

# Genome-Wide Expression Analysis in *Drosophila* Reveals Genes Controlling Circadian Behavior

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In *Drosophila*, a number of key processes such as emergence from the pupal case, locomotor activity, feeding, olfaction, and aspects of mating behavior are under circadian regulation. Although we have a basic understanding of how the molecular oscillations take place, a clear link between gene regulation and downstream biological processes is still missing. To identify clock-controlled output genes, we have used an oligonucleotide-based high-density array that interrogates gene expression changes on a whole genome level. We found genes regulating various physiological processes to be under circadian transcriptional regulation, ranging from protein stability and degradation, signal transduc-

tion, heme metabolism, detoxification, and immunity. By comparing rhythmically expressed genes in the fly head and body, we found that the clock has adapted its output functions to the needs of each particular tissue, implying that tissue-specific regulation is superimposed on clock control of gene expression. Finally, taking full advantage of the fly as a model system, we have identified and characterized a cycling potassium channel protein as a key step in linking the transcriptional feedback loop to rhythmic locomotor behavior.

**Key words:** oligonucleotide arrays; *Drosophila*; circadian outputs; *slowpoke*; locomotor behavior; gating

Circadian behaviors take place at regular intervals because of the action of a cell autonomous clock, which marks time even in the absence of environmental information. This molecular clock relies on negative feedback loops, in which transactivators drive the transcription of repressor proteins, which in turn block transcription at their own promoters (Young and Kay, 2001). It is important to note that this oscillation at the mRNA level is only the first step toward sustainable molecular rhythms, which are accomplished by introducing a number of “delays” affecting message stability (Suri et al., 1998) and protein stability (Price et al., 1998) or controlling subcellular localization (Saez and Young, 1996).

Although we have a relatively good understanding of how these molecular oscillations are generated in *Drosophila*, a clear link between the oscillator and the downstream biological processes under clock control is still missing. Previous attempts to describe the extent of circadian transcriptional regulation have identified a handful of clock-controlled “output” genes. A cDNA library screen identified ~20 oscillating mRNAs, most of them with unknown functions (Van Gelder et al., 1995). Alternative ap-

proaches included the screening of subtractive cDNA libraries, which retrieved *circadian regulated gene-1* (Rouyer et al., 1997) and *takeout* (Sarov-Blat et al., 2000) differential display, which identified *vri* (*vri*) (Blau and Young, 1999), and luminescence-based enhancer trap screens that uncovered additional clock-controlled genes (ccgs), among those *numb* and a putative nicotinamide adenine dinucleotide kinase (Stempfl et al., 2002) and *regular* (Scully et al., 2002).

To carry out a more global examination of clock-controlled transcription, we used high-density oligonucleotide-based arrays. This approach has been used successfully in *Arabidopsis* and mammals, uncovering an astonishing range of physiological processes under circadian control (Harmer et al., 2000; Panda et al., 2002; Storch et al., 2002). Recently three groups used a similar strategy to identify the extent of clock-controlled transcription in flies. McDonald and Rosbash (2001) described 134 genes that oscillate under free-running [constant dark (DD)] conditions and display altered levels in the *Clk<sup>ir</sup>* mutant background. Claridge-Chang et al. (2001), on the other hand, analyzed cycling profiles under driven [light/dark (LD) cycles] and free-running (DD) conditions in different genetic backgrounds. They identified 158 cycling genes, some of which are involved in learning and memory, vision, olfaction, locomotion, detoxification, and metabolism. Ueda et al. (2002) reported a very striking observation. Of 712 genes cycling in the fly head in LD, only 115 were also cycling in DD, and conversely, they identified 341 genes that were considered cycling only in DD.

To gain deeper insight into clock-controlled processes in the whole fly, we probed high-density arrays with RNA isolated from both head and body fractions. We found that although the number of cycling genes is similar between the two samples, only a small proportion of genes cycles in both structures, indicating that the clock controls different aspects of physiology throughout the

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organism. As is the case in mammals, we identified a large group of genes that although expressed to mid-high levels in both fractions are cycling in only one of them. Finally, taking advantage of the fly as a model genomics organism, we have characterized a rhythmic potassium channel as a key step in linking the transcriptional feedback loop to rhythmic locomotor behavior.

## MATERIALS AND METHODS

**Fly stocks.** Time courses for the DNA chipping experiments were performed using *y w* as wild-type control and *y w; Clk<sup>trk</sup>* (Allada et al., 1998) as the mutant with impaired clock function. The *slo 4* (Atkinson et al., 1991) stock was kindly provided by Dr. N. Atkinson (University of Texas, Austin, TX). The *stI slo I* stock (Elkins et al., 1986) was a gift from the Bloomington Stock Center. *slo I* was generated by ethyl methane sulfonate mutagenesis and *slo 4* was generated by gamma irradiation; the latter is a result of a chromosome inversion with one breakpoint within this gene (Atkinson et al., 2000). *slo 4* mutants have been shown to carry a null allele by a number of approaches, and it fails to complement the *slo I* mutation in electrophysiological studies in the dorsal longitudinal flight muscle (Atkinson et al., 1991). No molecular characterization of the *slo I* mutant is available.

**RNA and protein time courses.** Newly eclosed wild-type (*y w*) or mutant (*y w; Clk<sup>trk</sup>*) flies were entrained for 5 d to a 12 hr LD regime under constant temperature (25°C). Time courses were performed under either LD or DD conditions. Samples were collected every 4 hr for 2 consecutive days and immediately frozen in dry ice. Frozen flies were shaken and sieved to generate two fractions: head (without antennas) and body (decapitated, and without legs or wings). Heads and bodies were kept apart for RNA/protein extraction. For the DD time courses, flies were entrained to LD cycles during 5 d and then released into constant darkness; samples were taken during the first and second day in DD.

**RNA extraction and hybridization.** Total RNA was prepared from fly head or body homogenized in Trizol reagent (Invitrogen). RNA was purified using RNeasy kit (Qiagen). Total RNA (7.5 µg) was labeled for hybridization according to manufacturer's instructions (Affymetrix). For Northern analyses, 25 µg of total RNA was loaded into a 1.2% formaldehyde agarose gel and transferred to nylon (Hybond N, Amersham). Expressed sequence tags used as probes were obtained from ResGen (Invitrogen) and correspond to GH27053 (CT38753), LP 11415 (CT8171), AT29985 (CT18196), GH13172 (CT33647), and LD06553 (CT12127).

**DNA chip experiments.** cDNA synthesis, biotin labeling of cRNA, and hybridization of the chips were performed as recommended by the manufacturer (Affymetrix). Cel to text file condensation was performed using the MAS 4 algorithm in the Microarray Suite v.4 (Affymetrix) and visualized using the GeneSpring (Silicon Genetics) software package. To identify clock-controlled genes, we used a statistical program, COSOPT, which we have described in detail elsewhere (Harmer et al., 2000; Panda et al., 2002). Finally, we have constructed a publicly accessible database <http://expression.gnf.org/circadian>, where users can query for circadian-regulated genes in the fly head and body.

**Locomotor behavior analysis.** Newly eclosed flies were entrained to 12 hr LD cycles for 3 d, and adult males were placed in glass tubes and monitored for activity with infrared detectors and a computerized data collection system (Trikinetics) (Hamblen-Coyle et al., 1992). Activity was monitored in LD conditions for 3–4 d, when the flies were released into DD at least for 1 week. Data were analyzed using the Clocklab software package (Actimetrics, Evanston, IL) as in Yang and Sehgal (2001). Only those flies that were alive 2 d after analysis were taken into account. Periodogram analysis of flies that were scored as arrhythmic in Table 4 produced no strong peak that was statistically significant ( $p < 0.05$ ). Those flies that showed recognizable daily onsets and offsets of activity that was not consolidated, thus resulting in periodograms that displayed weakly significant periods, were classified as weakly rhythmic (Yang and Sehgal, 2001) in Table 4 and were not taken into account for average period calculations. Overall activity was calculated by averaging equivalent bins during LD or DD cycles for each fly and then taking an average of all the flies within each genotype.

**Western blots.** Time courses were performed as described. Approximately 10 µl of wild-type and mutant fly heads was loaded into precast 4–15% acrylamide gels (Bio-Rad). Gels were transferred in 15% methanol Tris-glycine buffer onto Nitrobind-supported nitrocellulose (Osmonics). Primary antibodies used were rabbit IgG anti-SLO (1:500) and mouse monoclonal IgG anti-hsp70 (1:2000; Sigma). Washes were per-

**Table 1. COSOPT assigned a *p* value to each putative cycling gene**

	Cycling genes		
	$p < 0.01$	$p < 0.025$	$p < 0.05$
Head	120	478	1206
Body	177	524	1144

formed in TBS (for SLO) or TBS 0.5% Tween (for hsp70). Appropriate secondary antibodies were detected using ECF (Amersham Biosciences).

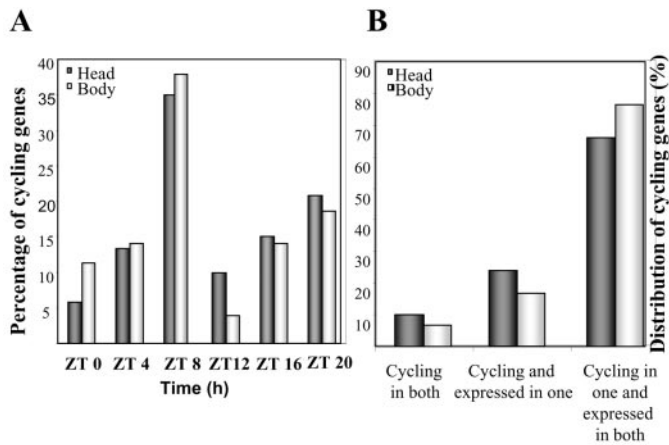
## RESULTS

### Clock-controlled transcription throughout the day

We monitored steady-state RNA levels using DNA GeneChips (Affymetrix) spanning the entire *Drosophila* genome. Temporal profiling of 13,500 probe sets was performed with a 4 hr time resolution during 2 consecutive days under entrained and free-running conditions. Each sample was hybridized to two DNA GeneChips to test the reproducibility of the technique (Harmer et al., 2000). The mean hybridization signal strength and the SEM for each probe set was calculated from the duplicate hybridizations.

To identify the cyclic mRNAs among the pool of expressed genes, we used COSOPT, an analytical algorithm that we developed for detection and statistical characterization of rhythmic gene expression in gene array experiments. Briefly, data were fit to 100,000 cosine test waves, and the significance of each fit was then determined empirically by temporally randomizing the data sets; those genes with traces that fit a cosine wave with a period between 20 and 28 hr were scored as cycling with a given probability (Panda et al., 2002). In the LD *y w* head time course, COSOPT scored 120 genes as cycling with  $p < 0.01$  (Table 1). This may be an underestimation of the total number of cycling genes because previously characterized clock components such as *per* (which cycles with  $p = 0.015$ ) and *Clk* ( $p = 0.036$ ) fall outside of this category. We chose a conservative *p* value for selecting clock-controlled genes for further study. Storch et al. (2002) used a similar criterion to minimize the overall number of genes in the array selected as cycling while maximizing the number of “guide genes” (known cccgs) present, which further validates our method. Internal validation of this approach toward the identification of novel output genes came from the observation that previously characterized cycling genes (such as *tim*, *vri*, *to*, and even low amplitude ones such as *cry*) displayed a circadian pattern of gene expression in our array experiment (data not shown). We also detected *dreg-5* (Van Gelder et al., 1995) with some element of low-amplitude cycling, although it was not identified as such by our algorithm. Recently, an enhancer trap-based screen retrieved four additional cccgs (Stempfl et al., 2002). Of those, COSOPT identified CG2207, CG13432, and CG3779 as cycling in the fly head, although none of them reached the significance level of  $p < 0.01$ . Interestingly, CG6145 appears to cycle robustly in the fly body (see below and Table 3).

To address type 1 and type 2 errors in our analysis, 1000 Gaussian random temporal patterns of 13 time points (analogous to 48 hr of 4 hr sampling) with no rhythmic component were analyzed with COSOPT using the same criteria applied to our experimental data ( $p < 0.01$ ; no multiple-measurement correction). COSOPT identified two cycling curves when it should have detected none, indicating that the false positive rate under these conditions is 0.2%. If we applied this percentage to the 14,000 genes assessed in the experiment, we would have 28 false posi-



**Figure 1.** *A*, Output genes peak throughout the day in the fly head and body. Genes were grouped according to the phase calculated by COSOPT; each cluster represents genes peaking at the specified time  $\pm$  2 hr. ZT0 refers to the time when lights were switched on. Cluster size is represented as percentage of the total number of cycling genes. *B*, The clock controls different subsets of genes in head and body fractions. Distribution of genes cycling in both tissues, cycling and expressed in only one, and expressed to mid-high levels in both but cycling in one is displayed.

tives. If it is more appropriately applied to only the  $\sim$ 5000 genes that are detectable, the number of false positives becomes 10. Determining the rate of false negatives is a more complex process because it imposes *a priori* assumptions on the quality of the data. We ran these simulations under two hypothetical conditions: a worse case scenario in which the signal/noise ratio is 1 (signal and noise are equal in magnitude), and a more likely scenario in which this ratio equals 2. When we analyzed 1000 cosine waves of amplitude equal to 1 SD above the noise level, COSOPT detected 445 (implying that the false negative rate under these conditions is 55%); interestingly, when the amplitude equals 2 SDs above the noise (still a conservative assumption), the false negative rate becomes negligible. COSOPT identified 997 of 1000 (that is, 0.3% error rate) of the test cosine waves, providing an exceptionally high degree of confidence that our approach is valid (data not shown).

Cycling genes were grouped in clusters according to the phase of peak expression. The phase distribution is shown in Figure 1*A*. This approach allowed the identification of several novel phases of peak expression [Zeitgeber time 4 (ZT4), ZT8, ZT12] and, more importantly, made possible a direct comparison between the different genes within the same experiment. All phases were similarly represented with the exception of ZT8, and to a lesser extent ZT20, where a larger proportion of the genes reach maximum levels of expression. This is similar to what has been reported by Ueda et al. (2002) and contrasts with the findings of Claridge-Chang et al. (2001), although this could be attributable partially to differences in the cluster analysis that was performed.

One of the hallmarks of clock-controlled activity is the persistence of the rhythms in the absence of environmental cues. To confirm the circadian nature of the newly identified target genes, we performed an independent experiment under free-running conditions. We tested which proportion of the novel outputs showed reliable cycling profiles under constant darkness. One of the limitations of this analysis in DD is the “dampening” of the amplitude of the oscillation, which could partially result from the desynchronization of individual cells in the absence of resetting

environmental cues, or it could reflect a direct effect of light boosting the amplitude of the oscillations (Hardin, 1994). Approximately 50% of the genes identified in the LD experiment were found to cycle robustly in DD as well (Table 2). Interestingly, the phase predicted by COSOPT for each individual cycling gene is very similar in LD and DD.

To examine the cycling profiles of the newly identified output genes in a behaviorally arrhythmic background, we entrained *y w;; Clk<sup>irk</sup>* and performed a time course with a 4 hr time resolution during 2 consecutive days in LD; this resolution was chosen to properly compare genes peaking at different phases in the wild-type condition with their counterparts in the mutant background. We found that 116 of the 120 identified output genes ceased to cycle in this mutant background, commensurate with the role that the CLOCK protein plays in sustaining the molecular oscillations. The remaining four genes were scored by COSOPT as cycling with  $p < 0.01$ . Closer inspection of the individual traces highlighted the direct effect of light and light/dark transitions on gene expression: three of the mRNAs appeared to slowly build up during the day and decrease during the night, whereas others appeared to be acutely driven by light and decreased thereafter (data not shown). To determine whether the levels of expression changed in the mutant background, we calculated the arithmetic average difference (subtracting the average level of expression in *y w;; Clk<sup>irk</sup>* from that in wild type) and corresponding SD. By this criteria we found that 5 genes were downregulated and 11 were upregulated ( $p < 0.025$ ) in the *y w;; Clk<sup>irk</sup>* mutant background (as indicated in the last column of Table 2).

### The clock regulates gene expression in a tissue-specific manner

To provide a more comprehensive view of clock control in a whole organism, we analyzed cyclic gene expression under entrained conditions using male bodies as the source of total RNA and compared these transcripts with those derived from heads. COSOPT identified 177 genes that cycle within a period between 20 and 28 hr with  $p < 0.01$  (Table 1); the data are summarized in Figure 1, *A* and *B*, and Table 3. As in the mouse (Panda et al., 2002), only a small proportion of cycling transcripts was found to overlap between the two tissues (12 genes), including some previously identified clock components (such as *tim*, *vri*, and *cry*). The analysis of the remaining genes revealed an even more interesting feature: a large number of genes that cycle solely in fly heads are still expressed at medium to high levels in bodies, pointing to differential transcriptional regulation in this subset of clock outputs (Fig. 1*B*). This finding is also in agreement with what has been found in the mouse (Panda et al., 2002; Storch et al., 2002).

To independently confirm the cyclic pattern of a subset of output genes, we performed Northern blot analysis on separate time courses. We chose genes spanning a range of  $p$  values; all were found to oscillate with a phase concordant to that predicted by the microarray experiments (Fig. 2).

### Circadian transcriptional regulation of physiology

#### Protein stability

Although cyclic transcription is a signature of clock activity, important levels of regulation take place downstream. Several genes involved in various aspects of protein stability were under rhythmic transcriptional control. We identified three genes cycling in the fly head with a peak at ZT8 that are part of the 26S proteasome complex (Fig. 3*A*). *pros26* encodes a multicatalytic endopeptidase (Saville and Belote, 1993). *pros26.4* and *rpn9* are

**Table 2. Cycling genes in fly heads ( $p < 0.01$ ) are categorized by known or predicted function**

Gene ID	Function	LD		DD		Levels in <i>Clk<sup>irk</sup></i>
		$p$ - $\beta$	Phase	$p$ - $\beta$	Phase	
<b>Cell adhesion</b>						
CT35785		8.3E-03	21.3			
<i>Trn</i>	tartan	7.7E-03	9.7			
<b>Metabolism</b>						
CT1116	Succinyl CoA transferase	4.6E-03	4.2			
CT22171	Isocitrate dehydrogenase (NADP <sup>+</sup> )	4.6E-03	20.6	2.4E-01	21.1	
CT34968	3-OH isobutyrate dehydrogenase	4.2E-03	3.2	3.1E-02	2.5	Up
CT12987	Short-branched chain acyl CoA dehydrogenase	4.8E-03	0.5	9.1E-02	21.1	
CT41571	Aldehyde dehydrogenase	9.5E-03	18.5	8.4E-02	22.0	
CT1187	Choline phosphate cytidil transferase	9.9E-03	8.8	2.3E-02	8.8	
CT11757	Anon 23-Da	4.0E-03	14.6			
<i>Hmgs</i>	Hydroxymethylglutaryl-CoA synthase	6.9E-03	21.6			
CT16527	Cholesterol O acyl transferase	8.0E-03	7.8			
CT40163	Angiotensin-converting enzyme	2.3E-03	13.4			
CT12411	Oxido reductase	7.5E-03	8.4			
<b>Development</b>						
CT27880	Yellow-d2	5.5E-03	8.8	5.0E-03	15.0	
<i>Idgf1</i>	Imaginal Disc Growth Factor 1	6.0E-03	20.7			Up
<i>mbc</i>	Myoblast city	5.0E-03	11.7			Up
<b>Detoxification</b>						
<i>cyp6a21</i>	Cytochrome P450 6a21	3.8E-03	21.2	6.8E-03	2.2	
<i>Ugt35b</i>	UDP-glycosyltransferase 35b	4.0E-03	3.7	9.8E-03	3.0	
CT38753	Glutathione S-transferase	4.9E-03	5.6			
CT38747	Glutathione S-transferase	7.5E-03	5.1			Down
<i>cyp4e3</i>	Cytochrome P450 4e3	3.3E-03	9.0	6.4E-03	14.2	Down
<i>cyp6d5</i>	Cytochrome P450 6d5	6.0E-03	21.6			
<i>cyp6a17</i>	Cytochrome P450 6a17	9.4E-03	2.9			Up
CT16545	Glutathione transferase	2.9E-03	7.8	1.6E-01	2.9	
<i>cyp6a2</i>	Cytochrome P450 6a2	3.1E-03	7.9	1.5E-02	13.0	Down
<i>cyp18</i>	Cytochrome P450 18	6.9E-03	17.1	6.9E-03	15.7	
<b>Heme metabolism</b>						
<i>Alas</i>	5-Aminolevulinatase synthase	1.0E-02	22.5			
CT34507	Heme oxygenase-like	8.8E-03	9.1	1.3E-02	14.2	
<b>Ligand binding/carrier</b>						
CT6764	Glucan $\alpha$ 1,4 glucosidase	4.2E-03	18.4	1.0E-01	22.5	
<i>glob1</i>	Globin 1	6.7E-03	20.1	1.2E-01	21.2	
CT33362		7.6E-03	20.4	2.4E-02	22.0	
CT28495		2.8E-03	13.9	2.9E-02	21.9	
<b>Neurotransmission</b>						
<i>ple</i>	Tyrosine 3-monooxygenase	4.5E-03	2.4	6.6E-03	1.6	
<i>Eaat2</i>	Excitatory amino acid transporter	4.4E-03	13.2			
<i>Dat</i>	Dopamine N-acetyltransferase	5.2E-03	9.5			
CT42497	Glycine transporter	5.6E-03	6.9	5.8E-03	13.1	
<i>b</i>	Black, glutamate decarboxylase	7.7E-03	16.2			Down
<b>Photoreceptor</b>						
<i>cry</i>	Cryptochrome	3.6E-03	1.1			
<b>Protease inhibitors</b>						
<i>serpin</i>	Serine protease inhibitor	8.3E-03	20.0			
CT37177		9.3E-03	14.6			Up
CT37195		8.2E-03	10.4			Up
<b>Protein synthesis/degradation</b>						
CT18196	Ubiquitin thiolesterase	2.2E-03	13.1	7.3E-04	16.5	
<i>pros26</i>	Proteasome 26 kDa protein	4.9E-03	8.7	1.7E-02	8.4	
<i>pros26.4</i>	Proteasome 26S subunit subunit 4 ATPase	7.1E-03	7.9			
CT28749	26S proteasome regulatory subunit p39A	6.2E-03	8.9	1.4E-02	10.7	
CT7240	Translation initiation factor (eIF-5)	7.4E-03	8.5	2.9E-02	9.5	
CT39962	tRNA synthase	6.5E-03	20.8			

(Table continues)

**Table 2.** *Continued*

Gene ID	Function	LD		DD		Levels in <i>Clk<sup>irk</sup></i>
		<i>p</i> -β	Phase	<i>p</i> -β	Phase	
<b>Protein folding?</b>						
CT16169	Heat shock protein 27-like	7.4E-03	21.9			
<i>Cnx99A</i>	Calnexin 99A	2.8E-03	17.4	6.6E-02	19.2	
<b>Transcription</b>						
CT30663		7.0E-03	11.6			
<i>tim</i>	timeless	4.7E-03	16.4	4.6E-03	17.4	
<i>Mad</i>	Mothers against dpp	5.0E-03	14.5			
<i>vri</i>	vriille	5.5E-03	13.6	1.6E-03	15.4	
CT31519		5.1E-03	2.8	3.4E-01	7.5	
CT19628		6.3E-03	16.6			
CT17296		4.8E-03	14.1	1.1E-02	17.4	
CT18631	TfII A-L	8.4E-03	9.3			
<i>140up</i>	Upstream of RpII14	9.1E-03	17.5	9.1E-02	15.4	
<b>Signal transduction</b>						
<i>Akap200</i>	Protein kinase A anchoring protein	6.2E-03	15.6			
<i>Pk61C</i>	Protein kinase 61C	2.9E-03	10.8	2.3E-02	14.1	
<i>Pka-C3</i>	Ser/Thr kinase	4.9E-03	15.3	5.7E-02	7.6	
CT35755		9.1E-03	21.5	1.9E-01	4.4	
CT27512	Ras small monomeric GTPase	8.1E-03	9.3	1.2E-03	12.4	
<i>Slob</i>	Slowpoke binding protein	3.0E-03	18.0	2.2E-03	16.3	
CT34849	Chd64	9.8E-03	5.5			
<b>Stress response</b>						
CT25938	Superoxide dismutase (Cu-Zn)	3.3E-03	8.6			
Hsp22	Heat shock protein 22	7.8E-03	17.6			
<i>to</i>	Takeout	8.3E-03	21.7	5.3E-02	2.1	
<b>Structural proteins</b>						
dlp	Heparin sulfate proteoglycan	7.4E-03	11.2			
Sulf1	<i>N</i> -acetylglucosamine-6-sultase	9.0E-03	8.8	1.3E-02	9.4	
βTub56D	βTubulin56D	8.7E-03	10.0	1.4E-01	22.9	Up
CT31310		8.9E-03	6.2			Down
CT31613	Thrombospondin	2.3E-03	10.3	2.4E-02	11.9	
CT15377		9.1E-03	5.2	2.3E-02	3.0	
<i>Cpn</i>	Calphotin	8.9E-03	7.2	3.0E-02	9.9	Up
<i>capu</i>	Cappuccino	1.0E-02	18.5			
<b>Transporters</b>						
CT42567	High-affinity inorganic phosphate:sodium transporter	3.8E-03	0.2	4.6E-02	22.9	Up
CT30701	Glucose transporter	8.2E-03	7.7			
<i>trpl</i>	Trp-like	6.7E-03	9.8			
CT16777		8.6E-03	8.6	1.3E-02	13.9	
<b>Unknown function</b>						
CT12588		8.9E-03	23.5	2.9E-02	17.9	
CT31141		9.9E-03	15.2			
CT28701		7.7E-03	8.3			
CT34631		8.3E-03	8.9			Up
CT6802		7.4E-03	9.4			
CT32204		3.9E-03	7.1	2.4E-03	7.7	
CT16557		9.3E-03	8.8	2.1E-03	16.2	
CT16503		7.9E-03	2.2	7.2E-03	4.9	
CT33900		7.9E-03	18.9			
CT22515		9.9E-03	14.1			
CT33647		4.1E-03	1.0	7.5E-03	19.6	
CT18564		9.9E-03	21.8			
CT25838		5.2E-03	8.7			
CT30256		8.0E-03	20.5			
CT33484		3.6E-03	18.4	6.1E-03	21.0	
CT32008		6.9E-03	8.3			
CT28709		8.7E-03	7.9			

(Table continues)

Table 2. Continued

Gene ID	Function	LD		DD		Levels in <i>Clk<sup>irk</sup></i>
		<i>p</i> - $\beta$	Phase	<i>p</i> - $\beta$	Phase	
CT31865		2.3E-03	9.2	1.2E-03	14.3	
CT32600		7.9E-03	22.7	1.0E-01	22.0	
CT15908		4.8E-03	8.8	2.4E-03	13.2	
CT32262		2.3E-03	8.3	1.0E-03	10.2	
CT34135		9.5E-03	5.5	7.1E-02	16.3	
CT34439		9.6E-03	3.3	1.7E-01	15.7	
CT10556		4.4E-03	8.7			Up
CT42539		5.5E-03	8.5			
CT35916		9.6E-03	5.9	1.1E-02	7.5	
CT33073		4.6E-03	7.2	3.8E-03	10.0	
CT37044		2.4E-03	20.6	7.0E-03	0.9	
CT29508		8.8E-03	19.1	1.4E-01	1.3	
CT29612		2.7E-03	19.5	3.4E-03	19.3	
CT35582		2.7E-03	8.5	1.0E-03	14.4	
CT32596		8.9E-03	10.8			
CT26517		1.0E-02	4.8	3.1E-01	17.4	
CT29500		4.7E-03	21.4	1.8E-02	2.3	
CT16351		4.8E-03	15.5			
CT24597		9.5E-03	4.5	2.8E-01	16.1	
CT26954		9.3E-03	8.7			
CT25972		8.5E-03	15.9			

Gene ID lists either the gene name (if known) or the Celera Transcript (CT) number, as well as the associated *p* value and phase under entrained (LD) and free-running (DD) conditions. The last column displays the genes that were found to change significantly in the *y w; Clk<sup>irk</sup>* background.

part of the 19S regulatory complex and correspond to subunit 4 of the AAA-ATPase and are required for assembly and stability of the proteasome. In *Drosophila*, different proteasome subunits are expressed throughout development, possibly to control specific processes such as cell division or morphogenesis (Haass and Klotzel, 1989). We also identified a putative de-ubiquitinating enzyme, the homolog of the mouse Usp8, with peak expression at ZT12 (Fig. 3A). The cyclic pattern of CG5798 was confirmed independently by Northern blot analysis (Fig. 2). De-ubiquitinating enzymes remove the polyubiquitin chain from conjugated proteins before their degradation by the proteasome. These enzymes either may regulate degradation by the proteasome or may be involved in ubiquitination processes regulating subcellular localization. In summary, these data suggest that temporal regulation of the proteasome may be important in *Drosophila* physiology.

#### Heme metabolism

The observation that the expression of both the rate-limiting enzyme in heme biosynthesis,  $\delta$ -aminolevulinic synthase, *alas*-, and the rate-limiting enzyme of heme degradation, heme oxygenase, cycle in *Drosophila* heads suggests that heme metabolism is tightly regulated by the clock (with a peak at ZT20 and ZT8, respectively) (Fig. 3B). Heme, or heme-containing proteins, is involved in respiration, oxygen transport, detoxification, and signal transduction processes, but as a chelator of iron it may promote deleterious cellular effects such as oxidative membrane damage. Thus, maintaining a proper balance between heme biosynthetic and degradative pathways is crucial for cellular homeostasis (Ryter and Tyrrell, 2000). Although *alas* has been found to cycle in the mouse liver, heme oxygenase has not been observed to cycle (Kornmann et al., 2001).

#### Detoxification/olfaction

Heme is also required for P450 function. In insects, P450 enzymes are thought to be involved in the biosynthetic pathways of ecdysteroids and juvenile hormones, and as such play a role in insect growth, development, and reproduction as well as in the metabolism of natural plant products and insecticides, resulting in bioactivation or detoxification (Feyereisen, 1999). This biotransformation process has been described as occurring in two phases. The initial compound can be transformed into a more reactive species (usually via redox reactions catalyzed by cytochrome P450s), whereas in the second phase, highly polar groups such as UDP-glucuronosyl or glutathione are added either to the products of the first phase or in some cases directly to the toxic chemicals. The enzymes involved in this second-stage phase are UDP-glucuronosyl transferases (UGTs) and glutathione *S*-transferases (GSTs). Products of phase II are highly hydrophilic, can no longer cross membranes, and are eliminated by secretion (Wang et al., 1999). We found that six different cytochrome P450 genes, *cyp4e3*, *cyp6a2*, *cyp6a17*, *cyp6a21*, *cyp6d5*, and *cyp18*, cycle with different phases in heads (ZT8, 4, 0, 20, 20, and 16, respectively) (Fig. 3C) and *cyp6g1*, *cyp9b2*, and *cyp18a1* cycle with different phases in the body (Table 3). The only functionally characterized enzyme thus far is *cyp6a2*, which is involved in the metabolism of organophosphorus insecticides. Interestingly, we also found that *ugt35b* and several GSTs also cycle in fly heads ("phase II" enzymes) (Fig. 3D), implying that multiple steps in the biotransformation process appear to be under circadian control.

In *Drosophila*, *ugt35b* is preferentially expressed in the third antennal segment where most of the olfactory sensilla are found (Wang et al., 1999), although it can be found in the fly head as

**Table 3. List of cycling genes in fly bodies ( $p < 0.01$ ), as in Table 2**

Gene ID	Function	LD experiment	
		$p$ - $\beta$	Phase
<b>Defense/immunity</b>			
<i>LysX</i>	Lysozyme X	9.50E-03	17.55
<i>Lectin-galC1</i>	Galactose-specific C-type lectin	3.60E-03	9.00
CT8705	PGRP-SC1b, peptidoglycan recognition protein	1.87E-03	22.20
Phas1	Eukaryotic-initiation-factor-4E binding protein	9.76E-03	23.00
<i>Ag5r</i>	Antigen 5-related	2.10E-03	21.31
<i>Chit</i>	Chitinase-like	6.84E-03	7.60
CT5624	Chitinase-like	3.01E-03	17.25
CT30310	Drosomycin-like	9.84E-03	15.12
<i>Idgf4</i>	Imaginal Disc Growth Factor 4, chitin binding protein	2.20E-03	3.20
CT29102	Tep 4, complement-like protein	3.51E-03	5.40
<b>Development</b>			
CT13185	dorso-ventral patterning in oogenesis	8.53E-03	4.90
<b>Detoxification</b>			
<i>cyp9b2</i>		4.91E-03	4.70
CT20826	Cyp18a1	4.07E-03	19.34
<i>Cyp6gl</i>		1.86E-03	8.03
CT38747	Glutathione transferase	2.29E-03	5.90
<i>GstD1</i>	Glutathione S-transferase B1	5.67E-03	7.30
CT35150	Glutathione S-transferase-like	4.46E-03	7.50
CT35071	UDP-glucuronosyltransferase	9.59E-03	4.90
<b>Electron transfer</b>			
Cyt-A5	Cytochrome A5-related	7.50E-03	7.90
<b>Ligand binding/carrier</b>			
CT3751	Triglyceride binding	6.09E-03	6.50
<i>Mp20</i>	Muscle protein 2	8.59E-03	18.85
<i>Dbi</i>	Diazepam-binding inhibitor	7.95E-03	8.60
PebIII	Ejaculatory bulb protein III	4.14E-03	19.62
CT33980	Neural Lazarillo	9.36E-03	19.72
CT31326	Antennal binding protein X-like	6.24E-03	3.90
CT32778	Odorant binding protein	2.78E-03	5.40
CT21061	Fatty acid binding protein-like	1.94E-03	9.00
<b>Metabolism</b>			
CT16885	Galactokinase	1.46E-03	8.50
<i>wun</i>	Phosphatidate phosphatase	6.99E-03	19.01
CT16187	3-Hydroxyisobutyryl-CoA hydrolase	6.41E-03	0.50
CT24308	Threonine dehydratase	6.48E-03	7.90
CT6492	Maltase L-like	2.28E-03	6.80
<i>Hmgs</i>	Hydroxymethylglutaryl-CoA synthase	6.40E-03	23.06
<i>LvpH</i>	Larval visceral protein H	4.80E-03	7.50
<i>Pepck</i>	Phosphoenolpyruvate carboxykinase	6.50E-03	11.00
<i>Scu</i>	3-Hydroxyacyl-CoA dehydrogenase	9.57E-03	8.90
CT17038	Malate dehydrogenase	3.07E-03	8.10
CT36683	$\alpha$ -Amylase-like	1.74E-03	8.40
CT6532	Maltase H-like	4.49E-03	7.30
CT22063	Triacylglycerol lipase-like	9.88E-03	7.60
CT22069	Hexokinase	2.84E-03	8.00
CT21171	Carbonate dehydratase-like	6.81E-03	4.70
CT12913	Ubiquinone biosynthesis	6.51E-03	21.39
CT34308	UDP-glucose epimerase	5.17E-03	8.50
CT18319	Pyrroline 5-carboxylate reductase-like	6.39E-03	7.30
CT33098	$\alpha$ -glucosidase II	3.93E-03	7.40
CT19053	Carbonate dehydratase-like	5.82E-03	16.25
CT41369		2.57E-03	4.70
CT28913	Glucosylceramidase-like	7.84E-03	9.45
<i>RfaBp</i>	Retinoid- and fatty-acid binding protein	2.28E-03	8.40
<i>RfaBp</i>	Retinoid- and fatty-acid binding protein	1.50E-03	9.60

(Table continues)

Table 3. Continued

Gene ID	Function	LD experiment	
		<i>p</i> - $\beta$	Phase
CT26924	Glucose dehydrogenase-like	6.49E-03	7.50
<i><math>\alpha</math>-Est5</i>	Carboxylesterase	8.47E-03	10.00
<i>pug</i>	Formate–tetrahydrofolate ligase	8.23E-03	7.60
<i><math>\alpha</math>-Est7</i>	$\alpha$ -Esterase-7	9.35E-03	9.70
<i>ade5</i>	Phosphoribosylaminoimidazole carboxylase; EC:4.1.1.21	3.38E-03	9.50
<i>Pdh</i>	Photoreceptor dehydrogenase	9.23E-03	6.90
CT30991	IMP cyclohydrolase	4.38E-03	9.80
CT24026	$\alpha$ -Galactosidase	7.24E-03	8.90
CT9666	Transaldolase	3.14E-03	8.50
CT39259	Oxidoreductase	3.46E-03	3.10
CT41283	Cholinephosphate cytidyltransferase	6.56E-03	0.42
CT33239	<i>N</i> -acetyl transferase	9.11E-03	15.44
CT31087	rRNA methyltransferase	4.32E-03	13.20
CT34977	Epoxide hydrolase	5.71E-03	6.30
Muscle contraction			
<i>Mhc</i>	Myosin II heavy chain	6.81E-03	17.56
<i>TpnC73F</i>	Troponin C at 73F	6.66E-03	17.79
Proteases			
CT15463	Zinc carboxypeptidase	7.73E-03	21.92
CT14806	Vitellogenic carboxypeptidase	7.54E-03	6.80
CT14378	Carboxypeptidase-like (inactive)	3.89E-03	1.50
CT4209	Chymotrypsin	3.30E-03	22.58
CT37183	Serine protease inhibitor	1.66E-03	9.00
CT25448	Chymotrypsin	8.12E-03	21.11
CT8699	Serine protease-like	7.10E-03	22.52
CT19724	Serine protease	6.97E-03	20.04
CT37173	Serine protease-like	1.98E-03	14.30
CT3996	Cathepsin	4.41E-03	9.30
CT20780	Cathepsin L	5.44E-03	13.40
<i>Ser99Da</i>	Serine protease 1	9.03E-03	23.59
<i>Ser99Db</i>	Serine protease 2	9.91E-03	23.18
CT29408	Serine protease	4.45E-03	22.77
$\gamma$ -Try	$\gamma$ -Trypsin	2.77E-03	20.66
Protein synthesis/folding			
CT25442	Ribosomal protein K11-like	7.71E-03	20.20
CT17486	Chaperone	2.69E-03	19.57
Signal transduction			
<i>pp1-13C</i>	Ser/Thr phosphatase	9.91E-03	5.80
CkII- $\beta$	Casein kinase II $\beta$ subunit	7.15E-03	16.77
<i>cry</i>	Cryptochrome	5.77E-03	4.10
<i>Lk6</i>	Heat shock construct of Kidd	4.74E-03	5.20
CT22109	Accessory gland peptide 36DE	9.84E-03	17.93
Stress response			
<i>Cat</i>	Catalase	1.53E-03	8.80
CT33074	Takeout	4.71E-03	0.65
CT9894	Heat shock protein 70	6.26E-03	23.78
Structural proteins			
<i>Gel</i>	Gelsolin	6.95E-03	7.80
Msp-300	Muscle-specific protein 3	8.02E-03	16.42
CT33880	Cuticle protein-like	9.68E-03	17.33
<i>Glt</i>	Glutaactin	4.72E-03	9.10
CT41348	A-band-protein-225	6.88E-03	17.09
CT39472	Peritrophin-15a	4.86E-03	20.12
Transcription			
CT15944		7.39E-03	17.21
CT6009		9.47E-03	13.50
<i>tim</i>	Timeless	3.98E-03	17.53

(Table continues)



Table 3. Continued

Gene ID	Function	LD experiment	
		<i>p</i> - $\beta$	Phase
<i>Transporter</i>			
<i>Vha13</i>	Hydrogen-transporting two-sector ATPase	6.26E-03	21.66
BY8473	ATP-binding cassette transporter	1.48E-03	22.77
CT27832	Sodium/phosphate cotransporter	4.17E-03	17.16
CT13124	zetaCOP	5.41E-03	20.56
CT14766	ATP-binding cassette transporter	9.08E-03	18.53
CT30783	Sugar transporter-like	6.99E-03	8.00
CT10168	Sodium/phosphate cotransporter	6.97E-03	18.12
CT19169	Sugar transporter	6.06E-03	6.70
CT33284		4.70E-03	8.90
CT15971	Amino-acid permease-like	9.52E-03	17.98
<i>Unknown function</i>			
CT22723	Senescence marker protein-3	3.39E-03	8.90
<i>Mst57Dc</i>	Male-specific RNA 57Dc	6.37E-03	16.93
<i>Gip</i>	Gip-like	8.12E-03	9.80
CT26166	Kisir	4.69E-03	21.50
CT19307		7.73E-03	20.48
CT21613		8.81E-03	17.97
CT35864		9.38E-03	14.41
CT14230		6.06E-03	6.50
CT14296		2.49E-03	6.60
CT23898		2.59E-03	9.30
CT23894		8.82E-04	7.50
CT21019		7.01E-03	5.70
CT9093		8.76E-03	18.52
CT35241		6.39E-03	9.30
CT25774		7.17E-03	8.00
CT27394		3.05E-03	9.00
CT35953		8.23E-03	3.00
CT34370		9.41E-03	17.70
CT22505		6.20E-03	7.60
CT12127		1.59E-03	7.70
CT35796		5.00E-03	17.12
CT26922		4.52E-03	8.90
CT33647		3.27E-03	0.00
CT27834		2.31E-03	7.80
CT18564		2.24E-03	23.50
CT35055		8.36E-03	7.50
CT35089		9.38E-03	5.00
CT8227		5.83E-03	20.29
CT18118		9.50E-03	20.02
CT42569		1.54E-03	22.14
CT40493		9.81E-03	9.00
CT33419		9.49E-03	1.95
CT34875		9.46E-03	15.36
CT32604		9.65E-03	19.09
CT39634		6.22E-03	8.40
CT22395		5.73E-03	21.98
CT33153		8.49E-03	5.20
CT12789		5.68E-03	21.28
CT1789		6.56E-03	2.80
CT40966		7.27E-03	6.00
CT36877		6.76E-03	23.39
CT26804		3.60E-03	21.31
CT26802		4.05E-03	21.61
CT28427		2.07E-03	5.40
CT38785		9.84E-03	2.50

(Table continues)

Table 3. Continued

Gene ID	Function	LD experiment	
		<i>p</i> - $\beta$	Phase
CT34717		4.17E-03	5.70
CT18134		3.68E-03	9.90
CT33073		5.64E-03	13.90
CT37044		4.08E-03	3.30
CT33083		8.21E-03	9.30
CT29612		9.10E-03	20.14
CT20261		4.25E-03	7.80
CT34939		1.41E-03	9.40
CT34936		8.21E-03	11.00
CT37419		8.18E-03	13.90
CT36793		3.98E-03	16.56
CT18390		3.53E-03	1.30
CT9987		9.65E-03	7.70
CT29500		4.76E-03	21.63
CT35842		6.17E-03	8.60
CT32316		7.80E-03	3.40
CT16297		9.32E-03	17.56
CT21565		9.20E-03	18.83
CT42272		5.07E-03	6.20

well. Given that olfaction is known to be under circadian control (Krishnan et al., 1999) and olfactory UGTs have been found to play a role in odorant signal termination (Lazard et al., 1991), we can speculate that *ugt35b* could potentially be a link between them.

#### Neurotransmission

Another gene that appears to be under circadian control is *ple*, implicating neurotransmission as a clock-controlled process. *ple* codes for the tyrosine 3-monooxygenase (also known as tyrosine hydroxylase), the first and rate-limiting enzyme in dopamine biosynthesis. Dopamine is an intermediate in cuticular sclerotization and also functions as a neurotransmitter in the fly nervous system. It has been shown to modulate certain forms of learning (such as female sexual receptivity and habituation), as well as motor neuron activity and neuromuscular function in larva (Neckameyer, 1998; Cooper and Neckameyer, 1999) and to exert circadian control over reflexive locomotion in decapitated flies as well (Andreatic and Hirsh, 2000). We found that *ple* cycles with high amplitude, peaking at ZT4 under both entrained and free-running conditions (Fig. 3E). *ple* expression falls below the level of detection in *y w;;Clk<sup>lrk</sup>* flies (a mutation that impairs clock function) (Allada et al., 1998). *ple* represents one of the several examples of homologous genes cycling in both flies and mammals (Panda et al., 2002), further stressing the significance of our observation. The oscillation of *ple* expression is unlikely to relate to its role in the head cuticle because the expression is undetectable in the body. Rather, the dramatic oscillation may contribute to circadian regulation of behavior.

#### Immunity

*Drosophila* is able to mount a rapid immune reaction in response to the infection by a pathogen. In fact, two distinct pathways are triggered depending on the nature of the pathogen involved, namely Gram-negative bacteria or fungi (Khush et al., 2001). We found a number of genes that play a role in different aspects of the immune response that cycle throughout the day in the fly body

(Fig. 3F, Table 3). These genes are involved in microbial recognition and phagocytosis (such as PGR-SC1b, *ldgf4*, *Tep4*, *Agr5*, and lectin-GalC), or they encode antimicrobial peptides (such as *lysX* and CT30310); others have unknown function but have been found to be induced specifically by infection (Phas1) (De Gregorio et al., 2001).

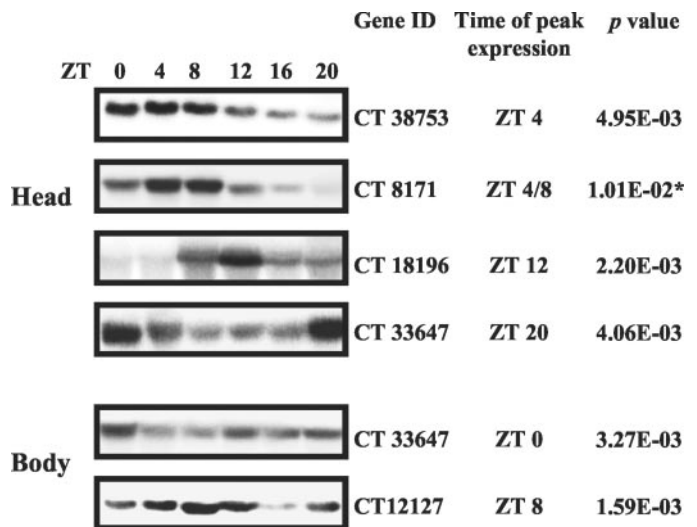
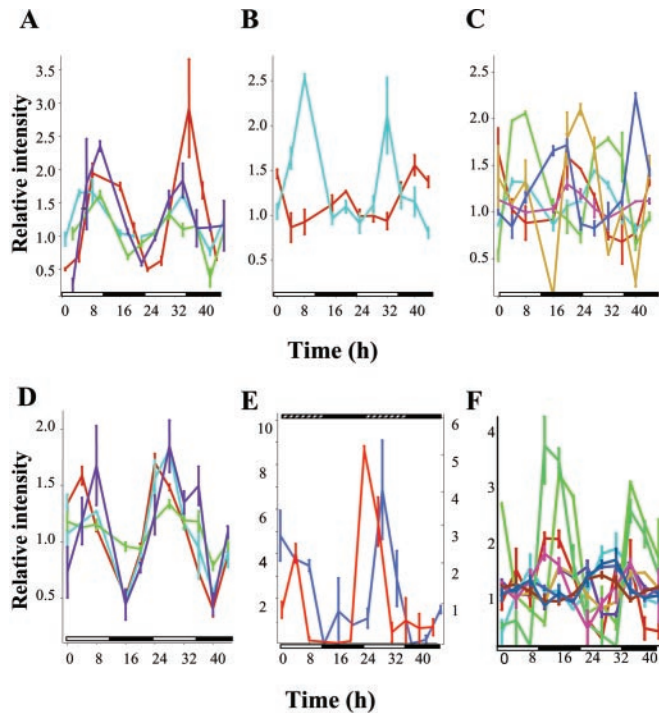


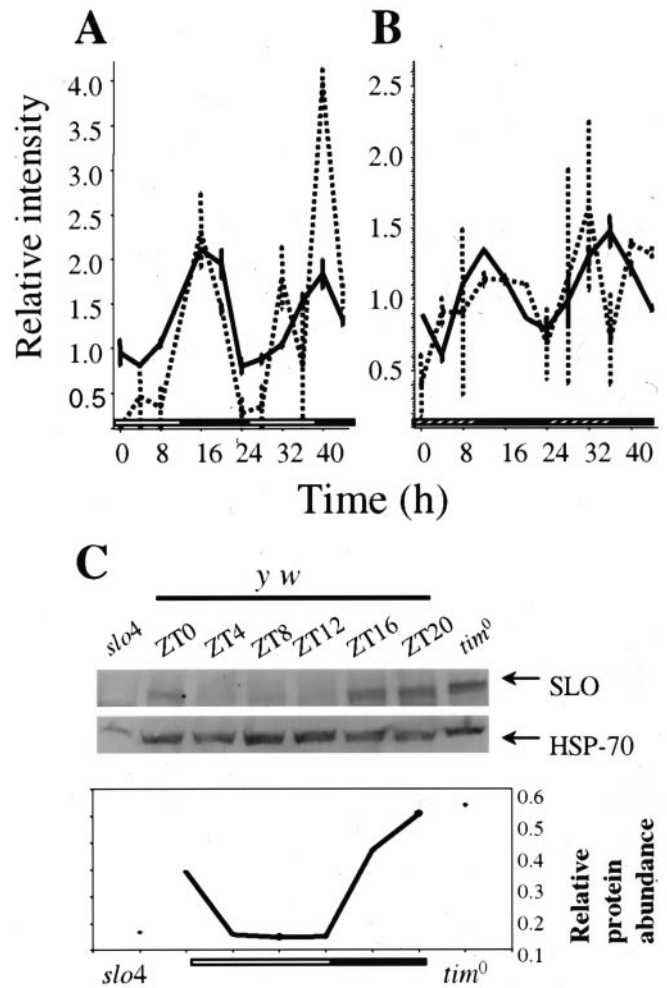
Figure 2. Northern blot analysis of several clock-controlled genes. Northern blot analysis of independent head and body time courses confirms both the cyclic nature of the candidates tested and their respective phases of expression. *Gene ID* refers to the cDNA used as probes. The *right columns* indicate the expected peak time and the corresponding *p* value predicted by the array experiment, respectively. The *asterisk* indicates a gene that is outside of the *p* < 0.01 cutoff and still appears rhythmic. According to our experiments, the CT18196 transcript has smaller size than predicted.



**Figure 3.** Cyclic patterns of clock-controlled genes in the fly head and body under entrained conditions. Complementary RNA samples were prepared as described and hybridized to duplicate DNA GeneChips. Data were normalized such that the mean expression level for each particular gene over the course of all time points equals 1. The average signal strength at each time point was then expressed as a ratio over the median signal strength for that particular gene. Representative traces of cycling transcripts implicated in various physiologies and metabolic pathways are shown in *A–F*. *A*, Clock control of protein stability. *pros26* (light blue), *pros26.4* (green), *rpn9* (purple), and ubiquitin thiolesterase (red). *B*, Heme metabolism. The gene encoding *alas* is in red; heme-oxygenase is in light blue. *C*, Genes implicated in detoxification are under circadian regulation. Phase I cytochrome P450s are colored as follows: *cyp4e3* (green), *cyp6a2* (light blue), *cyp6a17* (brown), *cyp6a21* (red), *cyp6d5* (dark blue), and *cyp18* (pink). *D*, Phase II genes: *ugt35b* in red, GST3 in purple, and two uncharacterized GSTs (CT38753 and CT38747) in light blue and green, respectively. *E*, Neurotransmission. *ple* expression under entrained and free-running conditions is depicted in red and blue, respectively. *F*, Immunity. A number of genes involved in different aspects of innate immunity are under circadian control and are indicated as follows: in recognition and phagocytosis: *Agr5* (light brown), a peptidoglycan protein (light green), lectin galC (aqua), *Idgf4* (brown), CT29102 (deep blue); antimicrobial peptides: *lysX* (red) and CT 30310 (pink); Chitinase-like molecules: *Chit* (light blue) and CT5624 (dark green). Relative intensities  $\pm$  SEM at each time point are shown. White and black boxes on the abscissa represent the duration of the light and dark periods. Hatched boxes (*E*) indicate subjective day.

**A potassium channel is an output of the clock involved in sustained rhythmic behavior**

The availability of a more complete description of clock-controlled genes enabled the selection of several candidates for the control of locomotor behavior. One of these candidates was slowpoke binding protein (*slob*), which binds to the Ca<sup>2+</sup>-dependent voltage-gated potassium channel *slowpoke* (*slo*) (Schopperle et al., 1998). A mutation in this channel causes behavioral defects (Atkinson et al., 2000) and an altered mating song, also a hallmark of certain clock components (Peixoto and Hall, 1998). *slowpoke* participates in the repolarization of the action potential in flight muscles and in motoneurons (Elkins et al., 1986; Gho and Mallart, 1986). SLOB has been shown to

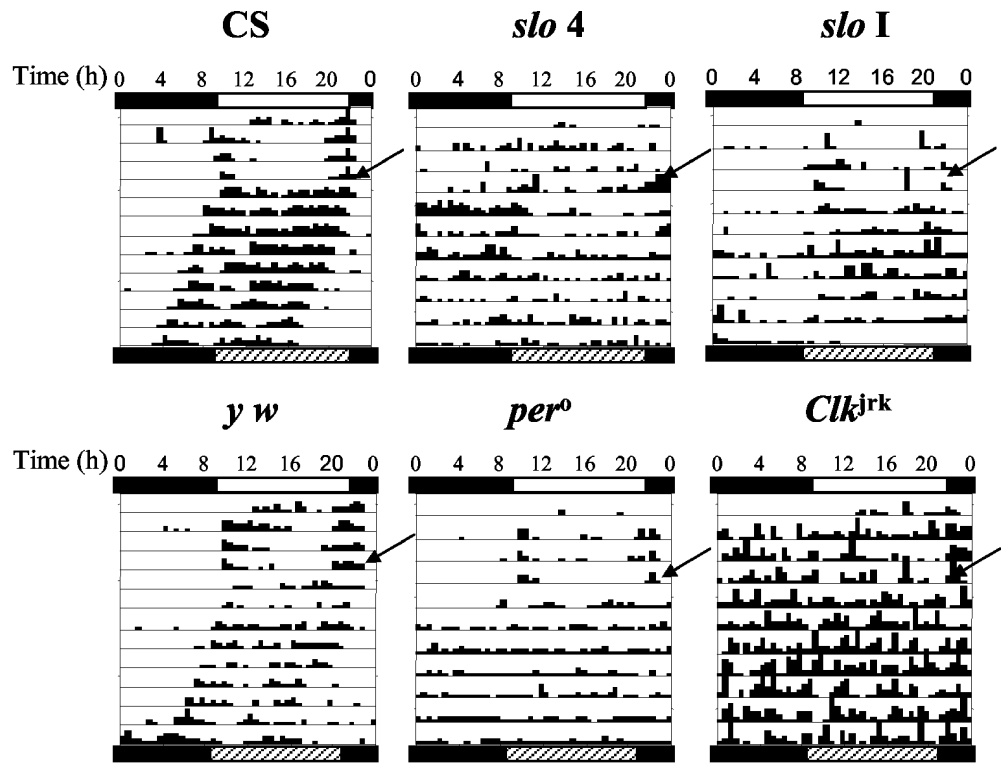


**Figure 4.** *slo* and *slob* cycle in LD and DD in wild-type flies. Shown is circadian pattern of expression of *slob* (gray) and *slo* (black) under entrained (*A*) and free-running (*B*) conditions. Relative intensities  $\pm$  SEM at each time point are shown. *C*, Representative example of a Western blot to detect the SLO protein under LD conditions. Flies were entrained and collected as described. The first and last lanes correspond to protein extracts collected at ZT16 from *slo4* and *tim*<sup>0</sup> flies, respectively. The graph indicates the quantification of SLO levels throughout the day (normalized to HSP-70).

modulate SLO activity per se, and through the formation of a complex with the  $\zeta$  isoform of 14-3-3 protein that acts downstream in several signaling pathways (Zhou et al., 1999).

We found that *slob* mRNA cycled robustly in fly heads in LD and DD (Fig. 4*A,B*, Table 2), consistent with recent reports (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ueda et al., 2002). This pattern was lost in the *y w; Clk<sup>jrk</sup>* mutant background. Although *slo* was not detected as cycling by COSOPT because of its low level of expression, we noticed that it appeared to cycle in phase with *slob* in both LD and DD (Fig. 4*A,B*). The cycling of *slo* was investigated by RT-PCR analysis (data not shown), and the protein was shown to cycle and peak at ZT20 by Western blot (Fig. 4*C*). The *slo* spatial expression pattern has been studied extensively (Becker et al., 1995) and has revealed that the *slo* mRNA is widely expressed in the adult brain. Furthermore, SLO protein has been localized both to neuronal cell bodies as well as to the neuronal projections.

Prompted by the speculation that SLO might be involved in



**Figure 5.** *slowpoke* is required for sustained rhythmic behavior. Representative actograms of wild-type CS and *y w* flies (left) and the mutants *per*<sup>0</sup>, *Clk*<sup>jrk</sup>, and arrhythmic *slo I* and *slo 4* flies are displayed. Flies were entrained for 5 d before the onset of the experiment. During the experiments, flies were kept in LD for 3–4 d and then switched to DD and monitored for at least another week. Rhythmicity and total activity in LD and DD conditions were determined using the Clocklab software package.

**Table 4.** A null mutation in the *slowpoke* potassium channel does not dramatically change overall activity levels

	Rhythmicity (%) LD			DD			DD period (analyzed)	Activity (total counts per day)	
	R	WR	AR	R	WR	AR		LD	DD
CS (70)	92.9	5.7	1.4	75.7	8.6	15.7	23.58 (51)	940	1055
<i>y w</i> (72)	87.5	9.7	2.8	87.5	12.5	0.0	23.46 (75)	633	863
<i>slo I</i> (59)	39.0	27.1	33.9	55.9	25.4	18.6	24.19 (33)	874	795
<i>slo 4</i> (78)	6.4	24.4	69.2	0.0	29.5	70.5		791	665
<i>slo 4/slo I</i> (81)	34.6	30.9	34.6	23.5	29.6	46.9	23.10 (19)	1152	731
<i>Clk</i> <sup>jrk</sup> (46)	30.4	30.4	39.1	8.7	39.1	52.2	24.56 (3)	1076	1491
<i>per</i> <sup>0</sup> (35)	60.0	31.4	8.6	0.0	37.1	62.9		695	1141

Number of flies analyzed within each genotype is expressed in parentheses. Actograms were analyzed as described in Materials and Methods. R, WR, and AR stand for strongly rhythmic, weakly rhythmic and arrhythmic flies.

circadian control of activity, we examined the locomotor activity in two *slo* mutants, *slo I* and *slo 4*. Wild-type flies show increased locomotor activity near dawn and dusk and remain quiescent the rest of the day (Fig. 5) (Hamblen-Coyle et al., 1992). These bursts of activity do not merely follow the next temporal transition, but instead anticipate it. Figure 5 shows individual representative actograms of wild-type and mutant flies. Wild-type Canton S (CS) and *y w* are rhythmic in LD and in DD, where the endogenous period becomes apparent. *per*<sup>0</sup> and *Clk*<sup>jrk</sup> mutants, which have defects in core clock components, behave differently under entrained conditions. Although *per*<sup>0</sup> flies still look mostly rhythmic in LD, *Clk*<sup>jrk</sup> is often not (Fig. 5) (Wheeler et al., 1993; Allada et al., 1998). This apparent rhythmicity in *per*<sup>0</sup> flies is caused by the so-called “startle effect,” an immediate behavioral response to the light/dark transitions. We found that most of the *slo 4* mutant flies display weak rhythms (defined as lacking a consolidated peak in the periodogram analysis) or no rhythms at all in LD. As expected, the lack of rhythmicity persisted under free-running conditions (Fig. 5, Table 4). Surprisingly, this arrhythmicity is

comparable to, if not worse than, the one displayed by *Clk*<sup>jrk</sup>. *slo I* mutants, on the other hand, displayed a milder phenotype, with only 40–55% of rhythmic flies in LD and DD, respectively, which is commensurate with a hypomorphic *slo* mutation (as opposed to a true null, as is the case for *slo 4*). Given the nature of the *slo 4* mutation and the difference in the strength of the phenotype observed between *slo I* and *slo 4* mutants, we tested *slo 4/slo I* trans-heterozygotes to rule out the possibility that other loci (also affected by the chromosomal inversion) could be contributing to the observed phenotype. We found that the *slo 4/slo I* mutants show a somewhat intermediate phenotype (especially obvious in DD) between that of *slo 4* and *slo I* (Table 4). We also tested a small number of *slo 4* heterozygotes (*slo 4/+*) and found that most are either strongly or weakly rhythmic; no arrhythmic flies were found (data not shown). This argues against an effect exerted by the other putative loci.

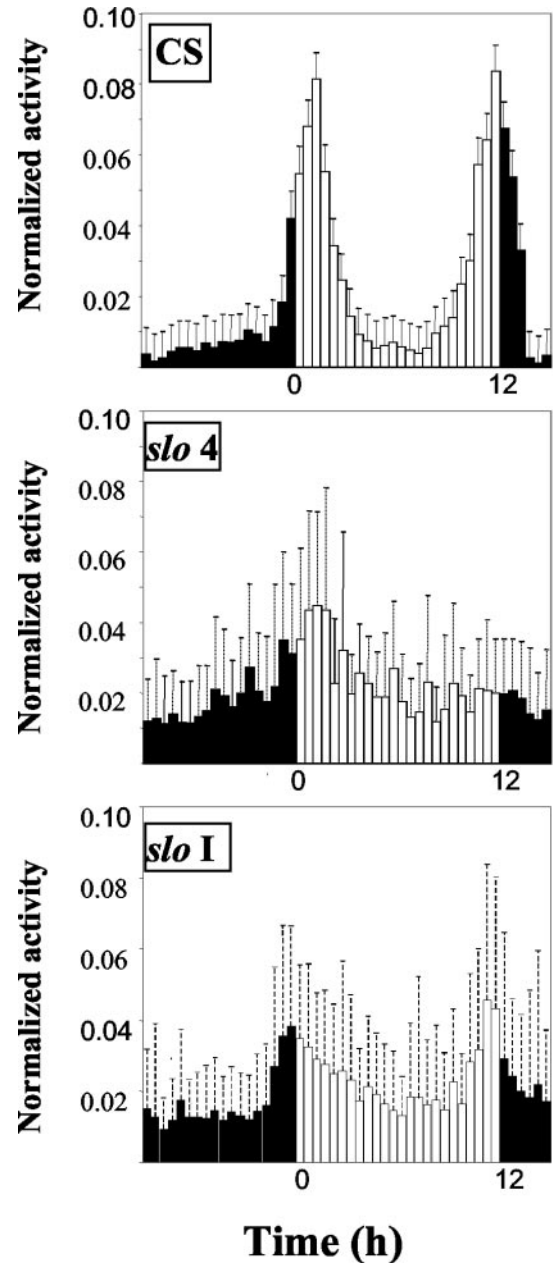
To determine whether this mutation caused a general decrease in motility, which by itself could result in arrhythmicity, we quantified the total locomotor activity displayed by the different

genotypes under LD and DD conditions. Although wild-type flies appear to be slightly more active under constant darkness, both *slo* mutants are impervious to the lighting regimen. More importantly, the overall levels of activity are not different from those of the wild-type flies (Table 4). We superimposed the actograms of wild-type, *slo 4*, and *slo I* mutant flies because the average activity plots are known to reveal features not apparent when individual flies are inspected. This analysis revealed that the most striking difference is the impaired anticipation of the transitions in the *slo 4* (null) mutant flies, indicating that the temporal gating that consolidates behavior around dawn and dusk is absent in flies lacking *slo* function (Fig. 6).

## DISCUSSION

Although a description of the core circadian oscillator exists in flies and mammals, a connection between the molecular basis of rhythmicity and physiology under clock control has been lacking. Because many clock mutants are transcription factors, a central approach in linking core mechanism to physiology is to examine mRNAs for rhythmic expression. To accomplish this on a whole genome scale, we and others have examined steady-state mRNA levels using high-density oligonucleotide arrays for circadian patterns of expression in the fly head and body. This analysis has identified several genes known to be rhythmically expressed, as well as several hundred genes of known and unknown function that are likewise under clock control. We found that a number of aspects of fly physiology ranging from basic cellular metabolism to neurotransmission, stress resistance, and detoxification appear to be under the control of the biological clock. Importantly, as in the mouse, few genes cycled in both heads and bodies, suggesting that tissue specificity is an important component of circadian transcriptional regulation. Furthermore, several cycling genes in the fly were also cycling in the mouse, suggesting that these were conserved output mediators, or perhaps even core clock components.

Recently, three groups reported the use of a similar strategy to identify output genes in *Drosophila* heads (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ueda et al., 2002). Each study identified >100 transcripts that cycle within a 24 hr period in the fly head, and the expression of these transcripts was affected in arrhythmic backgrounds. Similarly to our work, Claridge-Chang et al. (2001) confirmed a subset of the cycling genes by performing Northern blots on independent time courses. Instead, Ueda et al. (2002) used quantitative RT-PCR to confirm a number of cyclers. A direct comparison of the four datasets yields a surprising result: the overlap among lists represents a small fraction of the total number of cycling genes (14, among those the previously characterized clock components). Is this a reflection of differences in the experimental approaches, genotypes used, or variability of the techniques used? The four experiments have two major aspects in common: the use of total RNA prepared from fly heads and Affymetrix Genechips, although different genotypes have been used (*y w*, CS, *cn bw*, and *w*). Jackson and Schroeder (2001) proposed that the use of different methods to identify the cycling genes is responsible for the variability observed. We favor that hypothesis, coupled with the subtly different experimental designs used in each case [we performed independent analysis on 48-hr-long LD and DD time courses as did Ueda et al. (2002); meanwhile Claridge-Chang et al. (2001) concatenated three independent time courses, each representing the last day of entrainment and the first in free-



**Figure 6.** Mutations in *slowpoke* disrupt the consolidation of activity around the transitions. Average activity plots for CS, *slo 4*, and *slo I* mutant flies are shown. Activity records of the LD portion of the experiment for 53 wild-type, 28 *slo 4*, and 56 *slo I* flies were used for the analysis. To superimpose the separate animal records, the levels of activity were normalized per fly per day. Each vertical bar represents the mean  $\pm$  SD.

running conditions (LD + DD), and McDonald and Rosbash (2001) only looked at a single day in DD].

In that regard, it appears particularly informative to contrast our observations with those of Ueda et al. (2002) given the similarity in experimental approaches. If we grouped the cycling genes identified by COSOPT (with  $p < 0.01$ ) in the LD and DD fly head experiments as in Ueda et al. (2002), class I (LD and DD), class II (only LD), and class III (only DD) subsets would contain 24, 96, and 321 genes, respectively. The overlap between the two datasets is high, ranging from ~40% in class I to 20% in

class II genes. Conversely, the overlap between the genes that cycle exclusively in DD is minimal, which might allude to problems in the detection of low-amplitude cyclers or the dampening of the signal, which is characteristic of gene expression under free-running conditions. Nevertheless, the ultimate comparison that will address the limits of the experimental approach is to use a single algorithm to analyze all datasets. As in Ueda et al. (2002), we found a large proportion of cycling genes in DD that were not identified as such in the LD experiment. A direct effect of light on gene expression (by masking or suppression) could account for such an observation.

The important question to ask then is the following: how many of these transcripts are truly cycling? The answer can be derived only from independent observations. In that sense it is encouraging to find that ~67 of the cycling genes were identified in at least two datasets, which together with those that have been shown to cycle by independent means brings the number of confirmed cyclers to >80 in the fly head. Our results have also shown that looking into different tissues will help address the issue of which proportion of the genome is under clock control, because there is very minimal overlap between the ones that cycle in the head and the body, as is the case in mammals (Panda et al., 2002; Storch et al., 2002).

Microarray experiments are extremely powerful in their scope and should be taken as a starting point to delve into the specifics of different aspects of physiology that appear to be under control of the clock. We identified several genes potentially linked to behavior. Follow-up of one of them, *slo*, implicates it as a central regulator of locomotor activity, because a null mutation (*slo 4*) in this locus results in behavioral arrhythmicity without a major change in total activity levels. Several scenarios could account for these observations. A mutation in *slo* could cause arrhythmicity if it directly affects the output pathway controlling behavior by affecting the excitability of the neurons that control it, although if such were the case we would expect hyperkinetic or hypokinetic flies. Alternatively, the mutation could act at the level of the pacemaker neurons by reducing the synchronous firing between the lateral neurons, which would also cause the observed lack of behavioral rhythmicity. *slowpoke* could also be “gating” (McWatters et al., 2000) fly locomotor activity that would be regulated by additional unidentified components. The observation that *slo 4* mutants lack the consolidation of behavior around dawn and dusk clearly favors this hypothesis, although additional work will be required to rule out other plausible scenarios, such as its involvement in the light input pathway that conveys environmental information to the clock or the core oscillator itself.

The notion that a potassium channel is involved in the generation of rhythmic activity was proposed a number of years ago after the analysis of membrane conductance changes in isolated retinal neurons of the mollusk *Bulla* (McMahon and Block, 1987; Michel et al., 1993). This observation, together with the finding that potassium currents are under circadian regulation in the mouse (D. McMahon, unpublished observations) and that expression of *Kcma1*, the *slowpoke* mouse ortholog, cycles (Panda et al., 2002), strongly suggests that this mechanism of control of rhythmic activity could play a role in more complex organisms as well.

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