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

Russell N. Van Gelder1,2,3,4 and Mark A. Krasnow

1Department of Biochemistry and 2Basic Sleep Research Laboratory, Stanford University School of Medicine, Stanford, CA 94305, USA
3Present address: Department of Ophthalmology and Visual Sciences, Washington University Medical School, 660 South Euclid Avenue, St Louis, MO 63110, USA
4Corresponding 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 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 Loris 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...
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 per0) 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 per0 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 per0 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 ninaE) tested (data not shown). Light–dark cycles can drive rhythmic behavior in per0 flies (Wheeler et al., 1993), and other Dreg genes (such as Dreg-3) continued to display diurnal rhythms of mRNA expression in per0 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
**Circadian expression of Dreg-5 gene in Drosophila**

**A**

![Fraction maximal expression vs Hours since Zeitgeber Time 0](image)

**B**

![Fraction maximal expression vs Circadian Time](image)

**C**

![Western blot of Dreg-5, period, and rp49 mRNA](image)

**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 nonpolyadenylated and probably represents unprocessed transcript.

**Fig. 3.** Diurnal rhythmicity of (A) Dreg-5 and (B) *per* mRNA in wild-type (Canton-S) and *per*° 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 (McCung *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-
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.

While demonstration of the role of Dreg-5 in the genetics 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 flies keep 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 differ-
ent post-transcriptional controls. This dissociation of syn-
chronized 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 con-
ditions 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 ccc-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 ccc-1 and ccc-2 are
at least partially dependent on the frequency (frq) locus
for rhythmic expression (Arpia et al., 1993). However,
the temporal profiles of ccc-1 and ccc-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 addi-
tional per-dependent oscillating mRNAs provides a means
of biologically characterizing the output pathways of the
circadian pacemaker in Drosophila. Mutations in several
genes, including disconnected, produce behavior-
ally 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 ~6x10^4 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 per-
formed as described (Van Gelder et al., 1995). Northern blots were
probed with 10^4 c.p.m.ml^{-1} ^32P-labeled antisense RNA (10^4 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),
Isolation and sequencing of full-length Dreg-5 clones

The long cDNA of Dreg-5 was cloned using the original head-not-embryo collection A-SWAX cDNA as a probe on a fly head cDNA library (Hamilton et al., 1991). Recombinant phage (3×10⁵) were screened and 12 positive plaques were obtained. Four of these were plaque purified and tested for insert size. The longest resulting cDNA, pDREGSEXLL, was subcloned into pEXLX using the Cre-LoxP system (Palazzolo et al., 1990). Two Apal–SacI 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 pDREGSEXLL NarI–BcII fragment corresponding to amino acids 14–136 into the end-filled BamHI site of expression vector pATH-3 to generate pDSNTRP. A second C-terminal fusion of amino acids 136–282 was generated by cloning the Bell–BamHI fragment of pDREGSEXLL into the BamHI site of pATH-3 to generate pDSCTRPT. Antiserum 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 antiserum as described (Redding et al., 1991).

For Western blots fly head extracts were prepared by homogenizing 75 fly heads in 100 μl craking 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[III] 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


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* [Seghal, A., Rothenfluh-Hiticker, 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].