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Comparative Analysis of Pdf-Mediated Circadian Behaviors Between *Drosophila melanogaster* and *D. virilis*

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ABSTRACT

A group of small ventrolateral neurons (s-LN$_v$'s) are the principal pacemaker for circadian locomotor rhythmicity of *Drosophila melanogaster*, and the pigment-dispersing factor (Pdf) neuropeptide plays an essential role as a clock messenger within these neurons. In our comparative studies on Pdf-associated circadian rhythms, we found that daily locomotor activity patterns of *D. virilis* were significantly different from those of *D. melanogaster*. Activities of *D. virilis* adults were mainly restricted to the photophase under light:dark cycles and subsequently became arrhythmic or weakly rhythmic in constant conditions. Such activity patterns resemble those of *Pdf*^{01} mutant of *D. melanogaster*. Intriguingly, endogenous *D. virilis Pdf* (*DvPdf*) expression was not detected in the s-LN$_v$-like neurons in the adult brains, implying that the *Pdf*^{01}-like behavioral phenotypes of *D. virilis* are attributed in part to the lack of *DvPdf* in the s-LN$_v$-like neurons. Heterologous transgenic analysis showed that *cis*-regulatory elements of the *DvPdf* transgene are capable of directing their expression in all endogenous Pdf neurons including s-LN$_v$'s, as well as in non-Pdf clock neurons (LN$_d$'s and fifth s-LN$_v$) in a *D. melanogaster* host. Together these findings suggest a significant difference in the regulatory mechanisms of *Pdf* transcription between the two species and such a difference is causally associated with species-specific establishment of daily locomotor activity patterns.

PIGMENT-dispersing hormone (Pdh) was initially identified in crustaceans (Ferlund 1976) as a mediator for the dispersion of extraretinal screening pigments to enhance visual sensitivity (reviewed in Aréchiga *et al.* 1993; Rao 2001). Persistent daily fluctuations in the pigmentation pattern even under a constant condition have suggested that the pigment dispersion rhythm is under the control of an endogenous clock system, thus implicating Pdh as a hormonal factor that channels the central circadian clock functions.

Structural homologs of the crustacean Pdh have been found in diverse insect groups, and these insect peptides are referred to as pigment dispersing factors (Pdf) (Rao and Riehm 1993). Pdf-immunoreactive (ir) neurons in many insects are typically found in the lateral margin of the anterior protocerebrum (accessory medulla) that neighbors the medulla of the optic lobes (Homberg *et al.* 1991b; Sehadova *et al.* 2003). Experiments involving surgical extirpation suggested this protocerebral region as a site of circadian pacemaker activity in several insects (Page 1982; reviewed in Saunders 2002). In addition, a dosage- and time-dependent phase shift of locomotive activity in response to injection of Pdf peptide into the accessory medulla in cockroaches and crickets implies that Pdf plays a modulatory function in the insect biological clock system (Stengl and Homberg 1994; Petri and Stengl 1997; Singaravel *et al.* 2003).

Further understandings of Pdf functions associated with the insect clock came from molecular and neurogenetic studies in the fruit fly, *Drosophila melanogaster*. Since the identification of the *period* (*per*) as the first genetic locus associated with circadian clock functions (Konopka and Benzer 1971), extensive investigations have been undertaken to understand molecular and cellular bases of the endogenous time-keeping system in *D. melanogaster*. These studies unraveled a group of Per-producing ventrolateral neurons (LN$_v$'s) as a strong candidate for the circadian pacemaker (Ewer *et al.* 1992; Frisch *et al.* 1994). Subsequently, detection of Pdf immunoreactivity in the LN$_v$'s provided an important clue that Pdf functions in the circadian rhythms in this genetically amenable insect (Helfrich-Förster 1995). In line with this, lack of Pdf-ir neurons in behaviorally arrhythmic *disconnected* (*disco*) mutants and behavioral alteration by ectopic expression of the locust *Pdf* gene further supported Pdf as a regulator of circadian rhythms (Dushay *et al.* 1989; Helfrich-Förster 1998; Helfrich-Förster *et al.* 2000).

More decisive evidence for the role of Pdf came from genetic studies using a *Pdf*-null mutation (*Pdf*^{01}) and selective ablation of Pdf neurons. These types of genetic manipulations caused similar arrhythmic free-running

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. FJ043031 (*MdPdf*) and FJ154750 (*AgPdf*).

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locomotor activity under continuous darkness (Renn *et al.* 1999). Consistent with these findings, mutants lacking Pdf-receptor functions phenocopy behavioral defects of Pdf^{01}, thus confirming essential roles played by the Pdf-signaling pathway in the regulation of circadian rhythms (Hyun *et al.* 2005; Lear *et al.* 2005; Mertens *et al.* 2005). Multiple lines of evidence also suggest that Pdf is important for intercellular communication between Pdf neurons and non-Pdf clock neurons, and self-sustaining molecular oscillation in both types of neurons, particularly under constant darkness conditions (Peng *et al.* 2003; Lin *et al.* 2004; Wu *et al.* 2008).

Expression of the *D. melanogaster Pdf* (*DmPdf*) gene in the protocerebrum is evident in two distinct clusters of ventrolateral neurons, which are classified into large (l)-LN_v's and small (s)-LN_v's on the basis of their sizes of the somata and in a pair of neurons located in the tritocerebrum (Helfrich-Förster 1997). The latter group appears to be eliminated via programmed cell death shortly after eclosion, thereby leaving only the LN_v's in the mature adult brain (Renn *et al.* 1999). Various lines of evidence demonstrated that the s-LN_v neurons are particularly important for the circadian locomotor activity rhythms (Helfrich-Förster 2005; Taghert and Shafer 2006, and references therein). Moreover, *DmPdf* expression is differentially regulated between the two groups of neurons, as transcription of *DmPdf* is absent specifically in the s-LN_v's but not in the l-LN_v's in the $Clock^{Jrk}$ (Clk^{Jrk}) and $cycle^{0}$ (cyc^{0}) mutants, suggesting that Clk and Cyc central clock regulators are upstream factors of the *DmPdf* within the s-LN_v's (Park *et al.* 2000). In addition, circadian fluctuations of the Pdf immunoreactivity at the s-LN_v terminals indicate that the rhythmic release of the Pdf peptide from the s-LN_v neurons is an important cellular event for the circadian rhythmicity (Park *et al.* 2000; Nitbach *et al.* 2006).

Despite well-defined clock functions played by Pdf in *D. melanogaster*, it has not been determined whether Pdf's function as a circadian regulator is general in other insects. In the hawk moth, *Manduca sexta*, no Pdf-ir cells were found in the accessory medulla (Homberg *et al.* 1991a; Wise *et al.* 2002). Moreover, colocalization of Per and Pdf was not detected in this neuropil in most other insects (Závodská *et al.* 2003). The same studies also showed that Per immunoreactivity was found mainly in the cytoplasm, which is contrasted to nuclear-cytoplamic shuttling of this protein within the clock neurons of *D. melanogaster* during the course of a day (*e.g.*, Shafer *et al.* 2002). These observations raise the possibility that neural and molecular bases of the biological clock system have evolved uniquely among insect species, perhaps to maximize adaptive fitness to their natural environment.

To gain insight into the evolutionary aspects of circadian rhythmicity associated with Pdf, we examined locomotor activity behavior and *Pdf* expression patterns in *D. virilis*, a species distantly related to *D. melanogaster* and diverged from the *melanogaster* lineage about 63 million years ago (Tamura *et al.* 2004). We found significantly different circadian locomotor activity patterns between the two species and such dissimilar patterns likely stem from differential regulation of *Pdf* expression in the key pacemaker neuronal groups.

MATERIALS AND METHODS

Fly strains: Flies were raised in a food containing yeast-cornmeal-agar medium supplemented with 10% methyl paraben (Tagosept) as a preservative, and kept at room temperature. Canton-S or *yellow white* (*y w*) was used as controls, and $Clock^{Jrk}$, $cycle^{0}$, and Pdf^{01} mutants were described as previously (Allada *et al.* 1998; Rutila *et al.* 1998; Renn *et al.* 1999; Park *et al.* 2000). Wild-type *D. virilis* was obtained from Hall lab (University of Maine).

Cloning and characterization of the *Pdf* gene in *D. virilis*: See supplemental material.

Histology: Digoxigenin (dig)-labeled antisense DNA probe was produced by asymmetric PCR using full-length *DvPdf* cDNA template, single primer, and dig-tagged nucleotide, as described (Kim *et al.* 2006). The probe was incubated with the CNSs, and resulting mRNA-DNA hetroduplex was detected immunologically (Lee *et al.* 2000). Pdf immunohistochemistry (IHC) of whole-mounted CNS was performed in the same manner as reported previously using rat-derived anti-Pdf (Park *et al.* 2000). Polyclonal antisera specific to the DvPdf precursor were raised against a synthetic peptide within the PAP region in two rabbits (underlined in Figure 2D). The two antisera produced identical results.

Analysis of the circadian locomotor activity rhythm: Flies were entrained for 3–4 cycles of 12-hr light:12-hr dark conditions (12:12 LD) and then proceeded into constant darkness (DD) or constant light (LL). In some experiments, the second LD cycles were provided following constant conditions. Locomotor activity of individual flies was monitored using Drosophila activity monitors (Trikinetics). For the measurement of *D. virilis* locomotor activity, 7-mm-diameter locomotor monitors equipped with dual detectors were used to allow free moving of the flies (Trikinetics; Rosato and Kyriacou 2006). Data analysis was done with ClockLab software (Actimetrics).

RESULTS

Circadian behavioral rhythms of *D. virilis*: Locomotor activity rhythms are one of the best-characterized circadian behaviors in *D. melanogaster* (Konopka and Benzer 1971; Hamblen *et al.* 1986; Rosato and Kyriacou 2006). To understand whether the circadian clock system is conserved in other Drosophila species, we analyzed this type of behavior in *D. virilis*.

Interestingly, *D. virilis* adults showed substantial differences in daily activity patterns from those of *D. melanogaster*. As observed by other studies, under LD cycles, *D. melanogaster* displayed bimodal activity peaks, each at dawn and dusk, with gradual increase in activity prior to lights on and lights off (*e.g.*, Renn *et al.* 1999 and Figure 1). By comparison, *D. virilis* flies were quiescent during the entire nighttime, and a sudden burst of activity followed immediately after lights on. Activity levels gradually rose after midday, reaching the peak at ~2 hr prior to lights

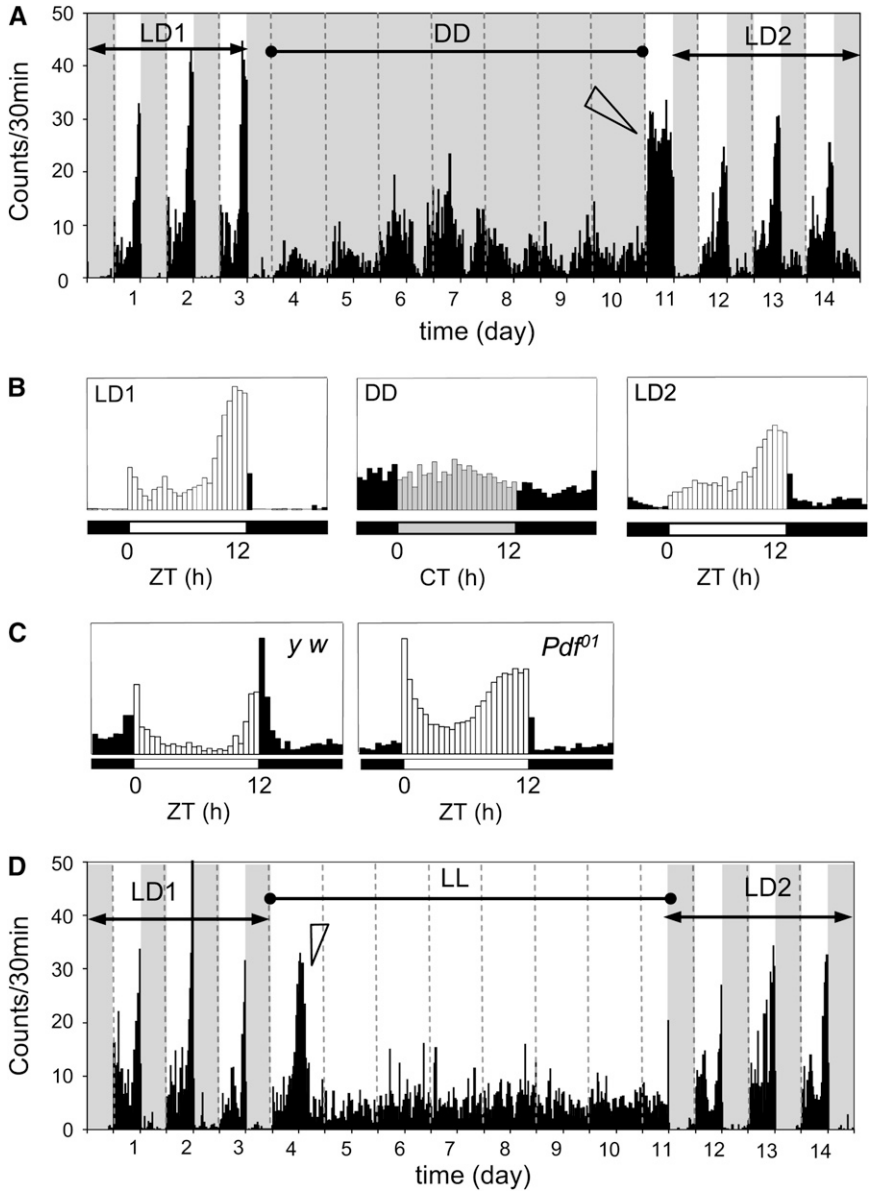

FIGURE 1.—Circadian locomotor activity rhythms in *D. virilis*. (A) The activities were measured during three 12:12 LD cycles (LD1) and subsequent 7 days of constant darkness (DD), followed by the second three LD cycles (LD2). Under LD cycling condition, flies show prominent evening activity peaks, but no anticipatory increase of activity levels before lights on. Activities are largely restricted to light phase. Arrowhead indicates persistent hyperactivity in the first daytime period following DD. (B) Average activity for each lighting regimen, as described in A. Bars indicate average activity events per 30-min bin per fly. Open bars indicate activities in daytime, solid bars in nighttime, and shaded bars in subjective day. (C) Average activity of *D. melanogaster* wild-type (*y w*) and Pdf^{01} mutants under 12:12 LD cycles. Note that Pdf^{01} behavior is somewhat comparable to that of *D. virilis*. (D) Circadian activity in constant light (LL) condition. Activity was monitored for 3 days of LD (LD1), 7 days of LL, followed by 3 days of LD (LD2). Arrowhead indicates gradual decline of activity levels in the first subjective night in LL.

off (Figure 1, A and B). Then, the evening activity vanished rapidly after lights were off and remained inactive throughout the night phase. As a result, 96% of total activity was concentrated in the photophase, indicating that this species is principally diurnal. Similar restriction of activity to the photophase was also observed for other flies (HELFRICH *et al.* 1985; CYMBOROWSKI *et al.* 1996). Such diurnally active patterns are in stark contrast to the crepuscular type of locomotor behavior displayed by wild-type *D. melanogaster*, which account for 53% of total activity in the dark phase (*e.g.*, Figure 1C).

Under DD conditions, a substantial fraction (54%) of *D. virilis* flies showed arrhythmic free-running locomotor activity, while the remaining flies displayed relatively weak rhythmicity with a short period length ($\tau = 23.2$ hr) (Table 1). This type of free-running rhythms is reminiscent of the *Pdf*-null mutant flies (RENN *et al.* 1999). When second LD cycles (LD2) were resumed after 7 days of DD, persistent hyperactivity was observed in the first light phase (arrowhead in Figure 1A), and then LD1-like activity patterns were restored, except for slightly increased night activity (Figure 1, A and B).

We also measured free-running locomotor activity in a constant light (LL) condition. Instead of a precipitous decrease of activity in response to lights off shown in LD condition, the activity levels gradually declined in the first subjective night (arrowhead in Figure 1D), and then became largely arrhythmic, which was similar to previous observations in *D. melanogaster* (KONOPKA *et al.* 1989). When flies were subsequently exposed to LD2, they were mostly inactive in the first night, and then typical diurnal activity patterns were resumed. In summary, locomotor activity of *D. virilis* is highly sensitive to

TABLE 1

Locomotor activity rhythms in DD condition

Genotype	*N*	*R* (%)	WR (%)	AR (%)	Period (hr) (mean ± SEM)	Power[a] (mean ± SEM)
y w	53	45 (84)	4 (8)	4 (8)	24.0 ± 0.5	59.4 ± 28.4
D. virilis	55	8 (15)	17 (31)	30 (54)	23.2 ± 3.3	8.3 ± 7.5
y w; Pdf^{01}	55	18 (33)	15 (27)	22 (40)	22.8 ± 1.8	20.7 ± 24.5
DvPdf^{S1a}	37	32 (86)	5 (14)	0	23.6 ± 0.5	71.9 ± 45.9
DvPdf^{S1a}; *Pdf*^{01}	25	19 (76)	5 (20)	1 (4)	24.4 ± 1.1	45 ± 40.6
DvPdf^{S2}*/CyO*	44	40 (91)	3 (7)	1 (2)	23.9 ± 2.3	60.2 ± 39.0
DvPdf^{S2}*/CyO; Pdf*^{01}	30	28 (94)	1 (3)	1 (3)	23.9 ± 0.5	75.0 ± 29.8
D. virilis (LL)	58	6 (10)	21 (36)	31 (54)	26.5 ± 7.2	6.1 ± 4.3

N, number of lines tested; *R*, rhythmic (power ≥10); WR, weak rhythmic (0 < power < 10); AR, arrhythmic; LL, locomotor activity in constant light condition.

[a] Power was defined as the amplitude of the peak above the significant line ($\alpha = 0.025$) in the chi-square periodogram (Liu *et al.* 1991).

lighting conditions, such that the flies seem to be motivated to move actively during daytime. Moreover, endogenous clock functions governing free-running activity rhythms are not as robust as *D. melanogaster* ones.

Characterization of the *D.* virilis Pdf (DvPdf) gene: The foregoing behavioral data of *D. virilis* showed strong resemblance to those of *Pdf*-null (*Pdf*^{01}) mutant flies of *D. melanogaster*: the lack of lights-on anticipation, slight phase advance of evening activity peak, and significantly arrhythmic free-running behavior in DD (Figure 1C; Renn *et al.* 1999). Such similarity led us to wonder whether the genome of our wild-type *D. virilis* carries a spontaneous *Pdf* loss-of-function mutation, as was the case for *Pdf*^{01} mutation that we described previously in *D. melanogaster* (Renn *et al.* 1999). Thus we first cloned and characterized *DvPdf* sequence (Figure 2A). The sequence data matched perfectly with the open reading frame (ORF) later published in the genome database for *D. virilis* (*Dvir\GJ23022*; Drosophila 12 Genomes Consortium 2007).

Results from RACE defined a 614-bp *DvPdf* transcriptional unit (supplemental Figure 1). Northern blot analysis agreed with this result, as it revealed ~0.8-kb *DvPdf* transcript expressed mainly in the head of male or female flies (Figure 2B). Comparison of the cDNA sequence with genomic sequence showed that the *DvPdf* is a single-exon gene, as is the case for the *DmPdf* gene (Park and Hall 1998). The Southern blot result suggests that the *DvPdf* gene is present in a single copy per haploid genome (Figure 2C).

Conceptual 99-amino-acid DvPdf peptide precursor consists of three distinct domains: signal sequence (24 aa) at the N terminus for secretory pathway, followed by Pdf-associated peptide (PAP, 53 aa), then by Pdf at the C terminus (Figure 2D). This tripartite structure is typical of Pdf precursors identified in other arthropod species (*e.g.*, Rao 2001). The presence of dibasic consensus cleavage site (KR) just before the Pdf indicates that the precursors are processed to produce mature 18-amino-acid-long Pdf peptide. Alignment of several dipteran Pdf precursors shows that the mature Pdf sequences are highly conserved, which points to the physiological significance of this domain (Figure 2D). In contrast, PAP regions are markedly diverged; for instance, a mere 22% of identity is observed in the precursor region between housefly (*Musca domestica*) and *D. virilis* (Figure 2E).

In two species outside the Drosophila genus, we found that the *Pdf* gene contains an intron within the open reading frame. Comparison of cDNA sequence (Matsushima *et al.* 2004) with PCR-amplified genomic DNA one revealed a 63-bp phase-0 intron (*i.e.*, between two codons) in the *M. domestica Pdf* (*MdPdf*) gene (GenBank accession no. FJ043031). *Anopheles gambiae Pdf* (*AgPdf*) also contains a larger 278-bp intron (also phase-0) at a comparable position on the basis of the reported genome database (GenBank accession no. FJ154750). Thus it seems that *Pdf* gene structure and sequence have changed significantly during the course of dipteran evolution, as was observed for another neuropeptide gene Corazonin (Choi *et al.* 2005).

Lack of *Pdf* expression in the s-LN$_v$-like neurons in *D. virilis* adults: Expression of the *D. melanogaster Pdf* (*DmPdf*) is limited to two neuronal groups, s-LN$_v$'s and l-LN$_v$'s, in the ventrolateral margin of the anterior protocerebrum of mature adults. These two groups are easily recognizable due to distinct sizes of their somata and axonal projection patterns unique to each group (Figure 3A; Park *et al.* 2000; Helfrich-Förster *et al.* 2007). DmPdf production from the s-LN$_v$'s is particularly important for the circadian rhythms in this species.

To investigate the causal relationship between *DvPdf* expression and *Pdf*^{01}-like activity patterns displayed by *D. virilis*, *DvPdf* expression was examined in the brain of *D. virilis* adults using anti-Pdf that detects a mature peptide. Surprisingly, Pdf immunoreactivity was found exclusively in a cluster of four neurons that gave rise to

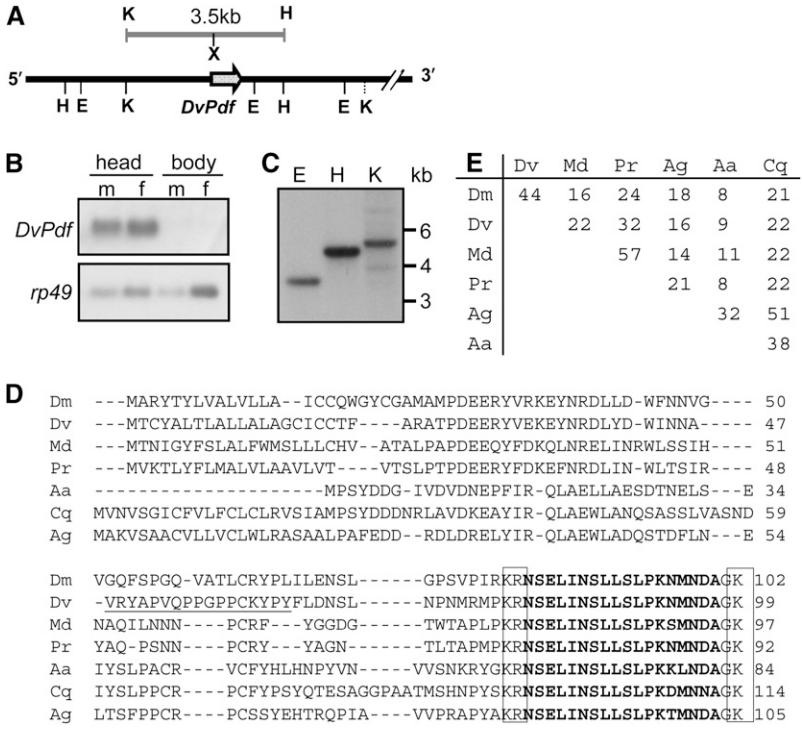

FIGURE 2.—Molecular characteristics of the *DvPdf* gene. (A) Restriction map of the 11.7-kb *D. virilis* genomic DNA fragment containing *DvPdf* gene (arrow). A 3.5-kb subfragment defined by *Kpn*I and *Hin*dIII (K–H) restriction sites was used for making *DvPdf* transgenic lines, and K–X upstream fragment (1.9 kb) for *DvPdf-gal4* drivers. The second *Kpn*I site designated by a broken line is not present in the phage DNA clone, but predicted to exist in the genome of our *D. virilis* flies on the basis of the Southern hybridization result. Such a difference is likely to reflect a polymorphic difference between the two genomic sources. E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; X, *Xcm*I. (B) Northern blotting. Total RNAs (20 μg/lane) were separately purified from *D. virilis* male (m) or female (f), heads and bodies, and then hybridized to ^{32}P-labeled *DvPdf* cDNA probe. A probe for the ribosomal protein 49 (*rp49*) gene was used as a loading control. (C) Southern blot analysis. *D. virilis* genomic DNA (30 μg/lane) was digested with restriction enzymes as indicated (E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I) and the blot was hybridized to ^{32}P-labeled *DvPdf* cDNA probe. Numbers on the right indicate size markers (kb). (D) Alignment of dipteran Pdf precursors by using Web-based ClustalW2 software (www.ebi.ac.uk/Tools/clustalw2/index.html). The numbers indicate amino acid length of each precursor. Boldface letters represent mature Pdf peptide, and the underlining indicates residues for producing DvPdf-specific antibody. Consensus prohormone proteolytic cleavage site (KR) and C-terminal amidation signal (GK) are indicated by boxes (*cf.* VEENSTRA 2000). Dm, *D. melanogaster*; Dv, *D. virilis*; Pr, *Phormia regina*; Md, *Musca domestica*; Ag, *Anopheles gambiae*; Aa, *Aedes agypti*; Cq, *Culex quinquefasciatus*. (E) Amino acid identity of the Pdf precursors. Consensus modification sites (boxes) and mature Pdf region are excluded for this comparison.

contralateral projections through the posterior optic tract (POT) and extensive arborization in the medulla of the optic lobes (Figure 3B, $n = 20$). These neuroanatomical features are comparable to those of the l-LN$_v$'s of *D. melanogaster*, suggesting that DvPdf-expressing neurons are equivalent to the l-LN$_v$'s. Furthermore, the absence of s-LN$_v$-like Pdf immunoreactivity as well as short and dorsal fibers deviated from the POT in the medial region (arrowhead in Figure 3B), are remarkably similar to the Pdf-ir patterns described for the *Clk^{Jrk}* and *cyc^{02}* mutants of *D. melanogaster* (PARK *et al.* 2000).

It is possible that s-LN$_v$-like neurons process DvPdf precursors differentially to contain PAP as a functional peptide, thereby lacking Pdf immunoreactivity. To test this, we performed IHC using antisera raised against the PAP region of the DvPdf precursor (anti-DvPdf), as indicated in Figure 2D. The results from this experiment were identical to those obtained with anti-Pdf (Figure 3, B *vs.* C, $n = 18$). In addition, *in situ* hybridization also produced signals only in the l-LN$_v$-like neurons, verifying a limited transcriptional activity of the *DvPdf* gene to these neurons (Figure 3, D and E, $n = 18$). We also confirmed the lack of s-LN$_v$ Pdf immunoreactivity in a different *D. virilis* strain that we obtained from the University of California San Diego Drosophila Stock Center (15010–1051.09) (data not shown, $n = 5$). Therefore, transcriptional regulatory mechanisms of the *Pdf* gene seem to have evolved differentially between the two species. Taking all of these data into consideration, we propose that the lack of *DvPdf* expression in the s-LN$_v$-like pacemaker neurons is responsible for the *Pdf^{01}*-like behavior phenotype of *D. virilis*.

Expression of the *D. virilis* Pdf gene in *D. melanogaster*: Since tissue-specific expression of a gene is regulated by an interaction between *cis*-acting elements and their cognate *trans*-acting factors, the loss of *DvPdf* expression in the s-LN$_v$-like neurons could result from nonfunctional *cis*-acting elements within the *DvPdf* regulatory region. If so, then *DvPdf cis*-acting elements would likely be inactive in the s-LN$_v$'s of *D. melanogaster* host as well. To test this hypothesis, we employed a heterologous transgenic assay in which a 3.5-kb genomic fragment, spanning from 1.9-kb 5′ upstream to 0.9-kb downstream of the *DvPdf* gene, was introduced into the *D. melanogaster* genome (Figure 2A). To detect *DvPdf* expression unambiguously in the *D. melanogaster* CNS, the transgene was recombined with the *Pdf^{01}* allele. In this genetic context, only *DvPdf* transgene would produce Pdf-ir materials, as *Pdf^{01}* mutant CNSs are devoid of Pdf completely (RENN *et al.* 1999). IHC using either anti-Pdf or anti-DvPdf revealed robust DvPdf immunoreactivity in both l-LN$_v$ and s-LN$_v$ groups (Figure 4A, arrowheads) as well as in the abdominal ganglionic neurons ($n = 8$, data not shown). These data suggest that *DvPdf*'s

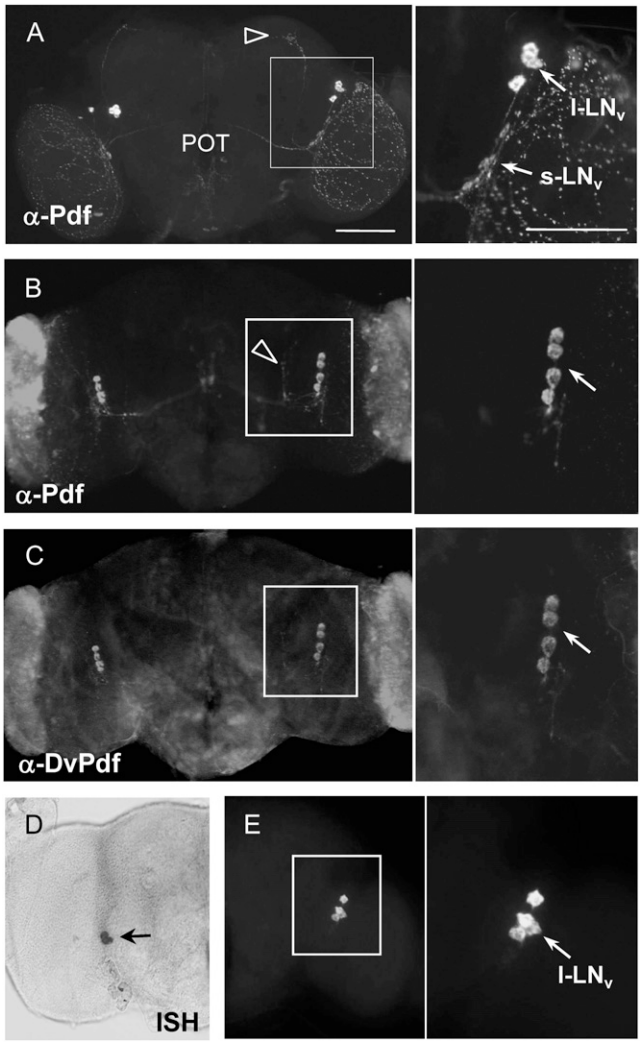

FIGURE 3.—*Pdf* expression patterns in *D. virilis*. (A) Pdf-ir patterns in *D. melanogaster* adult brain. Small and large ventrolateral neurons (s-LN_v and l-LN_v) are indicated by arrows and dorsally oriented projections from the s-LN_v's, by an arrowhead. Area marked by a box is enlarged in the right. (B and C) Pdf-ir patterns observed in *D. virilis* adult brain. IHC was done (B) with anti-Pdf or (C) anti-DvPdf raised against DvPdf specific region (Figure 2D). (D and E) *In situ* hybridization (ISH) of *DvPdf* mRNA in the *D. virilis* adult brain. ISH signal was detected (D) by a colorimetric reaction, or (E) fluorescence using tyramide signal amplification system (TSA). Both methods confirmed localization of the *DvPdf* mRNA only in l-LN_v-like neurons (arrows). Bar, 100 μm.

regulatory sequence is recognized by host transcription factors, thus capable of directing *DvPdf* expression in all endogenous neuronal groups in *D. melanogaster*.

Interestingly, we found transgenic *DvPdf* expression in two additional groups of neurons. One of them, consisting of three to four neurons per brain lobe, located in a region dorsal to the l-LN_v's (arrow in Figure 4A), while the other single neuron with relatively weak immunoreactivity was observed in the vicinity of the l-LN_v's. Due to weak staining intensities, the latter neuron was not always clearly distinguishable. These ectopic neurons were consistently identified in two independent transgenic lines, $DvPdf^{S1a}$ and $DvPdf^{T3}$ (Figure 4A, and data not shown, $n > 10$ for each transgene). To distinguish more clearly these ectopic neuronal groups from the l-LN_v's and s-LN_v's, *DvPdf* expression was examined in the CNS lacking the endogenous Pdf neurons. For this, selective ablation of the l-LN_v's and s-LN_v's was achieved through the expression of a proapoptotic gene, *reaper* (*rpr*) by using *DmPdf-gal4* driver (*cf.* RENN *et al.* 1999). The result confirmed two distinct groups of ectopic Pdf-ir neurons, while two groups of endogenous neurons were successfully removed (Figure 4B).

To further investigate whether such ectopic expression reflects transcriptional activity of the *DvPdf* transgene, *DvPdf-gal4* drivers using ~1.9-kb upstream sequence were employed to express GFP reporter gene. As a result, GFP expression was clearly detected in the s-LN_v's, l-LN_v's, as well as in the extra sets of neurons (numbered in Figure 4C). Thus it is apparent that *DvPdf* expression reflects *bona fide* transcriptional activity regulated by the *DvPdf* *cis*-acting sequences in the heterologous host.

The ectopic DvPdf neurons showed anatomical resemblance to those described for non-Pdf clock neurons, dorsolateral neurons (LN_d), and the fifth s-LN_v. To verify this, we carried out a double-labeling experiment in which *DvPdf-gal4*-driven GFP-marked neurons were labeled with anti-Timeless (Tim). Tim, as a clock protein, has been shown to be a marker of the LN_d and the fifth s-LN_v neurons, let alone s-LN_v's and l-LN_v's (KANEKO and HALL 2000; RIEGER *et al.* 2006; HELFRICH-FÖRSTER *et al.* 2007). Indeed, all GFP-positive neurons marked "1" (Figure 4C) were also labeled with anti-Tim (Figure 4D, $n = 12$), confirming that these neurons are a subset of the LN_d neurons. The other extra single neuron marked "2" in the vicinity of the l-LN_v's (Figure 4C) was also Tim-ir (Figure 4D), thus this neuron is comparable to the one previously described as the fifth s-LN_v (HELFRICH-FÖRSTER *et al.* 2007).

We previously demonstrated that two or three neurons out of six LN_d's per brain lobe (designated as L1-s) produce neuropeptide-F (npf) in the male brain (LEE *et al.* 2006). To examine whether DvPdf-LN_d's overlap with *npf*-expressing ones, *npf-gal4*-driven GFP-labeled L1-s neurons were immunostained with anti-Pdf. As a result, approximately two L1-s neurons were found to produce DvPdf (Figure 4E). Unlike *npf*, however, *DvPdf* expression is common to both sexes. These findings support functional diversity among the six LN_d's, as proposed previously (LEE *et al.* 2006; RIEGER *et al.* 2006; HELFRICH-FÖRSTER *et al.* 2007).

Regulation of the *DvPdf* gene by central clock factors: Selective loss of *DmPdf* expression in the s-LN_v's was observed in the brain of arrhythmic Clk^{Jrk} and cyc^{0} mutants, suggesting that Clk:Cyc heterodimer transcription factors are required for *DmPdf* transcription particularly in this neuronal group (PARK *et al.* 2000). This

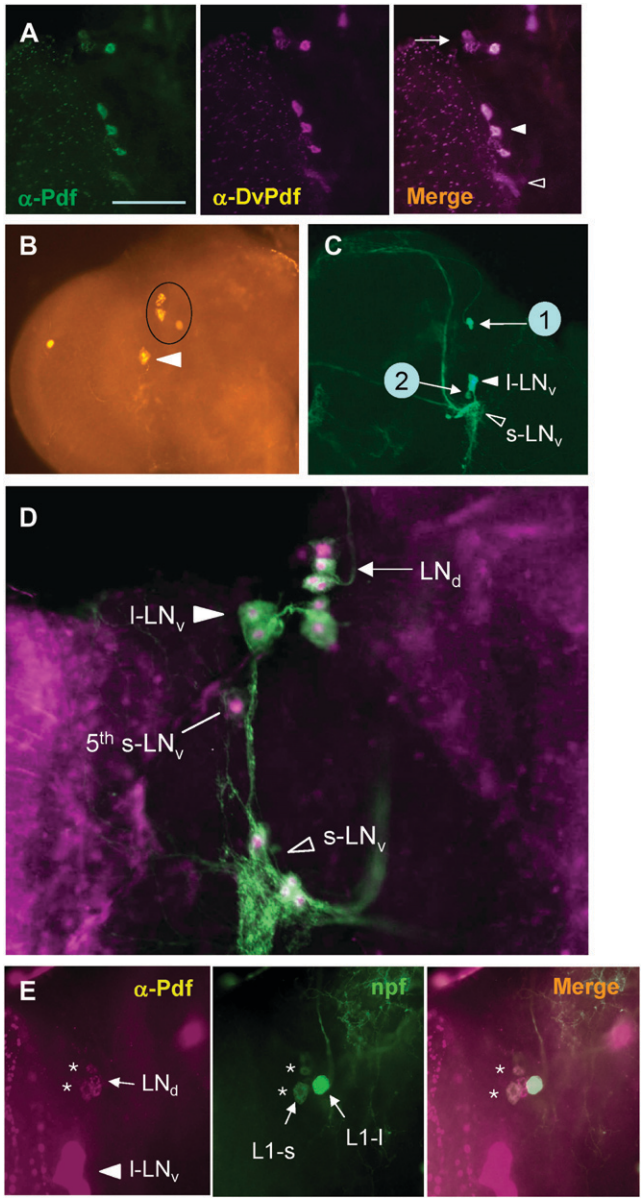

FIGURE 4.—Expression of the *DvPdf* transgene in *D. melanogaster*. (A) Expression of the *DvPdf*^{S1a} transgene in the *Pdf*-null (*Pdf*^{01}) mutant. Both anti-Pdf (green) and anti-DvPdf (magenta) detect all l-LN$_v$'s (solid white arrowhead) and s-LN$_v$'s (open arrowhead). In addition to these normal Pdf neurons, a group of Pdf-ir neurons are found in a region dorsal to the l-LN$_v$'s (arrow). (B) Pdf immunoreactivity of the progeny from {*DmPdf-gal4*; *UAS-rpr* × *DvPdf*^{S1a}; *DvPdf*^{T3}}. This type of transgenic manipulation kills all endogenous Pdf neurons. Only two groups of ectopic DvPdf neurons are stained (circle and arrowhead). (C) *DvPdf*-promoted GFP expression. Progeny from {*DvPdf-gal4* × *UAS-mCD8GFP*} cross was processed to visualize GFP signals in adult brains. Two groups of ectopic neurons are designated by numbers. (D) Immunostaining of GFP-labeled *DvPdf* neurons with anti-Tim at ZT20 (lights are off at ZT12 in 12:12 LD cycles). Nuclear anti-TIM immunoreactivity (magenta) is clearly observed within all of the *DvPdf*-producing neurons (green). (E) Double labeling of the LN$_d$'s with npf and DvPdf. Progeny from {*UAS-mCD8GFP*; *npf-gal4* × *DvPdf*^{S1a}; *DvPdf*^{T3}} were immunostained with anti-Pdf. On average, 1.9 of 2.4 npf neurons (*i.e.*, L1-s) are positive for DvPdf ($n = 10$ brain hemisphere), as indicated by asterisks. L1-l is another npf neuron, which is not part of LN$_d$'s.

prompted us to examine whether the Clk and Cyc similarly control the *DvPdf* transgene. To test this, the *DvPdf* transgene was recombined with *Clk*^{Jrk} or *cyc*^{0} alleles and *DvPdf* expression was examined in these mutants. As a result, the *DvPdf* expression was detected only in the l-LN$_v$'s in *Clk*^{Jrk}, *cyc*^{01}, and *cyc*^{02} mutant brains (Figure 5, C–F *vs.* A and B), suggesting that the mutant alleles abolish expression of endogenous *DmPdf* as well as *DvPdf* transgene in the s-LN$_v$'s. Of interest, the *Clk*^{Jrk} and *cyc*^{0} mutations also eliminated *DvPdf* expression in the LN$_d$'s and fifth s-LN$_v$ (Figure 5, B *vs.* C–F). Thus we concluded that Clk and Cyc are essential upstream components for the expression of *DmPdf* as well as *DvPdf* transgene in neurons outside the l-LN$_v$'s.

Rescue of arrhythmic *Pdf*^{01} mutant behavior by the *DvPdf* transgene: Since Pdf is essential for normal circadian locomotor activity rhythms in *D. melanogaster* (RENN *et al.* 1999), we investigated whether expression of the *DvPdf* transgene can rescue *Pdf*^{01} mutant phenotypes. While ~67% of the *Pdf*^{01} flies showed arrhythmic or weakly rhythmic locomotor activity in DD condition, two independent transgenic lines carrying both the endogenous *DmPdf* gene and the *DvPdf* transgene (*i.e.*, wild-type background) or only *DvPdf* (*i.e.*, *Pdf*^{01} mutant background) showed normal locomotor activity rhythms (Figure 6 and Table 1). These results suggest that DvPdf precursors are appropriately processed to produce the functional Pdf peptide in a *D. melanogaster* host and deliver clock information properly, despite significant sequence divergence in the PAP regions (Figure 2). Ectopic production of the Pdf in the LN$_d$'s and fifth s-LN$_v$ from the *DvPdf* transgene does not seem to influence normal circadian activity rhythms.

DISCUSSION

Circadian behavior and *Pdf* expression of *D. virilis*: In this study, we have characterized circadian locomotor activity rhythms of *D. virilis*, a species that has radiated from the *melanogaster* linage ~63 million years ago, which is approximately the beginning of the Cenozoic era. During this geological period, major paleoclimatic changes are proposed to drive speciation of Drosophila (TAMURA *et al.* 2004). In addition, studies indicate diversification of native habitats of Drosophila species, as *D. virilis* is suggested to be indigenous to eastern Asia (THROCKMORTON 1982), whereas *D. melanogaster* is believed to be African origin (KELLER 2007). Therefore, it is possible that *D. virilis* and *D. melanogaster* may have evolved unique biological clock systems that suit their endemic environment. This is supported by our behavioral data that revealed substantially different daily and circadian locomotor activity patterns between *D. virilis* and *D. melanogaster*.

The first insight that Pdf might be responsible for behavioral characteristics of *D. virilis* comes from the

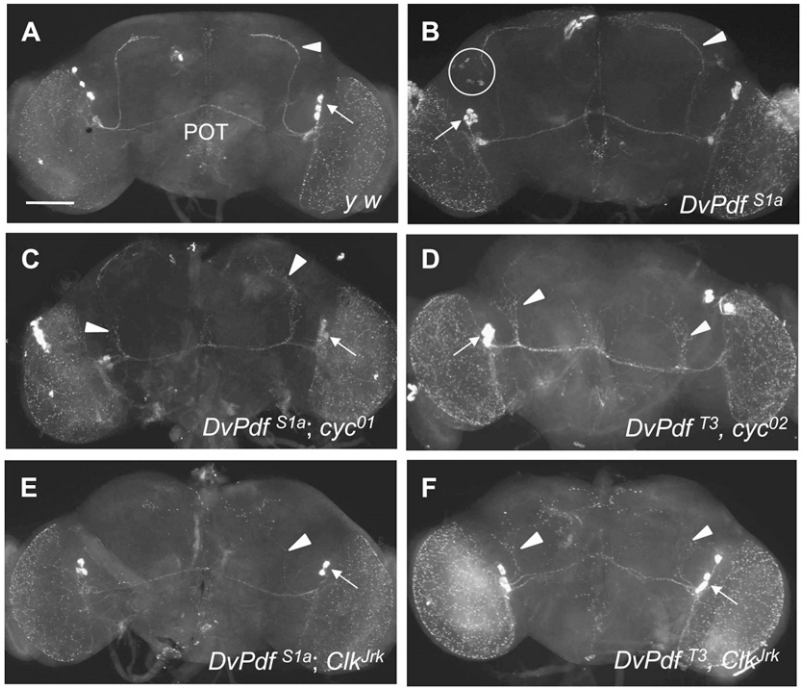

Figure 5.—Regulation of the *DvPdf* transgene expression in Clk^{Jrk} and cyc^{0} mutant. (A) Control (*y w*). Dorsally projected fibers stemming from the s-LN_v's are indicated by an arrowhead and l-LN_v's, by an arrow. POT, posterior optic tract. (B) $DvPdf^{S1a}$ transgenic line. Ectopic LN_d neurons are in the circle. (C and D) Pdf immunoreactivity in cyc^{0} mutant CNS, and (E and F) in Clk^{Jrk} mutant CNS. Two transgenic lines (S1a and T3) produce signals only in l-LN_v's (arrows). Short processes deviated from the POT are projected dorsally (arrowheads in C–F). At least 10 specimens per genotype were observed with consistent results.

uncanny resemblance in locomotor activity patterns between *D. virilis* and Pdf^{01} mutant flies of *D. melanogaster*. Under LD condition, activities of the Pdf^{01} flies are largely restricted to the daytime; such diurnally shifted activity of the Pdf^{01} flies is likely to be attributed to both prominently reduced morning anticipatory behavior and the slight phase advance of evening activity peaks to the photophase (Renn *et al.* 1999). Moreover, like *D. virilis*, Pdf^{01} flies are largely arrhythmic or weakly rhythmic in DD condition. Therefore, it is reasonable to suggest that the lack of *Pdf* expression in the s-LN_v equivalent neurons is intimately associated with behavioral characteristics of *D. virilis*. These results are also consistent with morning oscillator functions of s-LN_v's (Grima *et al.* 2004; Stoleru *et al.* 2004), and further support the importance of Pdf's role within the s-LN_v neurons for lights-on anticipatory behavior.

Daily locomotor activity patterns described for the housefly, *M. domestica* are notably similar to those of *D. virilis* (Helfrich *et al.* 1985), as both species display day-phase-restricted activity without lights-on anticipation. IHC using anti-Pdf showed both large and small LN_v-equivalent neuronal groups in the adult brain of *M. domestica* (Pyza and Meinertzhagen 1997; Pyza *et al.* 2003). In contrast to this result, *in situ* hybridization revealed *MdPdf* mRNA expression only in the l-LN_v-like neurons (Matsushima *et al.* 2004). We confirmed these results independently (data not shown, $n = 6$). A plausible explanation for this discrepancy is that the s-LN_v-like neurons contain materials that cross-react with the anti-Pdf. From these data, it is tempting to propose that lack of *Pdf* expression in s-LN_v-like neurons is also responsible for diurnally active locomotion displayed by *M. domestica*.

Regulation of *DvPdf* expression: In the heterologous host, expression of the *DvPdf* gene is evident in all of DmPdf-positive groups, suggesting that donor *DvPdf* regulatory elements are capable of interacting with host *trans*-acting factors to activate its expression in a manner similar to that of *DmPdf*. Although no useful information about potential elements emerged from simple sequence alignment between 0.5-kb upstream sequence of *DmPdf* (Park *et al.* 2000) and 1.9-kb of *DvPdf*, the *cis*-regulatory element(s) responsible for s-LN_v *Pdf* expression is likely conserved in both *DmPdf* and *DvPdf*. An important question raised from these studies is, then, Why do *D. virilis* flies lack *DvPdf* expression particularly in the s-LN_v-like neurons? It could be that *D. virilis* does not possess the s-LN_v-like neurons. However, this is unlikely, because a fly species (*M. domestica*), which is even more remotely related to *D. melanogaster*, contains Pdf-ir s-LN_v-like neurons (Pyza and Meinertzhagen 1997; Pyza *et al.* 2003), although such immunoreactivity likely originated from cross-reactivity, as mentioned earlier. We tried to confirm the presence of s-LN_v's in the *D. virilis* CNS using anti-Tim, as was done for *D. melanogaster* (Figure 4D). However, no immunosignals were detectable even in the l-LN_v's at two different time points. Although similarity of the Tim between the two species is substantial (76% overall amino acid identity; Ousley *et al.* 1998), perhaps diversity between the two proteins does not allow anti-Tim to detect *virilis* Tim protein.

In *D. melanogaster*, DmClk and DmCyc proteins are well-defined upstream positive factors responsible for *DmPdf* expression specifically in the s-LN_v's (Park *et al.* 2000). Our present study shows that these factors are also essential for the *DvPdf* transgenic expression in the

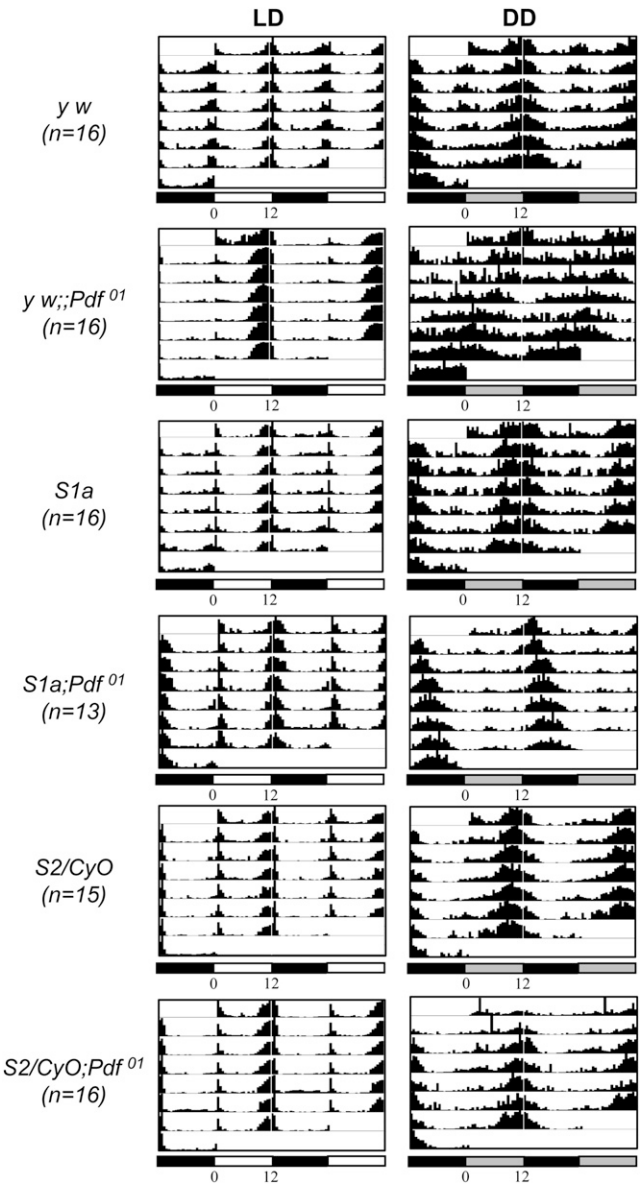

FIGURE 6.—Average actograms for indicated genotypes (*n*, number of flies). Open and solid bars, respectively, designate day and night phases in 12:12 LD, and shaded bars, subjective day in the constant dark (DD). See also Table 1 for the quantitative data analysis. Arrhythmic circadian locomotor activities displayed by Pdf^{01} mutant flies are restored by two independent *DvPdf* transgenes (S1a and S2).

s-LN_v's, LN_d's and fifth s-LN_v, suggesting that DvClk and DvCyc likely act as positive regulators for the *DvPdf* in the s-LN_v-like neurons in *D. virilis* brain. Thus the lack of *DvPdf* expression in the s-LN_v-like neurons might be due to a loss of function of these proteins in *D. virilis*.

According to the genome database, *DvClk* gene (*Dvir\GJ11427*) predicts to encode a protein of 988 amino acids. Our RT–PCR result suggests that *DvClk* from our flies encodes a 987-amino-acid product and has differences from the *Dvir\GJ11427* at three sites (data not shown). Two of them are within polyglutamate (Q) stretches, missing two Q's at one position and having the addition of one Q at another position. The other one is a homologous substitution of leucine to isoleucine (data not shown). Amino acid composition of the DvClk shows 70% identity to DmClk. For Cyc, our sequence of DvCyc deduced from RT–PCR matches perfectly to that from genome database (*Dvir\GJ14003*), and amino acid residues of the DvCyc share 85% identity with the DmCyc (data not shown). In other words, we did not find any significant mutations within the ORFs of both DvClk and DvCyc that might alter their functions. Moreover, robust activity rhythms displayed by *D. virilis* under LD cycles, in contrast to significantly abnormal LD behavior of *D. melanogaster* Clk^{Jrk} and cyc^{0} mutants (ALLADA *et al.* 1998; RUTILA *et al.* 1998), suggest that functions of the two clock proteins are unlikely defective in *D. virilis*.

Absence of s-LN_v-specific *DvPdf* expression could be accomplished through negative regulation. According to HELFRICH-FÖRSTER *et al.* (2007), l-LN_v's and LN_d's in *D. melanogaster* appear to be originated from the common precursor cells, as clusters of these cells are mixed without clear anatomical distinction in the ventral region of early pupal brain. As the pupal development progresses, presumptive LN_d's are separated from l-LN_v's, migrate dorsally, and start to develop their characteristic projections. Shortly after this stage, l-LN_v's become Pdf-positive, while LN_d's remain Pdf-negative. However, the authors found one exceptional specimen in which the migration of the LN_d's is impaired; interestingly, these neurons are Pdf-positive. We interpret these findings as follows: activation of the *DmPdf* in both l-LN_v's and LN_d's during pupal development is a default pathway, and then the suppression of the *DmPdf* is acquired during the maturation of the LN_d neurons, perhaps through the activation of repressors. These studies provide an interesting possibility of the transcriptional suppression of *DmPdf* in the LN_d's and fifth s-LN_v. This notion is supported by the ectopic *DvPdf* transgene expression in these neurons, as negative *trans*-acting factors might be unable to interact with *DvPdf*'s regulatory region due to sequence incompatibility, thus allowing ectopic expression of the *DvPdf*. As an extrapolation of these results, it would be interesting to investigate whether negative factors suppress *DvPdf* expression in the s-LN_v-like (and perhaps LN_d- and fifth s-LN_v-like) neurons in *D. virilis*. Transgenic dissection of the 1.9-kb *DvPdf* upstream region (*e.g.*, CHOI *et al.* 2008) will help reveal specific *cis*-acting elements that are necessary for such negative *DvPdf* regulation.

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