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Clockwork Orange is a transcriptional repressor and a new Drosophila circadian pacemaker component

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Many organisms use circadian clocks to keep temporal order and anticipate daily environmental changes. In Drosophila, the master clock gene Clock promotes the transcription of several key target genes. Two of these gene products, PER and TIM, repress CLK–CYC-mediated transcription. To recognize additional direct CLK target genes, we designed a genome-wide approach and identified clockwork orange (cwo) as a new core clock component. cwo encodes a transcriptional repressor that synergizes with PER and inhibits CLK-mediated activation. Consistent with this function, the mRNA profiles of CLK direct target genes in cwo mutant flies manifest high trough values and low amplitude oscillations. Because behavioral rhythmicity fails to persist in constant darkness (DD) with little or no effect on average mRNA levels in flies lacking cwo, transcriptional oscillation amplitude appears to be linked to rhythmicity. Moreover, the mutant flies are long period, consistent with the late repression indicated by the RNA profiles. These findings suggest that CWO acts preferentially in the late night to help terminate CLK–CYC-mediated transcription of direct target genes including cwo itself. The presence of mammalian homologs with circadian expression features (Dec1 and Dec2) suggests that a similar feedback mechanism exists in mammalian clocks.

[Keywords: Circadian; clk; Drosophila; transcriptional oscillations; chromatin]

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least in the fly system, there is good genetic evidence that this is the case (Allada et al. 2003).

A recent report suggests an additional “active” repression mechanism, as important changes in chromatin structure escort circadian transcriptional oscillations in mammals and Neurospora (Ripperger and Schibler 2006; Belden et al. 2007). In mammals, these modifications appear to follow the CRY-PER repression events and may enhance the oscillation amplitude of various cycling mRNAs. It is likely that similar phenomena take place in the Drosophila system.

Although this view advances transcriptional oscillations as an important feature of circadian timekeeping, recent evidence strongly indicates that they might be dispensable in the cyanobacterial system (Tomita et al. 2005). Moreover, there are transgenic fly strains in which behavioral and molecular (protein) rhythmicity can persist despite the absence of per and tim transcriptional oscillations (Yang and Sehgal 2001). Consequently, the role of cyclic transcription in generating the molecular and behavioral rhythms characteristic of wild-type flies is still uncertain.

Nonetheless, it is curious that all known bona fide direct targets of the master gene Clk are involved in transcriptional regulation (per, vri, tim, Pdp1). We therefore reasoned that finding additional CLK direct targets might identify new biochemical pathways relevant to central clock function or perhaps reinforce the centrality of transcriptional regulation. We report here the isolation and characterization of a new core clock component: clockwork orange (cwo). cwo transcription is activated by CLK–CYC and repressed by PER–TIM. As a consequence, cwo mRNA oscillates with an amplitude and phase comparable with other CLK direct targets; for example, vri, Pdp1, per, and tim. cwo is prominently expressed in circadian brain neurons and cooperates with PER to repress CLK–CYC-mediated transcription. Characterization of flies deficient in cwo activity demonstrates that the protein is essential for robust oscillations of core clock mRNAs as well as persistent behavioral rhythms with wild-type periods. Because the oscillation amplitude of direct clock transcripts is specifically affected in cwo-deficient strains, the core transcriptional feedback loop appears essential for circadian rhythms in Drosophila. As cwo orthologs (Dec1 and Dec2) are possible pacemaker components in mammals (Honma et al. 2002), this view may also extend to other animal systems.

cwo is expressed cyclically and in pacemaker neurons

Among putative direct CLK targets was a gene encoding a transcription factor with an ORANGE domain, CG17100, which we call clockwork orange (cwo). This gene was previously misannotated as stich1 [Salzberg et al. 1994; Dubruille et al. 2002]; cwo belongs to a family of transcriptional repressors [basic helix–loop–helix-O [bHLH-O]] involved in various aspects of cell physiology and metabolism [Davis and Turner 2001]. Importantly, two close mouse relatives, dec1 and dec2, are circadianly expressed in the suprachiasmatic nucleus (SCN). However, there is no evidence that they contribute to the generation or maintenance of circadian rhythms [Honma et al. 2002]. We previously reported that cwo mRNA oscillates in a circadian manner by microarray and RNAse protection assays [McDonald and Rosbash 2001]. We verified this observation with RT–PCR as well as with new 2.0 Drosophila Affymetrix microarrays. By both criteria, cwo mRNA cycles with a phase [peak around 14 h Zeitgeber time (ZT14)] [Fig. 2A; data not shown] that resembles those of per, tim, Pdp1, and vri mRNAs [Hardin et al. 1990; Sehgal et al. 1995; Blau and Young 1999; Cyran et al. 2003]. These CLK direct targets contain numerous CLK–CYC-binding elements [E-boxes] in their promoters. E-boxes are necessary for transcriptional oscillations and have been shown in some cases to mediate CLK activation followed by PER repression [McDonald et al. 2001; Wang et al. 2001]. There are six E-boxes within the promoter of cwo, 2 kb upstream of the transcriptional start site, and 15 E-boxes within the first intron [data not shown]. Moreover, cwo mRNA was regulated in clock mutant strains like characterized direct target genes [Fig. 2B], namely, low and high mRNA levels in the Clk mutant Jrk and the per01 mutant, respectively [Allada et al. 1998; Rutila et al. 1998b; Blau and Young 1999].

To examine cwo spatial expression, we crossed a UAS-
GFP line with an enhancer trap fly line (NP7492; NP Consortium, Kyoto, Japan) containing a GAL4-coding sequence in the promoter region of *cwo*. Costaining with anti-PDF antisera (PDF is a neuropeptide specific for pacemaker cells) showed strong GFP expression in brain pacemaker neurons (Fig. 2C). Recent independent reports confirm this observation: A LacZ enhancer trap in this same gene is prominently expressed in clock cells—that is, in all PER-expressing cells [Shafer et al. 2006]—and comparable data with an anti-CWO antibody are presented in the accompanying paper by Matsumoto et al. (2007).

**cwo is a new core clock component**

We next characterized two *cwo*-deficient fly strains (e4027 and f5073; Exelixis collection, Harvard University), which contain unique transposon insertions at the
beginning and at the end of the first intron (Fig. 3A, top). RT–PCR shows that both insertions reduce cwo mature mRNA levels below the level of detection, probably as a consequence of deficient splicing (Fig. 3A, bottom). Moreover, no cwo protein is detectable in the 5073 strain as shown in Matsumoto et al. (2007).

These strains have strong circadian locomotor activity phenotypes [Fig. 3B,C; Supplementary Fig. 1]. During the first 4 d in free running conditions [constant darkness, DD], more than half of these flies were arrhythmic (56% and 51% for strains 4027 and 5073, respectively); the remaining flies had weaker and longer rhythms than control strains, which have nearly 0 arrhythmic flies. After 4 d in DD, most mutant flies were arrhythmic (75% or 100% for 5073 and 4027, respectively) [Fig. 3B,C; Supplementary Fig. 1]. An indistinguishable phenotype, long and weak rhythms, was obtained when either of the two chromosomes was heterozygous over a deficiency that includes the cwo gene, and an identical phenotype was observed with the trans-heterozygous strain; that is, 5073/4027 (Fig. 3B; Supplementary Fig. 1). Moreover, expression of a cwo RNA interference (RNAi) construct in

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**Figure 2.** cwo gene expression is regulated in a circadian manner. (A) Expression profile for cwo and tim across six time points in wild-type (yw) flies. Data were obtained by Q-PCR. Expression values for each transcript and time point were generated by dividing the cwo or tim mRNA signal by the expression value for a control noncircadian mRNA [ribosomal protein 49, RP49]. A value of 1 was assigned to the maximum expression across the six time points, the values in the other time points were calculated as a fraction of this maximum value. The data are the average of the normalized cwo or tim expression values for three independent RNA samples. The error bars indicate the SEM. (B) cwo mRNA levels in control (yw), Clik^{lo}, and per^{01} fly strains. Flies were entrained for 4 d in LD conditions and then transferred to DD. After 2 d in DD conditions, flies were harvested at CT3 and CT15. RNA from both time points for each strain was combined, and Q-PCR was performed using primers for cwo and RP49. Expression values are reported as a ratio of cwo/RP49 expression. We assigned a value of 1 to the ratio obtained for control flies and proceed as in A. The experiment was performed twice and the results were averaged. The error bars indicate the SEM. (C) cwo localization in the fly brain examined with GFP. p[cwo-gal4 (NP7442-gal4)]; UAS-GFP flies were stained with PDF antiserum. Strong overlap between the PDF staining and GFP expression can be observed in the region corresponding to the position of the pacemaker neurons (sLNv). An amplified picture of the overlap in the sLNv region is shown in the bottom right panels.
circadian neurons produces a similar phenotype (Matsumoto et al. 2007).

The long period phenotype of the cwo-deficient genotypes is a consequence of lack of cwo expression in circadian cells, since expression of a UAS-cwo transgene with a timeless driver restores the circadian period to 24 h in the trans-heterozygous mutant background [Fig. 4A]. A slow clock is also apparent under light–dark (LD) conditions in 5073 flies, as there is a clear delay in morning anticipation (Fig. 4B). Moreover, 5073 flies also have an aberrant phase-response curve (PRC) [Fig. 4C], when subjected to a light pulse during the last night of LD entrainment. The mutant PRC is similar in the early night but then lags behind the wild-type PRC, which we interpret to indicate a slow clock even in these LD conditions [Fig. 4B,C].

An altered clock is also apparent from the 5073 six-time-point microarray data, which show a striking decrease in mRNA oscillation amplitude for some clock components (per, tim, and vri) [Fig. 5A,B]. Normalization of the data facilitates visualizing the delayed repression of these mRNAs in the 5073 strain [Fig. 5C]. The mRNA trough is two- to threefold elevated for direct CLK target genes in the cwo mutant strain (Fig. 5D), which fits with “incomplete” repression, even in LD conditions when behavioral rhythms are robust. However, lower peak levels also contribute to the low amplitude of the cwo-deficient strain. This effect on oscillation amplitude is accompanied by little or no effect on average mRNA levels in the 5073 strain [Fig. 5E]. Similar mRNA profiles for tim, vri, and per mRNAs were observed using RT–PCR (Supplementary Fig. 2; data not shown). It is intriguing
that an oscillation amplitude effect was less apparent on other cycling genes, including core oscillator genes that are not direct CLK targets; for example, Clk and cry [Fig. 5A; data not shown]. All of these data indicate that cwo has central pacemaker component characteristics. Similar data and a parallel conclusion are presented in Matsumoto et al. [2007].

cwo cooperates with PER in repressing CLK-mediated transcription

Is the function of CWO to repress CLK direct target gene transcription, as its bHLH-O motif and the mutant data suggest? To this end, we used direct target promoter-luciferase reporters and transfected them into *Drosophila* S2 cells with a *cwo* expression vector (pAc-cwo). Even without CLK coexpression, cwo expression reduced basal transcription from the vri, tim, cwo, and CG15095 promoters but had no effect on the actin promoter [Fig. 6A]. This is consistent with the notion that cwo encodes a transcriptional repressor.

Does *cwo* interact directly with CLK target genes? Close inspection of the *cwo* bHLH region reveals striking similarity with the CYC bHLH domain, suggesting that *cwo* could recognize clock gene E-boxes like the CLK partner CYC [Fig. 6B]. To assess this possibility, we first added the VP16 domain to the C terminus of *cwo* [Wysocka and Herr 2003]. *cwo*-VP16 is a potent transcriptional activator of CLK direct target genes, as it increased transcription of the vri-Luc and *tim*-luc reporters by 60- and 150-fold, respectively [Fig. 6C, left panel; Supplementary Fig. 3B]. Deletion of the three E-boxes in the *tim* promoter [McDonald et al. 2001] prevented activation [Fig. 6C, right panels], and similar data were obtained with vri-Ebox and vri-Ebox mutant promoters [Supplementary Fig. 3C; Blau and Young 1999]. Addition of cyc double-stranded RNA [dsRNA] had no effect [Supplementary Fig. 3D], indicating that *cwo*-VP16 activity does not require CYC, and ChIP data from S2 cells indicate direct binding of CWO to target gene E-boxes [Matsumoto et al. 2007; data not shown]. All of these considerations indicate that CWO functions by interacting directly with promoter E-boxes of CLK direct target genes.

However, *cwo* overexpression only mildly repressed CLK-mediated transcription in S2 cells [Fig. 6D, left]. This is probably not a consequence of CLK levels, since *cwo* expression has the same effect with lower amounts of pAc-Clk [Supplementary Fig. 3A]. Based in part on the similar mRNA profiles of *cwo* [Fig. 2A] and *per*, we assayed CWO-mediated repression in the presence of PER. Indeed, CWO now repressed CLK-mediated transcription fivefold to 10-fold [Fig. 6D, right; data not shown]. In a reciprocal manner, expression of a fixed amount of *cwo* plasmid strongly enhanced PER-mediated repression [Fig. 6E]. For example, low doses of pAC-per [e.g., 25 ng] are ineffective in reducing CLK-mediated expression without CWO coexpression. The two complementary experiments demonstrate that CWO and PER can cooperate to repress CLK-mediated transcription, and we suggest that they work through different mechanisms [Fig. 7].

Discussion

We have identified *cwo* as a new core clock component in *Drosophila*. It encodes a transcriptional repressor, which synergizes with PER and inhibits CLK-mediated activation. Consistent with this function, the mRNA profiles of CLK direct target genes manifest high trough values and low amplitude oscillations in mutant flies. Because rhythmicity fails to persist in DD and there is little or no effect on average mRNA levels in the 5073 strain, transcriptional oscillation amplitude appears linked to rhythmicity. Moreover, the mutant flies are
long period, consistent with the late repression indicated by the RNA profiles. These findings suggest that CWO acts preferentially in the late night to help terminate CLK–CYC-mediated transcription of direct target genes including cwo itself. The presence of cwo homologs (Dec1 and Dec2) in mammals suggests that a similar feedback mechanism exists in mammals.

cwo is a new core clock component

We used a genome-wide approach to identify candidate CLK targets from fly heads [Fig. 1A]. Intriguingly, a significant fraction of these genes are nonoscillating. Because the S2 cell assays predict that most of these genes are probably bona fide CLK targets, they may reflect a noncircadian role of CLK [Fig. 1C,D]. Accordingly, a recent study reported that CLK expression is not restricted to circadian neurons in the fly brain [Houl et al. 2006]. In contrast, cwo mRNA cycles and is expressed in circadian neurons [Fig. 2A,C; Matsumoto et al. 2007]. Moreover, the cwo mRNA profile is similar to that of the other core clock components, as the gene is activated by CLK–CYC and repressed by PER [Figs. 1D, 2A,B]. We suggest that cyclical transcription of cwo probably contributes to cir-
Cadian changes in the level of CWO similar to other direct CLK–CYC targets.

The two cwo insertion strains have no detectable cwo mRNA. Both also have long periods, which fail to persist after 4–5 d in DD. The penetrance of these two phenotypes, however, is not identical: 100% of flies have long periods, whereas 50%–75% are arrhythmic after 4 d in DD (Fig. 3B). This suggests that these two phenomena are separable and that the long periods are not due to the weak rhythms. The circadian phenotype is slightly more severe in the 4027 strain but is accompanied by a high mortality of flies (data not shown). In contrast, 5073 homozygous flies and 5073/4027 trans-heterozygous flies show no life-span effect (data not shown) and have comparable phenotypes, that is, long rhythms and 75% arrhythmic flies after 4 d in DD (Fig. 3B). Supplementary Fig. 1). This indicates that both rhythm features are determined by the absence of cwo expression.

The slow clock is not only manifest by a period phenotype in DD but also by a late activity phase in LD. More specifically, 5073/5073 flies have delayed anticipation of the lights-on transition (Fig. 4B). This is consis-
tent with cwo acting in the pdf-expressing neurons, since these cells are both responsible for the morning anticipation in LD and period determination in DD (Grima et al. 2004; Stoleru et al. 2004, 2005). We therefore prefer the parsimonious interpretation that the delayed phase is caused by a slow central molecular oscillator rather than an output defect. More support for this hypothesis comes from the delayed mRNA profiles as well as the delayed PRC. Although aberrant PRCs often reflect defects in light perception, we have previously suggested that they can also reflect a fast or slow central oscillator [Rutilla et al. 1998a]. In this view, the wider PRC delay zone reflects a slower clock and in particular the broader transcriptional peak (Fig. 4C). Taken together with the 5073 mRNA curves, CWO may preferentially function to repress transcription at the end of each cycle [see below]. In contrast, the more potent advance zone of the 5073 PRC may reflect an underlying weaker circadian oscillator [Vitaterna et al. 2006].

Expression of a cwo transgene in tim-expressing cells restored a 24-h period to the mutant genotype [Fig. 4A]. In contrast, cwo overexpression using the pdf-gal4 as well as the tim-gal4 driver had no effect on the period of an otherwise wild-type strain (data not shown). This adds to the evidence that the long period phenotype is due to the absence of functional cwo. Although the rescue also improves the rhythm strength of the cwo-deficient host strain, it is not as strong as that of wild-type flies [data not shown]. Moreover, cwo overexpression combined with heterozygosity for the 5073 or 4027 chromosomes also gives rise to weak rhythms [data not shown]. We suspect that rhythm strength is sensitive to the levels and timing of cwo expression. We note that the UAS transgene lacks the cwo 5' and 3' untranslated (UTR) regions, which are unusually long (2.6 and 1.5 kb, respectively) and probably contribute to post-transcriptional regulation of CWO expression.

Model for cwo function

In the current model, high-amplitude oscillations of tim, per, vri, and Pdp1 mRNAs levels are due to cyclical activation and repression of the CLK–CYC heterodimer. Recent reports from mammals suggest that there is a daily change in chromatin structure, which parallels the CLK–BMAL (CLK–CYC equivalent) activation cycle. Moreover, circadian chromatin remodeling has recently been reported in the Neurospora system [Belden et al. 2007]. How these changes are generated and/or linked to the activation–repression cycle is not known.

However, based on the link between bHLH-O proteins and histone deacetylase recruitment [Davis and Turner 2001], we suggest that CWO helps build a repressive chromatin structure during the end of a cycle not unlike the one observed at mammalian circadian promoters [Ripperger and Schibler 2006]. This explains the higher trough values as well as the long period and delayed mRNA decline in the 5073 strain [Fig. 5C,D]. PER probably recruits the kinase doubletime (DBT) to the CLK–CYC dimer, resulting in diminished CLK–CYC affinity for DNA [Lee et al. 1999; Yu et al. 2006], this should favor CWO binding to E-boxes and corepressor recruitment. Similarly, CWO activity may aid CLK–CYC inactivation by PER–DBT, as observed in the S2 cells experiments [Fig. 6F]. As closed chromatin structures are necessary for full activation by several transcription factors [Beato and Eisfeld 1997], this may also help explain the lower mRNA peak of most CLK direct targets in the 5073 strain [Fig. 5B]. This lower mRNA peak could also be an indirect or “system” effect, as peak direct CLK-target mRNA levels in this strain are comparable (~60%) with the levels observed in other repressor mutant strains, namely, per01 and tim01.

Role of transcriptional oscillations in generating circadian rhythms

Although mRNA and transcriptional oscillations were proposed long ago to be essential for circadian clock function, recent evidence strongly indicates that they are dispensable in cyanobacteria (Tomita et al. 2005). Consistent with this notion, there is evidence in the fly system that some rhythmicity persists without per and tim transcriptional cycling [Yang and Sehgal 2001]. Importantly, behavioral rhythms and probably other CLK direct target genes still undergo oscillations in these strains. We suggest that the CWO feedback system contributes to this residual rhythmicity.

As shown above, the absence of CWO has two effects on the mRNA profiles of tim, per, and vri: late repression and low-amplitude oscillations. We propose that the former is responsible for the phase change in LD and period change in DD, whereas the latter causes the weak rhythmicity phenotype. Because mRNA oscillation amplitude is affected with little or no effect on average mRNA levels in the 5073 strain [Fig. 5E], transcriptional regulation appears essential for persistent DD rhythms, which fail after several days in the cwo mutant genotypes. In this view, we interpret the weak mRNA amplitude to be the cause of the weak rhythms. This adds to the evidence supporting the direct involvement of tran-

Figure 7. Proposed role of CWO in the circadian molecular circuit.
scriptional oscillations in the timekeeping process [Al-
lada et al. 2003]. We note that we cannot rule out the
possibility that the weak rhythmicity is a consequence
of an additional role of cwo in the output pathway. Al-
though there are no comparable genetic results in mam-
malian systems, the similar expression profiles as well as
the conservation of Dec1 and Dec2 with cwo suggest
that a comparable feedback mechanism with behavioral
effects exists in mammals.

Materials and methods

Plasmids

pAc-clk, pAc-per, Copia-Renilla luciferase, tim-Luciferase,
timΔΔ2m-Luciferase, vri-Luciferase, vriBox-Luciferase, and
vrimut-Box-Luciferase have been previously described [Blau
and Young 1999; McDonald et al. 2001]. pAc-cwo was con-
structed by amplifying the coding region of cwo by PCR and
ligating in-frame into pAcB V5/His6 [Invitrogen]. The construc-
tion of reportors CG15095-Luciferase, picot-Luciferase, and
CG15095-Luciferase was done in two steps. First, a PCR frag-
ment containing the Luciferase gene was amplified from the
tim-Luciferase construct (McDonald et al. 2001) and ligated into
pBS-KS [Stratagene]. The corresponding promoters were added
upstream of the Luciferase gene by ligation of the appropriate
PCR fragments. pAc-Luciferase was constructed by ligation of
the PCR product containing the Luciferase gene and an artificial
poly-linker sequence into pAcB V5/His6 [Invitrogen]. pAc-
cwoVP16 was constructed by ligation of the PCR product con-
taining cwo in-frame into pAc-VP16 [a kind gift from Rebecca
Schoer, Columbia University, New York].

S2 cell transfection

S2 cells were maintained in 10% fetal bovine serum [FBS] [In-
vitrogen] insect tissue culture medium [HyClone]. Cells were
seeded in a six-well plate. Transfection was performed at 70%–
90% confluence according to the manufacturer’s recommen-
dations (12 µL of cellfectin [Invitrogen] and 2 µg of total DNA).
In all experiments, 50 ng of pCopia-Renilla Luciferase plus 50 ng
of the Luciferase firefly reporter were used. pBS-KS+ [Stratagene]
was used to bring the total amount of DNA to 2 µg.

Luciferase activity assay

Forty-eight hours after transfection, cells were assayed using the
Dual Luciferase Assay Kit [Promega] following the manufactur-
er’s instructions.

dsRNA synthesis and RNAi treatment

For both procedures, we followed the RNAi protocol in S2 cells
previously described [Nawathean et al. 2005]. Two different
dsRNAs were synthesized for each gene.

Analysis of gene expression by RT-PCR

Total RNA was prepared from adults from the indicated strains
using Trizol reagent [Invitrogen] according to the manufactur-
er’s protocol. cDNA derived from this RNA (using Invitrogen
SuperScript II) was used as template for quantitative real-time
PCR performed with the Corbett Research Rotor-Gene 3000
real-time cycler. The PCR mixture contained Platinum Taq
polymerase [Invitrogen], optimized concentrations of SYBR-
green, and the corresponding primers: tim, 5’-CCTTTYTCGTA
CACAGATGCC-3’ and 5’-GGTCCGCTGGTGGATCCCAG
3’; vri, 5’-GGCCTCGAAGTCTGTTCACT-3’ and 5’-CTTT
GTGTCGTGTGTTG-3’, P49, 5’-ATCCGCCCAGCA
TACG-3’ and 5’-TCCGACCAGGTACAGAA-3’, cwo, 5’-
GCTGTGGAAGAGGAGCAG-3’ and 5’-GGCATAATTCAG
CAGCTGTC-3’.

Cycling parameters were 3 min at 95°C, followed by 40 cycles
of 30 sec at 95°C, 45 sec at 55°C, and 45 sec at 72°C. Fluores-
cence intensities were plotted against the number of cycles by
using an algorithm provided by the manufacturer. mRNA levels
were quantified using a calibration curve based on dilution of
concentrated cDNA. mRNA values from heads were normal-
ized to that from ribosomal protein 49 (RP49).

Microarrays

Probe preparation Total RNA was extracted from S2 cells or
fly heads, using Trizol reagent [Invitrogen] according to the
manufacturer’s protocol. For heads, cDNA synthesis was car-
rried out as described in the Expression Analysis Technical
Manual (Affymetrix). The cRNA reactions were carried out us-
ing the BioArray High-Yield Transcript Labeling Kit [Enzo] or
IVT [Affymetrix] depending on whether the oligonucleotide mi-
croarrays to be used were first or second generation, respec-
tively. Affymetrix high-density arrays for Drosophila melano-
gaster were probed, hybridized, stained, and washed according
to the manufacturer’s protocol.

Data analysis

GeneChip CEL files were analyzed using R
[http://www.r-project.org] and the bioconductor package [gcrma
algorithm; http://www.bioconductor.org]. Anti-logarithm [base
2] was applied to the data to obtain the expression values. These
data have been deposited at NCBI GEO as series GSE7644,
GSE7646, GSE7651, GSE7652, and GSE7653. Heat maps were
done normalizing the gene expression value to the maximum
for each gene across the different conditions in the following
way: Normalized value = (Value – Maximum)/Maximum.

Determination of direct CLK targets

Experiment layout For the experiments in S2 cells, duplicate
experiments using Affymetrix Drosophila Genome oligo-
nucleotide microarrays and Drosophila Genome 2.0 oligo-
nucleotide microarrays were performed. In all cases, we per-
formed two conditions: cycloheximide only (CHX) or CHX plus
dexamethasone (Dex). In the case of microarrays from cultured
fly heads, we performed two sets of Drosophila Genome micro-
arrays [stimulation with Dex or vehicle in presence of CHX at
ZT16] and four sets of Drosophila Genome 2.0 microarrays
[stimulation with Dex or vehicle in presence of CHX at ZT4 and
ZT16].

Head culture Heads were dissected and cultured in the 12:12
LD condition as described before [Levine et al. 2002]. After 3 d
of LD entrainment, CHX (0.1 mg/mL) and either dexametha-
sone (15 µM) or vehicle was added for 6 h.

Data analysis The samples were obtained and processed as
described above. Expression values were obtained as described
above. For each probe set, fold changes between the control and
Dex plus samples were calculated as follows: ratio = Min(Dex)/
Max[No Dex], where Min[Dex] is the minimum value for each
probe across the samples with Dex, and Max[No Dex] is the
maximum value for each probe set across the control samples.
This calculation was performed on data obtained from Af-

Downloaded from www.genesdev.org on July 13, 2007
In situ hybridization and GFP detection were performed as described previously (Levine et al. 2002). We used autocorrelation and spectral analyses were performed with a signal-processing toolbox. Male flies were monitored for 4 d in LD conditions, followed by the locomotor activity peaks after the light pulse was determined separately. Those probes that pass one or more of the following criteria were selected as direct-CLK targets in S2 cells: (1) The ratio on both sets of chips (first and second generation) is >1.5. (2) The ratio on one of the sets is >2.

With these criteria, we found 46 probes (corresponding to 43 genes). To obtain the CLK targets from fly heads, we chose the probe sets that passed one or more of the following criteria: (1) The ratio is >1.5 for first-generation (one time point) and second-generation (both time points) oligonucleotide microarrays. (2) The ratio is >3 for one of the three sets of chips (one first generation or one of the time points from the second generation). Using these criteria, we found 73 probes sets (corresponding to 72 genes). To obtain the final targets, we selected probe sets that passed at least one of the following criteria: (1) They are on both the S2 and fly head lists. (2) They are in the S2 cell list and the ratio from fly head microarrays is >1.2, or they are in the fly head list and the ratio from the S2 cell microarray is >1.2. The Pet gene is not expressed in S2 cells upon CLK addition. However, we decided to included it in the final list, since it is a known CLK direct target and the second most-induced gene upon addition of dexamethasone from fly heads (22-fold).

For ranking the obtained targets, we built an index: Targetness = Average[(ratioHeads)/Max ratio Heads], [(ratioCell)/Max ratio Cell)].

Fly strains
The insertion lines 5073 and e4027 were obtained from the Exelixis Consortium, Harvard University. The insertion line NP7492 was obtained from the NP Consortium, Kyoto, Japan.

Construction of UAS-cwo transgenic lines
UAS-cwo plasmids were generated by cloning a PCR fragment from pAc-cwo into pUAST (Brand and Perrimon 1993). This construct was used to generate germine transformants by injecting yw; Ki p场所 / H9004 2 – 4027 into yw; H9004 2 – 4027.

PRC
Flies were entrained to a 12:12 LD cycle for 4 d. During the fifth dark phase of the cycle, flies were given a 10-min saturating white light pulse (2000 lux) at 13, 15, 17, 19, 21, and 23 h after the last light-on event. A separate control group of flies was not given a light pulse. Flies were then put into DD. The phase of the locomotor activity peaks after the light pulse was determined and compared with the no-light-pulse control.

Locomotor behavior
Male flies were monitored for 4 d in (LD) conditions, followed by 4–5 d in DD using Trikinetics Drosophila Activity Monitors. Analyses were performed with a signal-processing toolbox (Levine et al. 2002). We used autocorrelation and spectral analysis to estimate behavioral cycle durations (periods) and the Rhythm Index to assess rhythm strength (Levine et al. 2002).

In situ hybridization and GFP detection
In situ hybridization and GFP detection were performed as described previously (Zhao et al. 2003).

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References


