THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 16, Issue of April 19, pp. 14048–14052, 2002

Genome-wide Transcriptional Orchestration of Circadian Rhythms in *Drosophila**

Received for publication, December 27, 2001, and in revised form, February 15, 2002
Published, JBC Papers in Press, February 19, 2002, DOI 10.1074/jbc.C100765200

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Circadian rhythms govern the behavior, physiology, and metabolism of living organisms. Recent studies have revealed the role of several genes in the clock mechanism both in *Drosophila* and in mammals. To study how gene expression is globally regulated by the clock mechanism, we used a high density oligonucleotide probe array (GeneChip) to profile gene expression patterns in *Drosophila* under light-dark and constant dark conditions. We found 712 genes showing a daily fluctuation in mRNA levels under light-dark conditions, and among these the expression of 115 genes was still cycling in constant darkness, *i.e.* under free-running conditions. Unexpectedly the expression of a large number of genes cycled exclusively under constant darkness. We found that cycling in most of these genes was lost in the arrhythmic *Clock* (*Clk*) mutant under light-dark conditions. Expression of periodically regulated genes is coordinated locally on chromosomes where small clusters of genes are regulated jointly. Our findings reveal that many genes involved in diverse functions are under circadian control and reveal the complexity of circadian gene expression in *Drosophila*.

The use of *Drosophila* has been at the forefront of studies of the molecular and genetic basis of circadian rhythms (1). A number of clock genes have been identified in *Drosophila*, and interlocked *per-tim* and *Clk* feedback loops are now thought to underlie the central molecular machinery of circadian rhythms (2, 3). However, we still do not know how expression of the whole genome is orchestrated by the circadian mechanism nor have we identified all the genes involved. One comprehensive way to find out all the rhythmically expressed genes is to utilize microarray. A number of genes regulated in a circadian manner have been identified in *Arabidopsis* and mammalian cultured cells (4, 5). Since information about all the possible transcription units is available in *Drosophila* (6, 7), we can extensively analyze the data for all the genes relating to their function. Functions of identified genes can be analyzed using various genetic tool and databases (9–11) available in *Drosophila*.

EXPERIMENTAL PROCEDURES

Strain and Sample Preparations—white^{1118} was used as a wild-type strain, and *Clk^{Jrk}* was also used (11). Flies were reared in a regime of 12 h of light followed by 12 h of darkness (LD),[1] and collected every 4 h over 2 days. Total RNA was prepared from 100 heads of 1-week-old adult males and females using the Fast RNA kit (BIO 101, Inc.) followed by DNase treatment. Double-stranded cDNA was synthesized from 10 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) and was used as a template to synthesize biotin-labeled cRNA by *in vitro* transcription using an ENZO BioArray High Yield RNA transcript labeling kit. Amplified cRNA was fragmented and hybridized to GeneChip *Drosophila* arrays (Affymetrix, Santa Clara, CA) for 16 h at 45 °C. Hybridized arrays were washed, stained, and scanned using a Hewlett-Packard GeneArray Scanner. Affymetrix GeneChip software was used to determine the average difference between perfectly matched oligonucleotide probes and single base pair mismatches for each probe set. Data were then scaled globally such that the total intensity of each microarray is equal. The resulting hybridization intensity values reflect the abundance of a given mRNA relative to the total RNA population and were used in all subsequent analyses. Quantitative PCR was performed using the ABI Prism 7700 and SYBR Green reagents (Applied Biosystems).

Analysis of Cycling Genes—We examined gene expression profiles under LD using two successive filters: a periodic filter to extract genes with periodic expression patterns and a deviation filter to identify genes where the changes were above background level.

First, to extract genes with periodic expression pattern, we empirically tested for statistically significant cross-correlation between the temporal expression profiles of each probe set and cosine waves of defined period and phases. We prepared cosine waves of nine test periodicities (τ) from 20 to 28 h in increments of 1 h. Cosine waves of each test period were considered over 60 phases (*i.e.* peaks at 60 equally spaced times in the defined period), yielding a total of 540 test cosine waves. Statistical significance was assessed by an empirical procedure. We generated ~14,000 (the same number of probe sets) normally distributed random expression profiles. Then we calculated correlation between the random profiles and each of the 540 test cosine waves. Standard deviations and averages of cross-correlation were virtually equal for 540 cosine filters despite their different periods and phases. Thus, we searched for the common cut-off correlation of these cosine filters so that 95% of random expression profiles were filtered out after passing 540 parallel cosine filters. We determined this value as 99.8% probable correlation. This analysis is independent of signal strength and imposes no minimal change in amplitude.

Next, to further extract genes whose variation was above background, we determined the noise level associated with a series of ex-

* This work was performed as a part of a research and development project of the Industrial Science and Technology Program supported by NEDO (New Energy and Industrial Technology Development Organization) and was also supported by grants from the Ministry of Education, Science, Sports and Culture of Japan (to T. T. and A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Tables I–VIII.

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[1] The abbreviations used are: LD, 12 h of light followed by 12 h of darkness; DD, constant dark; RT, reverse transcription; GABA, γ-aminobutyric acid.

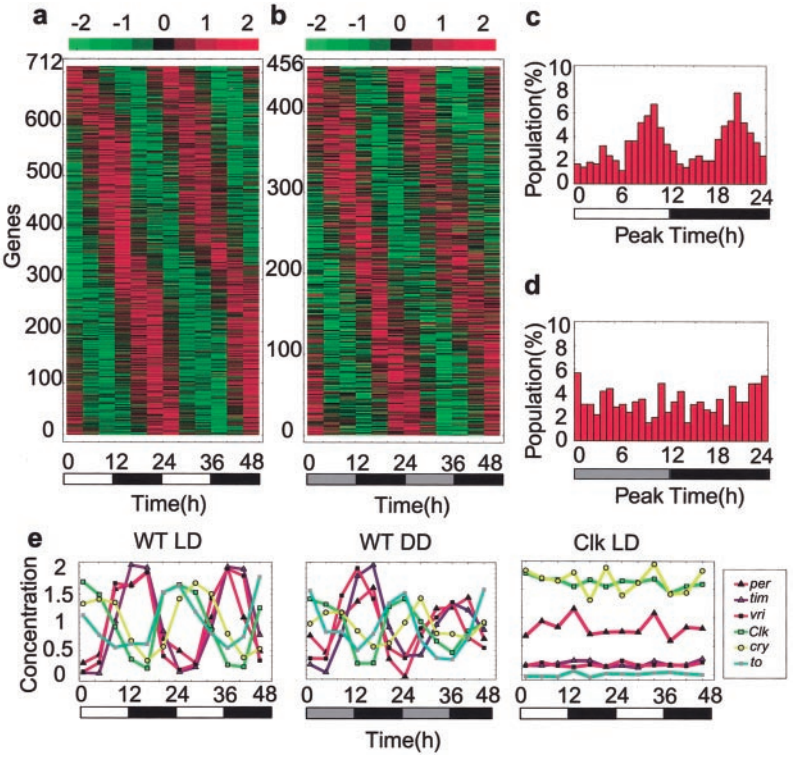

FIG. 1. **Cycling of gene expression in wild-type flies kept under LD and DD and in *Clk^{Jrk}* mutant flies under LD.** *a*, a cluster image of 712 cycling genes under LD. Data were normalized so that the average and the standard deviation of signal intensities of 12 time points are 0.0 and 1.0, respectively. For each gene, the *12 horizontal bars* along the *time axis* represent a 48-h series of data. The genes were ordered by their peak time to help to visualize the extensive pattern of cycling. *Bars* are colored *red* for positive values and *green* for negative values as shown in the *upper color code*. *b*, a cluster image of 456 genes whose expression is free-running under DD. The details of representation are similar to *a*. *c* and *d*, phase distributions of the peak expression times of periodically regulated genes under LD (*c*) and DD (*d*) derived from data on 712 and 456 periodically regulated genes under LD and DD, respectively. Two major populations have peaks at around ZT10 and ZT20 under LD. These peaks are not found in DD. *e*, periodic expression of *per*, *tim*, *vri*, *Clk*, *cry*, and *to* under LD and DD in wild-type and in *Clk^{Jrk}* mutant background flies under LD. Data of LD and DD were normalized so that the average signal intensity of 12 time points was 1.0. For data in *Clk^{Jrk}* background, signal intensities of these genes were divided by the average signal intensities under LD conditions. *WT*, wild type.

perimental procedures for each probe set. Two replicate samples (*i.e.* two sets of 100 fly heads collected independently at the same time of the day) were hybridized to two GeneChips. The standard deviation of the two signal intensities for each probe set was calculated and used as noise deviation (σ) in subsequent analysis. Expression profiles which, over the 12 time points, show a standard deviation (s) greater than noise deviation (σ) with 95% significance are classified as "changing." 95% probability cut-off values are determined from χ^2 (chi-square) distribution with 11 degrees of freedom ($(12 - 1)\, s^2/\sigma^2 > 19.6751$).

To estimate the false positive rate, we generated ~14,000 (the same number of probe sets) random expression profiles that were normally distributed using the noise deviations as determined above. Then we filtered these random expression profiles using two successive filters. Random profiles produced 27 genes classified as "periodically changing." We assume that this estimates the false positive rate (*i.e.* 3.8% of all genes identified would be false positives).

To analyze periodicity of gene expression profiles under constant dark (DD), we used damping cosine curves as test waves. We prepared damping cosine waves of four decay rates (k) from 0.0/h (no damping) to 0.03/h (half-life is 23.0 h) in increments of 0.01/h. Each was considered at nine test periodicities and over 60 phases as described above. We used the same cut-off cross-correlation values and the same deviation filters as in LD analysis.

Phase Analysis—To determine the phase of cycling genes, we tested for correlation between the temporal expression profiles of each gene and 24-h period cosine waves at 60 different phases. We estimated the phase of each cycling gene from the phase of the cosine wave with which it was correlated most closely.

Determination of Statistical Significance for Rhythmic Biological Processes and Periodically Regulated Molecules—We classified the cycling genes by biological process category in the Gene Ontology database (9). For each category, we calculated the probability of finding at least r periodically regulated genes from the category size (n) using the cumulative hypergeometric probability distribution. Probability is given by:

$$\text{Probability} = 1 - \sum_{l=1}^{r-1} \frac{\binom{n}{l}\binom{N-n}{R-l}}{\binom{N}{n}}$$

where N is the total number of genes within the genome, and R is the total number of periodically regulated genes. p values ($-\log_{10}$ (probability)) where the sum of probabilities is below 0.05 were considered significant.

We also performed similar analyses using the LIGAND metabolic database (9). We mapped cycling genes into the metabolic network in *Drosophila* and calculated the probability for observing at least r cycling genes within n enzymes metabolizing the same molecule using the cumulative hypergeometric probability distribution as above.

Analysis of Clk^{Jrk}—For each gene, expression levels in *Clk^{Jrk}* mutants were averaged with those in wild-type flies. Both were kept in LD conditions, and equivalent points in the light-dark cycle were compared. Genes were classified as "up-regulated" if expression was at least 2 times higher in the mutant and "down-regulated" if expression was least 2 times lower in the mutant. Otherwise genes were classified as "unchanged."

Mapping of Periodically Regulated Genes and Calculation of Chromosome Correlation Maps—Among 14,010 probes on the GeneChip, 44 probes are for control, and 299 probes map to multiple genes. The other 13,667 probes map to single genes. Among these, there are several redundant probe sets that map to the same gene, leaving 13,282 nonredundant probes. 12,795 of these match in FlyBase ID (10) to identified genes from Release 2 *Drosophila* genomic sequences (6, 7). We identified the chromosomal positions of all 12,795 genes using the BLASTN algorithm. Using the chromosomal positions obtained above, we mapped the genes belonging to each Class I, II, and III on chromosomes. To detect co-regulated regions, we calculated the correlation between expression patterns under LD conditions of genes on the same chromosome as described elsewhere (12). To identify significantly co-regulated regions, we calculated the average correlation of six adjacent genes and compared it with the average correlation of six nonadjacent genes as background. There were 140 chromosomal regions where the average correlation of the six adjacent genes was more than 3.5 standard deviations from background, *i.e.* the mean average correlation of six nonadjacent genes. This analysis showed that a substantial number of adjacent sets have correlated expression patterns in comparison with 25 expected co-regulated regions derived from a control set of nonadjacent genes. Similar results were obtained from analysis of 2–10 adjacent genes. Among 140 chromosomal regions, 38 clusters of genes included periodically regulated genes. We also analyzed co-expressed region under the DD condition and obtained similar results to the LD condition.

RESULTS AND DISCUSSION

We have examined temporal patterns of gene expression under LD and under DD using a GeneChip (Affymetrix) representing the entire genome (more than 13,500 genes) of *Dro-*

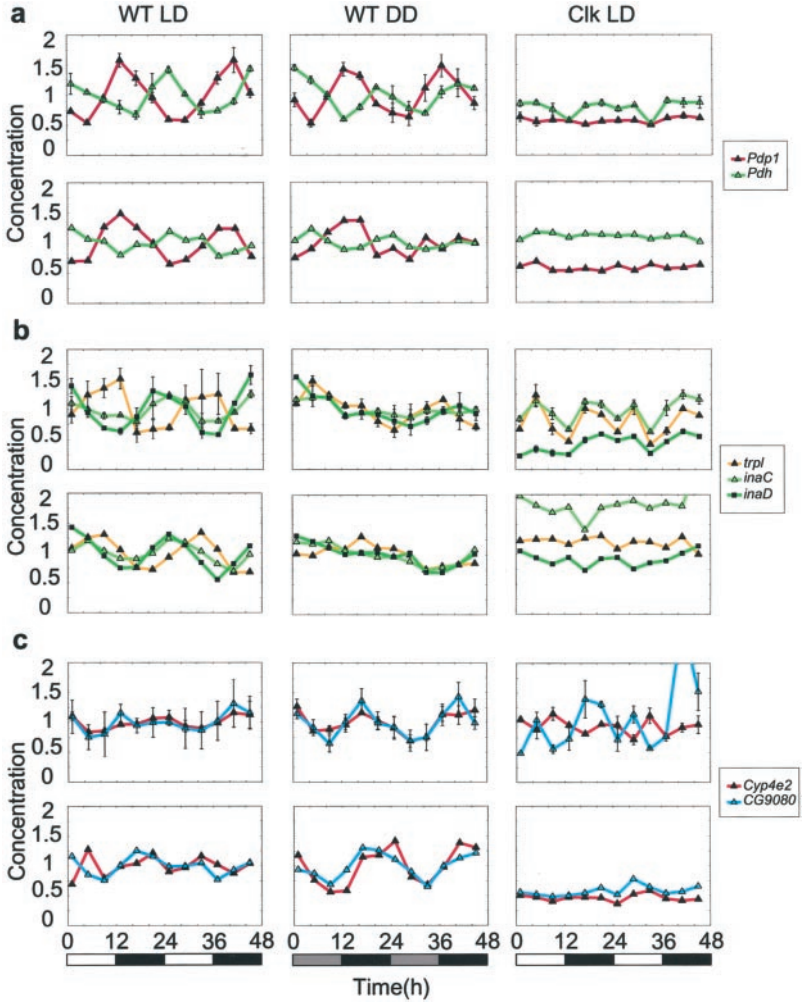

FIG. 2. **Three classes of periodically regulated genes and validation of GeneChip data by quantitative RT-PCR using wild-type flies (*WT*) under LD and DD and *Clk^{Jrk}* (*Clk*) mutant flies under LD.** *a*, Class I genes cycling both in LD and DD; *b*, Class II genes cycling only in LD; *c*, genes cycling in DD but not in LD. In each class, data are shown for two to three genes, the rhythmic expression of which was found in this study. *Upper curves* in *a*, *b*, and *c* are based on the GeneChip analyses; *lower curves* are based on the quantitative PCR analyses. *Pdp1*, PAR domain protein encoding a transcription factor; *Pdh*, photoreceptor dehydrogenase; *trpl*, transient receptor potential-like encoding a Ca^{2+} channel; *inaC*, inactivation no after-potential C encoding a protein kinase C; *inaD*, inactivation no after-potential D encoding a structural protein containing a PDZ domain; *Cyp4e2*, cytochrome P450-4e2.

sophila melanogaster. Flies were collected every 4 h over 2 days both in LD and DD, and biotin-labeled probes made from cDNA from 100 heads were used for hybridization. We estimate that the expression of 6,061 genes (43.4% of all genes) was detected on GeneChip. The number of genes detected here is thought to be delimited by the detection method using GeneChip, and there should be additional cycling genes expressed at a lower level or in a small number of cells. Data were analyzed through two sequential statistical filters, and 712 genes (5.3% of the whole genome) were classified as periodically regulated genes in LD (Fig. 1*a*). This is likely to be a minimum number for the genes that are periodically regulated; the number may increase if a different filtration analysis was applied. Our analyses might not detect genes that cycle in some cells but not in others, and moreover, it is technically difficult to monitor genes with very low levels of transcription. The number of periodically regulated genes in *Drosophila* is similar to that reported from *Arabidopsis* under constant light (4), in which 6% of genes investigated are rhythmic, but is in contrast to cyanobacteria, in which nearly all genes are expressed periodically (13). We found that genes implicated in circadian rhythms, including *period* (*per*) (14), *timeless* (*tim*) (15), *Clock* (*Clk*) (11), *vrille* (*vri*) (16), *cryptochrome* (*cry*) (17), and *takeout* (*to*) (18), cycled with high amplitude and in similar phase, as previously reported, validating our experimental and statistic procedures (Fig. 1*e*). We analyzed the phase of periodically regulated genes at a resolution of 0.4 h and found two peaks around ZT10 and ZT20 (Fig. 1*c*). The peaks may reflect the after-effect of the change from dark to light and light to dark since significant peaks were absent under the constant dark condition (Fig. 1*d*). The peak phases of the clock genes, *Clk*, *cry*, *per*, *vri, tim*, and *to*, were not at these times. We then analyzed the gene expression under DD (Fig. 1*b*) and found that 115 genes of 712 were still periodically regulated in the free-running condition (Class I, periodically regulated both in LD and DD). The remaining 597 genes were judged to be periodically regulated only in LD (Class II). Surprisingly 341 genes that were not judged as periodically regulated under LD were, however, periodically regulated under DD (Class III). Their cyclings might have been suppressed or masked under LD as suggested from behavioral experiments (19). In our classification of genes we should note that because we used a strict filter to identify periodically regulated genes, genes judged to be not cycling might in fact cycle with low amplitude. Lists of all genes in each class may be found in Supplemental Tables I–III. After completion of our work two similar works using GeneChip were published (20, 21). Their findings are similar to ours, but there are several differences, specifically the Class II and III genes were not mentioned in other studies. We think that the major differences are the numbers of sampling and statistical analyses. We analyzed data for 2 days both under LD and DD, while the previous studies analyzed data only for 1 day in each light condition. Several interesting periodically regulated genes were identified from the three classes, I, II, and III (Fig. 2). The reliability of GeneChip data was assessed by quantitative RT-PCR analyses, which confirmed that both methods yielded similar data. A novel candidate of clock genes, *Pdp1*, showing a robust cycling (Fig. 2*a*), encodes a transcription factor with

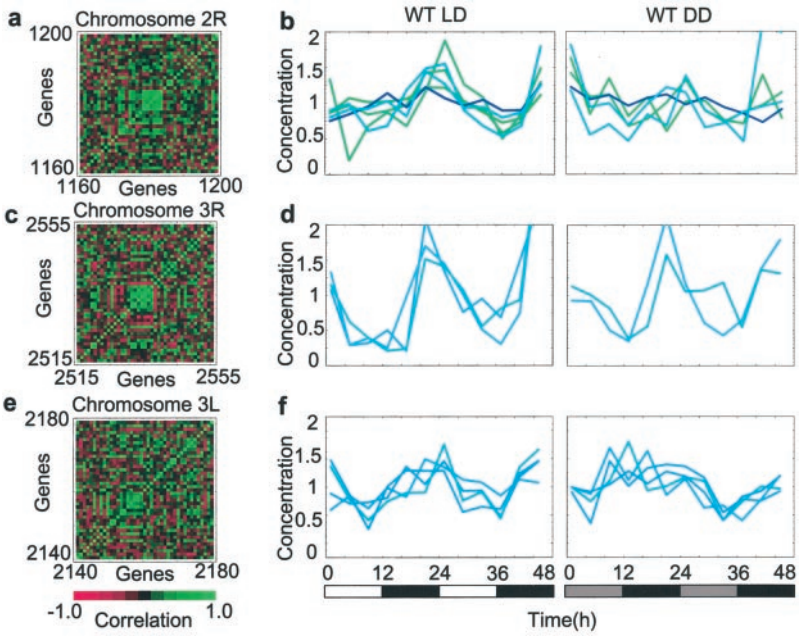

FIG. 3. **Global chromosomal profiles of periodic gene expression.** *a*, correlation map for gene clusters that included the Class I genes on the right arm of the second chromosome. The *green block* at the *center* indicates the group of six adjacent co-expressing genes, including cytochrome P450, *Cyp6a17* (Class I), *Cyp6a23*, *Cyp6a19*, *Cyp6a9*, *Cyp6a20*, and *Cyp6a21*. The *numbers* along the matrix represent the gene number along the chromosome. *Green squares* indicates a positive correlation; *red squares* indicate a negative correlation. *b*, the six cytochrome P450 genes that are periodically co-regulated under LD and DD. *Cyp6a17* is represented by the *green line* with the highest peak at a time point of 25 h. *c*, correlation map for gene clusters that included the Class I genes but that have no functional relatedness to each other. *d*, three genes on the third chromosome (CG11891, which belongs to Class I, CG11889, and CG10513) showed similar rhythmic expressions in LD, and periodic expression of these genes also persists under DD. Data for CG11889 under DD is not shown here as they included a few negative values. *e*, correlation map for gene clusters that included the three Class II genes on the left arm of the third chromosome. *f*, CG7646, CG7654, and CG7433 are Class II genes, and their neighboring gene, neurocalcin, showed a similar expression pattern in LD. *WT*, wild type.

homology to *vri* (16). Most of the genes we found to cycle could be classified according to the category of their "biological process" as defined in the Gene Ontology database (8) (Supplemental Tables IV and V). Phototransduction is one such category, which includes a significant number of periodically regulated genes. Two of these were Class I genes. Photoreceptor dehydrogenase, *Pdh*, also showed a clear cyclical expression (Fig. 2). One retinoid-binding protein (CG5958) was periodically expressed with peak at dusk, while the other retinoid-binding protein (CG10657) belonging to Class II was cycling with almost opposite phases. A number of genes belonged to Class II. Three opsins, *Rh3*, *Rh4*, and *Rh6*, which express in the central rhabdomeres of the compound eye's ommatidia, showed rhythmic expression. Another opsin, *Rh5*, belonged to Class III. The *ninaA* gene encoding cyclophilin, which transports opsins from endoplasmic reticulum to microvilli membrane, also showed rhythmic expression. Molecules associating with Ca^{2+} signal transduction in the photoreceptors, *inaC*, *inaD*, and *trpl*, also cycled (Fig. 2*b*). The expression of most genes listed above peaked in the morning, whereas *Rh6* and *trpl* peaked in the evening. Visual sensitivity is controlled by a circadian rhythm in insects (22), and it would therefore be interesting to know how cyclical changes in these genes influence photoreceptor structure and function.

We also used the LIGAND metabolic database (9) to examine the functional significance of periodically regulated genes (Supplemental Tables VI and VII). Enzymes and transporters involved in metabolism or function of glutamate and GABA were periodically regulated. *Eaat1*, CG5618, and CG7470 are Class I genes, and *Gdh*, *black*, CG4233, and CG7433 are Class II genes. *Gs1* belongs to Class III. All genes except CG4233 showed robust rhythmicity with peaks in the dark phase. In mammals, glutamate (23) and GABA (24) are neurotransmitters associated with clock function. These molecules mimic the dark pulse to a circadian rhythm in the optic lobe of *Musca* (25). In *Drosophila*, one type of glutamate receptor is highly enriched in pacemaker neurons (26), and our data suggest glutamate and GABA might have a role in the circadian mechanism. In the light of a recent finding that the redox state of NAD cofactors is involved in circadian rhythms (27), it is interesting that many enzymes related to NAD^+, NADH, $NADP^+$, and NADPH metabolism were periodically regulated. There are 16 periodically regulated genes directly associated to the synthesis of these nicotinamide nucleotides. Three are in Class I, and the remaining 13 are in Class II. Their peaks expression occurred in three phases under LD: noon, dusk, and night.

We next examined the cycling of gene expression in the arrhythmic mutant of the *Clock* (*Clk*) gene (11) under LD. Our study showed that many genes are cycling only in LD, and we then asked whether the cyclings of the Class II genes are merely a reflection of light responses. Homozygous Clk^{Jrk} mutants show completely arrhythmic locomotor behavior under DD (11). CLK is a transcription factor and activates clock-regulated genes (1, 2, 11). In the *Clk* mutant, periodically expression of all the clock genes disappeared (Fig. 1*e*), and only a few genes were judged to cycle under LD (7, 16, and 23 genes in Class I, II, and III, respectively; Supplemental Table VIII). It is possible that the cycling genes in the Clk^{Jrk} mutant may represent genes controlled by a possible CLK-independent mechanism. We did not analyze the cycling pattern with two peaks in a day. If cycling is simply controlled by light-on and -off, it shows a pattern with dual peaks in a day. We did not investigate this possibility, but genes belonging to the Class III may have such a property. Further studies are necessary to investigate this possibility.

Our results suggest that the cycling of the Class II genes is not merely a result of light exposure during LD but is under

circadian control. Under LD, the expression levels of *per*, *tim*, *vri*, and *to* were decreased relative to *Clk* (Fig. 1*e*), whereas *Clk* and *cry* continue to express at a high level as previously shown (11, 17). Fig. 2 shows the expression patterns of seven genes in Clk^{Jrk}. If the transcription level of a gene is lowered in Clk^{Jrk}, the gene might be up-regulated by *Clk*, whereas if the level is not affected, the gene might be regulated by a CLK-independent mechanism. The expression of about 6% of genes was decreased, while in about 3% of genes it was increased. In the latter case, their transcription might normally be down-regulated by genes controlled by CLK. These results indicate that CLK regulates transcription in many genes, but there are other genes in which transcription is not directly controlled by CLK. In addition some genes might be negatively regulated by CLK. We do not think that these changes are caused by the genetic background differences as we dealt with genes whose expression level changed over 2-fold or one-half. Our study thus shows that a single mutation in such a central gene regulator results in global but differential changes of gene expression.

We mapped the chromosomal locations of genes belonging to each class (I, II, and III) and found that they were not randomly distributed but clustered on chromosomes. There were 15 clusters where periodically regulated genes occupied a highly condensed chromosome interval. This suggests that temporal gene expression might be locally regulated on chromosomes. To confirm this possibility, we calculated the correlation of temporal expression pattern along the neighboring genes on all chromosomes and found 140 chromosomal regions where neighboring genes are expressed with a similar pattern to each other. Among them, 38 regions contained at least one periodically regulated gene. There were six genes in Class I, 24 in Class II, and eight in Class III. For example, the expression patterns of six neighboring genes belonging to the cytochrome P450 family, located on the right arm of the second chromosome, are similar (Fig. 3, *a* and *b*). There is a region where genes are co-regulated, but their functions are unknown (Fig. 3, *c* and *d*). Moreover, we found that functionally unrelated genes are co-regulated (Fig. 3, *e* and *f*). These results suggest that the temporal expression of neighboring genes is influenced by a periodically regulated gene. Searches of each class of periodically regulated genes so far failed to reveal any common motifs in the nucleic acid sequence along the putative regulatory region of each class of cycling genes. We found that the co-regulation of temporal expression occurs even more globally. The co-regulated regions were observed along the fourth chromosome at intervals of about 5–10 genes (data not shown). The regular spacing suggests control at the level of higher order chromatin structure as previously reported in suprachiasmatic nuclei neurons (28). These results suggest that gene expression on chromosomes is globally regulated by circadian mechanisms, although we still do not know their molecular bases. Coordinated gene regulation at the chromatin level might be an economical way in remodeling chromosome structures.

Our study thus reveals the complex transcriptional orchestration of genes under LD and DD conditions in *Drosophila*. Although cycling gene expression is not always essential for circadian function, in clock genes such as *cyc* (29) and *double-time* (30) our study has established the candidacy of many candidate genes that might be implicated in circadian mechanisms. Further work using genetic tools available in *Drosophila* should help to explore the function of these genes with the prospect of leading to a greater understanding of the molecular basis of circadian rhythms in all organisms.

Acknowledgments—We are grateful to Michael Rosbash and Jeffrey C. Hall for the Clk^{Jrk} strain. We thank Shigeru Iwase for computational analysis; Satoko Hayashi, Tomoko Kojima, Toshie Katakura, Yuko Mitsuiki, and Makiko Haruta for technical assistance; and Masami Horikoshi and Takashi Umehara for hospitality in the use of their laboratory. We also thank Hideo Iwasaki and Kenji Tomioka for discussion and Jennifer Hallinan and Ian Meinerzhagen for comments on the manuscript.

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