Jrk* homozygotes. X, cross-reacting band observed and unaffected in null per^{01} and tim^{01} extracts. On the graph, PER/TIM signal intensity is quantified relative to the cross-reacting band (X) and then normalized to the peak time point (=100). Intensity is plotted versus Zeitgeber time with error bars indicating the standard error of the mean. Each point represents the average of three experiments of which the blot above is representative.

rhythmicity was observed in all *Jrk*/+ individuals (data not shown). Thus, the dominant effects of *Jrk* on the molecular phenotype are fully penetrant, unlike the behavioral phenotype.

Jrk Maps to the Left Arm of the Third Chromosome at 66A

As *Jrk* has dramatic and specific effects on *per* and *tim* transcription, we sought to determine the nature of the

Table 2. Complementation Testing of *Jrk* with Various Deletions in the Third Chromosome Interval 65F–66B

Genotype	Period ± SEM[a]	% AR	N[b]
+/*pbl-X1*	25.6 ± 0.2	21	14
Jrk/pbl-X1	AR	100	7
+/*RM5-2*	25.7 ± 0.2	29	7
Jrk/RM5-2	AR	100	8
+/*D1*	25.9 ± 0.2	25	8
Jrk/D1	AR	100	6
+/*D2*	24.3 ± 0.2	16	16
Jrk/D2	25.9 ± 0.4	43	7

[a] Period of activity in constant darkness, given in hours ± the standard error of the mean.
[b] Number of animals tested.
AR, arrhythmic.

wild-type gene product. Meiotic mapping with morphologic markers indicated that *Jrk* was on the left arm of chromosome *3* near *hairy* (*h* is at map position, 26.0; Figure 4A, see Experimental Procedures). Initial complementation testing with deletions in this region revealed that only one, *Df(3L)pbl-X1*, completely uncovered the *Jrk* arrhythmic phenotype (Figure 4A). In addition, this deletion (which is homozygous lethal) manifested a long period phenotype (25.5–26.0 hr) as a heterozygote, indicating that a rhythm-relevant gene mapped within this region (Table 2). We further defined the cytogenetic interval by complementation testing with several smaller deletions. A lengthened period was apparent only in heterozygous deletion strains that also uncovered the *Jrk* phenotype. As a result of complementation testing (see Figure 4A legend), we refined the location of *Jrk* to 66A10–22. We were unable to generate a *Jrk* null strain as all deletions that failed to complement *Jrk* were homozygous lethal and lethal when heterozygous with each other.

Because of the semidominance of the *Jrk* behavioral phenotype and the general weakness of many deletion stocks, we verified our behavioral results by analyzing PER and TIM expression (Figure 4B). We observed that the heterozygous deletion *Df(3L)D1* still manifested TIM protein cycling. However, when heterozygous with *Jrk*, TIM levels failed to cycle and were comparable to those of *Jrk* homozygotes, i.e., a clear failure to complement. We observed similar results (low constitutive levels) when these blots were reprobed with anti-PER antibodies (data not shown).

Jrk Encodes *dClock*, a Homolog of *mClock*

Since *Jrk* is defective in clock gene transcription, a key clock promoter element is an E box, and a mouse circadian rhythm gene encodes a bHLH-PAS transcription factor, we reasoned that a candidate gene for *Jrk* is the *Drosophila* homolog of the mouse *Clock* gene. A search of the *Drosophila* expressed sequence tag (EST) database identified an EST, HLO4223 (Berkeley Drosophila Genome Project/HHMI EST Project, unpublished), with significant sequence identity to the PAS B region of *mClock*. As we had already obtained several P1 genomic clones corresponding to the 66A10–22 cytogenetic interval (Hartl et al., 1994; Kimmerly et al., 1996; Berkeley Drosophila Genome Project, personal communication), we performed PCR with EST-specific primers and obtained specific amplification from one P1 clone (DS07797). We confirmed the cytological location of 66A by in situ hybridization using a probe derived from DS07797 (data not shown).

The EST matched well the *mClock* sequence but was missing canonical bHLH and PAS A regions. We interpreted the absence to indicate that the clone was not full length. Sequencing of multiple cDNAs (see legend to Figure 5) indicated one ORF, which we call *dClock*. The ORF has at least two forms: one corresponds to the full-length protein of 1015 amino acids and the other to a protein missing the bHLH and PAS A regions. Preliminary Northern analysis shows several transcript forms, none of which demonstrate robust circadian oscillations (L. Sarov-Blat and R.A., unpublished observations). The full-length *dClock* protein contains all of the known subregions of *mClock*, including bHLH, PAS A, PAS B, and prominent Q-rich activation domain (Figure 6). Within a *dClock* intron, we also found a second ORF with significant similarity to the mammalian gene *EB1*, implicated in binding the adenomatosis polyposis coli (APC) C terminus (Figure 5A) (Su et al., 1995).

To determine if *Jrk* is mutated in *dClock*, we sequenced both strands of PCR amplification products of *dClock*-encoding *Jrk* genomic DNA. As our initial sequences (cDNA and P1-derived) were generated from strains with different genetic backgrounds, base differences could be mutagen-induced or polymorphic. Thus, where we identified mismatches, we sequenced these regions of *Clk* from a Jrk^+ line derived from the same mutagenesis screen (a sib strain from the same parental stock). This analysis identified a single base change, a C–T transition characteristic of EMS-induced mutations. Sequencing of this region from two additional Jrk^+ lines confirmed this result. The resulting base pair substitution changes a glutamine (776) to a stop codon (Figures 5B, 5C, and 6). The predicted protein is missing the C-terminal 1/4 of the protein, including most of the glutamines (Qs). Thus, like mouse *Clock*, *Jrk* results in a deletion of part of the transcriptional activation domain, resulting in a semidominant behavioral phenotype. Based on all of these results, we propose that *dClock* plays a crucial role in the circadian system of *Drosophila* and regulates the circadian transcription of *per* and *tim* (Figure 7).

Discussion

The cloning of the novel *Drosophila* gene defined by the *Jrk* mutation has identified a likely *Drosophila* homolog of the mouse gene *Clock*. Though we have yet to demonstrate transgenic rescue of *Jrk* with *dClock*, the finding that both *Jrk* and *dClock* map to 66A as well as the premature stop codon in *dClock* in *Jrk* flies strongly suggests that *dClock* is the *Jrk*-defined gene. Furthermore, the semidominant behavioral and transcriptional defects in *Jrk* are perfectly consistent with the significant deletion of the putative transcriptional activation domain of dCLOCK found in *Jrk* flies. dCLOCK shows impressive amino acid sequence identity with mCLOCK and both proteins are putative transcription factors and

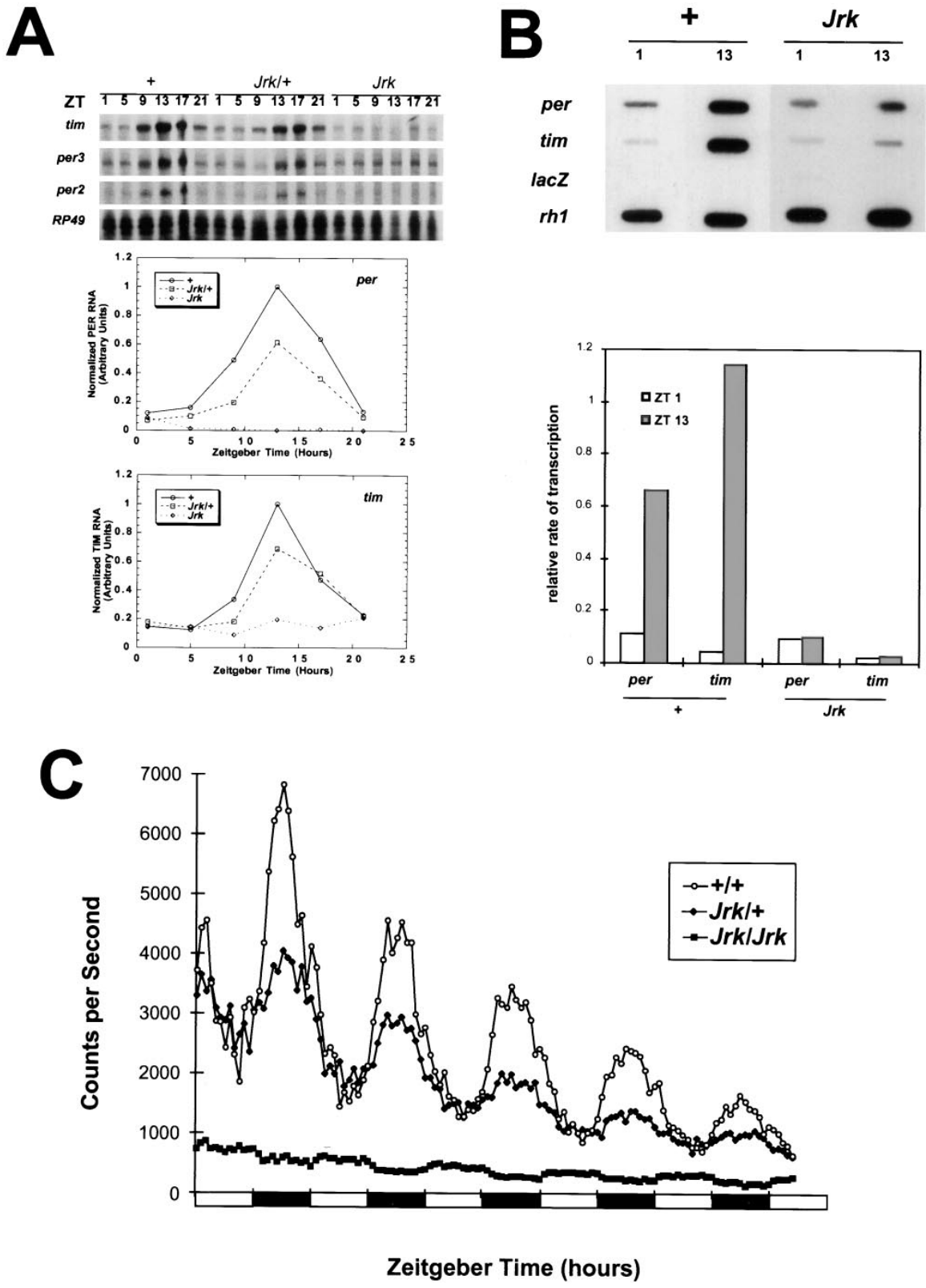


Figure 3. *period* and *timeless* Transcription Is Low in *Jrk*

(A) RNase protections of *per* and *tim* RNA. *per2* and *per3* refer to undigested RNA fragments corresponding to *per* exons 2 and 3. Quantitation of RNA levels is relative to that transcribed from a ribosomal protein gene (*RP49*) and normalized to peak RNA value (=100). *per2* signal was used to quantitate *per* (Hardin et al., 1990).

(B) Nuclear run-on assay of *per* and *tim*. Transcriptional activity was quantified relative to that of rhodopsin-encoding gene, *rh1*. *lacZ* signal

members of the bHLH-PAS family. A BLAST (Altschul et al., 1990) search (BLASTP) with the predicted full-length *Drosophila* sequence against the NCBI database revealed highest similarity with mouse *Clock* [P(N) = 4.9×10^{-120}], and a FASTA alignment of the full-length *dClock* ORF with *mClock* revealed 35% identity over the entire overlap (>800 amino acids). *dClock* also shows substantial sequence similarity to the mouse and human bHLH-PAS proteins NPAS2/MOP4 [P(N) $\approx 10^{-118}$], but the latter has no polyQ regions (Hogenesch et al., 1997; Zhou et al., 1997). In addition, there is no functional evidence linking NPAS2/MOP4 to circadian rhythms. Thus, based on their extensive homology, as well as their shared role in circadian rhythms, we will refer to the *Jrk*-defined gene as *Clock(Clk)*.

The sequence identity is even more impressive in the three subregions where one can infer a biochemical function. First, the bHLH domains, involved in DNA binding and protein dimerization, have 71% similarity and 60% identity. The basic region, involved in sequence-specific DNA contacts (Ma et al., 1994), is remarkably conserved with 11 out of 13 amino acids being identical; this suggests that the two proteins bind to similar if not identical DNA targets. In fact, 6 out of 9 are identical to a consensus generated for bHLH proteins that bind the CAC/GTG E box half-site, including the critical R residue at position 15 (Figure 6, amino acid 24; Swanson et al., 1995); this is consistent with the dramatic effect of the *Clk* mutant on *per* E box–mediated transcription. As expected, the *tim* gene also has an E box in its 5′ noncoding region (P. Emery and M. Rosbash, unpublished data). Finally, in-vitro experiments indicate that hCLOCK preferentially binds and activates transcription from DNA targets very similar to the *Drosophila per* E box (Hogenesch et al., 1998). Based on all of these observations, we propose that dCLOCK is an essential factor for the circadian transcription of *per* and *tim* and binds directly to these E boxes (Figure 7). Consistent with this model, we find *Clock* is epistatic to *per*: TIM levels are very low in a per^{0};*Clk* arrhythmic strain (data not shown), suggesting that *Clock* acts upstream of *per*. Although the direct targets of mCLOCK have yet to be identified, we also predict that *mper* genes contain one or more relevant E boxes and that *mClock* probably plays a similar role in mammals.

The PAS region, implicated in protein dimerization, is also strikingly conserved between the insect and murine genes. The PAS B repeat and the region within PAS just C-terminal to PAS B are particularly conserved; 79% identical and 91% similar over a span of 107 amino acids (Figure 6; amino acids 262–368). The conservation of PAS and its demonstrated role in dimerization suggest that dCLOCK and mCLOCK may have conserved heterodimeric partners. Indeed, it appears that another *Drosophila* clock gene encodes the relevant bHLH-PAS partner and that the same heterodimeric complex is functionally relevant in both systems (Hogenesch et al., 1998; Rutila et al., 1998 [this issue of *Cell*]). However, the PAS domain has been implicated in several other functions, including ligand binding and HSP90 interaction (Whitelaw et al., 1993). For example, DNA binding of the dioxin receptor, a bHLH-PAS protein, is ligand-gated and mediated by the PAS B region (e.g., Dolwick et al., 1993). Thus, as-yet-unidentified small molecules may affect dCLOCK activity and may be important components of these circadian systems. In addition, small molecule binding may link the PAS region with circadian photoreception (see below).

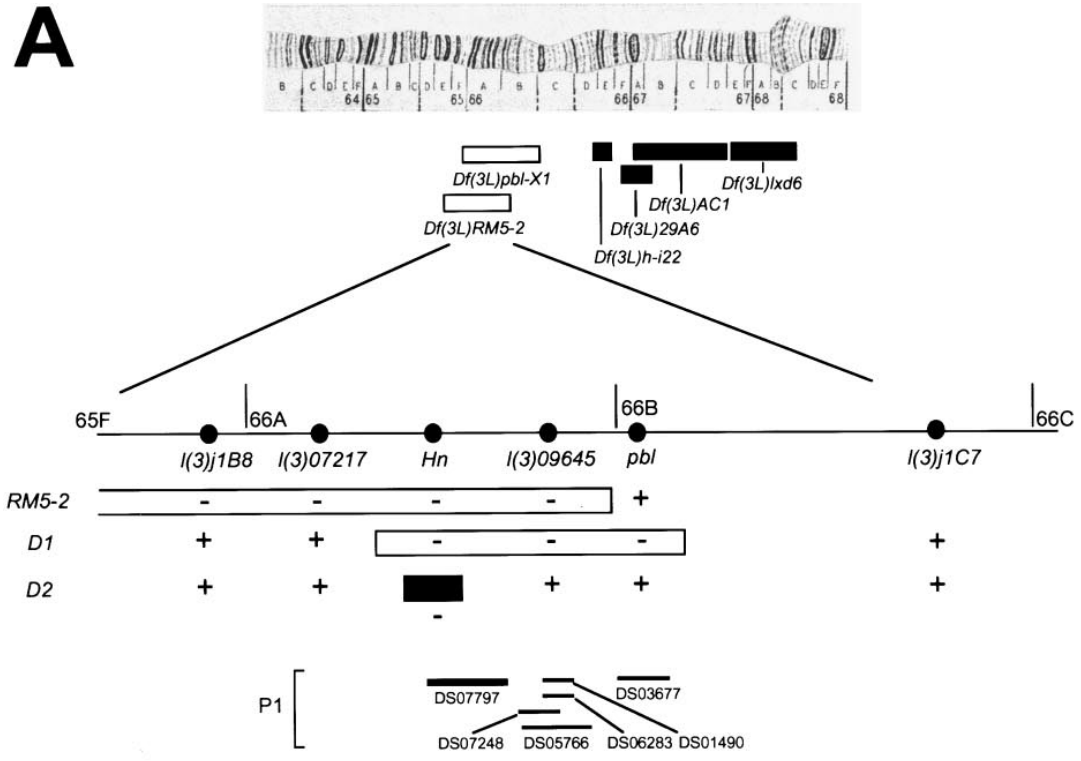
Relevant to the function(s) of the PAS region, we have identified one unusual transcript that contains the PAS B region but is missing the bHLH and PAS A domains. We do not know its abundance relative to the full-length canonical transcript, but the PAS B–only transcript was identified in two different libraries by colony filter hybridization and PCR. The product of this transcript may play an inhibitory role, sequestering the CLOCK partner or the putative ligand. Since the PAS B transcript may contain the entire EB1 ORF in the sense orientation, it is also possible that it is merely an EB1 transcript that does not terminate until after the *dClock* PAS B region. However, Northern-blot analysis indicates that this explanation is unlikely (data not shown).

The third conserved region is the glutamine (Q)-rich C terminus of the protein. Glutamine-rich regions, especially polyglutamine repeats, are known to function in transcriptional activation (Mitchell and Tjian, 1989). The *Clk* mutation would delete much of the glutamine-rich C terminus and polyQ repeats yet retain the putative dimerization and DNA-binding motifs (Figures 5B and 6). Thus, the cause of the *Clk* mutant reported here is somewhat similar to that of *mClock*. In both cases, removal of significant segments of the glutamine-rich activation domains gives rise to inactive or poorly active proteins, resulting in strong recessive phenotypes. But *mClock* homozygotes manifest some periodicity, which indicates that the mutant protein retains some biological activity (Vitaterna et al., 1994). In contrast, *Clk* homozygous flies are completely arrhythmic, and the *Clk* stop codon removes a greater fraction of the more extensive *dClock* glutamine-rich region. Since both mutants exhibit robust dominant phenotypes, we expect that the mutant proteins still heterodimerize well and sequester a DNA-binding partner in an inactive or poorly active complex (Figure 7). Other bHLH-PAS proteins lacking their glutamine-rich activation domains form transcriptionally inactive dimeric complexes in vivo (Whitelaw et al., 1994; Pollenz et al., 1996).

We also noted in sequencing *dClock* from different wild-type strains and cDNA libraries that the number of glutamines in the longest polyglutamine repeat (Figure 6; amino acids 798–822) was either 25 (in *Clk* and Clk^{+} sibs) or 33 (all other strains, libraries). The difference

is a negative control, indicating nonspecific background hybridization.

(C) Real-time luciferase monitoring in *per*-promoter luciferase transgenic flies in different *Jrk* backgrounds. Luciferase activity is quantified in counts/s. For each curve, data are averaged from 8 individually monitored flies. Twelve additional Jrk homozygotes were monitored and showed similar results, i.e., low and flat luciferase expression. Small fluctations of luciferase reflect changes in substrate availability apparent in *luc* with constitutive promoters (Stanewsky et al., 1997).



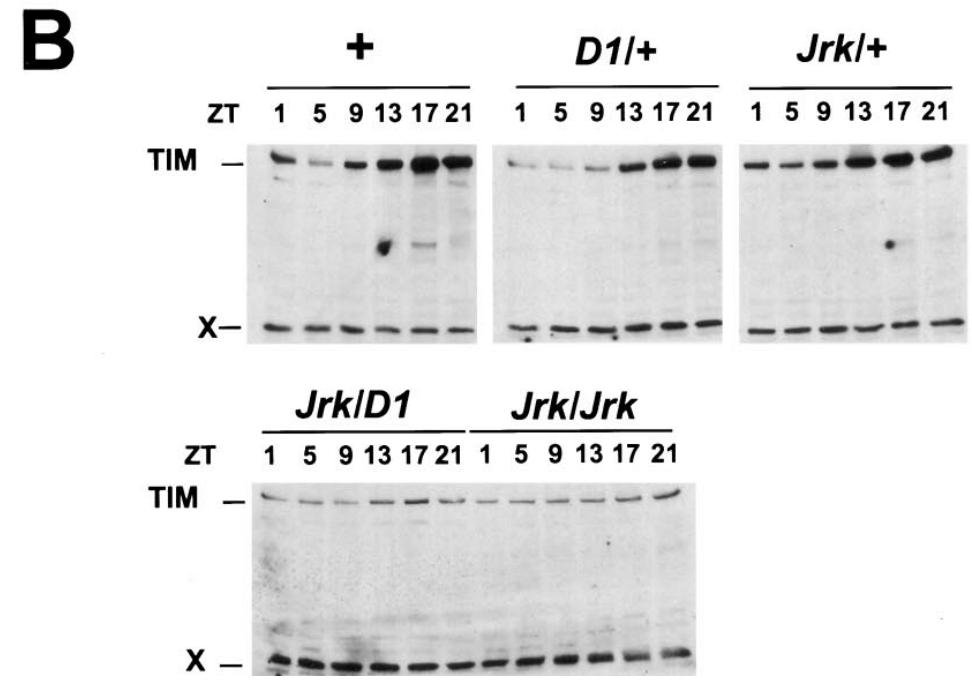


Figure 4. Mapping of *Jrk* to 66A

(A) Shown at the top is the diagramatic representation of the 64–68 region of the left arm of chromosome 3. Left arm = regions 61–80. Just below are deletions indicated by boxes, breakpoints from FlyBase Consortium, 1998, or Grasso et al., 1996. Open white boxes indicate deletions that fail to complement the *Jrk* phenotype by behavior and/or Western blotting for PER and TIM. Closed black boxes are deletions that complement *Jrk*. − (fails) and + (complements) indicate results of complementation testing of deficiencies with various lethal mutations (*l(3)*, *pbl* = *pebble*) or morphologic markers (*Hn* = *Henna*). None of the mutations fail to complement *Jrk*. *RM5-2*/*pbl* complementation result taken from Grasso et al., 1996. P1 indicates P1 genomic clones from the region. Extent of coverage of each clone taken from a map in the

appeared to be polymorphic as it did not affect locomotor activity rhythms (data not shown). However, we cannot exclude subtle effects on activity or an influence on other circadian outputs, and the contribution of the Q repeat to transcriptional activation suggests that some of the variation might make dominant contributions to phenotype. TG repeat-containing polymorphisms within the *Drosophila per* gene have been shown to influence behavioral rhythms (e.g., Sawyer et al., 1997), and it is known from genetic neurodegenerative diseases that CAG repeats are genetically unstable (Mitas, 1997). Mechanisms leading to such repeat-length polymorphism may contribute to variation at the *hClock* locus within the human population (Tut et al., 1997). It will be interesting to examine this region of the human *Clock* gene(s) for polymorphisms.

There are, however, differences between the insect and murine systems. The heterozygous deletion of *mClock* ($W^{19H}/+$) leads to normal ~24 hr periods (King et al., 1997). In contrast, the relevant *Drosophila* heterozygous deletions have a clear period phenotype (Table 2), resulting in an even longer period than heterozygous deletions of the other two well-characterized *Clock* genes *per* (24.5–25 hr; Smith and Konopka, 1981) and *tim* (24 hr, i.e., no change from wild-type; data not shown). As the circadian pacemaker is more sensitive to the dosage of *dClock* than to that of *per* and *tim*, we propose that *dClock* not only contributes to the general transcription of clock components like *per* and *tim*, but also participates in regulatory events necessary for circadian oscillations. The interspecific difference might reflect the presence of multiple *mClock* genes in mammals. The finding of a human gene (*KIAA0334*) (Nagase et al., 1997) with more similarity to *mClock* than to *hClock* suggests that there may be at least two human *Clock* genes and therefore at least two mouse *Clock* genes. Multiple genes might make the mouse less sensitive to reductions in gene dosage, without affecting the manifestation of dominant gain-of-function mutations. Furthermore, transgenic mice that carry extra copies of *mClock*-containing BACs exhibit period-shortening (Antoch et al., 1997). Thus, *mClock*, like *dClock*, also exhibits sensitivity to modest alterations in gene dose.

A second difference between systems is the altered light response of *Clk* flies, as a comparable effect has not been reported for the mutant mouse. The mutant flies fail to respond to the lights-on transition and are therefore even less photosensitive than arrhythmic per^0 or tim^0 flies (Figure 1). Even disruption of the standard phototransduction pathway (e.g., *norpA*) does not abolish this response (Wheeler et al., 1993). Thus, the absence of this light response may reflect a specific effect on a circadian photoreceptor or on one or more signal transduction components that reside between the photoreceptor and other clock molecules. We imagine two possible explanations: *dClock* is necessary for the transcription of one of these components, or it may itself participate in the light response. CLK may be a photoreceptor, it may be part of the signal transduction machinery, or it may be transcriptionally induced by light as an immediate-early gene. Another PAS protein, the photoactive yellow protein, is known to be directly light-sensitive, and its PAS domain binds the chromophore alpha-hydroxy cinnamic acid (Baca et al., 1994; Linden and Macino, 1997). Light induces the *Neurospora* clock gene *frq*, and this induction is mediated by a transcription factor, *white collar-1*, which also contains a PAS-like domain (Crosthwaite et al., 1997). In mammals, the circadian light response includes the transcription of immediate-early genes, which probably include *mper1* as well as a number of transcription factors (Kornhauser et al., 1990, 1992; Rusak et al., 1990; Albrecht et al., 1997; Shigeyoshi et al., 1997; Morris et al., 1998). Although *per* or *tim* RNA do not appear to respond acutely to light and light-inducible immediate-early genes have yet to be identified in *Drosophila*, we expect that such light-induced genes exist, are important to the insect circadian system, and may be relevant to CLK function.

The dramatic sequence conservation between *dClock* and *mClock* deepens the connection between the circadian systems of vertebrates and invertebrates. The PAS motif had previously connected *Drosophila*, mammals, and *Neurospora*—through the *period* genes and *Clock* genes on the one hand and the *white collar* genes of *Neurospora* on the other (Crosthwaite et al., 1997). But the relevant region of the *wc* proteins does not contain a canonical direct repeat PAS domain, and they manifest no other relationship to known metazoan clock proteins (Linden and Macino, 1997). In the case of the *mper1* and *mper2* genes, the similarity to *dper* extends across the full length of the proteins, suggesting they are true orthologs (Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997). But the interspecific sequence conservation is modest compared to the *dClock*/*mClock* comparison. Moreover, there are hints of some differences between *mper* and *dper*: *mper1* transcripts but not *dper* transcripts are rapidly induced by light, and the phase of *mper* RNA cycling is quite different from that of *dper* (Shearman et al., 1997; Sun et al., 1997 Tei et al., 1997). In contrast, the mutant phenotypes as well as the remarkable sequence conservation indicate highly conserved functions for *mClock* and *dClock*. The strong evolutionary conservation indicates that further progress in the *Drosophila* system will continue to provide insight into the circadian systems of mice and humans.

Experimental Procedures

Flies

Flies were raised and maintained on media consisting of cornmeal, dextrose, yeast, agar. $per^+;ry^{506}$ flies were used for mutagenesis, and Canton-S wild-type flies were used for behavioral and molecular controls as well as Jrk^+ sib strains from the mutagenesis as controls for sequencing. *Jrk* and *Jrk*/+ flies used in this study are *Jrk scarlet*

Encyclopedia of Drosophila (Berkeley Drosophila Genome Project/FlyBase), which links genetic and physical markers (Hartl et al., 1994; Kimmerly et al., 1996; Berkeley Drosophila Genome Project, personal communication). Thick bar (DS07797) is P1 clone that specifically amplifies with EST HL04223 primers; all other P1s failed to amplify with these primers.

(B) The deletion *Df(3L)D1* deficiency uncovers the *Jrk* phenotype by anti-TIM Western blotting. TIM indicates *timeless*-encoded protein band. X marks a cross-reacting band indicating relative loading of each lane (see Figure 2).

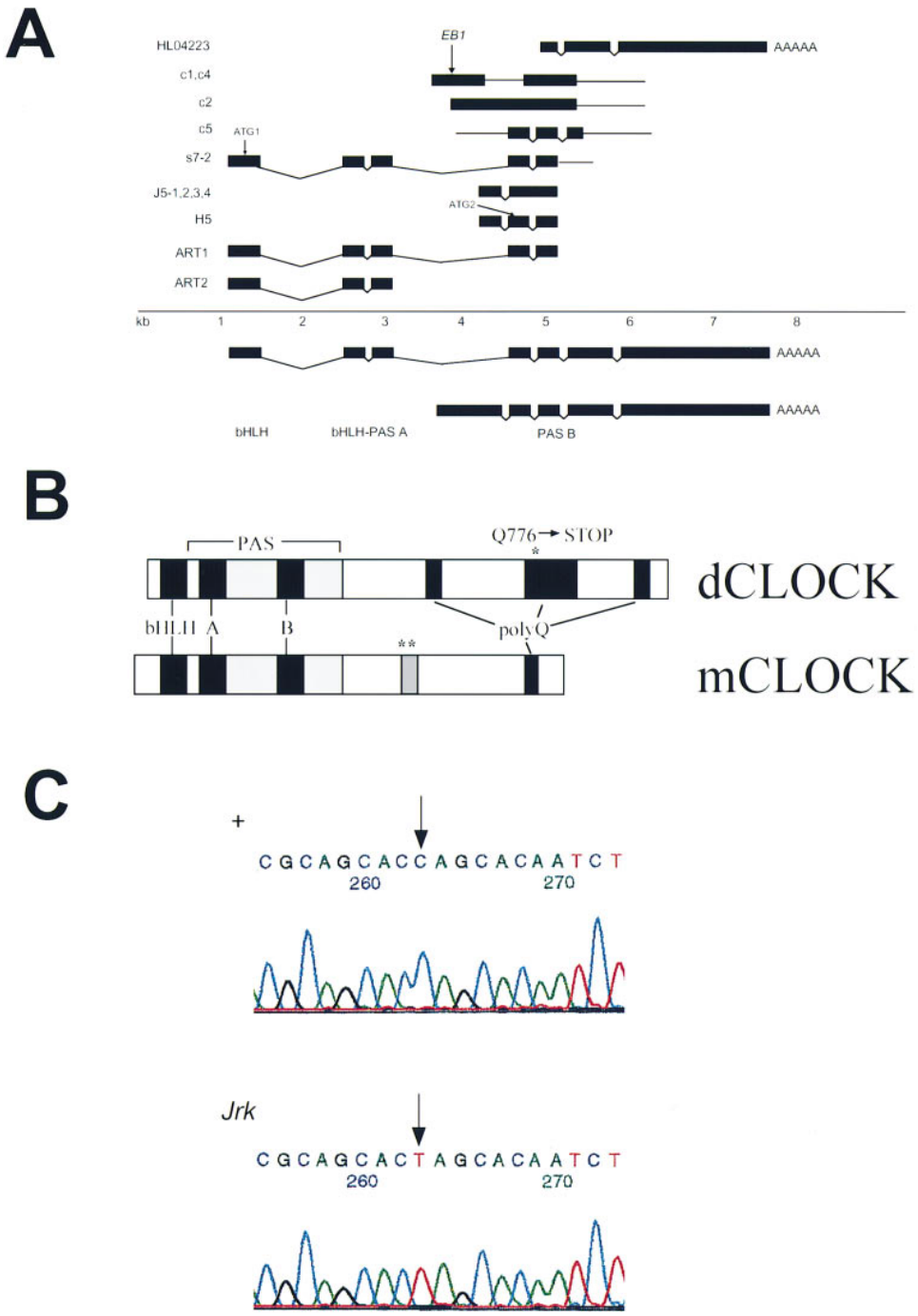


Figure 5. Analysis of *dClock* cDNAs and Sequence

(A) Black boxes denote exons determined by comparing sequence with that directly sequenced from P1 clone and PCR amplification products of genomic DNA. Bent diagonal lines between boxes indicate introns. The thin, straight horizontal lines extending from the end of exon boxes indicate continuing unsequenced cDNA. We initially tried to screen libraries using the PAS B region as a probe. However, sequencing of the 5′ end of these clones did not reveal a bHLH region. We subsequently attempted to PCR-amplify the 5′ end from cDNA libraries using library vector primers and gene-specific primers, followed by sequencing the products. Second, we directly sequenced the genomic P1 clone, DS07797, 5′ of the PAS B region, searching for open reading frames with homology to bHLH and PAS A, followed by designing new primers for PCR from libraries. HLO4223 is an EST clone derived from an adult head brain and sensory organ library (Berkeley *Drosophila* Genome Project/HHMI EST project, unpublished). Clones beginning with "c" were screened from an adult head cDNA library library (provided by T. Schwarz); S designates PCR products from the Schwarz library; H were PCR products from the Hamilton head library (Palazzolo et al., 1990); J designates PCR products from a yeast two-hybrid *Drosophila* head cDNA library (provided by J. Huang); ART, RT-PCR products. ATG1 is the putative initiation methionine for the full-length transcript. ATG2 is the putative initiation methionine for the PAS B–only transcript.

(B) Domain organization of *dClock* compared to *mClock*. PolyQ indicates polyglutamine stretches. * denotes the mutation in *dClock* that converts glutamine to stop at amino acid 776. ** denotes the splice mutant in *mClock* that deletes exon 18 (amino acids 484–513); the dotted box indicates the deleted exon.

(C) The *Jrk* mutation. Electropherogram of wild-type (+) and *Jrk* sequence. Arrow indicates location of C–T change.

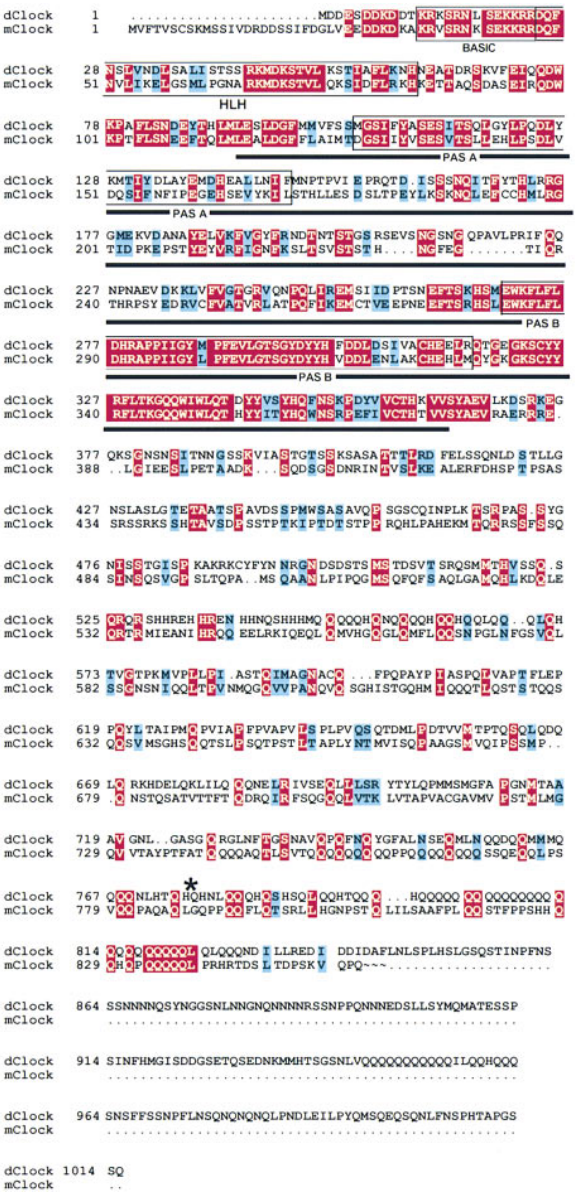


Figure 6. Predicted Amino Acid Sequence of *dClock* and Alignment with *mClock*

Alignment was performed using the PILEUP GCG (Genetics Computer Group) sequence alignment program. Identical amino acids are indicated by red box with white text. Similar amino acids are indicated by a blue box with black text. Amino acid change in *Jrk* noted by * at glutamine 776 to stop. Basic helix-loop-helix region indicated by boxes around the basic region (BASIC) and the helix-loop-helix region (HLH). Extent of PAS domain indicated by thick black line. PAS A and PAS B direct repeats indicated by boxes.

recombinants. The *per* promoter-luciferase transgenic strain has been previously described (Brandes et al., 1996). *per* promoter includes base pairs −4200 to +32 of a 13.2 kb genomic *per* clone (cf. Hardin et al., 1992). 66A deletions [Df(3L)RM5-2, *Df(3L)D1*, *Df(3L)D2*] are described in Grasso et al., 1996. All other fly stocks come from the Bloomington (IN) Stock Center.

Fly Mutagenesis

ry^{506} flies (Lewis and Bacher, 1968) were treated with 0.025 M ethyl methanesulfonate (EMS) in Whatman paper soaked with a 1% sucrose solution (Lewis and Bacher, 1968); food coloring was added

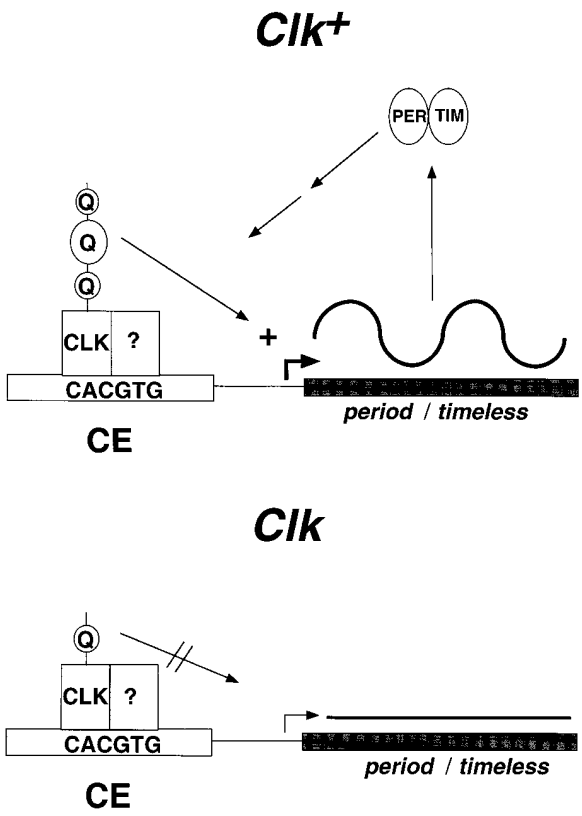


Figure 7. Model of dCLOCK Function in *Clock*$^{+}$ and *Clock*

CACGTG = E box from the circadian enhancer (CE) in the *per* and *tim* promoters. Q indicates polyglutamine repeats in the C terminus of the protein, thought to be responsible for transcriptional activation. ? indicates putative partner of CLOCK. In wild-type (*Clk*$^{+}$), CLOCK associates with its putative partner, binds the E box, thus mediating circadian transcription of *per* and *tim*. This transcription regulation is subject to direct or indirect control by PER and TIM. In the *Clock* mutant, truncation of the polyglutamine repeats results in low and noncycling transcription of *per* and *tim*.

as a measure to monitor the ingestion of the mutagen into the flies' abdomen. The males to be treated (age 2–3 days) remained in a glass vial with the EMS/sucrose solution–soaked Whatman paper at room temperature for 16–20 hr and were then crossed to females (see below) at 25°C, with these flies allowed to lay eggs in food-containing bottles for 3 days.

Mutagenized *ry*^{506} males were crossed en masse to balancer virgin females (*In (3LR) TM6,Ubx/Tp(3)MRS, ry Sb*). F1 males marked either with *Ubx* or *Sb* selected and backcrossed to *TM6/MRS* females this ensured that the X chromosome present during locomotor testing had not been mutagenized. F2 male and female siblings, each heterozygous for the mutated chromosome and the balancer *TM6*, were crossed to create a stock. If viable, four flies with homozygous mutant third chromosomes or four flies with heterozygous mutant chromosomes were tested for locomotor activity.

Behavioral Analyses

Flies were entrained for two 12 hr light:12 hr dark cycles before being assayed for locomotor activity in constant darkness at 25°C (Hamblen et al., 1986). Activity periods were determined by chi-square periodogram analysis, $\alpha = 0.01$ (Hamblen-Coyle et al., 1992). The single *Jrk* fly judged "rhythmic" by periodogram analysis was weakly rhythmic with a period of 18 hr. Similarly, a very low residuum of *per*^{0} flies exhibit formal "rhythmicity" with arbitrary periods in the range of 15–35 hr (Hamblen et al., 1986). LD activity monitoring, flies were monitored for 5–7 12 hr light:12 hr dark cycles for locomotor

activity. Eclosion rhythms were monitored at 25°C in a Trikinetics (Waltham, MA) monitoring system as described previously (Konopka et al., 1994). Optomotor testing was performed as in Stanewsky et al., 1996. The scores were: *Jrk scarlet* 69 ± 3 (SEM) (n = 11), positive control (wild-type) 67 ± 3 (n = 14), negative control (*optomotor-blind* [omb^{H31}]) 44 ± 3 (n = 15). Activity indices (vigor of behavior in these tests) are for wild-type set = 1.00, *Jrk* = 1.10, and *omb* = 0.99.

Mapping of the *Jrk* Mutation

Jrk ry^{506} was initially mapped relative to *spineless(ss)*: 16/140 recombinants with *ry* (chromosome 3—map position 52) and 24/140 with respect to *ss* (3–58). A subsequent experiment with respect to *h* (3–26) and *st* (3–44) demonstrated 0/9 recombinants with respect to *h* and 2/9 with respect to *st*. Further experiments with *hairy* revealed 0/20 recombinants total. Mapping with *Jrk st* was then done with respect to *Df(3L)29A6* (66F3;67B1) and demonstrated 4/101 recombinants. Subsequent recombination experiments were initially performed with lethal P elements to the right (centromeric) of *hairy*, including *l(3)01859* ry^{506}(67C5-8) (11/124 recombinants), *l(3)02240* ry^{506} (67C04-05) (7/71 recombinants) with *Jrk st ry*. All Jrk^{+} recombinants were *st* and all *Jrk* recombinants were st^{+}, suggesting *Jrk* was to the left of the lethal markers. Further mapping was done by complementation with deficiencies (see Results).

Western Blotting

Western blotting was performed as Edery et al. (1994) and So and Rosbash (1997). Flies were collected on dry ice at the appropriate time points. Thirty heads were homogenized in 45 μl of extraction buffer with 20 mM beta-glycerophosphate and 100 mM Na_3VO_4 added. Homogenate was spun at 4°C in microcentrifuge 14,000 × g for 30 min. Fifteen μl of 4× SDS sample buffer was added and the sample boiled for 5 min. Twenty microliters of boiled sample were loaded on 8% SDS-polyacrylamide gel (29.6:0.4 acrylamide: bisacrylamide ratio). Gels were electroblotted for 45 min at 0.15 A using a semidry blotting apparatus according to manufacturer's instructions (ISS). Gels were quantified using Bio-Rad chemiluminescence detector and Molecular Analyst software.

RNase Protection Assays

RNase protection assays were performed as described in Marrus et al., 1996. Gels were quantified on Bio-Rad phosphorimager with Molecular Analyst software.

Nuclear Run-On

Nuclear run-on analysis was performed as described in So and Rosbash, 1997. Quantification was performed by phosphoimager with Molecular Analyst software (Bio-Rad). *lacZ* is a negative control, indicating background nonspecific hybridization.

Real-Time Luciferase Reporting of *per* Expression

Single fly luciferase activity monitoring was performed as described in Brandes et al., 1996.

Polytene Chromosome In Situ Hybridization

Third instar larval salivary glands were squashed and fixed according to Engels et al., 1986. DNA probe was random prime-labeled using the DIG DNA Labeling and Detection Kit according to manufacturer's instructions (Boehringer Mannheim). Probe template was synthesized using PCR product from P1 clone (DS07797) using primers from EST (HL04223) (5′ nt 1324–1346, 3′ 3115–3095, GenBank AF065133). Probes were hybridized according to Blackman, 1996.

Isolation of cDNAs

Filter lifts of adult head cDNA libraries (total 3×10^6 phage/library) were screened using probes PCR amplified from a yeast two hybrid expression *Drosophila* adult head cDNA library (provided by J. Huang). These probes were amplified using primers corresponding to PAS B and products were random prime-labeled (Prime-It II, Stratagene). Five positive clones using PAS B probe were amplified by PCR directly on picked positive plugs with Bluescript vector T7 and T3 primers and PAS B primers (3′ nt 1386–1361, 5′ nt 1081–1102) and sequenced using ABI PRISM kit with same primers as PCR. cDNAs were also identified by PCR directly on the same libraries using vector and PAS B primer (3′ nt 1386–1361). First amplification products were reamplified using nested primer from the PAS B region (3′ nt 1222–1201) and sequenced using nested primers and vector primer. Sequencing of DS07797 5′ of PAS B was also performed with 2.5 μl of P1 DNA using ABI PRISM Kit.

Poly(A) RNA Preparation for RT-PCR/Northern Analysis

Total RNA was prepared as in Rutila et al., 1996. Poly(A)$^{+}$ RNA was prepared from total RNA using poly(A) Tract kit according to manufacturer's instructions (Promega). Using Superscript II RNase H^{-}, and initially PAS B primers, *dClock* cDNA was made. Northern analysis used 10 μg of poly(A)$^{+}$ RNA loaded per lane on a 1% agarose formaldehyde gel (Rouyer et al., 1997). A riboprobe corresponding to ~100 bp 3′ end of HL04223 was used for hybridization.

Identification of the *Jrk* Mutation

Genomic DNA was prepared from *Jrk*, sib strains, and an unrelated per^{L} ry^{506} strain. Briefly, 30 flies were frozen on dry ice then homogenized with motorized pestle in 500 μl of 5% sucrose, 80 mM NaCl, 0.1 M Tris (pH 8.0), 0.5% SDS, 50 mM EDTA. The homogenate was incubated at 70°C for 30 min. KOAc (8 M) is added to concentration of 160 mM and placed on ice for 30 min. The sample was spun for 5 min in microcentrifuge. The supernatant was extracted twice with phenol/chloroform and once with chloroform then precipitate with 0.75 vol of isopropanol. The precipitate was washed in 70% EtOH, dried, and resuspended in 100 μl of TE. The sample was then diluted to 100 ng/μl and boiled for 10 min. Genomic DNA (100 ng) was used for each PCR. PCR amplification products were purified by Qiagen PCR purification kit or gel purification kit according to instructions and 10 ng/100 bp of PCR product was used for ABI PRISM kit sequencing. Both strands of PCR products were sequenced.

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GenBank Accession Number

Sequence of *dClock* has been submitted to GenBank under accession number AF065133. All nucleotide (nt) numbers refer to this sequence.