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Functional Role of CREB-Binding Protein in the Circadian Clock System of *Drosophila melanogaster*[▽]

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Rhythmic histone acetylation underlies the oscillating expression of clock genes in the mammalian circadian clock system. Cellular factors that contain histone acetyltransferase and histone deacetylase activity have been implicated in these processes by direct interactions with clock genes, but their functional relevance remains to be assessed by use of appropriate animal models. Here, using transgenic fly models, we show that CREB-binding protein (CBP) participates in the transcriptional regulation of the *Drosophila* CLOCK/CYCLE (dCLK/CYC) heterodimer. CBP knockdown in *pigment dispersing factor*-expressing cells lengthens the period of adult locomotor rhythm with the prolonged expression of *period* and *timeless* genes, while CBP overexpression in *timeless*-expressing cells causes arrhythmic circadian behaviors with the impaired expression of these dCLK/CYC-induced clock genes. In contrast to the mammalian circadian clock system, CBP overexpression attenuates the transcriptional activity of the dCLK/CYC heterodimer in cultured cells, possibly by targeting the PER-ARNT-SIM domain of dCLK. Our data suggest that the *Drosophila* circadian clock system has evolved a distinct mechanism to tightly regulate the robust transcriptional potency of the dCLK/CYC heterodimer.

The circadian clock is an evolutionarily conserved system that perceives environmental time cues, synchronizes the organism's inherent clock with external time, and exhibits the circadian physiology of organisms (e.g., diurnal or nocturnal locomotor activities) (14, 40, 52, 59). At the cellular level of circadian clock systems in animals, pacemaker cells in a small subset of brain neurons display a robust oscillation of clock gene products and dominate the circadian behaviors of the organism by governing the peripheral clock systems. These core clock cells correspond to ventral lateral neurons (LN_vs) and the suprachiasmatic nucleus in *Drosophila* and mouse circadian clock systems, respectively (21, 23, 58). *Drosophila* LN_vs express the neuropeptide *pigment dispersing factor* (*pdf*) gene, which is implicated in the synchronization of clock cells (33, 41, 42, 44). At the molecular level, some core clock genes are periodically expressed, and their rhythmic expression is maintained under free-running conditions (i.e., exclusion of external time cues). This molecular clock system involves several transcription factors, protein kinases, phosphatases, and proteosomal pathway components, which together mediate the transcriptional regulation of clock transcripts and control the posttranslational localization, quantity, and quality of clock proteins (19, 45).

Published data suggest that two interlocking feedback loops maintain the oscillating expression of core clock genes in *Drosophila melanogaster* and mouse. In *Drosophila*, a heterodimer of the *dClock* (*dClk*) and *cycle* (*cyc*) gene products activates the transcription of the *period* (*per*), *timeless* (*tim*), *vrille* (*vri*), and *Par domain protein 1*ε (*Pdp1*ε) genes during subjective night by binding to E-box sequences within their promoters (2, 6, 11, 12, 48). The PER/TIM heterodimer or the PER monomer then translocates to the nucleus and inhibits the transcriptional activity of the dCLK/CYC heterodimer, subsequently repressing the transcription of the *per* and *tim* genes (8, 12, 29, 39, 47, 49). In the second feedback loop, VRI and PDP1ε bind to a site within the *dClk* promoter, repressing and activating the transcription of *dClk* gene, respectively (11, 17).

As far as the mammalian circadian clock system is concerned, it has been shown that the oscillating expression of the *mPer* and *mCry* genes is based on the rhythmic histone acetylation of their promoter regions (15). This acetylation may involve the intrinsic histone acetyltransferase activity of CLOCK protein (13) and/or transcriptional coactivators such as CREB-binding protein (CBP), p300, and p300/CBP-associated factor (p/CAF), since they have been shown to augment the transcriptional activity of the CLOCK/BMAL1 heterodimer (10, 56). In addition, it was recently reported that mCRY1 might attenuate the transcriptional activity of the CLOCK/BMAL1 heterodimer through interactions with the components of a corepressor complex that contains histone deacetylase activity (38). However, no appropriate animal model was available for investigating the functional role of either histone acetyltransferases or histone deacetylases in a circadian clock system. In this study, we adopted transgenic fly models in which *Drosophila* CBP expression can be up- or down-regulated in a tissue-specific manner via a GAL4/upstream activation sequence (UAS) system (7) and characterized their circadian behaviors as well as the molecular clocks in their pacemaker neurons. In contrast to the mammalian circadian clock system, both our in vivo and our in vitro data indicate that CBP may function as a negative regulator of the dCLK/CYC heterodimer, a *Drosophila* homolog of the mammalian CLOCK/BMAL1 heterodimer.

MATERIALS AND METHODS

Plasmids. Total RNA from adult fly heads was isolated using the TRIzol reagent (Invitrogen) and reverse transcribed using Moloney murine leukemia

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virus (M-MuLV) reverse transcriptase according to the manufacturer's instructions (Roche). *Pdp1ε* and *cyc* cDNAs were amplified by PCR using gene-specific primer sets, inserted into pAc5/V5-His (Invitrogen) for V5- and His-tagged expression in Schneider 2 (S2) cells, and confirmed by sequencing. The *dClk*, *per*, and mouse *PKA* catalytic subunit cDNAs were similarly cloned into pAc5/V5-His. The *dClk* cDNA was also inserted into pAc/FLAG, a modified version of pAc5/V5-His, to express N-terminally FLAG-tagged dCLK protein in S2 cells. Mammalian CBP cDNA with a C-terminal stop codon (32) was inserted into pAc5/V5-His and therefore could not express either the C-terminal V5 tag or the His tag. The *per*-luc, *tim*-luc, *dClk*-luc, and ATFx3-luc constructs have been described previously (11, 12, 31). The cDNAs corresponding to the deletion mutants of dCLK were amplified with the appropriate primer sets and inserted into pGEX 4T-1 (Amersham Biosciences) for expression of a glutathione *S*-transferase (GST) fusion protein in bacteria.

***Drosophila* stocks.** All flies were reared with standard cornmeal-yeast-agar medium at 25°C under light-dark (LD; 12 h of light and 12 h of darkness) cycles. *GMR*-GAL4, UAS-green fluorescent protein (GFP), and UAS-GFP^{RNAi} lines were obtained from the Bloomington *Drosophila* Stock Center. EP element insertion lines including EP1179 and EP1149, GAL4 driver lines including *pdf*-GAL4, *tim*-GAL4-62, *tim*-GAL4-86, Mz520-GAL4, Mai179-GAL4, and C929-GAL4, and GAL80 repressor lines including *pdf*-GAL80 and *cry*-GAL80 have been described previously (18, 23, 36, 44, 53). *pdf*-GAL4 is expressed in both large and small LN_vs (44). Two *tim*-GAL4 lines are expressed in all known clock neurons including both large and small LN_vs, dorsal LNs (LN_ds), and three types of dorsal neurons (DNs) (23). Mz520-GAL4 is expressed similarly to *pdf*-GAL4 (18). Mai179-GAL4 is expressed in a subset of LN_vs and LN_ds (18, 51). C929-GAL4 is expressed in large LN_vs as well as in 100 peptidergic noncircadian neurons of the adult fly brain (18, 55). For the construction of transgenic flies in which CBP expression is downregulated via a GAL4/UAS system, a double-stranded RNA (dsRNA) construct for CBP was designed according to the genomic cDNA hybrid method (22). The DNA fragment from bp 6721 to 7454 of CBP cDNA and genomic DNA including the cDNA with internal and adjacent 3′ introns were amplified by PCR from pDF378 (16) and fly genomic DNA, respectively. The fragments were ligated together into the pUAST vector, and the transgenic construct was injected with pUCHspπΔ2-3 into w^{1118} embryos, from which several germ line transformants were established. All experiments were performed using three independent lines containing the UAS-CBP^{RNAi} construct on the third chromosome, which gave consistent results. Data from a representative line are shown.

Behavioral analysis. The locomotor activities of individual male flies were measured using *Drosophila* activity monitors (Trikinetics). Monitoring conditions included LD cycles for 2 to 4 days, followed by constant-dark (DD) cycles for 4 to 7 days. Data were analyzed using ClockLab analysis software (Actimetrics). Rhythmic flies were defined as described previously (61), except that the significance level of the χ^2 periodogram was set at an α value of 0.05. Data were pooled from more than three independent experiments. The average locomotor activity profile for each genotype was analyzed using Microsoft Excel. Locomotor activity per half-hour bin was normalized by the average locomotor activity per day, and the relative locomotor activities per half-hour bin for individual flies were averaged for each genotype. For eclosion rhythm analysis, third-instar larvae or early pupae, reared under LD cycles, were individually placed in glass tubes. Their circadian rhythms were further entrained to LD cycles for 2 days and then subjected to DD cycles. Under DD conditions, the eclosion time was determined using *Drosophila* activity monitors and analyzed as described previously (35).

In situ hybridization. In situ hybridization using larval brains was performed as described previously (57) with minor modifications. RNA probes for in situ hybridization were transcribed in vitro using MAXIscript (Ambion) in the presence of digoxigenin (DIG)-11-UTP (Roche), precipitated with ethanol, dissolved in 1× hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], pH 5.0, 0.1% Tween 20, 50 μg/ml heparin, 50 μg/ml sonicated salmon sperm DNA, and 50% formamide), and stored at −70°C until use. The hybridizing region of the *tim* gene has been described previously (50). The probe for the *Pdp1ε* gene encompasses the mRNA region from nucleotide +565 to +765 relative to the transcription start site, while the probe for the *pdf* gene encompasses the full-length cDNA. After hybridization, DIG-labeled probes were detected colorimetrically using an alkaline phosphatase-conjugated anti-DIG antibody and a nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate stock solution (Roche).

Immunofluorescence assay (IFA) and image analysis. Adult flies were fixed in phosphate-buffered saline (PBS) containing 4% formaldehyde for 2.5 h, and their brains were subsequently dissected in PBS. Larval brains were dissected in PBS and fixed in PBS containing 4% formaldehyde for 1 h. After permeabilization with PBS containing 0.1% Triton X-100 (PBS-T), brain samples were blocked in PBS-T containing 2% bovine serum albumin and incubated overnight with primary antibodies in blocking solution at 4°C. After repeated washes, samples were incubated with secondary antibodies in PBS-T for 2 h, washed extensively, and mounted using Vectashield (Vector Laboratories). S2 cells were grown on coverslips and transfected using the calcium precipitation method. At approximately 40 h after transfection, cells were fixed in PBS containing 3.7% formaldehyde for 30 min and permeabilized with PBS containing 0.2% Triton X-100 at 4°C for 25 min. Blocking and antibody incubation were performed similarly with brain samples, except that the primary antibody was incubated at room temperature for 2 h. The primary antibodies used were a guinea pig anti-CBP antibody (30), a rat anti-PDF antibody (41), rabbit anti-PER, anti-dCLK, and anti-PDF antibodies (see below), rabbit anti-acetyl histone H3 and H4 antibodies (Upstate Biotechnology), and a mouse anti-V5 antibody (Invitrogen). The secondary antibodies used were rhodamine-conjugated anti-guinea pig, anti-rabbit, anti-mouse, and anti-rat antibodies and a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories). Samples were examined with a BX51 fluorescence microscope (Olympus) and a Pascal confocal laser scanning microscope (Carl Zeiss). For the quantitative analysis, the intensity of PER staining signals in each group of clock cells was quantified using ImageJ software. After subtraction of the background intensity, the total intensity value for each group of clock cells was averaged from the values of eight brains at each circadian time.

Cell culture, transfection, and semiquantitative RT-PCR. *Drosophila* S2 cells were maintained in Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Cells were transiently transfected using the standard calcium precipitation method. For semiquantitative reverse transcription-PCR (RT-PCR), total RNA from transfected cells was isolated using the TRIzol reagent (Invitrogen). After the removal of contaminating genomic DNA by DNase I digestion, RNA was reverse transcribed with M-MuLV reverse transcriptase and oligo(dT) primers according to the manufacturer's instructions (Promega). The cDNA for each clock gene was amplified by PCR with a gene-specific primer set under nonsaturating conditions. PCR products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed after exposure to UV light.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's instructions (Upstate Biotechnology) with minor modifications. S2 cells in 6-well plates were cotransfected with the expression vectors for V5-tagged dCLK and/or CBP. At approximately 40 h after transfection, cells were cross-linked with 1% formaldehyde at 25°C for 10 min. Cross-linked chromatin was sheared by sonication and immunoprecipitated with a rabbit anti-acetyl H3 antibody (Upstate Biotechnology) or a guinea pig anti-dCLK antibody (24). Immunoprecipitated DNA was analyzed by semiquantitative PCR with primers specific for each clock gene. The amplified region of the *vri* gene promoter was from −1.9 kb to −1.64 kb relative to the transcription start site, which includes two canonical E-box sequences. The amplified region of the *Pdp1ε* gene promoter was from −1.05 kb to −0.75 kb, which includes two canonical E-box sequences. The amplified region of the *dClk* gene promoter was from −0.2 kb to +0.18 kb, which includes no canonical E-box sequence. Representative data from two independent experiments are shown.

Antibody production, immunoprecipitation, and Western blotting. The C-terminal 544 amino acids of the PER protein, the N-terminal 388 amino acids of the PDP1ε protein, and the C-terminal 287 amino acids of the dCLK protein fused to GST were expressed in bacteria, purified using glutathione-Sepharose 4B beads (Amersham Biosciences), and used for the immunization of rabbits by subcutaneous injection. For the priming injection, the proteins were mixed with complete Freund's adjuvant (Sigma). After 1 month, the rabbits were injected four times, at 1-week intervals, with proteins in incomplete Freund's adjuvant (Sigma). Peptides corresponding to the C-terminal 20 amino acids of TIM (26) or PDF protein were synthesized (Anygen, Korea) and similarly used to immunize guinea pigs or rabbits, respectively. Polyclonal antibodies were affinity purified from rabbit antisera, dialyzed, and stored at −70°C. For immunoprecipitation from cultured cell extracts, transfected cells were lysed in a lysis buffer (25 mM Tris-Cl, pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 20 min. After clarification by centrifugation, soluble extracts were diluted to adjust salt concentration (final concentration, 100 mM NaCl) and then incubated with 2 μl of guinea pig anti-CBP serum (30) or control guinea pig serum at 4°C for 1.5 h. For immunoprecipitation from fly head extracts, approximately 50 μl of adult fly heads was homogenized in the same lysis buffer and incubated at 4°C for 20 min. After two clarifications by centrifugation, soluble extracts were similarly diluted and then incubated with 3 μl of guinea pig anti-dCLK serum (24) or control guinea pig serum at 4°C for 1.5 h. After the addition of preequilibrated

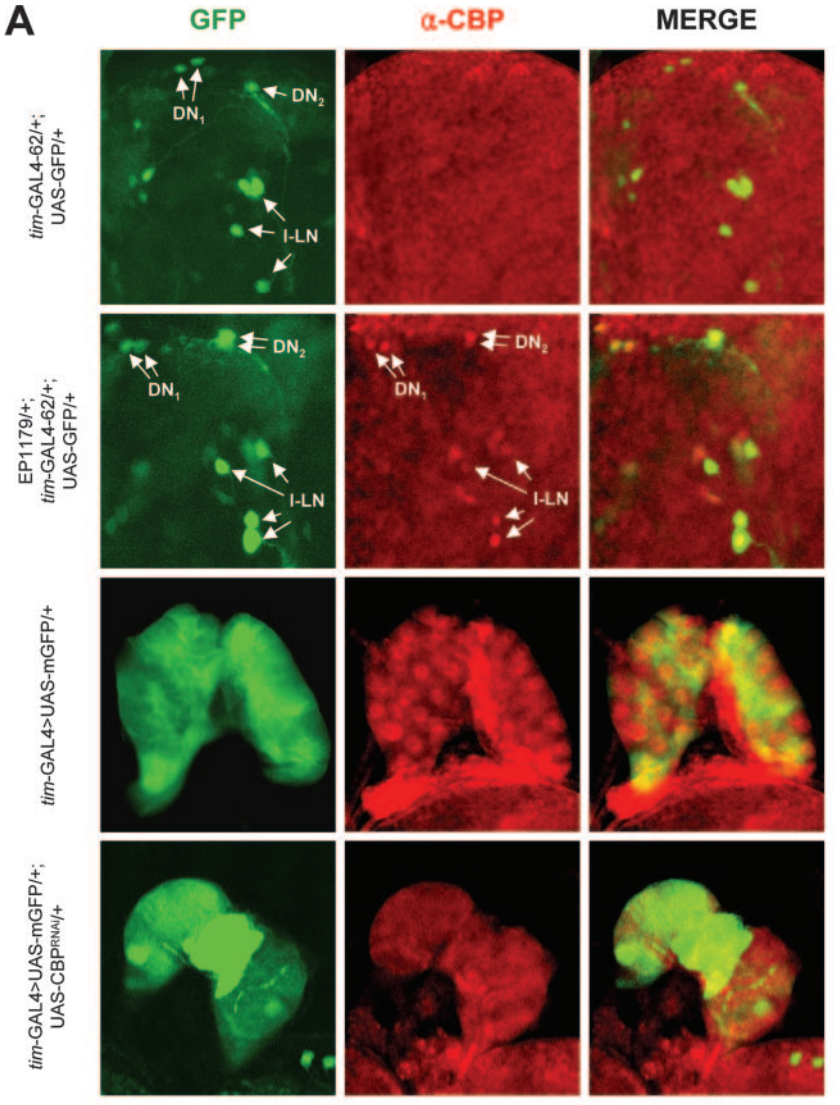
A
GFP
α-CBP
MERGE
tim-GAL4-62/+; UAS-GFP/+
EP1179/+; tim-GAL4-62/+; UAS-GFP/+
tim-GAL4>UAS-mGFP/+
tim-GAL4>UAS-mGFP/+; UAS-CBPRNAi/+
DN1
DN2
l-LN
B
GMR-GAL4/+
GMR-GAL4/+; UAS-CBPRNAi/+
EP1179/Y; GMR-GAL4/+
EP1179/Y; GMR-GAL4/+; UAS-CBPRNAi/+
LM
SEM

TABLE 1. Locomotor activity analysis of transgenic flies in which CBP knockdown is driven by specific GAL4 drivers[a]

Genotype	No. of flies tested	No. analyzed (no. dead)[b]	No. (%) of rhythmic flies during DD cycles	Period ± SEM (h)
UAS-GFP^{RNAi}	46	38 (8)	36 (97)	24.3 ± 0.1
pdf-GAL4/+; UAS-GFP^{RNAi}	45	43 (2)	43 (100)	24.2 ± 0.1
UAS-CBP$^{RNAi/+}$	20	19 (1)	18 (95)	23.6 ± 0.1
UAS-CBP^{RNAi}	58	56 (2)	48 (86)	23.4 ± 0.0
pdf-GAL4/+	44	43 (1)	43 (100)	23.5 ± 0.1
pdf-GAL4	32	31 (1)	25 (81)	23.7 ± 0.2
pdf-GAL4/+; UAS-CBP$^{RNAi/+}$	15	15 (0)	14 (93)	23.6 ± 0.1
pdf-GAL4/+; UAS-CBP^{RNAi}	54	50 (4)	50 (100)	25.3 ± 0.1
pdf-GAL4; UAS-CBP^{RNAi}	64	62 (2)	60 (97)	25.6 ± 0.1
tim-GAL4-62/+; UAS-CBP$^{RNAi/+c}$	28	0 (28)	ND[e]	ND
tim-GAL4-86/+; UAS-CBP$^{RNAi/+c}$	16	0 (16)	ND	ND
Mz520-GAL4/+; UAS-CBP$^{RNAi/+d}$	61	56 (5)	55 (98)	23.4 ± 0.1
Mz520-GAL4/+; UAS-CBP^{RNAi}	45	34 (11)	31 (91)	25.4 ± 0.1
Mai179-GAL4/+; UAS-CBP$^{RNAi/+d}$	16	15 (1)	15 (100)	23.5 ± 0.3
Mai179-GAL4/+; UAS-CBP^{RNAi}	59	39 (20)	34 (87)	24.9 ± 0.1
C929-GAL4/+; UAS-CBP$^{RNAi/+d}$	16	15 (1)	15 (100)	23.5 ± 0.1
C929-GAL4/+; UAS-CBP^{RNAi}	29	28 (1)	26 (93)	23.8 ± 0.1

[a] Female flies homozygous for the UAS-CBP^{RNAi} transgene were crossed with male flies homozygous or balanced for each GAL4 driver. To increase the copy numbers of transgenes, double balancer lines were intermediately used. Their male progenies were used in locomotor activity analysis. After entrainment for 3 days in LD cycles, data were further collected for a week under DD conditions. Period and rhythmicity were calculated using ClockLab software.

[b] No. of surviving (dead) flies during locomotor activity analysis.

[c] These flies displayed shortened longevity, excluding them from locomotor activity analysis.

[d] Flies homozygous for both the GAL4 driver and the UAS-CBP^{RNAi} transgene were not produced.

[e] ND, not determined.

protein A Sepharose (Amersham Biosciences), extracts were further incubated at 4°C for 1.5 h. The beads were then washed three times in the same buffer. Bound proteins were eluted, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a Hybond-C membrane (Amersham Biosciences), and analyzed by Western blotting. Proteins were detected using rabbit anti-CBP (43), anti-dCLK, anti-PER, and anti-PDP1ε sera, a mouse anti-V5 antibody, and guinea pig anti-TIM serum with ECL Plus reagents (Amersham Biosciences).

In vitro binding assay. GST fusion proteins were incubated with [^{35}S]methionine-labeled proteins synthesized by the TNT T7 coupled transcription-translation reticulocyte lysate system according to the manufacturer's instructions (Promega). After a 30-min incubation at room temperature in a binding buffer (25 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride), glutathione-Sepharose 4B beads were added and incubated further for 30 min at room temperature. The beads were washed four times in the same buffer. Bound proteins were eluted, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to autoradiography. For competition experiments, 2 to 5 μg of maltose-binding protein (MBP) or the cysteine-histidine-rich 3 (C/H3) region of CBP fused to MBP was preincubated with GST-dCLK protein for 10 min at room temperature before the addition of in vitro-translated CYC protein.

RESULTS

Construction of CBP mutant flies via a GAL4/UAS system. To investigate the possible role of CBP in the *Drosophila* circadian clock system, we first established transgenic fly models in which CBP expression is up- or downregulated. It was previously reported that two EP lines, EP1179 and EP1149, contain an EP element insertion in the promoter of the *CBP* gene and can be used for CBP overexpression by a GAL4 driver (36). Meanwhile, we found that the EP1149 line contains an SLIH mutation in the *per* gene (reference 20 and data not shown), and this line was excluded from further study. Flies hemizygous for a *CBP* null mutation have been shown to display embryonic lethality (1), making it difficult to study their circadian behavior. To knock down CBP expression in a cell type-specific manner, we made a transgenic fly in which dsRNA for CBP was expressed using a GAL4/UAS system. Accordingly, the EP1179 and UAS-CBP^{RNAi} lines were crossed with circadian clock-related GAL4 drivers, and their progeny was characterized.

Using an IFA, we first confirmed GAL4 driver-specific CBP overexpression and knockdown in these transgenic flies. As shown in Fig. 1A, CBP was overexpressed in *tim*-expressing cells of the EP1179 line by *tim*-GAL4. Endogenous CBP was barely identifiable in *tim*-expressing cells of control flies, making it difficult to directly assay the knockdown by CBP dsRNA in these clock cells. However, we found that a nuclear staining signal with the anti-CBP antibody was barely detectable in the

FIG. 1. Construction of transgenic flies in which CBP expression is either up- or downregulated via a GAL4/UAS system. (A) Clock cell-specific overexpression and knockdown of CBP by a *tim*-GAL4 driver. Larval brains were immunostained with a guinea pig anti-CBP antibody and a rhodamine-conjugated anti-guinea pig antibody (red), while *tim*-expressing cells were visualized by GFP expression (green). Confocal images of one hemisphere (upper panels) and the ring gland (lower panels) in each larval brain are shown. The genotypes of transgenic flies are given on the left. l-LN, larval lateral neuron; mGFP, myristylated GFP. (B) Eye-specific overexpression and knockdown of CBP by a *GMR*-GAL4 driver. Eye-specific expression of CBP dsRNA results in an abnormal phenotype, which is rescued by coexpression of CBP. CBP overexpression by a *GMR*-GAL4 driver also causes a smooth-eye phenotype. Images from light microscopy (LM) (upper panel) and scanning electron microscopy (SEM) (lower panel) are shown. The genotypes of transgenic flies are given at the top.

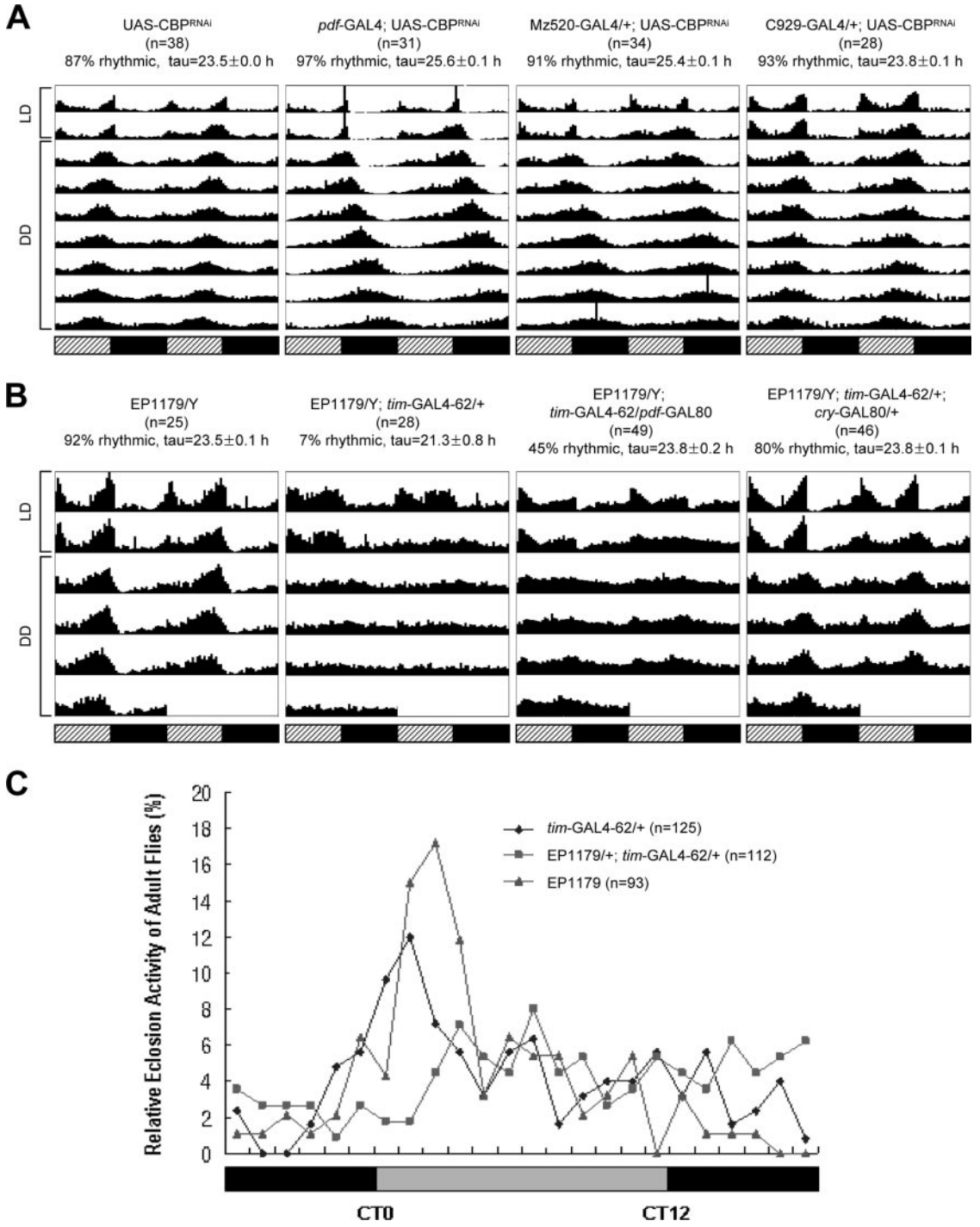

FIG. 2. Circadian behaviors of *CBP* mutant flies. (A) Actogram analysis of transgenic flies in which CBP expression is downregulated by LN_v-specific GAL4 drivers. The locomotor activities of flies with the same genotype were averaged and double plotted. The genotypes and numbers of transgenic flies used in the analysis are given at the top. The rhythmicity and the period (with the standard error of the mean) of adult locomotor activity are also shown. (B) Actogram analysis of transgenic flies in which CBP is overexpressed in *tim*-expressing cells. Where indicated, CBP overexpression by a *tim*-GAL4 driver is suppressed by either a *pdf*-GAL80 or a *cry*-GAL80 transgene. (C) Eclosion activity profile analysis of transgenic flies in which CBP is overexpressed in *tim*-expressing cells. After entrainment to LD cycles, the eclosion activities of individual pupae were monitored during subsequent DD cycles. The relative eclosion activity at each circadian time was calculated by normalizing to the total eclosion activity.

ring gland by expression of CBP dsRNA, suggesting that endogenous CBP expression was reduced in the transgenic fly. In addition, using the abnormal eye phenotype induced by CBP overexpression and knockdown (27, 34), we genetically validated that the CBP dsRNA used in our study specifically reduces CBP expression. An eye-specific expression of CBP dsRNA but not CG17100 dsRNA by *GMR*-GAL4 rescued the smooth-eye phenotype induced by CBP overexpression, while

TABLE 2. Locomotor activity analysis of transgenic flies in which CBP overexpression is driven by specific GAL4 drivers[a]

Genotype	No. of flies tested	No. analyzed (no. dead)[b]	No. (%) of rhythmic flies during DD cycles	Period ± SEM (h)
pdf-GAL4/+	39	38 (1)	34 (89)	23.7 ± 0.1
tim-GAL4-62/+	37	37 (0)	27 (73)	24.1 ± 0.1
tim-GAL4-86/+	35	35 (0)	26 (74)	24.4 ± 0.2
Mz520-GAL4/+	36	33 (3)	33 (100)	23.8 ± 0.1
Mai179-GAL4/+	39	37 (2)	35 (95)	23.9 ± 0.1
C929-GAL4/+	38	38 (0)	37 (97)	23.9 ± 0.1
EP1179/Y	63	63 (0)	57 (90)	23.4 ± 0.0
EP1179/Y; *pdf*-GAL4/+	57	57 (0)	46 (81)	24.1 ± 0.1
EP1179/Y; *tim*-GAL4-62/+	55	54 (1)	3 (6)	21.7 ± 0.6
EP1179/Y; *tim*-GAL4-86/+	45	41 (4)	2 (5)	21.3 ± 0.2
EP1179/Y; Mz520-GAL4/+	34	33 (1)	30 (91)	23.5 ± 0.2
EP1179/Y; Mai179-GAL4/+	33	33 (0)	18 (55)	23.8 ± 0.3
EP1179/Y; C929-GAL4/+	39	32 (7)	13 (41)	24.1 ± 0.4
tim-GAL4-62/+; UAS-GFP/+	32	32 (0)	30 (94)	23.8 ± 0.1
tim-GAL4-62/*pdf*-GAL80	36	35 (1)	35 (100)	24.4 ± 0.1
tim-GAL4-62/+; *cry*-GAL80/+	40	40 (0)	35 (88)	24.2 ± 0.1
tim-GAL4-62/UAS-PDF	16	16 (0)	14 (88)	24.7 ± 0.2
EP1179/Y; *pdf*-GAL4	55	40 (15)	36 (90)	23.2 ± 0.1
EP1179/Y; *tim*-GAL4-62	56	38 (18)	1 (3)	23.5
EP1179/Y; *tim*-GAL4-62/+; UAS-GFP/+	40	39 (1)	3 (8)	26.2 ± 1.4
EP1179/Y; *tim*-GAL4-62/*pdf*-GAL80	50	49 (1)	22 (45)	23.8 ± 0.2
EP1179/Y; *tim*-GAL4-62/+; *cry*-GAL80/+	46	46 (0)	37 (80)	23.8 ± 0.1
EP1179/Y; *tim*-GAL4-62/UAS-PDF	16	16 (0)	0 (0)	ND[c]

[a] Female flies homozygous for EP1179 or w^{1118} were crossed with male flies homozygous or balanced for each GAL4 driver. Female flies double homozygous for the EP1179 and *tim*-GAL4-62 transgenes were also generated and crossed with male flies homozygous or balanced for the UAS-GFP, UAS-PDF, *pdf*-GAL80, or *cry*-GAL80 transgene. Their male progenies were used in locomotor activity analysis. After entrainment for 3 days in LD cycles, data were further collected for 4 days under DD conditions. Period and rhythmicity were calculated using ClockLab software.

[b] No. of surviving (dead) flies during locomotor activity analysis.

[c] ND, not determined.

CBP overexpression but not PDP1ε overexpression rescued the abnormal eye phenotype by CBP knockdown (Fig. 1B; also data not shown). Taken together, we verified two transgenic fly models in which cell type-specific CBP expression could be up- or downregulated by a GAL4 driver, and we subsequently analyzed their respective circadian behaviors.

Circadian behaviors of CBP mutant flies. For behavioral analysis, we crossed several GAL4 drivers with the UAS-CBP^{RNAi} and EP1179 lines, and their progeny was monitored for adult locomotor activity under both LD and DD cycles. Expression from a single copy of the UAS-CBP^{RNAi} transgene by circadian clock-related GAL4 drivers had no apparent effect on circadian behavior (Table 1). Of note, *tim*-GAL4-mediated expression of CBP dsRNA decreased longevity, making it impossible to estimate the rhythmicity and period of adult locomotor activity under free-running conditions. However, CBP knockdown from two copies of the UAS-CBP^{RNAi} transgene by LN_v-specific GAL4 drivers (e.g., *pdf*-GAL4, Mz520-GAL4, and Mai179-GAL4) lengthened the period of adult locomotor activity (Fig. 2A). Because *pdf*-GAL4-mediated expression of GFP dsRNA did not significantly affect the adult locomotor rhythm, it seems unlikely that the altered circadian behavior by CBP knockdown would be an artifact arising from the nonspecific overexpression of dsRNAs in the pacemaker cells. In contrast, CBP overexpression in *tim*-expressing cells dramatically reduced the rhythmicity of adult locomotor activity under DD cycles (Fig. 2B). Although transgenic flies in which CBP was overexpressed by Mai179-GAL4 and C929-GAL4 showed more arrhythmic behavior than did control flies (Table 2), the averaged actograms of these transgenic flies were comparable to those of control flies (data not shown). When *tim*-GAL4-mediated expression of CBP was suppressed in *pdf*-expressing cells by the *pdf*-GAL80 transgene (53), the locomotor rhythm was partially rescued. However, the suppression of *tim*-GAL4-mediated CBP overexpression in *cryptochrome* (*cry*)-expressing cells by the *cry*-GAL80 transgene dramatically recovered rhythmicity. A profile analysis of locomotor activity revealed that CBP overexpression by *tim*-GAL4 resulted in a dampened evening peak of locomotor activity during LD cycles, which was also rescued by the *cry*-GAL80 transgene (data not shown). CBP overexpression in *tim*-expressing cells also disrupted eclosion rhythm, an additional circadian behavior of *Drosophila*, suggesting that CBP overexpression may generally disturb the *Drosophila* circadian clock system (Fig. 2C).

Rhythmic expression of clock genes is altered in clock neurons of CBP mutant flies. To dissect the molecular mechanisms underlying the circadian behaviors of CBP mutant flies, we analyzed clock gene expression in these transgenic fly models. Since a transgene under the control of the GAL4/UAS system would be heterogeneously expressed by a circadian clock-related GAL4 driver, we reasoned that clock gene expression in GAL4-expressing cells could be differentially affected by the transgene. More importantly, it has been shown that the altered expression of clock genes only in LN_vs can significantly affect the period and rhythmicity of adult locomotor rhythm (18, 53). Because a quantitative analysis using head extracts would mask any subtle changes in clock gene expres-

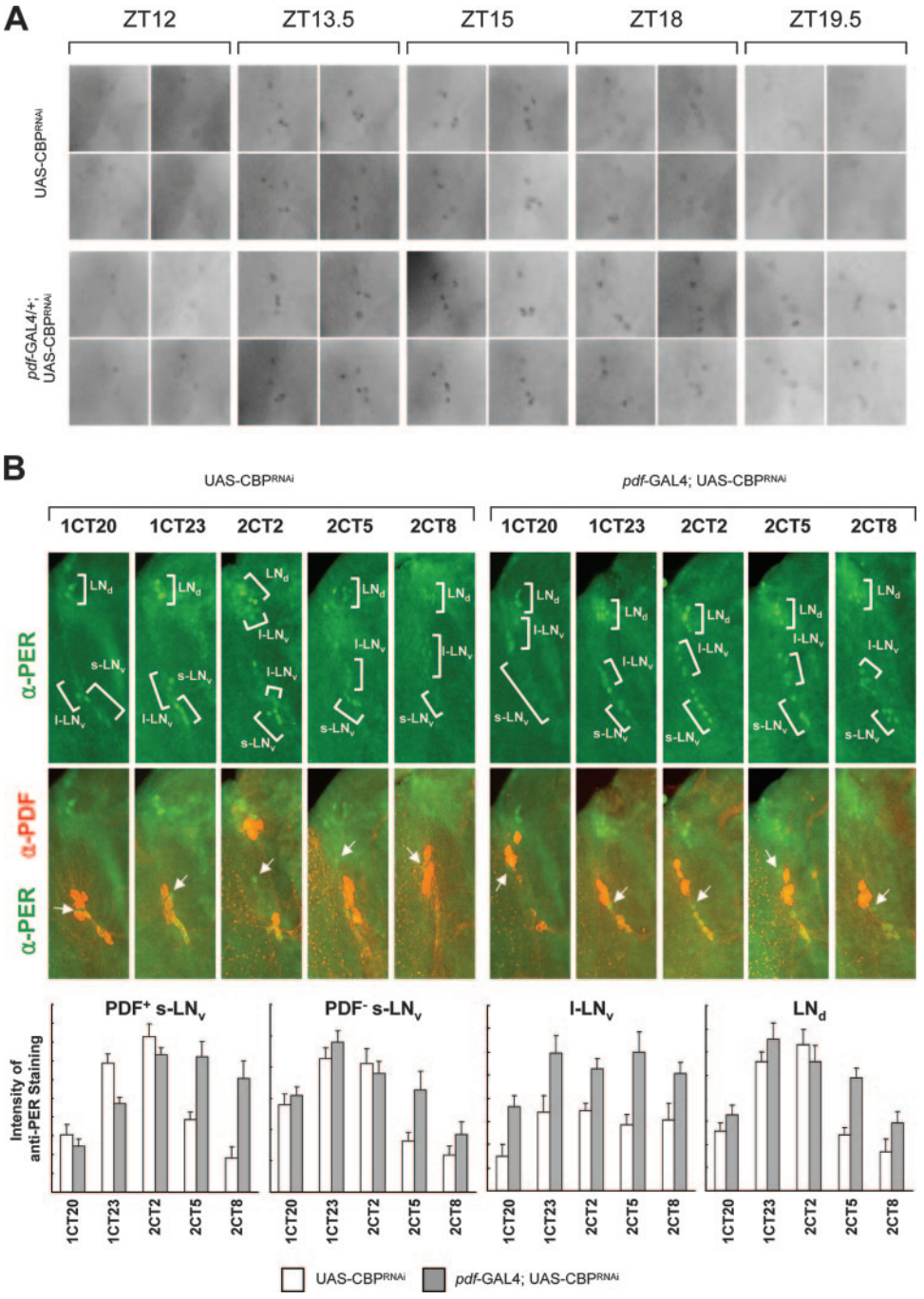

FIG. 3. Rhythmic expression of clock genes is prolonged by CBP knockdown. (A) Oscillating expression of a *tim* transcript is detectable in larval LNs expressing CBP dsRNA throughout later ZT times during LD cycles. Male flies homozygous for both the *pdf*-GAL4 and the UAS-CBP^{RNAi} transgene were sterile (data not shown). For the simplicity of the assay, female flies homozygous for both the *pdf*-GAL4 and the UAS-CBP^{RNAi} transgene were crossed with male flies homozygous for the UAS-CBP^{RNAi} transgene, and their progeny was subsequently analyzed. Of note, these transgenic flies also display a long period rhythm of locomotor activity (see Table 1). Third-instar larvae were dissected at the indicated times during the LD cycle. In situ hybridization was performed using a antisense probe for the *tim* gene. The genotypes of transgenic flies are given on the left. Four representative images of larval LNs at each ZT are shown. (B) Oscillating expression of PER protein is delayed and elevated by CBP knockdown. Adult flies were entrained to LD cycles, transferred to DD conditions, and fixed at the indicated times during the first and second DD cycles. Dissected brains were immunostained with a rabbit anti-PER antibody and an FITC-conjugated anti-rabbit antibody (green) and with a rat anti-PDF antibody and a rhodamine-conjugated anti-rat antibody (red). Representative confocal images of the anterior brains are shown (upper panels). The genotypes of transgenic flies are given at the top. PDF-negative small LN_vs are indicated by white arrows. The intensity of PER staining signals in each group of clock cells was quantified using ImageJ software and averaged from the values for eight brains at each circadian time (lower panel; graph). Error bars, standard errors of the means. l-LN_v, large LN_v; s-LN_v, small LN_v.

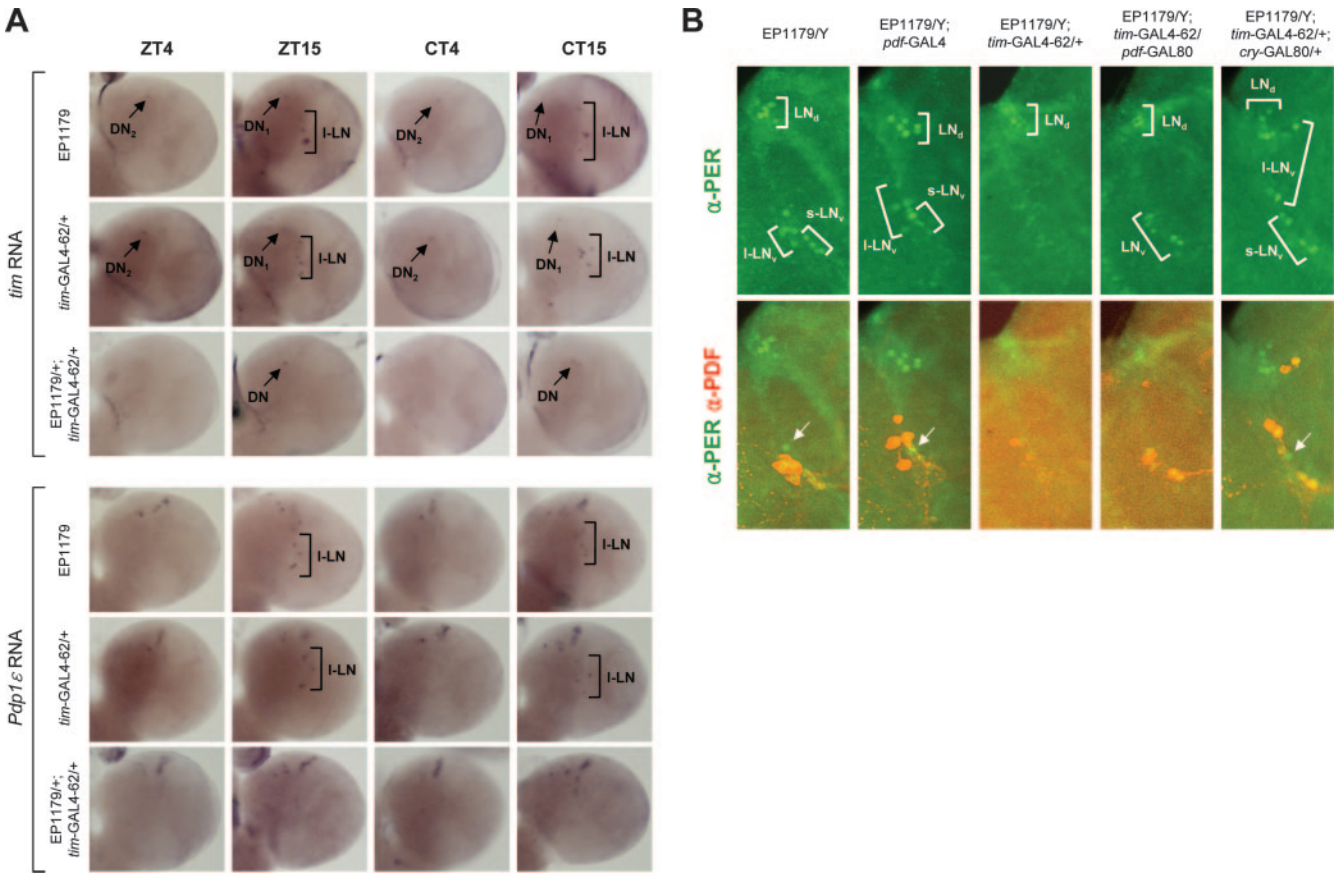

FIG. 4. Rhythmic expression of clock genes is abolished by CBP overexpression. (A) Oscillating expression of *tim* (upper panel) and *Pdp1*ε (lower panel) transcripts is not detectable in larval LNs of CBP-overexpressing flies. Third-instar larvae were dissected at the indicated times during the LD cycle or the first DD cycle. In situ hybridization was performed using antisense probes for the *tim* and *Pdp1*ε genes. Of note, some DNs of CBP-overexpressing flies also did not display oscillating expression of the *tim* transcript. It was difficult to accurately identify DN_1s and DN_2s among several DNs expressing the *Pdp1*ε transcript. Only one hemisphere of each larval brain is shown. l-LN, larval LN; DN_1 and DN_2, DNs that express the *tim* transcript in phase and out of phase, respectively, with l-LN. (B) The expression level of PER protein is reduced by CBP overexpression. Adult flies were fixed at ZT0 during LD cycles. Dissected brains were immunostained with a rabbit anti-PER antibody and an FITC-conjugated anti-rabbit antibody (green) and with a rat anti-PDF antibody and a rhodamine-conjugated anti-rat antibody (red). Representative confocal images of the anterior brains are shown. White arrows indicate PDF-negative small LN_vs. l-LN_v, large LN_v; s-LN_v, small LN_v.

sion in each group of pacemaker cells, we focused on clock gene expression in individual clock cells of CBP mutant flies.

Since the initial phase of clock gene expression would be daily synchronized by the light during LD cycles, we thought that the effect of CBP knockdown on circadian gene transcription could be directly evaluated under these conditions. Therefore, we first examined the rhythmic expression of the *tim* transcript in larval LNs during LD cycles. An in situ hybridization assay revealed that the *tim* transcript in larval LNs expressing CBP dsRNA persisted throughout later zeitgeber (ZT; lights on at ZT time zero and lights off at ZT12 during LD cycles) times, suggesting that the rhythmic expression of the *tim* transcript may be prolonged by CBP knockdown (Fig. 3A). Also, we examined and quantified the rhythmic PER expression in clock cells of adult brains at different circadian times during DD cycles. We concentrated on PER expression in LN_vs and LN_ds, because these clock cells function as the predominant pacemakers of the *Drosophila* circadian clock system (18, 53). As shown in Fig. 3B, CBP knockdown by *pdf*-GAL4 delayed and sustained the phase of cycling PER expression in PDF-positive small LN_vs, while it constitutively elevated PER expression in large LN_vs. Of note, the initial phase of rhythmic PER expression in PDF-negative small LN_vs and LN_ds of mutant flies was similar to that of control flies, but its phase was extended during the time that PER expression in PDF-positive small LN_vs of mutant flies displayed its peak level.

In contrast to CBP knockdown, the rhythmic expression of *tim* and *Pdp1*ε transcripts in larval LNs of CBP-overexpressing flies was barely detectable during both LD and DD cycles (Fig. 4A). We also performed an IFA to monitor the rhythmic expression of clock proteins in adult brains of CBP-overexpressing flies. The PER protein in adult brains was not observed at ZT8 (data not shown), but it was strongly expressed in a different subset of clock cells at ZT0 during LD cycles (Fig. 4B). CBP overexpression by two copies of *pdf*-GAL4 slightly reduced PER expression only in *pdf*-expressing cells, whereas CBP overexpression by a single copy of *tim*-GAL4 abolished PER expression in all LN_vs. In addition, the number and intensity of PER protein signals in LN_ds and DNs were decreased by *tim*-GAL4-mediated CBP overexpression (Fig. 4B;

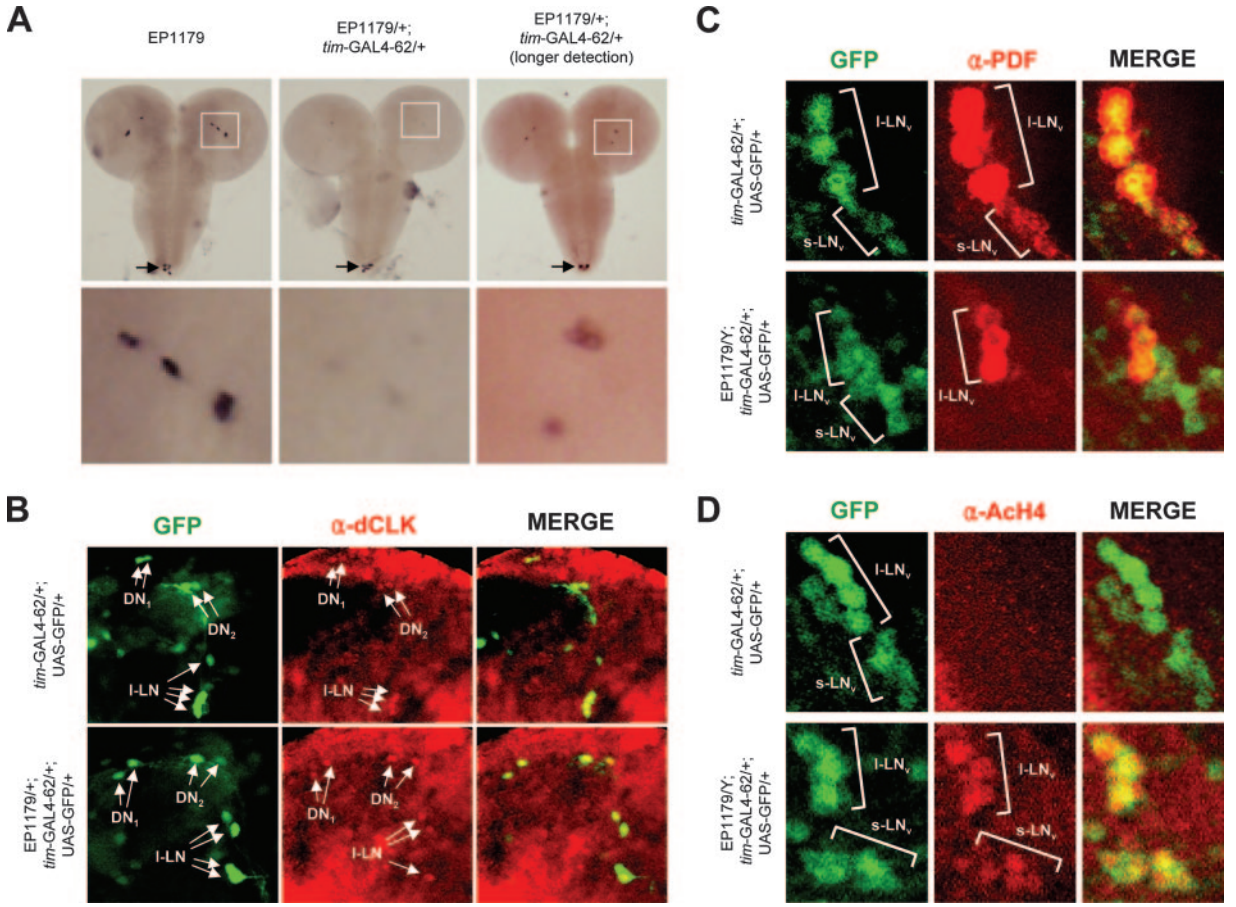

FIG. 5. Pacemaker cells are normally developed in larval and adult brains of CBP-overexpressing flies. (A) The expression level of the *pdf* transcript is reduced by CBP overexpression. In situ hybridization using an antisense probe for the *pdf* gene was performed for larval brains. While the *pdf* transcript was detected comparably in the ventral ganglion cells of control and CBP-overexpressing flies (upper panel, black arrows), larval LNs expressing the *pdf* transcript were barely identifiable in CBP-overexpressing flies under the same conditions (upper panel, white rectangles). However, weak expression of the *pdf* gene in larval LNs of CBP-overexpressing flies was observed during a longer detection time. The genotypes of transgenic flies are given at the top. Images outlined in the upper panel are shown at a higher magnification in the lower panel. (B) The expression level of dCLK protein is not altered by CBP overexpression. Dissected larval brains were immunostained with a rabbit anti-dCLK antibody and a rhodamine-conjugated anti-rabbit antibody (red), while *tim*-expressing cells were visualized by GFP expression (green). Under our experimental conditions, we observed dCLK expression in larval LNs, DN_1s, and DN_2s. Also, weak signals of anti-dCLK antibody staining were detected in some *tim*-negative cells. l-LN, larval lateral neuron. l-LN, DN_1, and DN_2 are as defined for Fig. 4. (C) Reduced expression of PDF protein is not due to an absence of LN_vs in CBP-overexpressing flies. Dissected adult brains were immunostained with a rabbit anti-PDF antibody and a rhodamine-conjugated anti-rabbit antibody (red), while *tim*-expressing cells were visualized by GFP expression (green). (D) The acetylation state of histone H4 protein is elevated by CBP overexpression. Dissected adult brains were immunostained with a rabbit anti-acetyl histone H4 antibody and a rhodamine-conjugated anti-rabbit antibody (red), while *tim*-expressing cells were visualized by GFP expression (green). l-LN_v, large LN_v; s-LN_v, small LN_v.

also data not shown). In the rescue experiment, the *pdf*-GAL80 transgene rescued PER expression only in *pdf*-expressing cells, while the *cry*-GAL80 transgene recovered it in all clock cells of CBP-overexpressing flies. Similar results were obtained during DD cycles, except that PER expression in large LN_vs was significantly weakened (data not shown). Meanwhile, we found that the expression level of PDF protein was also reduced in LN_vs of CBP-overexpressing flies, making their neural projections undetectable by immunostaining with an anti-PDF antibody.

The impaired expression of clock genes raised the possibility that these clock cells would be absent or abnormally developed in CBP-overexpressing flies. An in situ hybridization assay revealed that LNs were present in larval brains of CBP-overexpressing flies but that the *pdf* gene transcript was weakly expressed in the clock cells, as was observed for *Clk* and *cyc* mutant flies (6, 41) (Fig. 5A). In contrast, dCLK protein displayed comparable expression levels in larval brains of control and CBP-overexpressing flies (Fig. 5B). The presence of LN_vs in adult brains of CBP-overexpressing flies could be also visualized either by coexpression with GFP or by detecting the elevated histone acetylation state with an anti-acetyl histone H4 antibody (34) (Fig. 5C and D). Taken together, these data suggest that the abnormal circadian behaviors of *CBP* mutant flies may arise from alteration of dCLK/CYC-dependent clock gene expression in clock cells but not from absence of the pacemaker cells or reduced expression of dCLK protein.

The transcriptional activity of dCLK/CYC is inhibited by CBP overexpression. Our in vivo analysis of clock gene expression suggested that CBP may function as a negative regulator

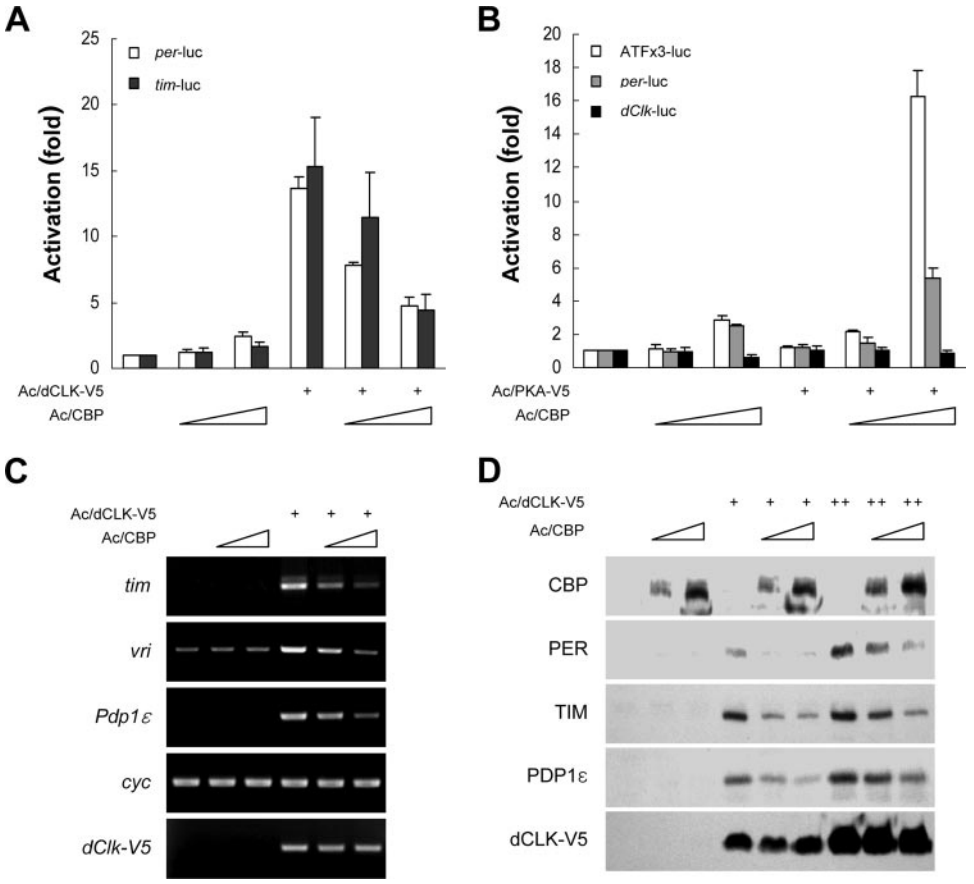

FIG. 6. The transcriptional activity of dCLK/CYC is inhibited by CBP overexpression. (A) CBP overexpression inhibits the transcriptional activation of the *per* and *tim* reporter plasmids by dCLK expression. S2 cells in 6-well plates were cotransfected with a reporter plasmid (1 μg), a V5-tagged dCLK expression vector (10 ng for *per*-luc and 1 ng for *tim*-luc), and increasing amounts of a CBP expression vector (0.1 μg and 1 μg). Extracts from transfected cells were prepared at approximately 40 h after transfection, and luciferase assays were performed. The cell extracts were also used in Western blotting to confirm that CBP overexpression does not affect the expression level of V5-tagged dCLK protein (data not shown). Levels of activation were calculated by normalizing values to the luciferase activity in the presence of the reporter plasmid, which was set to 1. Results are averages from three independent experiments. Error bars, standard deviations. (B) CBP and PKA synergically activate transcription from ATF/CREB-responsive reporter plasmids. S2 cells in 6-well plates were cotransfected with a reporter plasmid (1 μg), a V5-tagged PKA expression vector (100 ng), and increasing amounts of a CBP expression vector (0.1 μg and 1 μg). Luciferase assays, Western blotting, and calculation of increases in activation were performed as for panel A. (C) CBP overexpression inhibits the transcriptional activation of endogenous clock gene promoters by dCLK expression. S2 cells in 6-well plates were cotransfected with a V5-tagged dCLK expression vector (10 ng) and/or increasing amounts of a CBP expression vector (0.25 μg and 1 μg). Total RNA was prepared at approximately 40 h after transfection, and cDNAs were synthesized using M-MuLV reverse transcriptase. Semiquantitative PCR was performed using the clock gene-specific primers shown on the left. (D) CBP overexpression inhibits the dCLK-induced expression of endogenous clock proteins. S2 cells in 6-well plates were cotransfected with a V5-tagged dCLK expression vector (+, 10 ng; ++, 50 ng) and/or increasing amounts of a CBP expression vector (0.25 μg and 1 μg). Total extracts from transfected cells were prepared at approximately 40 h after transfection, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted with the clock gene-specific antibodies shown on the left.

of the dCLK/CYC heterodimer. To validate this hypothesis, we adopted an in vitro cell culture system comprised of the S2 cell line derived from *Drosophila* embryos. It was previously shown that S2 cells endogenously express the *cyc* gene, and therefore the ectopic expression of dCLK is sufficient for the activation of dCLK/CYC-dependent transcription from both reporter plasmids and endogenous promoters (12, 37). Because CBP knockdown in S2 cells retarded cell growth and significantly inhibited the transient expression of dCLK under our experimental conditions (data not shown), it was difficult to directly evaluate the effect of CBP knockdown on the transcriptional activity of the dCLK/CYC heterodimer. As shown in Fig. 6A, CBP overexpression suppressed dCLK-activated transcription from reporter plasmids containing *per* and *tim* gene promoters in a dose-dependent manner. Under similar conditions, CBP and protein kinase A (PKA) synergically activated transcription from a reporter plasmid containing tandem CREB/ATF-binding sites (Fig. 6B, ATFx3-luc). We could also observe moderate activation of *per*-luc by CBP and PKA. In view of the fact that CBP functions as a transcriptional coactivator of the classic CREB/PKA pathway (28), these data are consistent with the previous observations that *per*-luc reporter expression is reduced in *dCREB2* mutant flies and that there are three putative CREB-binding sites within 4 kb to 1.2 kb upstream of the transcription start site for the *per* gene (5). The dCLK-induced expression of endogenous clock genes in S2 cells was also repressed

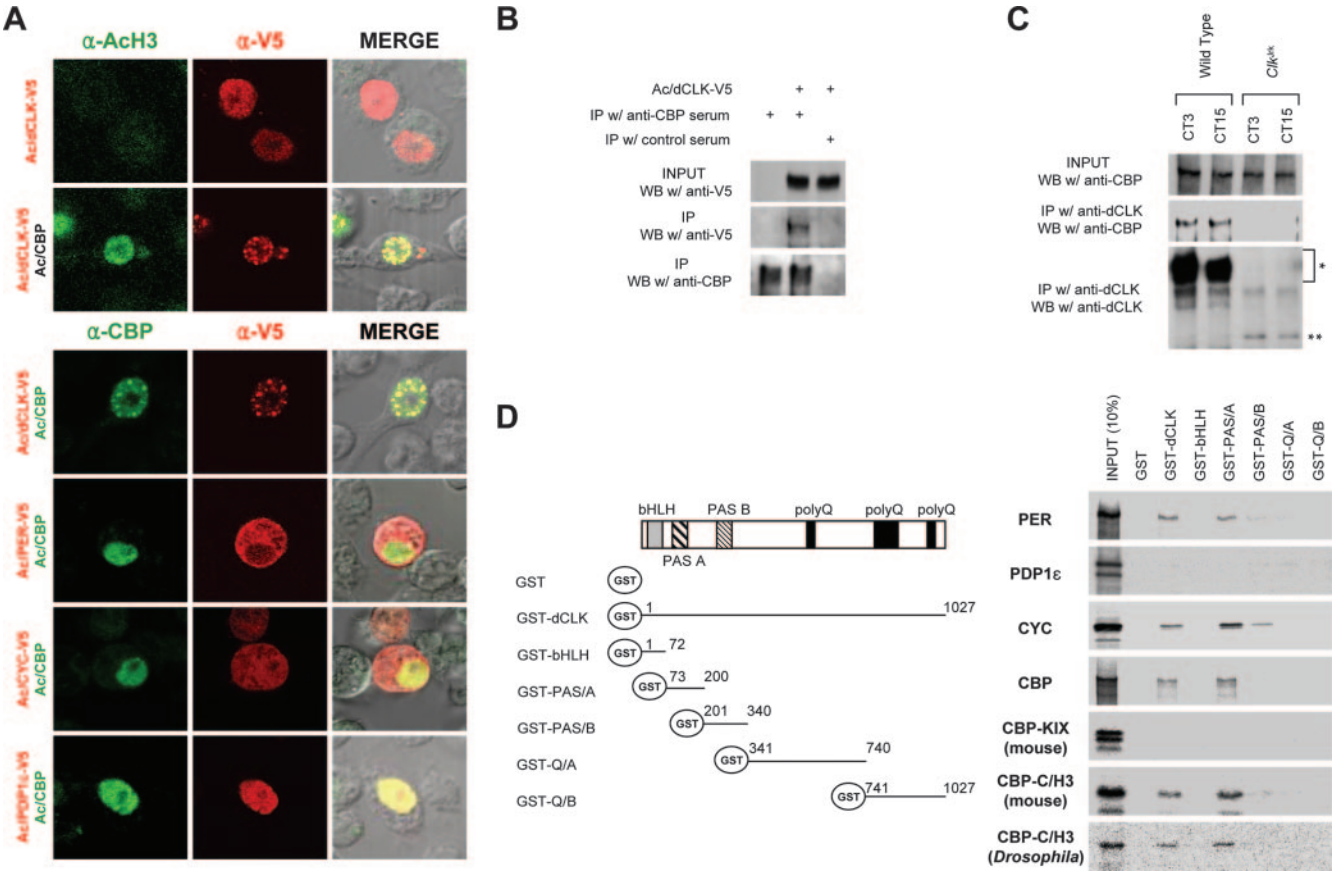

FIG. 7. CBP physically interacts with dCLK. (A) dCLK colocalizes with CBP in the punctate nuclear spots of transfected cells. S2 cells grown on coverslips were cotransfected with expression vectors for V5-tagged clock proteins and/or a CBP expression vector. An IFA was performed at approximately 40 h after transfection. (Upper panel) V5-tagged dCLK was immunostained with a mouse anti-V5 antibody (rhodamine; red), while acetylated histone H3 proteins were immunostained with a rabbit anti-acetyl H3 antibody (FITC; green). (Lower panel) V5-tagged proteins were immunostained with a mouse anti-V5 antibody (rhodamine; red), while CBP was immunostained with a rabbit anti-CBP antibody (FITC; green). Images were obtained using confocal laser scanning microscopy. Differential interference contrast images are overlaid with merged confocal images. (B) dCLK protein associates with endogenous CBP in transiently transfected S2 cells. S2 cells were transfected with a V5-tagged dCLK expression vector or a blank vector. At approximately 40 h after transfection, soluble extracts from transfected cells were immunoprecipitated with a guinea pig anti-CBP serum or a control serum. Coimmunoprecipitated proteins were immunoblotted with mouse anti-V5 and rabbit anti-CBP antibodies. INPUT, approximately 4% of soluble extracts used for immunoprecipitation. (C) CBP is coimmunoprecipitated with dCLK from fly head extracts. Adult flies were entrained to LD cycles, transferred to DD conditions, and harvested at the indicated times during the first DD cycle. Soluble extracts from adult fly heads were prepared and immunoprecipitated with a guinea pig anti-dCLK antibody. Bound proteins were immunoblotted with rabbit anti-CBP and anti-dCLK antibodies. The genotypes of adult flies are given at the top. Wild-type dCLK and mutant $dCLK^{Jrk}$ proteins are indicated by single and double asterisks, respectively. INPUT, approximately 5% of soluble extracts used for immunoprecipitation. (D) The PAS A domain of dCLK is targeted by CBP. ^{35}S-labeled in vitro-translated proteins were incubated with dCLK and with its deletion mutants fused to GST protein. Proteins pulled down by glutathione-Sepharose 4B were subjected to autoradiography. A schematic diagram of dCLK deletion mutants used in this assay is shown on the left. bHLH, basic helix-loop-helix; polyQ, glutamine repeat; KIX, CREB-binding domain; INPUT, 10% of in vitro-translated protein used in the binding assay.

by CBP overexpression at their transcript and protein levels (Fig. 6C and D), further confirming our in vivo observations. Taken together, these data suggest that CBP overexpression may specifically inhibit dCLK/CYC-dependent transcription.

CBP physically interacts with dCLK. Recently, it was reported that dCLK localized to nuclear spots in transiently expressed S2 cells (25). We also found the nuclear spots of dCLOCK in a subset of transfected cells highly expressing dCLK (data not shown), but they became more obvious in the presence of CBP (Fig. 7A, upper panel). For the present, it is difficult to evaluate their physiological significance. Since CYC overexpression weakened the punctate localization of dCLK in the presence of CBP (Fig. 8B), one possibility would be that the nuclear spots serve as a subnuclear reservoir for CYC-free dCLK. Interestingly, CBP colocalized with dCLK protein but not with other clock proteins in the nuclear spots of transfected cells (Fig. 7A, lower panel), suggesting that dCLK may associate with CBP in transiently expressed cells. As expected, dCLK protein was coimmunoprecipitated with an anti-CBP antibody, but not with a control antibody, from transfected cell extracts (Fig. 7B). At two different circadian times, we could also observe a physical interaction between CBP and dCLK in adult head extracts of wild-type but not Clk^{Jrk} flies (Fig. 7C), indicating that the reduced expression level and/or aberrant conformation of the dCLK mutant protein would diminish its association with CBP. To map the interaction domain between

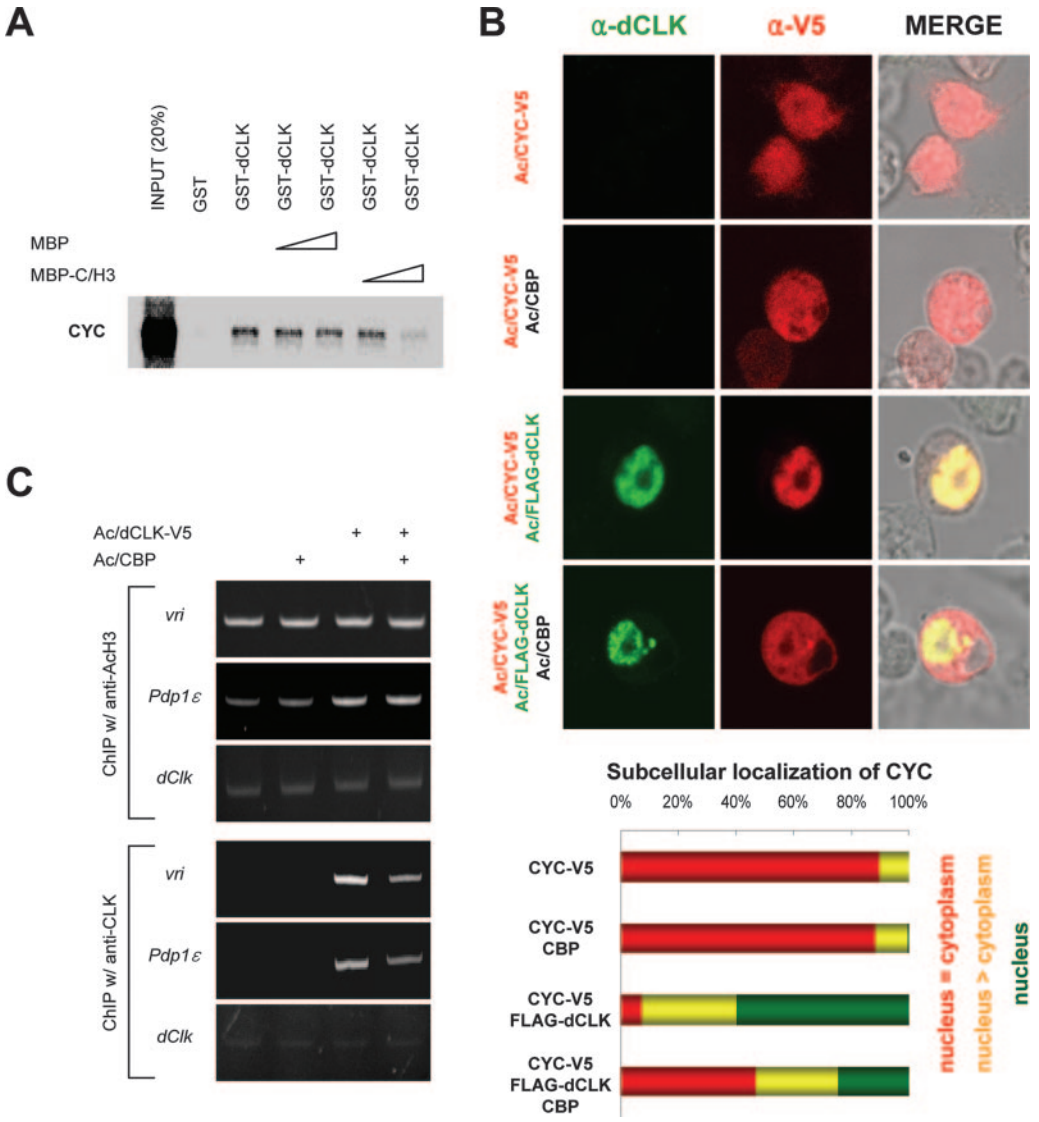

FIG. 8. CBP disrupts the association of dCLK with CYC. (A) The C/H3 region of CBP is sufficient for the competition with CYC for binding to dCLK protein. GST or GST-dCLK was preincubated with increasing amounts of MBP or the C/H3 region of CBP fused to MBP (2 μg and 10 μg) and was then further incubated with ^{35}S-labeled in vitro-translated CYC. After pulldown with glutathione-Sepharose 4B, bound proteins were subjected to autoradiography. INPUT, 20% of in vitro-translated CYC protein included in the binding assay. (B) CBP overexpression inhibits the nuclear translocation of CYC by dCLK in transiently expressed S2 cells. S2 cells grown on coverslips were cotransfected with a V5-tagged CYC expression vector (50 ng), a FLAG-tagged dCLK expression vector (50 ng), and/or a CBP expression vector (1 μg). IFA was performed at approximately 40 h after transfection. V5-tagged CYC was immunostained with a mouse anti-V5 antibody (rhodamine; red), while FLAG-tagged dCLK was immunostained with a rabbit anti-dCLK antibody (FITC; green). Transfected cells were examined using confocal laser scanning microscopy. Representative results are shown in the upper panel, and differential interference contrast images are overlaid with merged confocal images. In addition, the subcellular localization of V5-tagged CYC protein in the absence or presence of dCLK and CBP was scored for each cell (n = 460 to 520) and quantitatively analyzed. The percentages of cells in which CYC protein is evenly expressed in both the nucleus and cytoplasm, is expressed more strongly in the nucleus than in the cytoplasm, and is expressed exclusively in the nucleus are indicated by red, yellow, and green bars, respectively, in the lower panel. (C) CBP overexpression inhibits the association of dCLK with endogenous clock gene promoters. S2 cells in 6-well plates were cotransfected with a V5-tagged dCLK expression vector (10 ng) and/or a CBP expression vector (1 μg). Cross-linked chromatin was prepared at approximately 40 h after transfection, immunoprecipitated with an anti-acetyl histone H3 (upper panel) or an anti-dCLK (lower panel) antibody, and amplified by PCR with each clock gene-specific primer shown on the left. PCR products were resolved by 6% acrylamide gel electrophoresis and visualized by ethidium bromide staining.

CBP and dCLK, an in vitro binding assay was performed using wild-type dCLK protein and deletion mutants. As shown in Fig. 7D, the PER-ARNT-SIM (PAS) A domain of dCLK commonly interacted with in vitro-translated PER, CYC, and CBP proteins. In a reciprocal in vitro binding assay, we found that dCLK protein associates with the evolutionarily conserved C/H3 region of CBP (data not shown), which was sufficient for binding to the PAS A domain of dCLK.

CBP disrupts the association of dCLK with CYC. Since both CBP and CYC target the PAS A domain of dCLK, we rea-

soned that they could compete with each other for association with dCLK. As shown in Fig. 8A, a molar excess of the C/H3 region of CBP fused to MBP, but not MBP itself, inhibited the heterodimerization of dCLK and CYC proteins in an in vitro binding assay. We also observed that the exclusive nuclear localization of CYC in the presence of dCLK (25) was suppressed by CBP overexpression (Fig. 8B), suggesting that CBP may disrupt the association of dCLK with CYC in transiently expressed S2 cells. Finally, a ChIP assay revealed that the association of dCLK with the *vri* and *Pdp1*ε gene promoters was reduced by CBP overexpression (Fig. 8C, lower panel). Interestingly, the acetylation state of histone proteins in the endogenous promoter of the *Pdp1*ε gene, but not the *vri* gene, was increased by dCLK expression (Fig. 8C, upper panel). Under our experimental conditions, the *vri* transcript was readily detectable even in the absence of dCLK, and its expression was moderately elevated by dCLK, while expression of the *Pdp1*ε transcript, in contrast, was dramatically induced by dCLK (Fig. 6C). Therefore, it is possible that the striking activation of the *Pdp1*ε gene promoter by dCLK would be accompanied by an increased acetylation state of histone proteins. Taken together, these data suggest that CBP may inhibit dCLK/CYC-induced transcription by disrupting the formation of the functional heterodimer capable of binding to target promoters and activating their transcription.

DISCUSSION

To elucidate the functional role of *Drosophila* CBP in the circadian clock system, we generated transgenic fly models in which CBP expression can be up- or downregulated in clock cells using a GAL4/UAS system, and we characterized their circadian behaviors and molecular clocks. Our behavioral analyses revealed that CBP knockdown in *pdf*-expressing cells resulted in a long period rhythm of adult locomotor activity, with a concomitant prolongation and delay of the phase of rhythmic clock gene expression. Although the possibility that CBP knockdown may increase the stability of the *tim* transcript and/or PER protein cannot be excluded, our in vivo data support the hypothesis that dCLK/CYC-dependent clock gene expression may be derepressed by CBP knockdown. We propose that the extended phase of clock gene expression due to CBP knockdown during the subjective night of the LD cycle may be reset daily by the light but that it may subsequently delay the following phase of clock gene expression and adult locomotor rhythm during DD cycles. In addition, we found that the phase of rhythmic PER expression in PDF-negative small LN_vs and LN_ds was similarly delayed at a specific circadian time by CBP knockdown in *pdf*-expressing cells, supporting the previous observations that (i) molecular clocks in PDF-negative small LN_vs and LN_ds are in phase and (ii) PDF-positive LN_vs control the phase of the circadian clock in LN_ds (46, 54).

CBP overexpression in *tim*-expressing cells causes arrhythmic circadian behaviors, but CBP overexpression in LN_vs had no apparent effect. Since *tim*-GAL4 is believed to express more GAL4 proteins in LN_vs than *pdf*-GAL4 (54), these results suggest two possibilities: (i) that CBP is not sufficiently overexpressed by LN_v-specific GAL4 drivers to affect the locomotor rhythm and/or (ii) that clock cells other than LN_vs may be responsible for the arrhythmic circadian behavior. In the rescue experiment, the suppression of *tim*-GAL4-mediated CBP expression by *pdf*-GAL80 partially rescued the arrhythmic behavior of mutant flies, while suppression by *cry*-GAL80 recovered a normal locomotor rhythm, indicating that both *pdf*- and *cry*-expressing clock cells contribute to the arrhythmic behavior due to CBP overexpression. In addition, the average profile of adult locomotor activity showed that CBP overexpression by *tim*-GAL4 dampens the evening peak of locomotor activity during LD cycles, which was rescued only by *cry*-GAL80. Accumulating data suggest that the morning and evening peaks of adult locomotor activity are governed by *pdf*-expressing LN_vs (PDF^+ cells) and by clock cells that express the *cry* gene but not the *pdf* gene (CRY^+/PDF^- cells), respectively (18, 53, 54). With regard to this model, our profile analyses support the hypothesis that the oscillator function of CRY^+/PDF^- cells is disrupted by CBP overexpression.

Molecular analysis of clock gene expression in CBP-overexpressing flies revealed that CBP overexpression abolishes dCLK/CYC-dependent clock gene expression. Although CBP overexpression by 2 copies of *pdf*-GAL4 reduced PER expression in *pdf*-expressing cells, CBP overexpression by a single copy of *tim*-GAL4 completely abolished it, suggesting that CBP may not be sufficiently overexpressed by *pdf*-GAL4 to affect the molecular clock as well as circadian behavior. However, we cannot exclude the possibility that aberrant clock gene expression in CRY^+/PDF^- cells due to CBP overexpression influences PER expression in PDF^+ cells. We also showed that PER expression in PDF^- small LN_vs and LN_ds was recovered only by *cry*-GAL80, in agreement with a recent report that these clock cells may be responsible for the evening peak of locomotor rhythm (46). Of note, LN_vs were more sensitive to the effect of CBP overexpression than LN_ds and DNs. We reason that the expression level of CBP by *tim*-GAL4 and/or the nature of the circadian clock system differs among clock cells, resulting in the differential effects of CBP overexpression. It is also possible that CBP may have different roles in the central and peripheral clock systems, as recently exemplified by the *cry* gene (9).

Flies homozygous for the *pdf* null mutation gradually lose the rhythmicity of adult locomotor activity under free-running conditions (44), in contrast to the locomotor activity of CBP-overexpressing flies, which becomes arrhythmic immediately after transfer to DD cycles. In addition, the rhythmicity of adult locomotor activity was not recovered when the *pdf* gene was ectopically coexpressed with CBP by *tim*-GAL4 (Table 2), suggesting that the reduced expression of the *pdf* gene due to CBP overexpression may not be primarily responsible for the arrhythmic behavior of the mutant fly, which may instead result from the impaired activity of the dCLK/CYC heterodimer (6, 41). In contrast to CBP overexpression, *pdf* gene expression was negligibly affected by CBP knockdown. Considering that the *pdf* gene is strongly expressed in LN_vs (41), it is possible that *pdf* gene expression may already be saturated in wild-type flies and/or that the sensitivity of our assay systems may be insufficient for detecting the subtle difference. Taken together, our data indicate that CBP overexpression abolishes the oscillating expression of clock genes by the dCLK/CYC heterodimer, thereby eliminating the locomotor rhythm of adult flies immediately after transfer to DD cycles. This notion is

further supported by the previous observations that behavioral and molecular clocks in *Clk* mutant flies similarly display the arrhythmic phenotype (2, 3).

By in vitro experiments, we showed that CBP directly targets the PAS domain of dCLK, thereby inhibiting the dimerization, DNA binding, and transcriptional activity of the dCLK/CYC heterodimer. These unexpected data contrast with those for the mammalian circadian clock system, in which CBP/p300 associates with the CLOCK/BMAL1 heterodimer and augments its transcriptional activity (10, 15, 56). Although the protein interaction domains of CLOCK/BMAL1 and CBP/p300 were not extensively determined in the mammalian circadian clock system, this difference may originate from the evolutionary divergence of *Clock* orthologs in the circadian clock system. The PAS A domains of mammalian and *Drosophila Clock* genes (41% identity; 61% similarity) are less conserved than the PAS B domains (64% identity; 80% similarity), while the C-terminal glutamine-rich domains display little homology. Transient reporter assays in previous studies showed that the dCLK/CYC heterodimer activates transcription from E-box-containing reporters more robustly than the mammalian CLOCK/BMAL1 heterodimer (10, 12, 15, 56). When dCLK is fused to a GAL4 DNA-binding domain and subsequently tethered to a minimal promoter downstream of tandem GAL4-binding sites, it also strongly activates transcription from the artificial promoter (our unpublished observation); in contrast, mammalian CLOCK displays no transactivation activity under similar conditions (56). Furthermore, the ectopic expression of dCLK can induce the rhythmic expression of clock genes in misexpressed cells of adult fly brains or can cause developmental lethality (61), suggesting that the transcriptional activity of the dCLK/CYC heterodimer would be tightly controlled in a wild-type fly. A prior work showed that dCLK is bound to CYC throughout a daily cycle (4). However, dCLK may not be efficiently extracted from head homogenates under the experimental conditions of that study, since it was recently revealed that dCLK is constitutively expressed with the rhythmic phosphorylation state in fly head extracts (25, 60). We also observed that the extractability of dCLK protein from head homogenates is largely dependent on the buffer composition (our unpublished observation). Therefore, the expression level of dCLK would have been underestimated in previous reports. We propose that CBP constitutively restricts dCLK so that only a subpopulation of dCLK would form a functional complex with CYC and participate in transcriptional activity. This strategy would block the catastrophic induction of dCLK/CYC target genes before PER protein is sufficiently expressed to inhibit the transcriptional activity of the dCLK/CYC heterodimer, and it would enable dCLK/CYC target genes to maintain their highly oscillating expression throughout a daily cycle.

Taken together, our results provide genetic and molecular evidence that CBP may function as a negative regulator of the dCLK/CYC heterodimer. Since CBP participates in diverse biological processes, we cannot exclude the possibility that it also plays a functional role in other transcriptional feedback loops of core clock genes or in the input and output pathways of the *Drosophila* circadian clock system.

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