**The EMBO Journal** vol.15 no.7 pp.1625–1631, 1996

# A novel circadianly expressed *Drosophila melanogaster* gene dependent on the *period* gene for its rhythmic expression

**Russell N.Van Gelder[1,2,3,4] and Mark A.Krasnow[1]**

[1]Department of Biochemistry and [2]Basic Sleep Research Laboratory, Stanford University School of Medicine, Stanford, CA 94305, USA

[3]Present address: Department of Ophthalmology and Visual Sciences, Washington University Medical School, 660 South Euclid Avenue, St Louis, MO 63110, USA

[4]Corresponding author

**The *Drosophila melanogaster period* (*per*) gene is required for expression of endogenous circadian rhythms of locomotion and eclosion. *per* mRNA is expressed with a circadian rhythm that is dependent on Per protein; this feedback loop has been proposed to be essential to the central circadian pacemaker. This model would suggest that Per protein also controls the circadian expression of other genetic loci to generate circadian behavior and physiology. In this paper we describe *Dreg*-5, a gene whose mRNA is expressed in fly heads with a circadian rhythm nearly identical to that of the *per* gene. *Dreg*-5 mRNA continues to cycle in phase with that of *per* mRNA in conditions of total darkness and also when the daily feeding time is altered. Like *per* mRNA, *Dreg*-5 mRNA is not expressed rhythmically in *per* null mutant flies. *Dreg*-5 encodes a novel 298 residue protein and Dreg-5 protein isoforms also oscillate in abundance with a circadian rhythm. The phase of Dreg-5 protein oscillation, however, is different from that of Per protein expression, suggesting that *Dreg*-5 and *per* have common transcriptional but different post-transcriptional control mechanisms. These results demonstrate that the *per* gene is capable of modulating the rhythmic expression of other genes; this activity may form the basis of the output of circadian rhythmicity in *Drosophila*.**

*Keywords*: circadian/*Drosophila*/gene expression/*period*

## Introduction

The *period* (*per*) gene of *Drosophila melanogaster* is required for expression of circadian rhythms of eclosion and locomotion (reviewed in Hall and Rosbash, 1993; Jackson, 1993). Different alleles of this gene can lengthen or shorten the endogenous circadian period of the fly, while null alleles produce aperiodic animals (Konopka and Benzer, 1971). The biochemical mechanism by which a single gene exerts such a profound influence on the organization of behavior and physiology is not known. Several recent observations have suggested a role for the *per* gene in control of gene expression. Per protein is predominantly nuclear in the fly head (Liu *et al.*, 1992). The protein shares an ~200 amino acid region of homology with several DNA binding proteins, including the product of the neurogenesis gene *singleminded* and two subunits of the dioxane receptor (Crews *et al.*, 1988; Hoffman *et al.*, 1991; Burbach *et al.*, 1992). The shared motif, called the PAS domain, can mediate dimerization between Per and these proteins *in vitro* (Huang *et al.*, 1993). These observations suggest that Per protein may act as a partner to an as yet unknown DNA binding protein and thereby affect gene expression.

There is substantial genetic evidence that the *per* gene product modulates its own gene expression. The abundance of *per* mRNA and the protein product oscillate with a free running circadian rhythm (Hardin *et al.*, 1990, 1992c; Zerr *et al.*, 1990). The mRNA oscillation is generated by rhythmic transcription and requires functional Per protein. In the absence of Per protein *per* mRNA oscillation ceases. Thus the *per* gene product is formally involved in a feedback loop to regulate its own circadian transcription. Recent models of circadian function have suggested that this feedback loop may constitute the fundamental circadian pacemaker (Hall and Rosbash, 1993; Takahashi, 1993; Page, 1994). A similar feedback loop involving the *frequency* gene has been shown to be essential to circadian rhythmicity in *Neurospora crassa* (Aronson *et al.*, 1994).

If circadian rhythmicity in *D.melanogaster* is generated by the feedback of Per protein on its own transcription, one way circadian information could be transduced into timed behavior and physiology is through the Per protein controlling circadian expression of other genes. Many genes expressed with diurnal rhythms have been identified in other organisms and some of these appear to be controlled by the endogenous clock (see for example Loros *et al.*, 1989; Kay and Millar, 1993; Takahashi, 1993; Liu *et al.*, 1995). Only recently, however, have candidate clock-controlled genes been identified in *D.melanogaster*, where the clock itself is best understood. In a collection of several hundred anonymous cDNAs expressed in the adult fly head (Palazzolo *et al.*, 1989) we found 20 that demonstrated significant diurnal rhythms of mRNA expression (Van Gelder *et al.*, 1995). We call these genes the *Dregs* (for Drosophila rhythmically expressed genes). A subset of the *Dreg* genes that have been analyzed in detail show a complex dependence on *per* and various environmental cues for their daily expression pattern (Van Gelder *et al.*, 1995). In this paper we report the circadian and molecular characterization of *Dreg*-5, the only gene analyzed to date whose mRNA is expressed in phase with *per* mRNA under many different environmental conditions and whose rhythmic RNA expression is dependent on function of the *per* gene.

## Results

### *Dreg-5 mRNA oscillates in abundance with a waveform identical to per mRNA in the presence and absence of zeitgebers*

*Dreg*-5 was initially found to be expressed approximately in phase with the *per* gene at zeitgeber times 2, 8, 14 and

© Oxford University Press

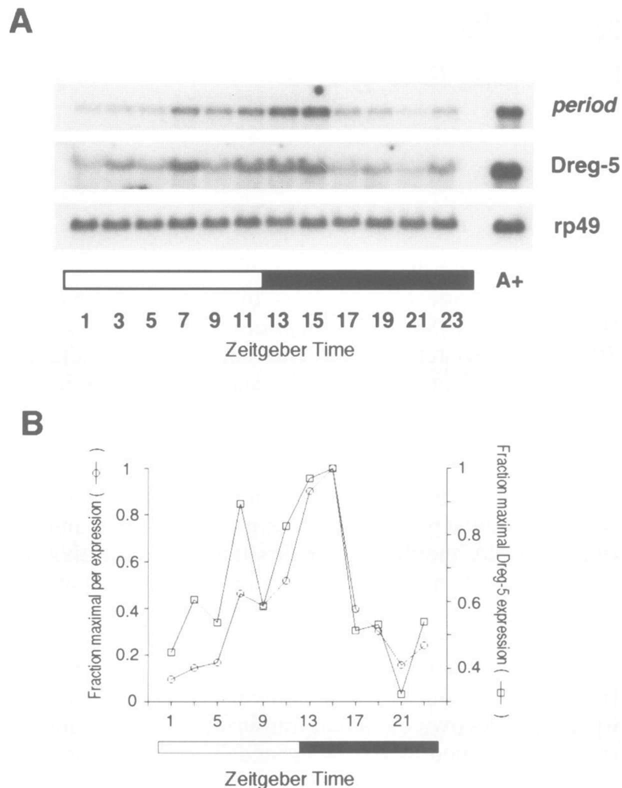


**Fig. 1.** Diurnal oscillations of *Dreg*-5 and *per* mRNA expression. (**A**) Autoradiograms of identical Northern blots of total fly head RNA (20 μg) taken from the zeitgeber times indicated and hybridized to radiolabeled *per*, *Dreg*-5 or *rp49* (control ribosomal protein) probes. Autoradiographic exposure times were: *Dreg*-5, 5 days with intensifying screen; *per*, 3 days without intensifying screen; *rp49*, 3 h without intensifying screen. The bar beneath blots indicates the lighting cycle (lights on from ZT 0 to ZT 12). The lane marked A+ is oligo(dT)-selected fly head RNA, demonstrating that each of the detected RNAs is polyadenylated. (**B**) Quantitation of (A). Quantitation was performed on a PhosphorImager. Values at each time point have been normalized to the *rp49* control mRNA. Data are plotted on separately scaled ordinate axes to facilitate comparison of the waveforms.

20 h in a 12 h light/12 h dark (LD 12:12) cycle (zeitgeber time is the time elapsed in hours from the dark–light transition). To determine how closely correlated expression of *Dreg*-5 mRNA is to that of *per*, *Dreg*-5 and *per* mRNA were analyzed by Northern blotting of total RNA purified from fly heads collected at 2 h intervals in an LD 12:12 cycle (Figure 1). Both *per* and *Dreg*-5 mRNAs demonstrated increasing expression through the daylight hours, with peak expression at ZT 15. Both also underwent a rapid decline after ZT 15. The amplitude of *Dreg*-5 mRNA circadian oscillation was approximately half that of *per* mRNA under these conditions. *Dreg*-5 mRNA levels varied more than *per* mRNA levels and suggested that there might be a superimposed, lower amplitude ultradian (~4 h) rhythm in *Dreg*-5 expression (see also results with Dreg-5 protein below). However, the overall kinetics and waveform of the *Dreg*-5 mRNA circadian oscillation were the same as those of *per* mRNA (Figure 1B), indicating that their circadian time courses do not differ by >1 h.

In addition to the endogenous circadian clock, two external time cues were present in these initial experiments that might influence *Dreg*-5 mRNA oscillation: light and feeding time. We compared expression of *per* and *Dreg*-5 mRNA under conditions of LD 12:12 with conditions of constant darkness (DD). As shown in Figure 2A, *Dreg*-5 mRNA continued to oscillate with the same phase and amplitude as *per* mRNA in the transition from LD to DD conditions. *Dreg*-5 mRNA oscillation continues for at least 44 h of DD (Figure 2B). Thus the *Dreg*-5 mRNA oscillation does not require rhythmic light cues.

The flies used in the initial experiments were housed in large population cages, which require daily introduction of fresh food. To determine if the timed daily food introduction was responsible for the observed rhythms in *Dreg*-5 mRNA expression we shifted the feeding time from ZT 17 to ZT 1. There was no effect on the phase of *Dreg*-5 or *per* mRNA oscillation with this alteration, although the amplitude of *Dreg*-5 cycling was decreased (Figure 2C). In contrast, changing of feeding time had a very large effect on the phase of expression of many of the other rhythmically expressed genes tested (Van Gelder *et al.*, 1995). We also tested oscillation of *Dreg*-5 mRNA in flies kept in small bottles, where no additional food was introduced over the course of the experiment. Although this condition resulted in decreased amplitude of both *Dreg*-5 and *per* mRNA oscillation, the phase of *Dreg*-5 expression remained the same as that of the *per* gene (Figure 3, Canton-S flies).

### *Diurnal Dreg-5 mRNA oscillation is dependent on per*

Given the tight temporal correlation observed between cycling of *Dreg*-5 and *per* mRNAs under various environmental conditions, we sought to determine whether *Dreg*-5 mRNA cycling was dependent on function of the wild-type *per* gene product. Bottled wild-type (Canton-S) and *per* null mutant (*y* $per^{01}$) flies kept on an LD 12:12 cycle were collected at fixed time points and RNA was purified from the fly heads. As shown in Figure 3, neither *Dreg*-5 nor *per* mRNAs demonstrated any oscillation in homozygous $per^0$ flies. The relative transcript levels of *Dreg*-5 compared with the *rp49* control were lower at all times of day in Canton-S flies than in $per^0$ flies, suggesting that the *per* gene product represses *Dreg*-5 expression. Such a difference in transcript levels appears specific for *Dreg*-5, since it was not observed for any of the other *Dregs* or negative control mRNAs (*rp49* and *nina*E) tested (data not shown). Light–dark cycles can drive rhythmic behavior in $per^0$ flies (Wheeler *et al.*, 1993), and other *Dreg* genes (such as *Dreg*-3) continued to display diurnal rhythms of mRNA expression in $per^0$ flies in this experiment (Van Gelder *et al.*, 1995). Thus it appears that neither oscillating light–dark conditions nor rhythmic behavior is sufficient to drive the cyclic expression of *Dreg*-5 mRNA in the absence of *per* gene function.

### *Dreg-5 encodes a novel 298 residue protein*

The *Dreg*-5 gene was mapped to cytological position 100C in the *D.melanogaster* genome by *in situ* hybridization to polytene chromosomes. This position does not correspond to the location of any genes known to affect circadian rhythms. *Dreg*-5 mRNA was detected as a ~1.7 kb species on Northern blots. It is a rare mRNA present at ~20% of the abundance of *per* mRNA at peak levels in total fly

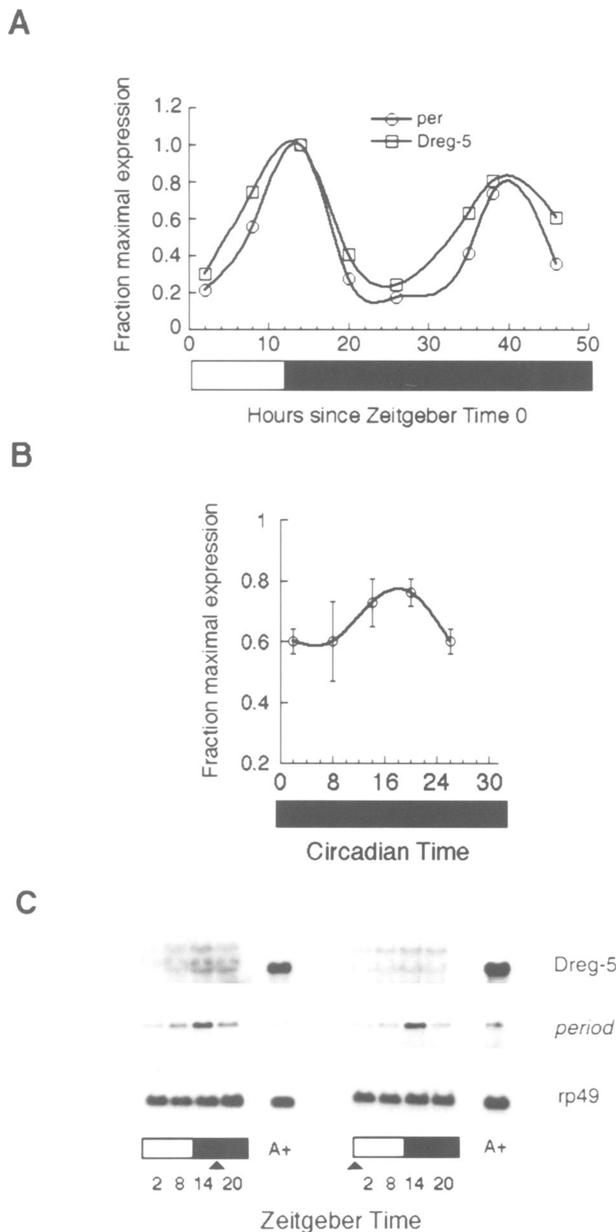


**Fig. 2.** Cycling of *Dreg*-5 mRNA under different environmental conditions. (**A**) *Dreg*-5 mRNA continues to oscillate in the same phase as *per* in the transition from LD to DD lighting. The experiment was performed as in Figure 1, except the lights remained off after ZT 24 and flies were collected at the indicated times over a 48 h period. Curve fitting was by cubic spline. (**B**) *Dreg*-5 mRNA oscillates in the absence of all zeitgebers. The experiment was performed as in Figure 1, except the flies were housed in small bottles with food available *ad libitum*. After three cycles of LD 12:12 the lights remained off and flies were collected beginning at 26 h of DD (circadian time 2). Data were quantified and normalized as in Figure 1. Values shown are the mean ± SE of three repetitions of the experiment. Curve fitting was by cubic spline. The CT 2 data are double plotted (at CT 2 and CT 26) to facilitate visualization of the circadian waveform. (**C**) Changing the timing of feeding tray placement does not alter the phase of *Dreg*-5 or *per* mRNA cycling. The experiment was performed as in Figure 1, except the daily feeding tray introduction for one set of cages was altered from ZT 17 (left) to ZT 1 (right). Arrowheads indicate time of food tray swap. The upper band in the *Dreg*-5 panels is non-polyadenylated and probably represents unprocessed transcript.

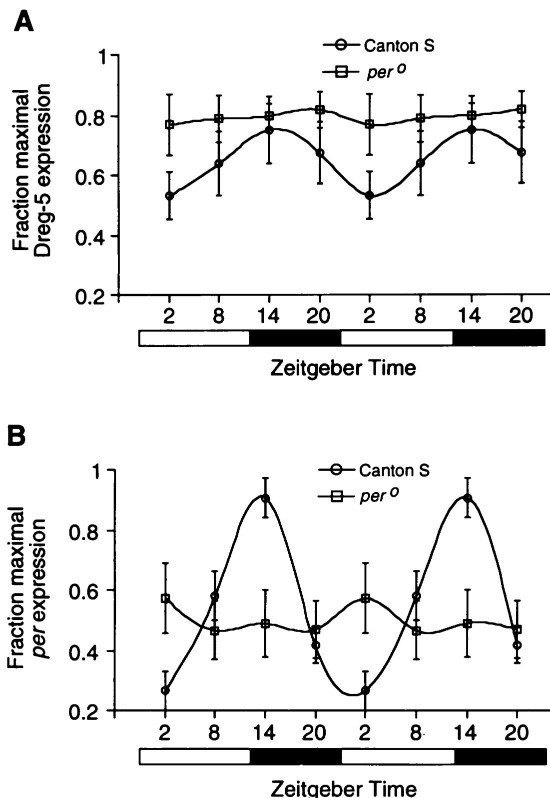


**Fig. 3.** Diurnal rhythmicity of (**A**) *Dreg*-5 and (**B**) *per* mRNA in wild-type (Canton-S) and *y per*$^{01}$ flies. The experiments were performed as in Figure 1 except the flies were maintained in small bottles (see Materials and methods). Values given are the mean ± SE from six independent repetitions of this experiment. Data are double plotted to more easily visualize the circadian waveform.

head RNA. It is expressed in the eyeless mutant *eyes absent* (Bonini *et al.*, 1993), demonstrating expression in the fly head outside the eyes. Transcript was not detected in poly(A)$^+$ RNA from fly bodies (data not shown). A fly head cDNA library was screened for full-length *Dreg*-5 clones using the original 0.5 kb *Dreg*-5 cDNA as probe. The sequence of the longest *Dreg*-5 cDNA clone obtained (1.6 kb) is shown in Figure 4. This cDNA encodes a predicted 298 residue protein. Searches of the SWISS-PROT, PIR and GenBank databases did not reveal any significant homologies with known proteins. The predicted protein has a hydrophobic 11 amino acid domain (residues 7–17) which is consistent with a short potential transmembrane domain or possibly a signal sequence. The C-terminus is predicted to be a PEST domain (Rechsteiner *et al.*, 1987), which is found in proteins with a rapid turnover rate, including Per (as determined by the PESTFIND algorithm) and the *frequency* gene product (McClung *et al.*, 1989).

### *Dreg-5 protein isoforms oscillate with a phase different from that of Per protein*

The full-length *Dreg*-5 cDNA clone was used to generate bacterial Dreg-5–TrpE fusion proteins, using non-overlapping N-terminal and C-terminal protein fragments. The fusion proteins were purified and used to raise polyclonal rabbit antisera to Dreg-5. On Western blots of whole fly head extract the affinity-purified N-terminal and C-ter-

```
   1  attccgcatgatgattatcaagaatctctatgtgatctgacgactcactgacatcgccgc
  61  gatcggttgttcgacatagtactgcaagattatagtgctcgctccgtttccgtttcgctt
 121  ggaattcgccgttttcgtgatcgactggtgggcgcccctctccctagaccgactgactcc
 181  cccatcttgccgagaaaccgatgtgtgaaacaagcttttgcgtttcaagctgagacacac
 241  gtacacccgaaaacagcgataatacgaattggccacgctcgttgtttggttaattcaatt
 301  aagtgcgtcggacagaagaaagccacttggctagccaccgaaaccaagtgataatctatc
 361  aagccgccacaaaagcgcttttattgggccctaaaaaaaaagaaagtcgccacacgaagg
 421  ttacacaaccgaacagtgcaattgacccacggaacacggatcacagatcacggagcacga
 481  atcccctgaactctccgtgtagtgcggagtacgcaacataaaagcagcaacgacaccATG
                                                               M    1
 541  ACGACTGCGGCGAAAGTAATCTTGGCCTGCTGCCTGCTGGGCGCCTTCCACATTCAGATC
       T  T  A  A  K  V  I  L  A  C  C  L  L  G  A  F  H  I  Q  I   21
 601  AGCTCGTCATCGGCCATTCCCATCTGGGAGTTCCTGACCCGCAACGAGAAGATGTCCCAC
       S  S  S  S  A  I  P  I  W  E  F  L  T  R  N  E  K  M  S  H   41
 661  CTCTACTCAACATTCGCTCAATTGGTAAGCGTGCACTGCAAGTCAACAGCCGCCGTCGGA
       L  Y  S  T  F  A  Q  L  V  S  V  H  C  K  S  T  A  A  V  G   61
 721  GGCCTCCCAGTGAACCAGTGCAAGCACAACCTGCTCGGTTACGGGTCCGCCAAGCTGCAG
       G  L  P  V  N  Q  C  K  H  N  L  L  G  Y  G  S  A  K  L  Q   81
 781  ACGCTCTCCGACGTACAGCTGGAGGCACTAGATCCGTATCAGCGCGATGCCAACGAGCTG
       T  L  S  D  V  Q  L  E  A  L  D  P  Y  Q  R  D  A  N  E  L  101
 841  ATCTGGTCATCGATTATGGGGGACCATCCGAGCGGAGCCAGCCTGGTGACAACGAGGCAG
       I  W  S  S  I  M  G  D  H  P  S  G  A  S  L  V  T  T  R  Q  121
 901  CCGCTCCAACAGCCCCTGCCAACTCCTCCTGCGTCCTCTCTGATCATCCTAACGCGCCAG
       P  L  Q  Q  P  L  P  T  P  P  A  S  S  L  I  I  L  T  R  Q  141
 961  CAACTTCCGCACGGAGCATCCCATGCCCACCCCATCCAGAGCTCCGGCTCCGCCACCAAT
       Q  L  P  H  G  A  S  H  A  H  P  I  Q  S  S  G  S  A  T  N  161
1021  CCAATCTTCGAGAGCGGCGAGCAGAAGCACAAATACGCCATGGACATGGACAAGGCCTAC
       P  I  F  E  S  G  E  Q  K  H  K  Y  A  M  D  M  D  K  A  Y  181
1081  GGCTACGGGCCGCAGTCCAGCAGTGAGCTCCCAGTGGCCGCCGCACTGACTTCAGAACCG
       G  Y  G  P  Q  S  S  S  E  L  P  V  A  A  A  L  T  S  E  P  201
1141  TCCAAAAGATTCCTCACCGGACCTTTGGTGATCCGGGTGCGACCCGATGGCTCCCCGGTG
       S  K  R  F  L  T  G  P  L  V  I  R  V  R  P  D  G  S  P  V  221
1201  GAGGAGGACAAGATGATGCCACTGCCGCGGGACGAAGACCTGCCCTACCTTTCATCTTGG
       E  E  D  K  M  M  P  L  P  R  D  E  D  L  P  Y  L  S  S  W  241
1261  TCTGGCCGCCGCTCAGCCCAACAGGCACCGCAAGATCGCCACAATCAGCTCCTTAAAGCA
       S  G  R  R  S  A  Q  Q  A  P  Q  D  R  H  N  Q  L  L  K  A  261
1321  GCAGCACTTCGCCTCCATCCTGCAGAGCGACCTCCAGCCACCCCACCAGACGCAGAGGCG
       A  A  L  R  L  H  P  A  E  R  P  P  A  T  P  P  D  A  E  A  281
1381  TCTGTTCCGCCAGCAACAGGCGTAAGATCGCGATCAGAGGATCCGCAGGCCTAGacctaa
       S  V  P  P  A  T  G  V  R  S  R  S  E  D  P  Q  A  *        298
1441  gtccttgtgtgaatgtgatagagcagatgaggagatcctgcatcatagagtgcgagagtg
1501  aacgtgtgagtcttctagttgtagccgtttgaggagttttcttgaatgcacttgagcttt
1561  aatggaatttcgtatcgtattcgtttaagtttttaattttatttattcgcgtgcattag
1621  tcgtaagatcacatagagattcttgagtaataaaccgcatgatgattatcattgttacga
1681  acgtaaaaaaaa
```

**Fig. 4.** Sequence of a *Dreg*-5 cDNA and the predicted protein. The bold region in the N-terminus of the protein highlights an 11 residue hydrophobic region. The underlined region in the C-terminus highlights the PEST domain.

minal antisera both detected two major bands of ~58 and ~64 kDa and two minor species of ~46 and ~48 kDa (Figure 5). In addition, several other higher molecular weight species were weakly detected by only one of the sera; we assume these are cross-reacting proteins. Both the 58 and 64 kDa proteins oscillated strongly with a diurnal rhythm. Maximal expression was observed at ZT 15, which was also the peak of mRNA expression; however, protein levels did not decline as rapidly as mRNA levels. Additionally, there appeared to be a weaker ~4 h ultradian rhythm of both protein levels and electrophoretic mobility, in phase with the observed weak mRNA ultradian rhythm (see Figure 1). The phase of the circadian rhythm of Dreg-5 protein expression is markedly different from that described for Per protein, which shows increasing expression levels from ZT 15 to ZT 22 in LD 12:12 (Edery *et al.*, 1994; Zeng *et al.*, 1994). Thus although *Dreg*-5 and *per* are transcriptionally very tightly coupled, they demonstrate markedly divergent protein expression rhythms.

## Discussion

In this paper we have described the isolation and characterization of *Dreg*-5, a novel gene whose mRNA oscillates closely in phase with that of *per* mRNA in fly heads. Unlike all the other *Dreg* genes that have been analyzed, *Dreg*-5 is dependent on *per* but not external time cues for its rhythmic expression. These results demonstrate that the *per* gene is capable of modulating the rhythmic expression of genes other than itself. This may form the basis of the output of circadian rhythmicity in *Drosophila.*

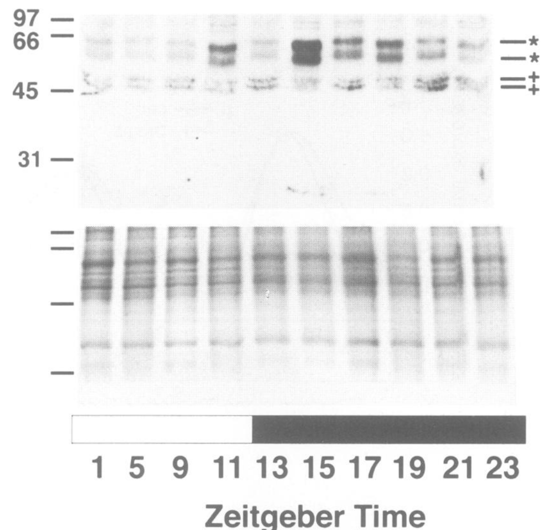


**Fig. 5.** Diurnal oscillation of Dreg-5 protein immunoreactivity. Aliquots of extracts from the same fly heads used in Figure 1 were prepared by homogenization in SDS–urea buffer and analyzed by SDS–PAGE. (Top) Western blot of fly head extract (corresponding to ~5 fly heads/lane) probed with a 1:500 dilution of affinity-purified anti-N-terminal Dreg-5 primary antibody, followed by a 1:1000 dilution of horseradish peroxidase-coupled donkey anti-rabbit secondary antibody. Detection was by enhanced chemiluminescence (Amersham) with 8 min exposure. Mobilities of markers (in kDa) are indicated on the left side of the blot. *, 58 and 64 kDa Dreg-5 bands; +, 46 and 48 kDa bands. Identical oscillations of both the 58 and 64 kDa protein bands were observed using the anti-C-terminal Dreg-5 antibody (not shown). Note that the 58 and 64 kDa species migrate slightly faster at the CT 11, 15, 19 and 23 time points than at other times, indicative of an ultradian post-translational modification. (Bottom) Coomassie Blue staining of the same region of an identically prepared 10% SDS–PAGE gel as above. Lines at the left of the gel are the same size standards as in the Western blot.

While demonstration of the role of *Dreg*-5 in the genesis of circadian rhythms of behavior or physiology must await isolation of mutations in this gene, a plausible function is suggested by its interaction with the *per* locus. As *Dreg*-5 mRNA is dependent on *per* gene function for its rhythmic expression, it must lie either genetically downstream of *per* or, if *per* mRNA rhythmic expression is reciprocally dependent on *Dreg*-5 function, in a feedback loop with Per protein. Since $per^0$ flies kept on a normal light cycle (and thus behaviorally rhythmic) did not demonstrate oscillating expression of *Dreg*-5 mRNA, its rhythmic expression is independent of light and locomotor activity. As rhythmic *Dreg*-5 mRNA expression is dependent on *per* but independent of rhythmic behavior, this suggests that genetically *Dreg*-5 may lie between *per* and the circadian functions it controls and thus may function in transducing phase information based on *per* gene function into timed behavior or physiology. We do not know whether the effect of Per protein on *Dreg*-5 expression is direct or cell autonomous. Indeed, although Zeng *et al.* (1994) have demonstrated that *per* feedback is likely to be a local phenomenon (as neural oscillation of *per* mRNA can be dissociated from ocular oscillation), it is not yet known if the effects of the *per* gene on its own transcription are direct or cell autonomous or whether they require production of an extracellular (perhaps paracrine) signal with subsequent feedback. Given the very close temporal association between *Dreg*-5 and *per* mRNA expression,

however, it is likely both are controlled by the same Per-dependent mechanism.

The means by which Dreg-5 protein might transduce phase information is not revealed by its sequence, as the predicted protein is novel and without any obvious motifs suggestive of function. Interestingly, Dreg-5 protein does not show the same phase of expression as Per protein. This observation implies that *per* and *Dreg*-5 mRNAs share common transcriptional mechanisms but have different post-transcriptional controls. This dissociation of synchronized transcription from later steps in gene expression suggests a means by which daily events with different phases of activity could be synchronized under the phase control of the *per* locus. Transcription of the gene products necessary for timed behaviors or physiological functions could occur in phase with *per* mRNA, dependent on the Per protein and the *per* autoregulatory circuit. Indeed, 17 of the 20 cDNAs identified in our initial screen for diurnally regulated transcripts detected mRNAs expressed in phase with *per* mRNA (Van Gelder *et al.*, 1995). Individual proteins could then be expressed at various phases during the circadian cycle by virtue of different *per*-independent post-transcriptional delays.

Our data suggest that exogenous factors, including feeding time and caging conditions, can modulate the amplitude but not the phase of circadian control of *Dreg*-5 expression. There are several possible mechanisms for the decreased amplitude of *Dreg*-5 mRNA oscillations observed in flies maintained in bottled versus caged conditions. Alterations in environmental conditions could trigger additional, constitutive expression of *Dreg*-5 mRNA (in normal *Dreg*-5 expressing cells or elsewhere), thereby masking the cycling of *Dreg*-5 in RNA prepared from whole fly heads. However, *Dreg*-5 transcript levels relative to *rp49* were lower at all times of day in the bottled flies compared with flies maintained in cages (data not shown), arguing against induction of non-oscillating *Dreg*-5 mRNA. Alternatively, varying environmental conditions might affect the strength of the effect of *per* on *Dreg*-5 mRNA oscillation. Under this hypothesis, a mechanism analogous to an automobile clutch would allow dissociation of the forced oscillation of *Dreg*-5 mRNA from *per* mRNA and Per protein. Such a facultative mechanism would permit coupling of *Dreg*-5 expression to *per* under most environmental conditions where this might be advantageous, while allowing dissociation under conditions where circadian oscillation of the *Dreg*-5 gene product would be deleterious.

Of the 20 *Dreg* genes isolated from a collection of several hundred independent cDNAs, *Dreg*-5 showed the closest temporal correlation with *per* mRNA cycling and it is the only *Dreg* gene tested whose phase of rhythmic expression could not be dissociated from expression of *per* mRNA by environmental manipulation (Van Gelder *et al.*, 1995). Given that the head-not-embryo collection of cDNAs is thought to represent a significant fraction of all mRNAs expressed in the head and not the early embryo (Palazzolo *et al.*, 1989), it would appear that the number of mRNAs like *Dreg*-5 (i.e. tightly coupled to *per* and dependent on *per* for their rhythmicity) is small, ~1% of the genes expressed in the head but not the early embryo or of the order of 10–20 genes. However, this estimate does not include genes that oscillate in only a subset of tissues in which they are expressed and do not therefore show strong rhythms of expression in mRNA isolated from whole fly heads.

Genes essential to the production of circadian rhythms of behavior or physiology have now been identified in six organisms: *D.melanogaster* (*per*, Konopka and Benzer, 1971; *timeless*, Sehgal *et al.*, 1994); the mold *N.crassa* (*frq*, Feldman and Hoyle, 1973); the hamster (*tau*, Ralph and Menaker, 1988); the mouse (*clock*, Vitaterna *et al.*, 1994); cyanobacteria (Kondo *et al.*, 1994) and *Arabidopsis thaliana* (Millar *et al.*, 1995). Of these genes only *per* and *frq* have been molecularly cloned and characterized. Both *per* and *frq* mRNAs oscillate with circadian rhythms. Many other diurnally and circadianly expressed mRNAs have been described in many divergent species since the first description of circadian control of mRNA expression in *Arabidopsis* (reviewed in Kay and Millar, 1993; Takahashi, 1993). Of these genes, however, only the clock-controlled genes (*ccg*-1 and *ccg*-2) in *Neurospora* (Loros *et al.*, 1989, 1993) have been identified in an organism where a genetic component of the circadian oscillator has also been characterized and thus can be placed in a genetic pathway for circadian rhythms. Both *ccg*-1 and *ccg*-2 are at least partially dependent on the *frequency* (*frq*) locus for rhythmic expression (Arpaia *et al.*, 1993). However, the temporal profiles of *ccg*-1 and *ccg*-2 expression in relation to *frq* expression under varied environmental conditions have not been described. It is thus not known whether these genes behave similarly to *Dreg*-1, *Dreg*-2 and *Dreg*-3 (Van Gelder *et al.*, 1995), which are partially dependent on *per* but can be phase dissociated from *per* expression by certain environmental stimuli, or whether, like *Dreg*-5, they are phase locked to expression of the clock molecule.

Further analysis of *Dreg*-5 and identification of additional *per*-dependent oscillating mRNAs provides a means of biochemically characterizing the output pathways of the circadian pacemaker in *Drosophila.* Mutations in several genes, including *disconnected*, produce behaviorally aperiodic flies, but do not affect the circadian rhythm of *per* mRNA expression (Hardin *et al.*, 1992a,b). By analyzing expression of *Dreg*-5 and other *per*-dependent genes in these mutant flies one can begin to elucidate the genetic pathway from the *per* gene to behavior. Likewise, behavioral analysis of *Dreg*-5 mutants provides a means of assessing the role of this gene in circadian behavior and physiology.

## Materials and methods

### *Fly strains, maintenance and collection*

The *D.melanogaster* strains used in these experiments, their housing and collection were as described previously (Van Gelder *et al.*, 1995). For the 2 h time point experiment ~$6\times10^4$ flies were seeded into each of 12 20 l population cages. These flies were maintained on LD 12:12 and food trays were introduced daily at ZT 17. One cage of flies was collected every 2 h by $CO_2$ anesthesia, followed by rapid freezing in liquid $N_2$.

### *RNA purification and Northern blotting*

Fly head purification, RNA isolation and Northern blotting were performed as described (Van Gelder *et al.*, 1995). Northern blots were probed with $10^6$ c.p.m./ml $^{32}$P-labeled antisense RNA ($10^9$ c.p.m./μg) synthesized by T7 RNA polymerase from either pDreg-5BS (containing the 3′ 0.5 kb of the original head-not-embryo *Dreg*-5 phage cDNA),

pPER0.5SK (bases 3965–4523 of 'A' form *per*; Citri *et al.*, 1987) or pRP49BSSK (the 640 bp *Eco*RI–*Hin*dIII fragment of *rp49*; O'Connell and Rosbash, 1984). Hybridization conditions were 50% formamide, 6× SSC, 5× Denhardt's reagent, 0.2% SDS, 100 μg/ml salmon sperm DNA at 65°C. Washing was to a final stringency of 0.1× SSC, 0.2% SDS at 65°C. Quantitation was performed by analyzing PhosphorImager-exposed blots with ImageQuant v. 3.22 software (Molecular Dynamics).

### Isolation and sequencing of full-length Dreg-5 clones

The long cDNA of *Dreg-5* was cloned using the original head-not-embryo collection λ-SWAJ cDNA as a probe on a fly head cDNA library (Hamilton *et al.*, 1991). Recombinant phage ($3\times10^5$) were screened and 12 positive plaques were obtained. Four of these were plaque purified and tested for insert size. The longest resulting cDNA, pDREG5EXLX, was subcloned into pEXLX using the Cre-LoxP system (Palazzolo *et al.*, 1990). Two *Apa*I–*Sac*I fragments were subcloned into pMOB and sequenced on both strands by transposon-facilitated sequencing (Strathman *et al.*, 1991). The sequence of the junction region between the two fragments was confirmed by sequencing this region of the original clone using custom oligonucleotide primers.

### Generation of Dreg-5 antibodies and Western blotting

An N-terminal Dreg-5–TrpE fusion protein was constructed by cloning the pDREG5EXLX *Nar*I–*Bcl*I fragment corresponding to amino acids 14–136 into the end-filled *Bam*HI site of expression vector pATH-3 to generate pD5NTRP. A second C-terminal fusion of amino acids 136–288 was generated by cloning the *Bcl*I–*Bam*HI pDREG5EXLX cDNA fragment into the *Bam*HI site of pATH-3 to generate pD5CTRP. Antisera to the purified fusion proteins were raised in rabbits by Josman Laboratories (Napa, CA) as previously described (Koelle *et al.*, 1991). Pooled antisera were affinity purified by negative selection against TrpE (inclusion body extract) and against the heterologous Dreg-5 fusion protein (i.e. the C-terminal fusion protein was negatively selected against the N-terminal fusion protein and vice versa), followed by positive selection against the fusion protein used to generate the antisera as described (Redding *et al.*, 1991).

For Western blots fly head extracts were prepared by homogenizing 75 fly heads in 100 μl cracking buffer (4 M urea, 2% SDS, 50 mM HEPES, pH 7.4, 0.5% β-mercaptoethanol, 10 mM EDTA) and clearing the solution by centrifugation. Protein concentrations of the extracts were estimated by Bradford assay. Aliquots of the extracts, corresponding to ~5 fly heads, were electrophoresed on 10% SDS–polyacrylamide gels and transferred to nitrocellulose by electroblotting overnight at 30 V.

## Acknowledgements

We were greatly assisted in these experiments by H.Bae. We thank N.Hacohen for performing *in situ* chromosome mapping. We are indebted to M.Palazzolo, B.Hamilton and E.Meyerowitz for provision of cDNA clones and cDNA libraries used in this study, J.C.Hall and M.Rosbash for the *per* cDNA and M.Young for the *y per*$^{01}$ flies used in these studies. We thank J.C.Hall, C.S.Pittendrigh and members of the Krasnow laboratory for valuable discussions. R.V.G. was supported by the Medical Scientist Training Program of the National Institutes of General Medical Sciences. M.A.K. was supported by a Presidential Young Investigator Award. This work was supported by a grant from the Smith-Kline Beecham Corporation.

## References

Aronson,B., Johnson,K., Loros,J. and Dunlap,J. (1994) Negative feedback defining a circadian clock: autoregulation of the clock gene frequency. *Science*, **263**, 1578–1584.

Arpaia,G., Loros,J., Dunlap,J., Morelli,G. and Macino,G. (1993) The interplay of light and the circadian clock. Independent dual regulation of clock-controlled gene *ccg-2* (*eas*). *Plant Physiol.*, **102**, 1299–1305.

Bonini,N., Leiserson,W. and Benzer,S. (1993) The *eyes absent* gene: genetic control of cell survival and differentiation in the developing *Drosophila* eye. *Cell*, **72**, 379–395.

Burbach,K., Poland,A. and Bradfield,C. (1992) Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl Acad. Sci. USA*, **89**, 8185–8189.

Citri,Y., Colot,H., Jacquier,A., Yu,Q., Hall,J., Baltimore,D. and Rosbash,M. (1987) A family of unusually spliced biologically active transcripts encoded by a *Drosophila* clock gene. *Nature*, **326**, 42–47.

Crews,S.T., Thomas,J.B. and Goodman,C.S. (1988) The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. *Cell*, **52**, 143–151.

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

Feldman,J. and Hoyle,M. (1973) Isolation of circadian clock mutants of *Neurospora crassa*. *Genetics*, **75**, 605–613.

Hall,J.C. and Rosbash,M. (1993) Oscillating molecules and how they move circadian clocks across evolutionary boundaries. *Proc. Natl Acad. Sci. USA*, **90**, 5382–5383.

Hamilton,B., Palazzolo,M. and Meyerowitz,E. (1991) Rapid isolation of long cDNA clones from existing libraries. *Nucleic Acids Res.*, **19**, 1951–1952.

Hardin,P.E., Hall,J.C. and Rosbash,M. (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature*, **343**, 536–540.

Hardin,P., Hall,J. and Rosbash,M. (1992a) Behavioral and molecular analyses suggest that circadian output is disrupted by *disconnected* mutants in *D.melanogaster*. *EMBO J.*, **11**, 1–6.

Hardin,P., Kaneko,M., Mestel,R., Hall,J. and Rosbash,M. (1992b) The restless (rls) mutant disrupts circadian activity without affecting the circadian pacemaker. *Drosophila Res. Conf. Abstr.*, **33**, 15.5.

Hardin,P.E., Hall,J.C. and Rosbash,M. (1992c) Circadian oscillations in period gene mRNA levels are transcriptionally regulated. *Proc. Natl Acad. Sci. USA*, **89**, 11711–11715.

Hoffman,E., Reyes,H., Chu,F., Sander,F., Conley,L., Brooks,B. and Hankinson,O. (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science*, **252**, 954–958.

Huang,Z., Edery,I. and Rosbash,M. (1993) PAS is a dimerization domain common to *Drosophila period* and several transcription factors. *Nature*, **364**, 259–262.

Jackson,F.R. (1993) Circadian rhythms mutants of *Drosophila*. In Young,M.W. (ed.), *Molecular Genetics of Biological Rhythms*. Marcel Dekker, New York, NY, pp. 91–122.

Kay,S.A. and Millar,A.J. (1993) Circadian-regulated *cab* gene transcription in higher plants. In Young,M.W. (ed.), *Molecular Genetics of Biological Rhythms*. Marcel Dekker, New York, pp. 73–89.

Koelle,M., Talbot,W., Seagraves,W., Bender,M., Cherbas,P. and Hogness,D. (1991) The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell*, **67**, 59–77.

Kondo,N., Tsinoremas,N., Golden,S., Johnson,C., Kutsuna,S. and Ishiura,M. (1994) Circadian clock mutants of cyanobacteria. *Science*, **266**, 1233–1236.

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

Liu,X., Zwiebel,L., Hinton,D., Benzer,S., Hall,J. and Rosbash,M. (1992) The *period* gene encodes a predominantly nuclear protein in adult *Drosophila*. *J. Neurosci.*, **12**, 2735–2744.

Liu,Y., Tsinoremas,N., Johnson,C., Lebedeva,N., Golden,S., Ishiura,M. and Kondo,T. (1995) Circadian orchestration of gene expression in cyanobacteria. *Genes Dev.*, **9**,1469–1478.

Loros,J., Denome,S. and Dunlap,J. (1989) Molecular cloning of genes under the control of the circadian clock in *Neurospora*. *Science*, **243**, 385–388.

Loros,J.J., Lichens-Park,A., Lindgren,K.M. and Dunlap,J.C. (1993) Molecular genetics of genes under circadian temporal control in *Neurospora*. In Young,M.W. (ed.), *Molecular Genetics of Biological Rhythms*. Marcel Dekker, New York, pp. 91–121.

McClung,C., Fox,B. and Dunlap,J. (1989) The *Neurospora* clock gene *frequency* shares a sequence element with the *Drosophila* clock gene *period*. *Nature*, **339**, 558–562.

Millar,A., Carre,I., Strayer,C., Chua,N. and Kay,S. (1995) Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science*, **267**, 1161–1163.

O'Connell,P. and Rosbash,M. (1984) Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Res.*, **12**, 5495–5513.

Page,T. (1994) Time *is* the essence: molecular analysis of the biological clock. *Science*, **263**, 1570–1572.

Palazzolo,M.J., Hyde,D.R., VijayRaghavan,K., Mecklenburg,K., Benzer,S. and Meyerowitz,E. (1989) Use of a new strategy to isolate and characterize 436 *Drosophila* cDNA clones corresponding to RNAs detected in adult heads but not early embryos. *Neuron*, **3**, 527–539.

Palazzolo,M., Hamilton,B., Ding,D., Martin,C., Mead,D., Mierendorf,R., Raghavan,K., Meyerowitz,E. and Lipshitz,H.D. (1990) Phage lambda

cDNA cloning vectors for subtractive hybridization, fusion-protein synthesis and Cre-loxP automatic plasmid subcloning. *Gene*, **88**, 25–36.

Ralph,M. and Menaker,M. (1988) A mutation in the circadian system in golden hamsters. *Science*, **241**, 1225–1227.

Rechsteiner,M., Rogers,S. and Rote,K. (1987) Protein structure and intracellular stability. *Trends Biochem. Sci.*, **12**, 390–394.

Redding,K., Hokomb,C. and Fuller,R. (1991) Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in yeast *Saccharomyces cerevisiae*. *J. Cell Biol.*, **113**, 527–538.

Seghal,A., Price,J.L., Man,B. and Young,M.W. (1994) Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant timeless. *Science*, **263**, 1603–1606.

Strathman,M., Hamilton,B., Mayeda,C., Simon,M., Meyerowitz,E. and Palazzolo,M. (1991) Transposon-facilitated DNA sequencing. *Proc. Natl Acad. Sci. USA*, **88**, 1247–1250.

Takahashi,J. (1993) Circadian-clock regulation of gene expression. *Curr. Opin. Genet. Dev.*, **3**, 301–309.

Van Gelder,R., Bae,H., Palazzolo,M. and Krasnow,M. (1995) Extent and character of circadian gene expression in *Drosophila melanogaster*: identification of twenty oscillating mRNAs in the fly head. *Curr. Biol.*, **5**, 1424–1436.

Vitaterna,M. *et al.* (1994) Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science*, **264**, 719–725.

Wheeler,D., Hamblen-Coyle,M., Dushay,M. and Hall,J. (1993) Behavior in light–dark cycles of *Drosophila* mutants that are arrhythmic, blind, or both. *J. Biol. Rhythms*, **8**, 67–94.

Zeng,H., Hardin,P.E. and Rosbash,M. (1994) Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling. *EMBO J.*, **13**, 3590–3598.

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

*Received on August 10, 1995; revised on November 24, 1995*

## Note added in proof

The *timeless* gene was recently cloned. Its mRNA cycles in phase with *per* and *Dreg*-5 and is dependent on *per* [Sehgal,A., Rothenfluh-Hifiker,A., Hunter-Ensor,M., Chen,Y., Myers,M. and Young,M. (1995) Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation. *Science*, **270**, 811–815].