

Rhythmic E-Box Binding by CLK-CYC Controls Daily Cycles in *per* and *tim* Transcription and Chromatin Modifications^{∇†}

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Received 31 August 2007/Returned for modification 29 October 2007/Accepted 5 May 2008

The *Drosophila melanogaster* circadian oscillator comprises interlocked *per/tim* and *Clk* transcriptional feedback loops. In the *per/tim* loop, CLK-CYC-dependent transcriptional activation is rhythmically repressed by PER or PER-TIM to control circadian gene expression that peaks around dusk. Here we show that rhythmic transcription of *per* and *tim* involves time-of-day-specific binding of CLK-CYC and associated cycles in chromatin modifications. Activation of *per* and *tim* transcription occurs in concert with CLK-CYC binding to upstream and/or intronic E-boxes, acetylation of histone H3-K9, and trimethylation of histone H3-K4. These events are associated with RNA polymerase II (Pol II) binding to the *tim* promoter and transcriptional elongation by Pol II that is constitutively bound to the *per* promoter. Repression of *per* and *tim* transcription is associated with PER-dependent reversal of these events. Rhythms in H3-K9 acetylation and H3-K4 trimethylation are also associated with CLOCK-BMAL1-dependent transcription in mammals, indicating that the mechanism that controls rhythmic transcription is a conserved feature of the circadian clock even though feedback repression is mediated by different proteins.

In *Drosophila melanogaster*, circadian oscillator function is initiated by the basic helix-loop-helix PERIOD (PER)-ARNT-SIM transcription factors CLOCK (CLK) and CYCLE (CYC). These two proteins form a heterodimer that binds CACGTG E-box elements to activate transcription of target genes including *per*, *tim*, *vri*, and *Pdp1ε* during midday (zeitgeber time ~6 h [ZT 6]) (2, 7, 9, 19, 34, 48). At this time, CLK-CYC is expected to promote histone modifications associated with transcriptional activation such as acetylation and H3-K4 trimethylation. As *per* mRNA accumulates to peak levels around dusk, PER accumulates in the cytoplasm, where it associates with TIM (11, 16, 21, 23, 35, 37, 43, 50, 51) and then enters the nucleus and inhibits CLK-CYC activity (5, 9, 35, 44). This inhibition is expected to involve histone modifications such as deacetylation and H3-K9 dimethylation, which are typically involved in transcriptional repression. PER is degraded early in the light phase to relieve transcriptional inhibition, thereby allowing the next cycle of CLK-CYC transcription to begin (11, 17, 26, 50, 51).

The mouse homologs of *Drosophila* CLK-CYC, CLOCK-BMAL1, have been shown to bind rhythmically to E-box elements in *Dbp* upstream and intronic sequences (41) but are constantly bound to E-boxes upstream of *Per1* (28). Apparently, time-specific interactions between CRYPTOCHROME (CRY) or PER-CRY (PER-CRY) and CLOCK-BMAL1 inhibit transcription either by removing CLOCK-BMAL1 from E-boxes or by blocking CLOCK-BMAL1 transactivation. In

Drosophila, CLK-CYC binds rhythmically to E-boxes in the *per* circadian regulatory sequence (CRS) and the *tim* upstream sequence (49), indicating that PER (or PER-TIM) inhibits transcription by removing CLK-CYC from E-boxes. Additional E-boxes in the *per* upstream, *per* intron 1, and *tim* intron 1 regions may also contribute to the proper phase and/or amplitude of *per* and *tim* mRNA cycling (19, 34, 48), though CLK-CYC binding to these E-boxes has not been determined. In mice, CLOCK-BMAL1 binding is associated with histone modifications that promote transcriptional activity including acetylation of histones H3 and H4 (6, 10, 12, 38, 41) and trimethylation of H3-K4 (41). Transcriptional repression by CRY is associated with methylation of H3-K27 (13), histone deacetylation (38), and dimethylation of H3-K9 (41). Given that PER and CRY are the key feedback repressors in flies and mammals, respectively, it is possible that some of the rhythmic transitions in histone modifications may be different between flies and mammals.

Histone modifications regulate transcription by controlling RNA polymerase II (Pol II) binding and activity. In mice, CLOCK-BMAL1 activity and associated rhythms in chromatin modifications occur in concert with Pol II binding to the *Per1*, *Per2*, and *Cry1* promoters (6, 12). The C-terminal domain (CTD) of Pol II consists of multiple heptad repeats that mediate phosphorylation-dependent changes in Pol II activity. The Pol II CTD is phosphorylated at serine 5 (S5) by TFIIF as transcription is initiated at the 5' end of the transcribed region and is phosphorylated at S2 by Ctk kinase as Pol II moves toward the 3' end of the transcription unit (3). The phosphorylated Pol II CTD recruits factors important for productive Pol II elongation, RNA processing, and mRNA export (18). Constitutive binding of Pol II to rhythmically expressed genes might have suggested that Pol II activity is regulated, but this was not seen for *Per1*, *Per2*, and *Cry1* in mice.

Circadian transcriptional regulation corresponds well with

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† Supplemental material for this article may be found at <http://mcb.asm.org/>.

∇ Published ahead of print on 12 May 2008.

CLK-CYC binding to the *per* CRS and *tim* upstream E-boxes (49). Here we show that CLK-CYC rhythmically binds E-boxes in *per* intron 1 but doesn't bind other E-boxes in the *per* upstream region or in *tim* intron 1. These rhythms in CLK-CYC binding are associated with H3-K9 acetylation and H3-K4 trimethylation, thus demonstrating that these histone modifications are a conserved feature of the clock mechanism. PER-dependent transcriptional inhibition coincides with the reversal of these histone modifications. Rhythms in H3-K9 acetylation and H3-K4 trimethylation coincide with Pol II binding to the *tim* promoter. However, constitutive binding of Pol II to the *per* promoter suggests that rhythms in *per* transcription are controlled at the level of Pol II elongation.

MATERIALS AND METHODS

Fly stocks. The Canton Special strain was used as the wild type for all experiments. The *per⁰¹* and *cyc⁰¹* fly strains were used to assess chromatin modifications when CLK-CYC is constitutively bound or not bound to E-boxes, respectively (49).

Antibodies. Antibodies against histone modifications were acetyl-histone H3-K9 (Abcam; ab4441), acetyl-histone H3-K14 (Millipore; 07-353), acetyl-histone H4 (Millipore; 06-866), dimethyl-histone H3-K9 (Millipore; 07-441), trimethyl-histone H3-K4 (Abcam; ab8580), and phosphoryl-histone H3-S10 (Millipore; 05-817). Either rabbit immunoglobulin G (IgG; Sigma; I-5006) or rabbit serum (Sigma; R-9133) was used as a negative control for chromatin immunoprecipitation (ChIP) experiments for these antibodies depending of whether the selective antibody was purified IgG or was in serum. For purified antibodies, 3 μ g antibody or control IgG was used for ChIP experiments. For antibodies in serum, 5 μ l antibody or control serum was used for ChIP experiments.

Pol II, clone H14 (Covance; MMS-134R), which recognizes the heptapeptide repeat phosphorylated at serine 5 in the CTD, and Pol II, clone 8WG16 (Upstate, 05-952), which recognizes the unphosphorylated heptapeptide repeat in the CTD, were used for ChIP analysis. The H14 antibody was also used for Western blot analysis along with actin (Abcam; ab8224) as a loading control. For clone H14, rabbit anti-mouse IgM (Zymed; 61-6800) was used as a linker. Mouse IgG (Sigma; I-5318) was used as a negative control for 8WG16, and no IgG was used as a negative control for H14 for these ChIP experiments.

ChIP protocol. ChIP experiments were done as described by Yu et al. (49) with some modifications. For ChIP with Pol II H14, a rabbit anti-mouse IgM linker was needed. After the cross-linking was reversed, the DNA was purified using a QIAquick PCR purification kit (Qiagen; 28104). For the positions and sequences of primers used for semiquantitative PCR and quantitative real-time PCR (qPCR) of ChIP samples see Table S1 in the supplemental material.

Semiquantitative PCR. Semiquantitative PCR was carried out as described by Yu et al. (49) except that the PCR was performed for 18 cycles using a concentration of 0.5 μ M of hot primers throughout the reaction.

qPCR. ChIP samples and controls were diluted 1:10 and inputs were diluted 1:100 in Qiagen elution buffer. The qPCR was prepared as described previously (1), with the following modifications. For a 1 \times reaction the following were combined: 5 μ l Sybr green PCR Master Mix (Applied Biosystems; 4309155), 0.66 μ l forward primer (10 μ M), 0.66 μ l reverse primer (10 μ M), 1.68 μ l water, 2 μ l of DNA. The ABI 7500 real-time PCR system was run in fast 7500 mode and set up as follows: stage 1, 1 cycle for 10 min at 95°C; stage 2, 40 cycles of 30 s at 95°C, 30 s at 53 to 60°C (depending upon the primer set), and 30 s at 60°C. The data collection step was set to stage 2, step 3, at 30 s. For each plate, the average of the cycle threshold values (C_{75}) was calculated for each input, sample, and control. The input C_{75} was subtracted from the corresponding sample and control C_{75} s. The following formula was then applied: power \cdot 1.9 \cdot negative ln (subtracted value). This value was used for further calculations. Each sample and control was normalized by dividing both numbers by the highest value so that each ChIP experiment was scaled from 0 to 1. For sample minus control values, a negative number was replaced with a zero.

Western blot analysis. Whole-head protein samples were prepared from wild-type flies collected at ZT 2, 6, 10, 14, 18, and 22 by extraction in RBS buffer (49). Sonicated cross-linked nuclear extract (SXN) samples used in ChIP experiments were also used for Western analysis. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 100 μ g of whole-head sample or 10 μ l of SXN sample was run on Criterion precast gels (Bio-Rad) and transferred to Hybond-P membranes (Amersham). To determine Pol II S5 levels, immunoblots were probed

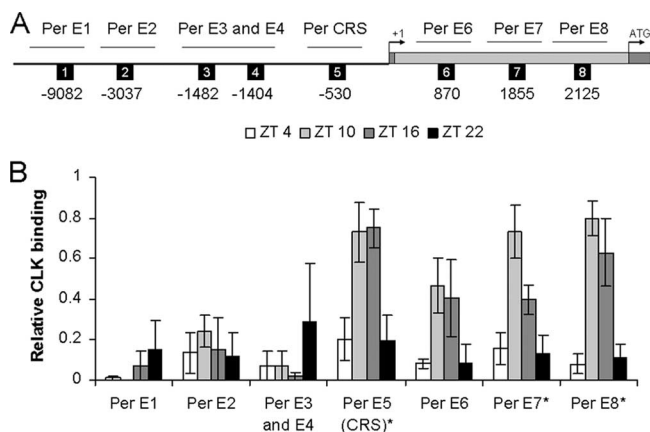


FIG. 1. CLK rhythmic binding to *per* E-boxes. (A) Schematic representation of *per* indicating regions analyzed for CLK binding E-boxes. Upstream sequence, thick horizontal line; transcription start, arrow with +1; exons, dark gray bars; intron 1, light gray bar; translation start, arrow with ATG; E-boxes, black squares numbered 1 through 8 and marked by the position of the first nucleotide relative to the transcription start site; regions analyzed by ChIP, thin horizontal lines marked by the E-boxes they contain. (B) ChIP was performed in wild-type flies collected at ZT 4, ZT 10, ZT 16, and ZT 22 during a 12:12 LD cycle. The relative level of CLK binding for each E-box-containing region was determined by qPCR analysis of samples immunoprecipitated with anti-CLK or guinea pig serum (GPS). The relative levels of CLK binding have been corrected for nonspecific binding by subtracting GPS values from CLK values. These data were derived from three independent experiments. *, statistically significant ($P < 0.05$) rhythm.

with Pol II H14 antibody at a dilution of 1:5,000 and a peroxidase-conjugated goat anti-mouse IgM (Sigma; A8786) secondary antibody at a dilution of 1:1,000 or probed with actin antibody at a dilution of 1:50,000 and a peroxidase-conjugated goat anti-mouse IgG (Sigma; A5278) secondary antibody at a dilution of 1:1,000 as a loading control. Phosphoryl-histone H3-S10 antibody was also used at a dilution of 1:2,000 and a peroxidase-conjugated goat anti-rabbit IgG (Sigma; A6154) secondary antibody at a dilution of 1:1,000. All probing was done for 1 h at room temperature. Immunoblots were visualized with ECL Plus (Amersham). Protein levels were calculated by densitometry analysis using Quantity One software (Bio-Rad). Background was removed by subtracting an area of the lane with no bands from an equal-size area containing the specific band. Levels were normalized to the actin loading control value. Relative levels were calculated by dividing each sample value by the peak sample value for each time course.

Statistical analysis. For statistical analysis STATISTICA 7 software was used. The sample values minus control values were used in one-way analysis of variance, with post hoc analysis using the Fisher least significant difference to determine significant differences.

RESULTS

CLK-CYC binding to *per* and *tim* E-boxes. Previous work has demonstrated that CLK-CYC binds an E-box upstream of *per* in the CRS and to an E-box upstream of *tim* (49). Several other canonical CACGTG E-boxes are present in *per* upstream and intron 1 sequences (Fig. 1), and a second canonical E-box is present in *tim* intron 1 (Fig. 2). To determine which *per* and *tim* E-boxes are bound by CLK-CYC in vivo, ChIP was done using CLK antiserum. ChIP analysis was performed on flies collected during light-dark (LD) cycles since *per* and *tim* mRNA cycling amplitude is high under these conditions yet reflects regulatory processes occurring in constant darkness (21, 43, 49).

As shown previously, strong binding to the *per* CRS E-box was detected and displayed significant ($P < 0.02$) diurnal cy-

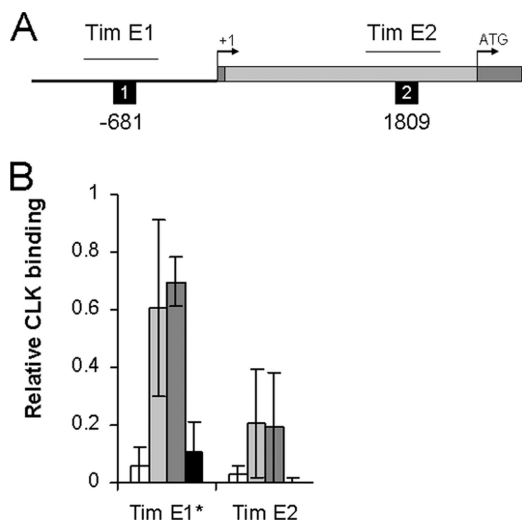


FIG. 2. CLK rhythmic binding to *tim* E-boxes. (A) Schematic representation of *tim* indicating regions analyzed for CLK binding E-boxes. Symbols are as described in the Fig. 1A legend. (B) ChIP was performed as described for Fig. 1B. The relative level of CLK binding for each E-box-containing region was determined and quantified as described for Fig. 1B. These data were derived from three independent experiments. *, statistically significant ($P < 0.05$) rhythm.

cling (Fig. 1) (49). However, CLK showed little if any binding to the E-boxes upstream of the CRS but exhibited moderate rhythmic binding to Per E-box 6 and strong rhythmic binding to Per E-box 7 ($P < 0.001$) and Per E-box 8 ($P < 0.01$), all of which are located in intron 1 (Fig. 1). The resolution of the ChIP assay is not sufficient to distinguish between CLK binding to Per E-box 7 only, to Per E-box 8 only, or to both Per E-box 7 and 8. CLK also strongly binds the E-box upstream of *tim* in a rhythmic manner ($P < 0.03$) (Fig. 2), as was shown previously (49). In contrast, only weak rhythmic binding of CLK to the E-box in *tim* intron 1 was detected (Fig. 2). These two E-boxes were far enough away from each other (>1 kb) to be distinguishable by the resolution of the ChIP assay. The strong binding of CLK to the E-box upstream of *tim* may not be due solely to the canonical CACGTG E-box since noncanonical E-boxes in *tim* upstream sequences also contribute to *tim* mRNA cycling (34). These results demonstrate that CLK, and by extension CLK-CYC, bind rhythmically to multiple E-boxes located upstream of, and within, the *per* gene and to an E-box upstream of *tim*.

Histone modifications in wild-type flies. CLK-CYC strongly binds to *per* and *tim* E-boxes at ZT 10 and/or ZT 16 but weakly at ZT 4 and ZT 22 (Fig. 1 and 2) (49). Such binding coincides with high levels of target gene transcription; thus, chromatin associated with target gene DNA is expected to contain modifications that promote transcriptional activity. The acetylation and methylation of histones are known to alter transcriptional activity and occur in parallel to transcriptional rhythms in *Per1*, *Per2*, *Cry1*, and *Dbp* in mammals (6, 10, 12, 38, 41). To determine if histone modifications are also associated with rhythmic transcription in *Drosophila*, ChIP was performed in wild-type flies at ZT 4, 10, 16, and 22 to assess rhythmic histone modifications. Primers were designed to analyze regions near or including E-boxes and sites of transcriptional and translational

initiation for *per* and *tim* (Fig. 3 and 4). Acetylation, trimethylation of H3-K4, and phosphorylation were expected to promote transcriptional activation, and dimethylation of H3-K9 was expected to be favorable for transcriptional repression (14). Antibodies were used to probe acetylation of H3-K9, H3-K14, and H4; dimethylation of H3-K9; trimethylation of H3-K4; and phosphorylation of H3-S10.

Phosphorylation of H3-S10 was not detected for any *per* or *tim* region (data not shown), whereas acetylation of H3-K14 and H4 was relatively constant for every *per* and *tim* region tested (Fig. 3 and 4). Acetylation of H3-K9 was rhythmic, with a peak at ZT 10 for most *per* and *tim* sites analyzed, though rhythmicity was significant for only the Per Up 2 ($P < 0.03$), Per 67 ($P < 0.04$), and Tim Up 2 ($P < 0.02$) regions (Fig. 3 and 4). Given that acetylation is associated with transcriptional activation, this result is consistent with high levels of CLK-CYC binding and *per* and *tim* transcription at ZT 10 (45, 49). Trimethylation of H3-K4 was rhythmic, constant, or very low, depending upon the region analyzed (Fig. 3 and 4). Rhythmic trimethylation of *per* and *tim* peaked at ZT 10 or ZT 16 and was significant for Per E-box 6 ($P < 0.02$), Per 67 ($P < 0.01$), Per Translation ($P < 0.04$), and Tim Up 1 ($P < 0.02$). The peak phase of trimethylation coincides with CLK binding and transcriptional activation of *per* and *tim* (45, 49), consistent with the known role of H3-K4 trimethylation (14). Dimethylation of H3-K9 was found to be rhythmic only in the first introns of *per* and *tim* (Fig. 3 and 4). Although the rhythm amplitude was relatively low and not statistically significant, a consistent peak was observed at ZT 16. Since this time corresponds to the end of CLK-CYC activation of *per* and *tim*, it is not clear whether this modification is involved in activation or repression. Dimethylation of H3-K9 within the coding region has been shown in some cases to be associated with Pol II elongation (47), but experiments with mammals have shown that dimethylation is associated with the repressed state of the circadian gene *Dbp* (41). Thus, in wild-type flies, H3-K14 and H4 acetylation is constantly high and S10 phosphorylation is not detected in *per* and *tim* upstream or intron 1 sequences, whereas H3-K9 acetylation, H3-K9 dimethylation, and H3-K4 trimethylation are rhythmic in some *per* and *tim* upstream or intron 1 sequences and not others (Fig. 5). Importantly, rhythms in H3-K9 acetylation, H3-K9 dimethylation, and H3-K4 trimethylation parallel rhythms in *per* and *tim* transcriptional activity.

Histone modifications in *per*⁰¹ and *cyc*⁰¹ flies. As an independent means of identifying sites of H3-K9 acetylation, H3-K9 dimethylation, and H3-K4 trimethylation associated with *per* and *tim* transcription, ChIP experiments were carried out with *per*⁰¹ and *cyc*⁰¹ flies. In *per*⁰¹ flies, PER is not present and can't interact with and inhibit CLK-CYC activation of target genes. Consequently, histone modifications associated with CLK-CYC-dependent transcriptional activation would be expected in *per*⁰¹ mutants. In contrast, since *cyc*⁰¹ flies lack CYC and thus CLK-CYC activity, histone modifications associated with transcriptional inactivity would be expected. Measurements of H3-K9 acetylation, H3-K9 dimethylation, and H3-K4 trimethylation were made at a time point during the light (ZT 10) and a time point during the dark (ZT 22). Light-induced differences in *per* and *tim* transcription and CLK-CYC-dependent E-box binding have not been detected in *per*⁰¹

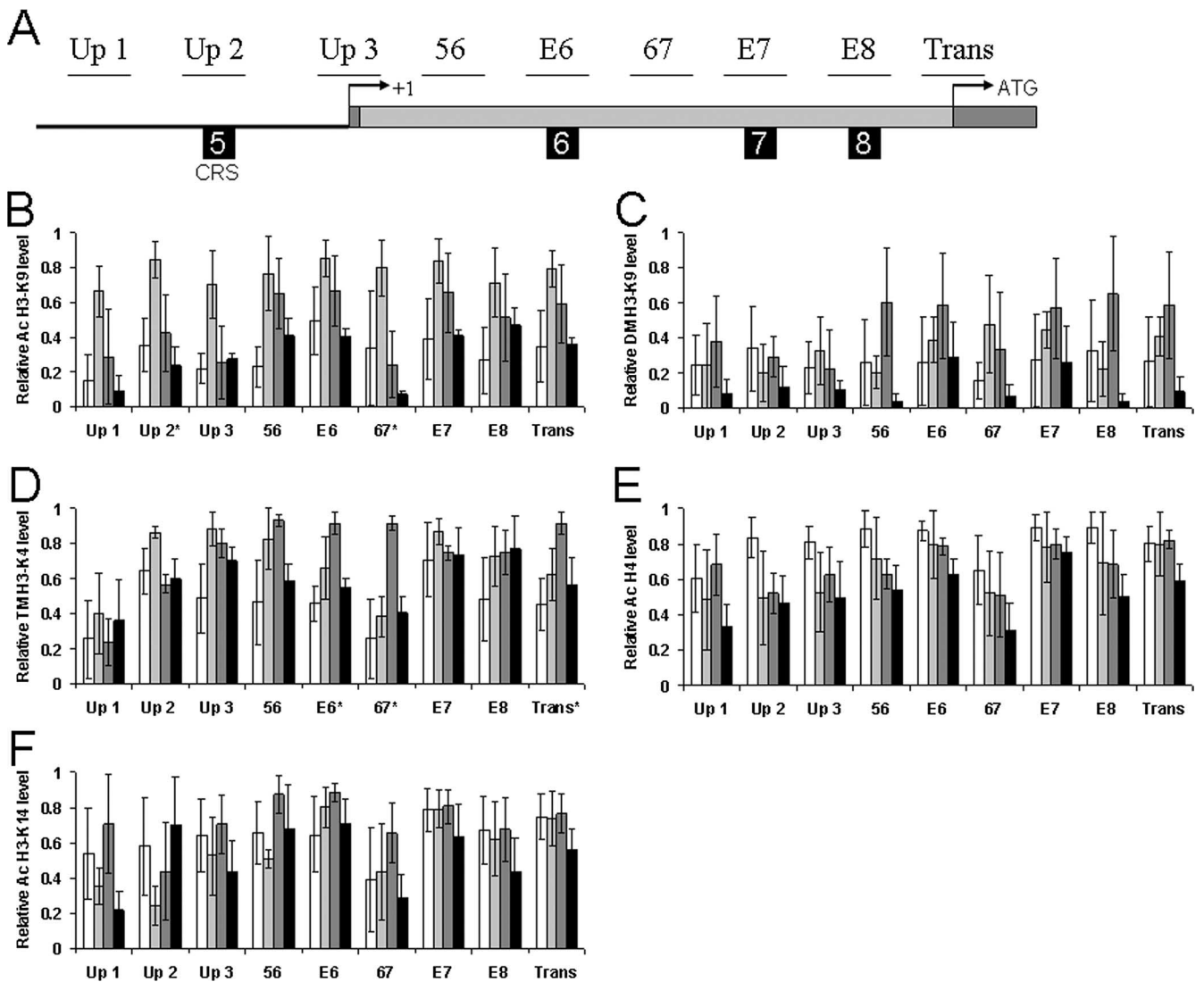


FIG. 3. Histone modifications in the *per* upstream and intron 1 regions. (A) Schematic representation of sites analyzed for histone modifications. The upstream sequence, transcription start, exons, intron 1, translation start, and E-boxes are depicted as described for Fig. 1A. Only E-boxes that show rhythmic CLK binding are shown. The thin horizontal lines labeled Up 1, Up 2, Up 3, 56, E6, 67, E7, E8, and Trans represent *per* genomic regions analyzed by ChIP. (B to F) ChIP was performed as described for Fig. 1B. Relative levels of (B) acetyl (Ac)-H3-K9, (C) dimethyl (DM)-H3-K9, (D) trimethyl (TM)-H3-K4, (E) acetyl-H3-K14, and (F) acetyl-H4 were determined by qPCR analysis. The levels of each histone modification have been corrected for nonspecific binding by subtracting the rabbit IgG or rabbit serum ChIP values at each time point. These data were derived from three independent experiments. *, statistically significant ($P < 0.05$) rhythm.

flies (45, 49); thus, no differences in histone modifications are expected in *per*⁰¹ and *cyc*⁰¹ flies at ZT 10 and ZT 22.

For most *per* and *tim* upstream/intron 1 regions, H3-K9 acetylation was higher in *per*⁰¹ flies than in *cyc*⁰¹ flies (Fig. 6 and 7). However, the only regions in which H3-K9 acetylation was significantly higher in *per*⁰¹ flies than in *cyc*⁰¹ flies at both times were Per 67 ($P < 0.04$) and Tim Up 1 ($P < 0.001$). Likewise, H3-K4 trimethylation in most *per* and *tim* upstream/intron 1 regions was higher in *per*⁰¹ flies than in *cyc*⁰¹ flies but was only significantly higher in the Per 56 ($P < 0.04$), Per E-box 6 ($P < 0.002$), and Tim Up 1 ($P < 0.001$) regions at both times (Fig. 6 and 7). The levels of H3-K9 dimethylation in *per*⁰¹ and *cyc*⁰¹ flies were similar; none of the *per* or *tim* regions tested showed significant differences (Fig. 6 and 7). Although

H3-K9 acetylation, H3-K9 dimethylation, and H3-K4 trimethylation levels were similar within a given region of *per* and *tim* at both time points in *cyc*⁰¹ and *per*⁰¹ flies, the levels of H3-K9 acetylation and H3-K4 trimethylation tended to be somewhat higher at ZT 22 in *cyc*⁰¹ flies and somewhat lower at ZT 22 in *per*⁰¹ flies.

H3-K9 acetylation and/or H3-K4 trimethylation was significantly higher in *per*⁰¹ than *cyc*⁰¹ mutants for sites within the *per* transcribed region (i.e., Per 56, Per E-box 6, and Per 67). Two of these sites (Per E-box 6 and Per 67) coincide with those that showed high levels of H3-K9 acetylation and/or H3-K4 trimethylation when *per* is highly transcribed in wild-type flies, further implicating these modifications in promoting *per* transcription. Moreover, the location of these chromatin modifi-

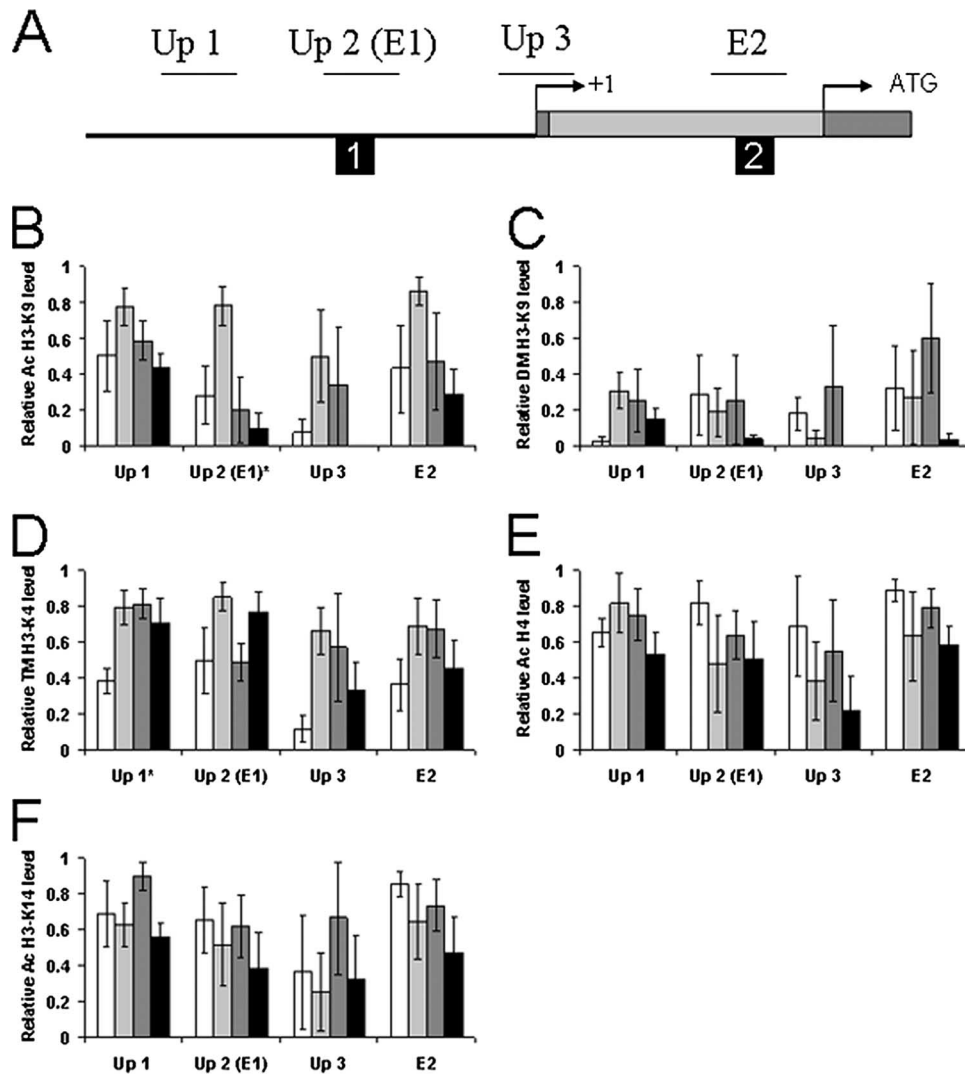


FIG. 4. Histone modifications in the *tim* upstream and intron 1 regions. (A) Schematic representation of sites analyzed for histone modifications. Symbols are depicted as described for Fig. 3A, except that the regions analyzed by ChIP are labeled Up 1, Up 2, Up 3, and E2. (B to F) ChIP was performed as described for Fig. 1B. Histone modifications are abbreviated as in Fig. 3B. Relative levels of (B) acetyl-H3-K9, (C) dimethyl-H3-K9, (D) trimethyl-H3-K4, (E) acetyl-H3-K14, and (F) acetyl-H4 were determined by qPCR analysis. The levels of each histone modification have been corrected for nonspecific binding as described for Fig. 3B. These data were derived from three independent experiments. *, statistically significant ($P < 0.05$) rhythm.

cations within the *per* transcribed region suggests that they may affect Pol II elongation. In contrast, H3-K9 acetylation and H3-K4 trimethylation were significantly higher in *per*⁰¹ than *cyc*⁰¹ mutants for a site (Tim Up 1) in the *tim* upstream region. This corresponds well with high levels of H3-K9 acetylation and/or H3-K4 trimethylation at Tim Up 1 and Tim Up 2 during *tim* transcription in wild-type flies, indicating that these sites are involved in activating *tim* transcription.

Pol II binding. CLK-CYC binding to E-boxes coincides with H3-K9 acetylation, H3-K4 trimethylation, and activation of *per* and *tim* transcription. Consequently, these histone modifications are expected to promote Pol II recruitment and/or activity. Pol II binding and activity at the *per* and *tim* loci were assessed with antibodies that recognize either the non-actively transcribing form of Pol II having an unphosphorylated CTD (Pol II CTD) or the actively tran-

scribing form of Pol II having an S5 phosphorylated CTD (Pol II S5 CTD). The Pol II S5 CTD antibody (H14) has been reported to bind specifically to either S5 or to S5 plus S2 and not to S2 alone, and the Pol II CTD antibody (8WG16) has been reported to bind with highest affinity to the unphosphorylated CTD and with weaker affinity to the phosphorylated forms (24). Both antibodies are able to recognize Pol II at the promoter region, indicating that 8WG16 can recognize either the unphosphorylated or S5-phosphorylated CTD (36). Neither of these antibodies has been reported to recognize Pol II in the transcribed region, where S2 phosphorylation predominates (36). These Pol II antibodies were used for ChIP analysis of various regions of *per* and *tim* in wild-type flies collected at ZT 4, 10, 16, and 22. For these experiments two gene-specific primer sets were used for the *per* and *tim* promoters (Up 3 and Up 3-2) and

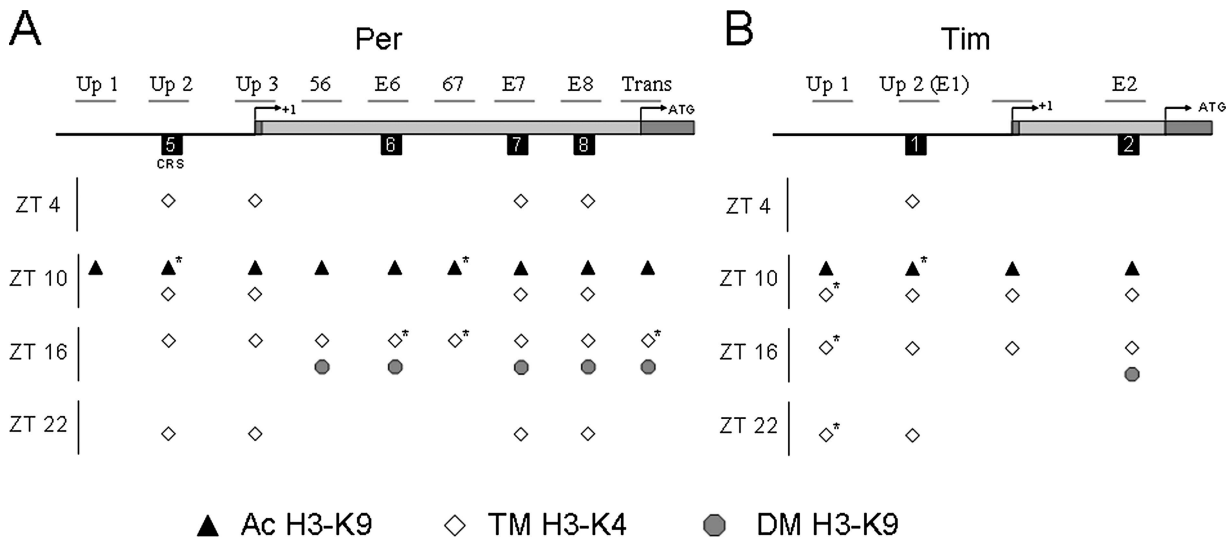


FIG. 5. Summary of *per* and *tim* histone modifications in wild-type flies. Histone modifications present at ZT 4, 10, 16, and 22 are shown for the upstream and intron 1 regions of *per* (A) and *tim* (B). Ac H3-K9, acetylation of H3-K9; TM H3-K4, trimethylation of H3-K4; DM H3-K9, dimethylation of H3-K9. *, modification with statistically significant ($P < 0.05$) rhythm. Schematic representations of the *per* and *tim* gene regions that were analyzed by ChIP are labeled as in Fig. 3A and 4A, respectively.

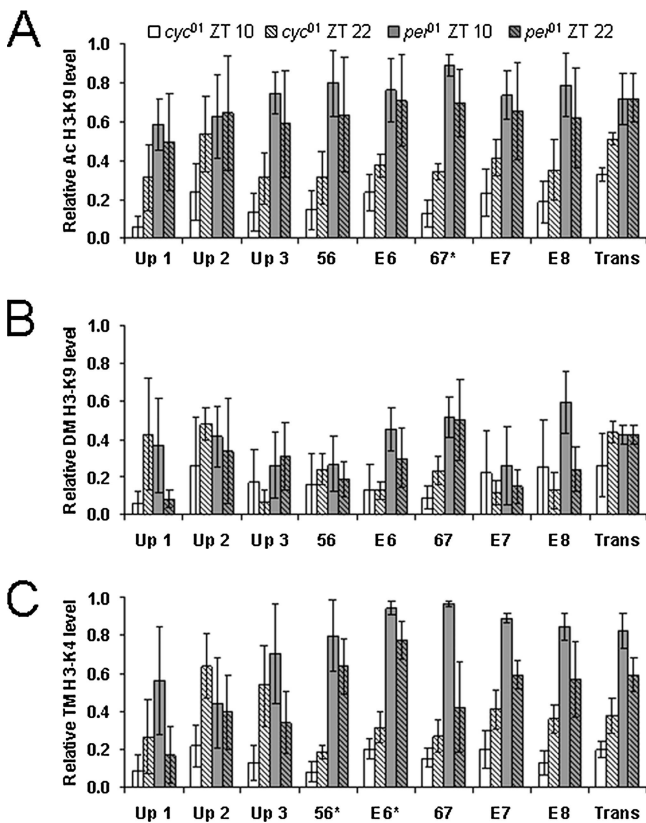


FIG. 6. Histone modifications in *per* upstream and intron 1 regions from *per^{D1}* and *cyc^{D1}* flies. ChIP was performed in *per^{D1}* and *cyc^{D1}* flies collected at ZT 10 and ZT 22 during a 12:12 LD cycle. Relative levels of (A) acetyl (Ac)-H3-K9, (B) dimethyl (DM)-H3-K9, and (C) trimethyl (TM)-H3-K4 binding were determined for the regions depicted in Fig. 3A by qPCR analysis. The levels of each histone modification have been corrected for nonspecific binding by subtracting the rabbit IgG ChIP values at each time point. These data were derived from three independent experiments. *, statistically significant ($P < 0.05$) difference.

single primer sets were used for the upstream (Up 1) and coding (MT or LE) regions.

Relatively high levels of Pol II CTD are bound to the *per* promoter region at all times tested, where Per Up 3 shows a low-amplitude rhythm (albeit not statistically significant) with a peak at ZT 16 and Per Up 3-2 shows no rhythm in binding (Fig. 8A). Compared to those for the Per Up 3/Per Up 3-2 region, low binding levels were detected in the other *per* regions tested (Fig. 8A). Similarly, Pol II S5 CTD is constantly bound to the Per Up 3 region, though no rhythm in binding is apparent (Fig. 8B). The background of the Pol II S5 antibody makes comparisons of levels of binding to various regions difficult; however, comparisons can be made when using the negative control as a reference. For the Per Up 3/Per Up 3-2 region, Pol II S5 binding is >10-fold higher than that for the negative control, whereas it is <4-fold higher than that for the negative control for all other regions tested (see Fig. S2 in the supplemental material). Neither of these antibodies has been reported to recognize Pol II beyond the promoter region (36); thus, negative results are expected for the transcribed region. In contrast, Pol II CTD binds the *Tim* Up 3/*Tim* Up 3-2 region rhythmically; high levels of binding are seen at ZT 10 and 16, and significantly ($P < 0.03$) lower binding levels (for the *Tim* Up 3-2 primer set) are seen at ZT 4 and ZT 22 (Fig. 8C). Likewise, Pol II S5 CTD binding to *Tim* Up 3 shows a significant daily cycle ($P < 0.03$), though both *Tim* Up 3 and *Tim* Up 3-2 primer sets show peak levels of binding at ZT 16 and low levels of binding at both ZT 4 and ZT 22 (Fig. 8D), consistent with nuclear run-on experiments (45). Regions within the *tim* transcribed region show low Pol II CTD binding, while regions upstream show rhythmic Pol II CTD binding with statistically significant daily cycles for *Tim* Up 1 ($P < 0.04$) and *Tim* Up 2 ($P < 0.05$), suggesting that Pol II interacts with proteins associated with these regions (Fig. 8C). In comparison with Pol II S5 binding, negative control levels are much lower for the *Tim* Up 3 region and the *Tim* Up 1 region (see Fig. S3

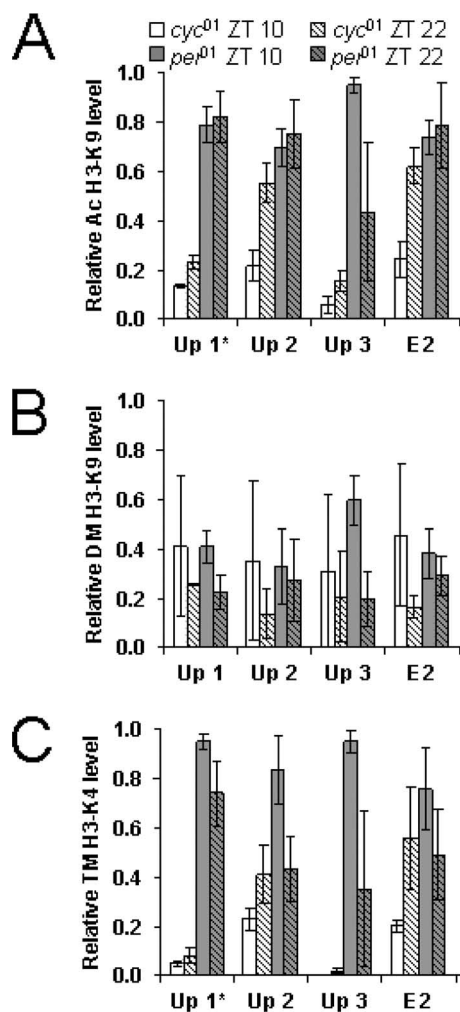


FIG. 7. Histone modifications in *tim* upstream and intron 1 regions from *per*⁰¹ and *cyc*⁰¹ flies. ChIP was performed as described for Fig. 6. Relative levels of (A) acetyl (Ac)-H3-K9, (B) dimethyl (DM)-H3-K9, and (C) trimethyl (TM)-H3-K4 binding were determined for the regions depicted in Fig. 4A by qPCR analysis. The levels of each histone modification have been corrected for nonspecific binding as described for Fig. 6. These data were derived from three independent experiments. *, statistically significant ($P < 0.05$) difference.

in the supplemental material). Pol II S5 binding does not seem to correlate with protein levels, as Pol II S5 levels do not appear to be rhythmic (see Fig. S4 in the supplemental material), indicating that binding is regulated. Since Pol II was not expected to constitutively bind the *per* promoter region, additional ChIP assays were carried out to compare levels of Pol II CTD binding via semiquantitative PCR in the Per Up 1, Per Up 2, and Per Up 3 regions. Although little or no Pol II CTD binding to Per Up 1 or Per Up 2 was detected, strong binding to Per Up 3 was detected at all time points (Fig. 8E). These ChIP analyses demonstrate strong constitutive Pol II binding that is specific to the *per* promoter. These results also suggest that Pol II does not interact with proteins in the Per Up 1 and Per Up 2 regions; otherwise, Pol II would pull down those regions, as was seen with Tim Up 1 and 2 (Fig. 8).

DISCUSSION

CLK-CYC binding to E-boxes is essential for rhythmic transcription of the core circadian oscillator genes *per* and *tim* in *Drosophila*. Previous analysis demonstrated that CLK-CYC binds rhythmically to the *per* CRS E-box in wild-type flies and that this binding is PER dependent (49). Although the *per* CRS E-box is sufficient to drive circadian transcription (8, 20, 32), other canonical CACGTG E-boxes present in *per* upstream and intron 1 sequences may also contribute to *per* transcriptional rhythms. ChIP analysis shows that CLK (and by extension CLK-CYC) binds rhythmically to E-boxes in *per* intron 1 (Fig. 1), indicating that multiple E-boxes are involved in rhythmic *per* transcription. This observation may explain rescue of behavioral rhythmicity by constructs that lack the *per* promoter but still contain E-boxes in intron 1 (15, 31). CLK-CYC binding to a region containing the *tim* upstream E-box is strongly rhythmic (Fig. 2) (49), in contrast to binding to the E-box-containing region in *tim* intron 1, which is weak (Fig. 2). These results are consistent with studies showing that the *tim* upstream region is sufficient to drive circadian transcription and that *tim* intron 1 is not able to activate CLK-CYC-dependent transcription in S2 cells (25, 34, 48). Although the canonical CACGTG E-box upstream of *tim* is important for rhythmic transcription, noncanonical E-boxes close to the canonical E-box contribute substantially to high-amplitude rhythmicity (34). Thus, rhythmic CLK binding to *tim* upstream sequences is likely due to canonical and noncanonical E-box elements. Taken together, these results suggest that cooperative binding of CLK-CYC to canonical and noncanonical E-boxes promotes high-amplitude cycling of *tim* transcription. We speculate that cooperative binding of CLK-CYC to multiple canonical and possibly noncanonical E-boxes also mediates high-amplitude cycling of *per* transcription.

Binding of CLK-CYC to *per* and *tim* regulatory sequences coincides with histone modifications that are known to alter transcriptional activity. Acetylation of H3-K9 was rhythmic, with a peak at ZT 10 for each *per* and *tim* region tested (Fig. 3 to 5). Such acetylation is consistent with transcriptional activation since CLK-CYC binding, *per* and *tim* transcription, and *per* and *tim* RNA accumulation are all high at ZT 10. Indeed, relatively low levels of H3-K9 acetylation are detected within/upstream of *per* and *tim* in *cyc*⁰¹ flies (Fig. 6 and 7), which exhibit constant low levels of CLK-CYC binding and express little or no *per* and *tim* mRNA (42, 49). Similar results are seen for *Dhb* in mice, where circadian expression is associated with rhythmic CLOCK-BMAL1 binding to multiple extra- and intragenic E-boxes and acetylation of H3-K9 (41). Likewise, *Per1* activation is coincident with H3 acetylation (6, 10, 12, 38), and CRY-mediated repression likely occurs through histone deacetylation since histone deacetylase (HDAC) is recruited into the complex by CRY and CBP overexpression relieves repression by CRY (6, 38). A similar situation is likely to exist in *Drosophila* since H3-K9 acetylation decreases as PER accumulates, though an interaction between PER and an HDAC has not been identified. The levels of H3-K9 acetylation in *per*⁰¹ flies are consistent with PER-dependent recruitment of HDAC. In *per*⁰¹ flies H3-K9 acetylation is constantly high, indicating that deacetylation is PER dependent (Fig. 6).

Our results suggest that CLK-CYC binding leads to histone

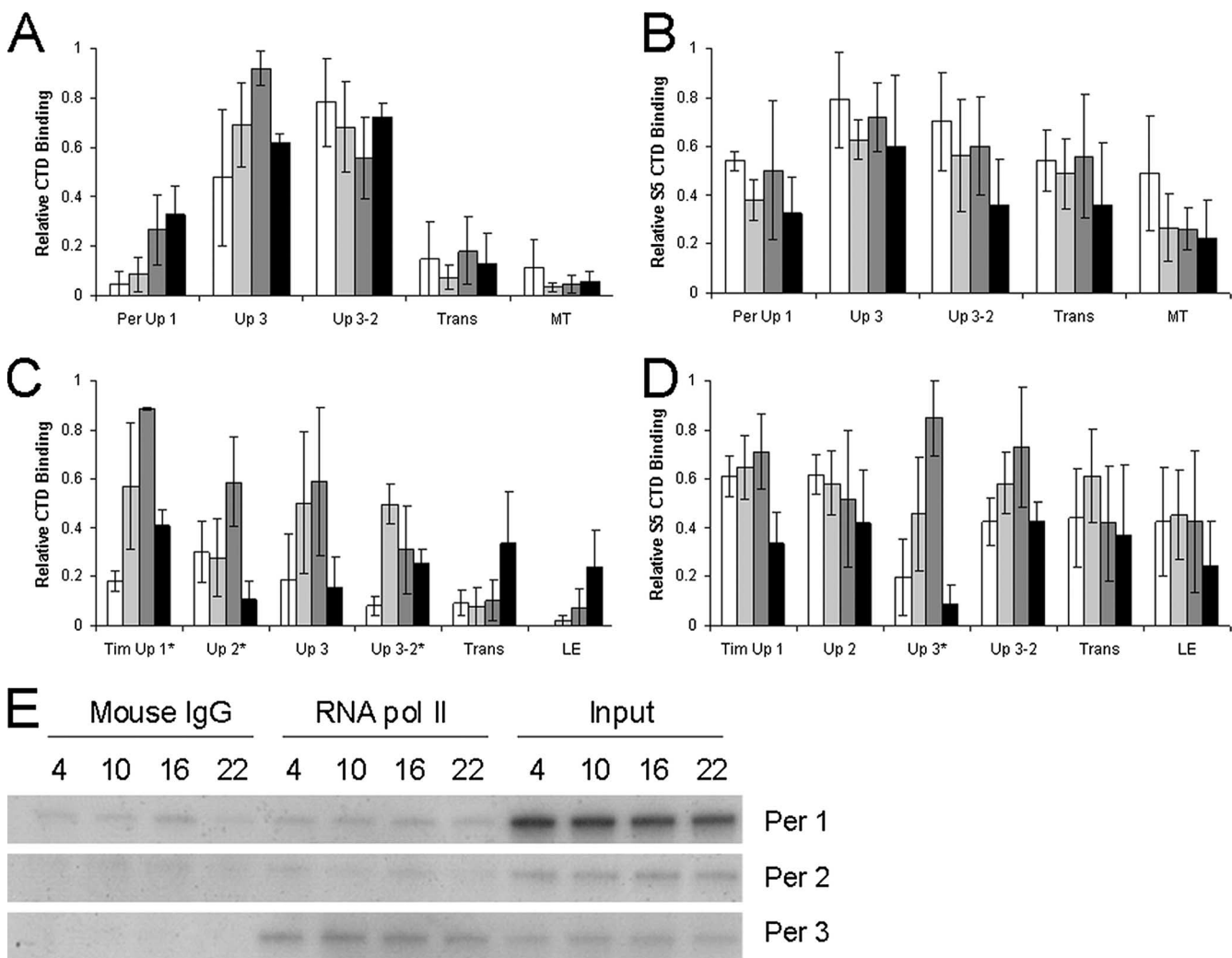


FIG. 8. Pol II binding to the *per* and *tim* genes. ChIP was performed in wild-type flies collected at ZT 4, 10, 16, and 22 during a 12:12 LD cycle. Relative levels were determined by qPCR analysis and are shown for samples immunoprecipitated with antibodies against Pol II CTD (A and C), with mouse IgG as a negative control, or Pol II S5 CTD (B and D), with no IgG as a negative control. The *per* (A and B) and *tim* (C and D) gene regions that were analyzed by ChIP are labeled as in Fig. 3A and 4A, respectively, with an additional primer set for the *per* and *tim* promoters, Per Up 3-2 and Tim Up 3-2, a primer set for the *tim* translation start (Trans), a primer set for the *per* translated region flanking intron 5 (MT), and a primer set for the last exon of *tim* (LE). The levels of each histone modification have been corrected for nonspecific binding by subtracting the mouse IgG or no IgG ChIP values at each time point. These data were derived from three independent experiments. *, statistically significant ($P < 0.05$) rhythm. (E) Semiquantitative PCR results for unphosphorylated CTD of Pol II binding to *per*. Shown are regions from Per Up 1, Per Up 2, and Per Up 3.

acetylation. Recent work has provided information as to the histone acetyltransferases (HATs) that may be involved. One possibility is that CLK itself is a HAT, as has been shown to be the case with the mammalian CLOCK (10). Another possibility is that CLK interacts with other HATs. One HAT shown to interact with CLK is the CBP ortholog NEJIRE (22, 29). In one case NEJIRE/CBP was found to act as a transcriptional coactivator (22), but other evidence shows that overexpression of NEJIRE has a negative effect on CLK-CYC-mediated transcription (29). It is possible that the reduced levels of CLK-CYC-dependent gene expression observed after *nejire* overexpression are due to circadian feedback compensation and that *nejire*/CBP is indeed a coactivator (22).

As with H3-K9 acetylation, a strong rhythm in H3-K4 tri-

methylation was observed just after the first exon of *per* (Per 56, Per E6, Per 67) and near the *tim* transcription initiation site (Tim Up 1, Tim Up 3). This rhythm in H3-K4 trimethylation consistently peaked at ZT 16, which was 6 h after the peak in H3-K9 acetylation and just after the peak in *per* and *tim* mRNAs. Rhythmic H3-K4 trimethylation in these regions suggests that this modification may be involved in Pol II elongation for *per* and Pol II recruitment for *tim*. Although trimethylation of H3-K4 is expected to be involved in transcriptional activation, analysis of cross talk among histone modifications indicates that this modification can also be present during the repressed state (14). This modification may allow for HATs, HDACs, histone methyltransferases, or other modifiers to selectively target histones in a key regulatory position. Rhythmic

trimethylation of H3-K4 in wild-type flies was observed in several upstream/intron 1 regions of *per* and *tim* but was either constantly high or constantly low in others. However, regions of *per* and *tim* where H3-K4 trimethylation is rhythmic in wild-type flies showed either constant high or low levels of H3-K4 trimethylation in *per*⁰¹ and *cyc*⁰¹ flies, respectively (Fig. 6 and 7), consistent with a role in activating transcription.

Another modification analyzed was dimethylation of H3-K9. Although no dimethylation was detected in upstream regions of *per* and *tim*, low-amplitude rhythmic dimethylation was observed in introns 1 of *per* and *tim*, peaking at ZT 16. Cycling of H3-K9 dimethylation was antiphase to H3-K9 acetylation and was associated with repression of *Dbp* in mice (41), but this was not the case for *per* and *tim* in flies. Since dimethylation of H3-K9 in the transcribed region is associated with transcriptional elongation in mice (47), somewhat higher levels of H3-K9 dimethylation in the first introns of *per* and *tim* are expected, as their transcription peaks might suggest that dimethylation of H3-K9 promotes elongation. The levels of H3-K9 dimethylation in *per*⁰¹ and *cyc*⁰¹ mutants were lower, higher, or unchanged compared to that for wild-type flies depending on the time point assayed (Fig. 3, 4, 6, and 7), thus making it difficult to correlate this modification with transcriptional activation or repression. Low-amplitude H3-K9 dimethylation rhythms in wild-type flies prevented meaningful interpretations regarding the function of this chromatin modification. Similarly, both H3-K9 acetylation and dimethylation at the *Dbp* locus are also reduced in arrhythmic *Per1*^{-/-}; *Per2*^{Brdm/Brdm} mutant mice, though a high-amplitude antiphase cycling of H3-K9 acetylation and dimethylation suggests that H3-K9 dimethylation represses transcription (41).

In contrast to the rhythmic chromatin modifications in *per* and *tim* upstream/intron 1 sequences, H3-K14 and H4 acetylation was generally at constant high levels. Acetylation of these sites may be involved in creating a chromatin structure that allows access to proteins that regulate circadian transcription, perhaps as a developmental modification that occurred during clock cell determination. Recent work has shown that histone modifications such as acetylation and methylation have a crucial role in development (30, 33). For instance, sequential H3-K4 methylation, H3 acetylation, and H4 acetylation are associated with the expression of HoxD4 genes during mouse neurogenesis (40) and sequential H3-K9 deacetylation, H3-K4 demethylation, and H3-K9 methylation are associated with downregulation of the mouse terminal transferase gene during thymocyte maturation (46). Although the phospho-histone H3-S10 antibody has been validated for use in ChIP experiments and phosphorylation of H3 often accompanies H3 acetylation (4, 39), phosphorylation of H3-S10 was also negative for all regions of *per* and *tim* tested. These negative results could mean that either H3-S10 is not phosphorylated or that the antibody could not detect phosphorylation at levels present in fly head nuclear extracts. This antibody recognizes a band corresponding to H3 (17 kDa) on an immunoblot containing the sonicated SXN samples used in ChIP experiments (see Fig. S1 in the supplemental material). This result suggests that the phospho-histone H3-S10 antibody recognizes phosphorylated H3-S10 in fly extracts, and thus the negative ChIP result is more likely a result of no phosphorylation of H3-S10 in the transcriptional regulation of *per* or *tim*.

The levels of H3-K9 acetylation and H3-K4 trimethylation in *per* and *tim* upstream/intron 1 sequences were constitutively high in *per*⁰¹ flies and constitutively low in *cyc*⁰¹ flies. However, the levels of these modifications were consistently, though not significantly, different at ZT 10 and ZT 22 in each mutant. In *cyc*⁰¹ flies, H3-K9 acetylation and H3-K4 trimethylation were higher at ZT 22 than at ZT 10, but in *per*⁰¹ flies, H3-K9 acetylation was lower at ZT 22 than at ZT 10 for several regions (the transcribed region of *per* and Tim Up 3) and H3-K4 trimethylation was also lower at ZT 22 than at ZT 10 (Fig. 6 and 7). ChIP was carried out on mutants collected in LD cycles, suggesting that light may alter the levels of H3-K9 acetylation and H3-K4 trimethylation. Since time-dependent differences in H3-K9 acetylation and H3-K4 trimethylation are not unidirectional in *per*⁰¹ and *cyc*⁰¹ flies, they appear to be regulated rather than acute responses to light. These time-dependent differences in H3-K9 acetylation and H3-K4 trimethylation do not lead to changes in transcription since *per* and *tim* mRNAs are expressed at constant levels in *per*⁰¹ and *tim*⁰¹ flies collected during LD cycles (21, 43).

High levels of Pol II are always present on the *per* promoter, which suggests that rhythms in *per* transcription are regulated by events associated with Pol II elongation. One such event is H3-K4 trimethylation, which exhibits high-amplitude rhythms in *per* intron 1. Deleting a specific regulator of H3-K4 trimethylation in *Saccharomyces cerevisiae*, Bur2 kinase, significantly impairs the recruitment of the PAF elongation factor, thus linking H3-K4 trimethylation with Pol II elongation (27). Since cross talk among chromatin modifications can occur at the level of a single histone tail or at the level of nucleosomes (14), H3-K4 trimethylation along with H3-K9 acetylation may promote *per* transcription by promoting Pol II elongation, whereas H3-K4 trimethylation along with H3-K9 deacetylation may inhibit *per* transcription by promoting Pol II pausing. The coincidence between CLK-CYC binding and high levels of H3-K9 acetylation and H3-K4 trimethylation suggests that there may be cross talk between these modifications. Although previous results from cultured mammalian cells suggested that the antibodies used in these experiments would not detect high levels of nonphosphorylated and S5-phosphorylated Pol II in *per* and *tim* transcribed regions, it is possible that the Pol II CTD modification associated with *per* and *tim* transcription elongation is S2 phosphorylation of Pol II (36).

In contrast to the situation with *per*, Pol II binding is rhythmic in the *tim* promoter region. Since H3-K4 trimethylation and H3-K9 acetylation are strongly rhythmic in *tim* upstream and promoter sequences and show more-modest rhythms in *tim* intron 1, it is possible that both Pol II recruitment and elongation are involved in regulating *tim* transcriptional rhythms. The rhythmic recruitment of Pol II, possibly in combination with Pol II elongation rhythms, may account for the high rate and amplitude of *tim* transcription compared to those of *per* transcription. In mammals, rhythmic histone acetylation at the *Per1*, *Per2*, and *Cry1* promoters correlates with Pol II binding and transcriptional cycling (6, 12), indicating that rhythmic recruitment of Pol II is a conserved mechanism for regulating transcriptional rhythms in mammals. Pol II was also shown to interact with *tim* upstream sequences (Tim Up 1 and Tim Up 2), presumably through Pol II interactions with proteins associated with these regions (Fig. 8). In addition to

rhythmic H3-K9 acetylation and/or H3-K4 trimethylation in wild-type flies, there is strong H3-K9 acetylation and H3-K4 trimethylation of Tim Up 1 and Tim Up 2 in *per⁰¹* flies and weak H3-K9 acetylation and H3-K4 trimethylation in *cyc⁰¹* flies. The resolution of ChIP is about 500 bp, and Tim Up 2 is less than 500 bp from Tim Up 1 and Tim Up 3; therefore, results showing Pol II interaction with the Tim Up 2 region may be due to crossover. This is consistent with lower Pol II CTD levels for Tim Up 2 than for Tim Up 1 (Fig. 8) and levels of Pol II S5 CTD that are closer to negative control levels for Tim Up 2 than for Tim Up 1 or 3 (see Fig. S3A to C in the supplemental material).

Results from these experiments demonstrate rhythmic binding of CLK-CYC to *per* intronic E-boxes; rhythmic and constitutive histone modifications in *per* and *tim* upstream, promoter, and transcribed regions; and rhythmic or constitutive Pol II binding to the *tim* and *per* promoters. The activation and repression of these genes may be directly or indirectly dependent upon CLK-CYC binding or other circadian proteins that interact with CLK-CYC, such as PER. Many of the histone modifications associated with rhythmic transcription in flies (e.g., H3-K9 acetylation, H3-K4 trimethylation, and H3-K9 dimethylation) are conserved in mammals, which suggests that PER and CRY repress transcription through similar mechanisms. Our results imply that histone-modifying enzymes such as HATs, HDACs, histone methyltransferases, ubiquitinases, and sumoylases and Pol II recruitment and elongation factors rhythmically interact with the CLK-CYC complex to regulate transcriptional cycling. Identifying proteins that interact with CLK-CYC during times of transcriptional activity or repression will advance our understanding of the mechanisms that regulate circadian transcription.

ACKNOWLEDGMENTS

We thank X. Li from M. Biggin's lab for advice on which Pol II antibodies to use for ChIP assays and the linker antibody to use with anti-S5 CTD. We are grateful for advice from F. Ng on qPCR assays used to quantify ChIP assays.

This work was supported by Public Health Service grant NS052854 from the National Institute of Neurological Disorders and Stroke.

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