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Circadian Regulator CLOCK Is a Histone Acetyltransferase

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SUMMARY

The molecular machinery that governs circadian rhythmicity comprises proteins whose interplay generates time-specific transcription of clock genes. The role of chromatin remodeling in a physiological setting such as the circadian clock is yet unclear. We show that the protein CLOCK, a central component of the circadian pacemaker, has histone acetyltransferase (HAT) activity. CLOCK shares homology with acetyl-coenzyme A binding motifs within the MYST family of HATs. CLOCK displays high sequence similarity to ACTR, a member of SRC family of HATs, with which it shares also enzymatic specificity for histones H3 and H4. BMAL1, the heterodimerization partner of CLOCK, enhances HAT function. The HAT activity of CLOCK is essential to rescue circadian rhythmicity and activation of clock genes in *Clock* mutant cells. Identification of CLOCK as a novel type of DNA binding HAT reveals that chromatin remodeling is crucial for the core clock mechanism and identifies unforeseen links between histone acetylation and cellular physiology.

INTRODUCTION

Several histone modifications contribute to chromatin remodeling and thereby to the control of a large array of nuclear processes (Cheung et al., 2000; Felsenfeld and Groudine, 2003). Histone acetylation is believed to play a pivotal role in the modulation of chromatin structure associated with transcriptional activation (Grunstein, 1997; Kuo and Allis, 1998; Struhl, 1998; Wade and Wolffe, 1997; Workman and Kingston, 1998). In support of this notion, a wide variety of nuclear proteins involved in transcriptional control have been demonstrated to possess intrinsic histone acetyltransferase (HAT) activity (Kouzarides, 1999; Roth et al., 2001; Sterner and Berger, 2000). In particular, a number of transcriptional coactivators, including GCN5 (Brownell et al., 1996), PCAF (Yang et al., 1996), CBP/p300 (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), SRC-1 (Spencer et al., 1997), and ACTR (Chen et al., 1997) are known to acetylate histones, thereby facilitating the transactivation exerted by a number of DNA binding transcription factors. Furthermore, HAT function is not limited to coactivators. Indeed, HAT function has been ascribed also to TAFII250, a component of the TATA box binding TFIID complex of the basal transcription machinery (Mizzen et al., 1996), and to ATF-2, a sequence-specific DNA binding transcription factor (Kawasaki et al., 2000).

Amino acid sequence analyses of HAT proteins reveal an important feature: HATs fall into distinct families that share relatively poor sequence similarity. For example, ACTR/SRC-1 is thought to constitute a unique class of HATs (Chen et al., 1997; Spencer et al., 1997), whereas p300/CBP displays only limited homology to the GCN5-related *N*-acetyltransferase superfamily (Martinez-Balbas et al., 1998). The MYST family of HATs is particularly interesting as these proteins show similarity with other acetyltransferases exclusively within the acetyl-coenzyme A binding motif (denominated "motif A"; Yamamoto and Horikoshi, 1997). Accumulating evidence indicates that histone acetylation exerted by various classes of HATs contributes to plasticity in transcriptional control by increasing the dynamic changes in chromatin structure (Fischle et al., 2003).

Finely controlled transcriptional regulation is the central feature of circadian clock function. About 10% of all mammalian transcripts undergo circadian fluctuations in abundance (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002). These oscillations are driven by cell-autonomous pacemakers present in the central clock structure, the suprachiasmatic nucleus (SCN) of the hypothalamus, and in most peripheral tissues (Schibler and Sassone-Corsi, 2002). Such a unique temporal regulation of transcription elects the cellular clock as a prominent model for the study of dynamic regulations of chromatin remodeling (Crosio et al., 2000). Moreover, as circadian rhythms are tightly coupled to physiological and metabolic control (Rutter et al., 2002; Schibler and Naef, 2005), clock-controlled chromatin reorganization is likely to reveal yet unexplored pathways linking histone modifications to cellular metabolism.

The molecular framework of the circadian clock machinery is constituted by a network of transcription/translation-based autoregulatory feedback loops (Cermakian and Sassone-Corsi, 2000; Dunlap, 1999; King and Takahashi, 2000; Reppert and Weaver, 2002; Young and Kay, 2001). The basic helix-loop-helix-Per/Arnt/Sim (bHLH-PAS) transcription factor, CLOCK acts as master controller serving as a positive regulator in the primary feedback loop (Darlington et al., 1998; Gekakis et al., 1998). In mice, mCLOCK forms heterodimers with mBMAL1 and drives transcription of *Period* (*mPer1*, *mPer2*, and *mPer3*) and *Cryptochrome* (*mCry1* and *mCry2*) genes through E box elements located in their promoter regions. The mPER and mCRY proteins then negatively feedback to repress their own transcription by acting on the mCLOCK:mBMAL1 complex. This negative-feedback regulation also drives gene expression cycles of a variety of circadian output genes (Lowrey and Takahashi, 2004).

The activation of clock-controlled genes by mCLOCK:mBMAL1 was recently shown to be coupled to circadian changes in histone acetylation at their promoters (Curtis et al., 2004; Etchegaray et al., 2003; Ripperger and Schibler, 2006), indicating that transcription-permissive chromatin states are dynamically established in a circadian time-specific manner. The molecular dissection of the mCLOCK:mBMAL1-mediated transactivation mechanism is likely to provide significant information on how circadian regulation of histone acetylation is achieved. Several lines of genetic evidence indicate that the carboxy-terminal glutamine-rich region of CLOCK exerts a central function in the circadian transactivation of target genes in flies and mice (Allada et al., 1998; Antoch et al., 1997; Gekakis et al., 1998; Jin et al., 1999; King et al., 1997). Yet, the molecular mechanisms linking CLOCK to circadian histone acetylation and a transcription-permissive chromatin structure has remained elusive.

Here, we show that CLOCK has intrinsic HAT activity. Analysis of the primary structure of CLOCK revealed that the carboxy-terminal glutamine-rich region presents high sequence similarity to ACTR, a member of SRC family of HATs. The acetyl-coenzyme A binding motif shows high similarity to Esa1, a yeast protein of the MYST family of HATs. Mutations of this putative acetyl coenzyme A binding site generated mutant CLOCK proteins that display a drastic reduction in histone acetyltransferase activity. Differently from normal CLOCK, ectopic expression of CLOCK mutants is unable to rescue circadian gene rhythmicity in *Clock* mutant MEF cells. These data underscore the physiological relevance of CLOCK-mediated histone acetylation as a chromatin-remodeling event required for circadian clock function.

RESULTS

Conserved Structural Features between CLOCK and HATs

We have been studying the transcriptional activation function of CLOCK and analyzed the protein primary structure, focusing our attention on the carboxy-terminal glutamine-rich region that was previously shown to be implicated in transactivation function (Allada et al., 1998; Gekakis et al., 1998). Except for a polyglutamine stretch, no characteristic structural motifs were previously described in this region (Figure 1A). Our extensive search has revealed that the carboxy-terminal region of CLOCK displays a significant sequence homology with the carboxy-terminal domain of ACTR (Figure 1B), a domain previously described to have intrinsic HAT activity (Chen et al., 1997). In this region at least six independent amino acid regions are found to share significant sequence similarity between the two proteins. Importantly, the amino acid residues common to CLOCK and ACTR are evolutionarily conserved in both proteins (Figure 1B). It is also noteworthy that CLOCK and ACTR share a number of other structural features outside of the carboxy-terminal glutamine-rich region. These include the highly conserved bHLH-PAS domain at the amino termini, a NRID (nuclear receptor interaction domain), as well as serine-rich regions within the middle portion of both proteins (Figure 1A). Although CLOCK is a significantly smaller protein as compared to ACTR, these common features result in a strikingly similar organization overall. Analysis of the primary sequence shows that BMAL1, the heterodimeric partner of CLOCK, which is also a bHLH-PAS domain-containing protein (Hirayama and Sassone-Corsi, 2005), displays no similarity to the HAT region of ACTR.

CLOCK Has HAT Activity

The presence of conserved structural features common to CLOCK and ACTR prompted us to test whether the CLOCK protein has the ability to acetylate histones. We transiently expressed a Myc-tagged mCLOCK (Myc-mCLOCK) in cultured mammalian cells and prepared cell extracts. CLOCK was found to have significant HAT activity in immunoprecipitates obtained using a specific anti-Myc antibody (Figure 2A, right). In contrast, Myc-mBMAL1 yielded little or no HAT activity, comparable to that observed in immunoprecipitates prepared from mock-transfected cells. Immunoblot analysis confirmed that the expression levels of Myc-mBMAL1 and Myc-mCLOCK proteins were equivalent (Figure 2A, left). Thus, the immunoprecipitated HAT activity is specific to mCLOCK and not to its heterodimeric partner mBMAL1.

To unequivocally establish that CLOCK is a protein exhibiting HAT activity in the Myc-mCLOCK immunoprecipitate, we performed in-gel HAT assays using a mixture of purified histones as substrate (Brownell et al., 1999). After transient expression in mammalian cells, Myc-mCLOCK was immunoprecipitated and purified. Proteins resolved by SDS-PAGE were subjected to in-gel enzymatic reaction. Detection of histones [^{14}C]-acetylated in situ demonstrated that acetylation took place specifically in a position corresponding to where the Myc-CLOCK protein migrated (Figure 2B, lanes 2 and 5). To confirm this result, and to rule out the possibility that a contaminant HAT comigrating with Myc-CLOCK would be responsible for the acetylation, we used an N-terminally truncated mCLOCK

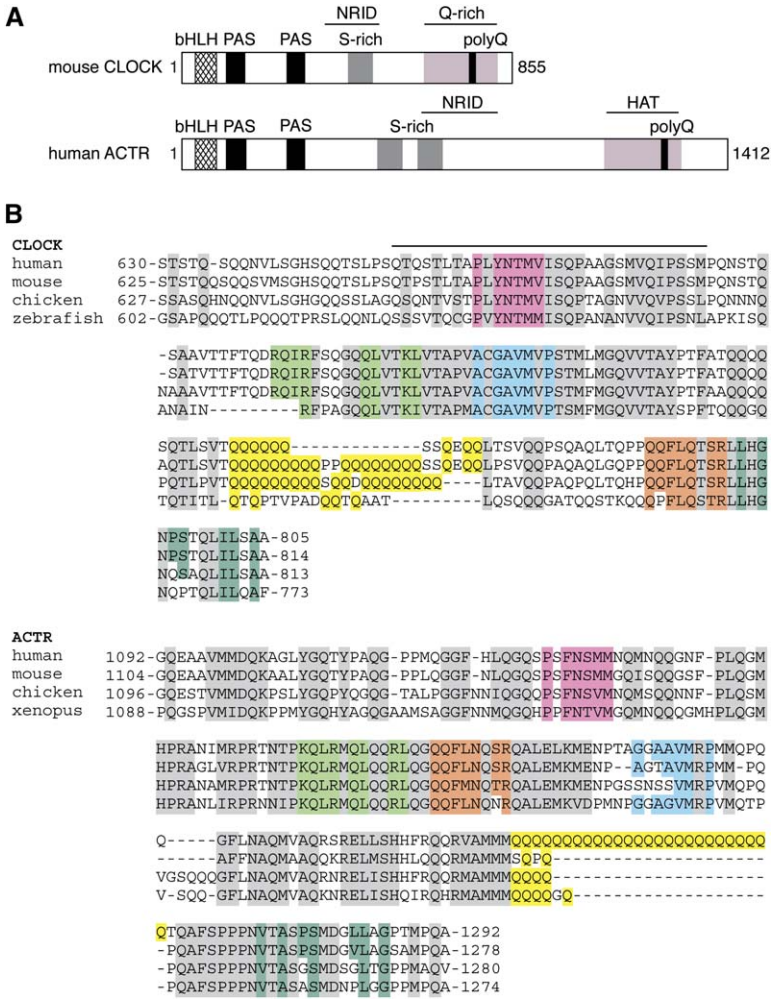

Figure 1. Comparison of Primary Structures of CLOCK and ACTR

(A) Schematic representation of the primary structures of mouse CLOCK and human ACTR with common features; a basic helix-loop-helix (bHLH) motif, Per-Arnt-Sim (PAS) domains, serine-rich (S-rich) regions, a nuclear receptor interaction domain (NRID), a glutamine-rich (Q-rich) region containing a poly-glutamine (polyQ) stretch. A horizontal line above hACTR indicates a region known to have HAT activity. (B) Amino acid sequences of the carboxy-terminal glutamine-rich region of CLOCK from various species (upper; shown for zebrafish is zCLOCK3) and amino acid sequences of the HAT domain of ACTR from various species (lower). Identical or chemically similar residues shared by CLOCK and ACTR proteins are shown by colored backgrounds, with each of the homologous amino acid stretches highlighted by distinct colors. Residues evolutionally conserved only within CLOCK or ACTR proteins are shown on gray-shaded backgrounds. A horizontal line above the CLOCK sequences indicates a region homologous to the acetyl-CoA binding motif.

(Myc-mCLOCKΔN) protein in the in-gel HAT assay. This truncated CLOCK protein lacks the N-terminal residues 1–242 but has an intact C-terminal region and still displays efficient HAT activity in the gel (Figure 2B, lanes 3 and 6). These results demonstrate that CLOCK is a HAT.

BMAL1 might modulate CLOCK-dependent HAT activity. To test this possibility, Myc-mCLOCK and Myc-mBMAL1 were transiently coexpressed and then coimmunoprecipitated. In the presence of mBMAL1, the relative HAT activity of mCLOCK was enhanced by about 4-fold (Figure 2C). This is particularly interesting as binding to E boxes and additional regulatory levels of control require the formation of the CLOCK:BMAL1 heterodimer (Gekakis et al., 1998; Hogenesch et al., 1998; Rutter et al., 2001). It should be noted that protein analysis revealed that CLOCK levels are generally reduced by the presence of BMAL1 (not shown), as predicted since CLOCK:BMAL1 heterodimerization induces protein degradation (Kondratov et al., 2003).

Acetyl-CoA Binding Motif in CLOCK

Acetyl-coenzyme A (CoA) binding motifs are hallmarks of HAT proteins (Sterner and Berger, 2000). Detailed sequence comparison between the acetyl-CoA binding motifs of various HATs revealed that CLOCK contains a motif within the carboxy-terminal glutamine-rich region (Figure 3A). This amino acid sequence stretch shares significant similarity to the so-called "motif A" in the HAT family denominated MYST (for its founding members MOZ, Ybf2/Sas3, Sas2, and Tip60). In particular, mCLOCK shows high sequence similarity to yeast Esa1 and other MYST members, including yeast Sas3, fly MOF, and human Tip60. Importantly, the same residues that have been demonstrated by crystal structure analysis of the Esa1 protein to be involved in acetyl-CoA interaction (Yan et al., 2000) are shared with mCLOCK (Figure 3A, indicated by open circles above the sequence). It is significant that these residues are all fully conserved in CLOCK proteins of various species (Figure 1B). One remarkable feature of the motif A in CLOCK, when compared to MYST family members, is the insertion of five amino acids—also fully conserved among species (Figure 1B). As compared to Esa1, the additional five amino acids would lengthen the loop comprised between β9 and α3, a region that is demonstrated to be exposed at the protein surface

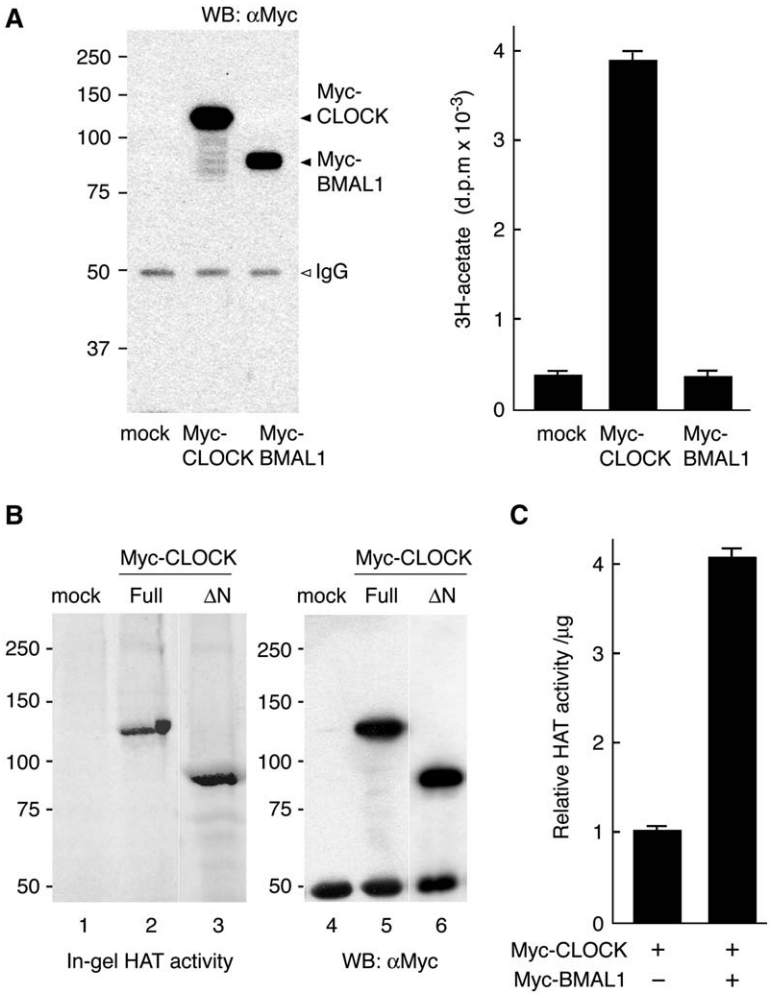

Figure 2. HAT Activity Displayed by Immunoprecipitated Myc-mCLOCK

(A) Myc-mCLOCK-specific immunoprecipitation of HAT activity. Myc-mCLOCK or Myc-mBMAL1 were transiently expressed in JEG3 cells and then immunoprecipitated with anti-Myc 9E10 antibody. After extensive washing, the resulting immunoprecipitates were incubated with [^{3}H] acetyl-CoA and a mixture of histone H3 and H4 amino-terminal tail peptides. The incorporated [^{3}H] acetate was detected by filter binding assays. As a control, cells transfected with an empty vector (mock) were also subjected to the immunoprecipitation-HAT assay. Representative Western blot, illustrating the protein levels of the immunoprecipitated Myc-tagged proteins, is shown on the left. Data shown are the means ± range of variation from two independent experiments.

(B) In-gel HAT activities of Myc-CLOCK. Either a full-length (Full) or an N-terminally truncated (ΔN) mCLOCK protein was expressed in JEG3 cells and immunoprecipitated as described in (A). The immunoprecipitates were resolved on a 7.5% SDS-PAGE gel containing core histones and processed to detect acetyltransferase activity (left). Identical immunoprecipitated samples were electrophoresed in a parallel SDS-PAGE gel and immunoblotted with anti-Myc 9E10 antibody (right).

(C) Positive effect of BMAL1 coexpression on CLOCK HAT activity. Myc-mCLOCK was expressed in JEG3 cells in the presence or absence of Myc-BMAL1. Immunoprecipitation-HAT assays were done as described in (A). The relative amount of Myc-CLOCK protein added to each HAT reaction was determined by immunoblot analyses with anti-Myc 9E10 antibody. The HAT activities shown were normalized with the amounts of Myc-CLOCK protein after subtraction of the background HAT activities derived from the mock immunoprecipitates. Data shown are the means ± range of variation from two independent experiments.

(Yan et al., 2000). Predictions indicate that changes in the length of these loops can be well accommodated without gross perturbation of the protein structure (Protein Data Bank, http://rutgers.rcsb.org/pdb/), yet they might be associated to differences in the physiological function of these HAT molecules. Intriguingly, the putative acetyl-CoA binding motif in *Drosophila* CLOCK lacks the five extra amino acids present in the vertebrate counterparts (Figure 3A), which increases its similarity to Esa1 and other MYST family members. Finally, a number of acetyl-CoA binding motifs from various *N*-acetyltransferases also show similarities.

Mutations in the Motif A Reduce HAT Activity

To validate the functional role of the acetyl-CoA binding motif, we generated a mCLOCK mutant with a short deletion that removes part of the homology (ΔA, residues 656–665, indicated by a horizontal line above the sequence in Figure 3A). We assayed purified recombinant proteins generated with the baculovirus system (Figure 3B). Full-length mCLOCK and the mutant mCLOCKΔA were tagged with hexahistidine residues together with a FLAG epitope (His-FLAG-mCLOCK). Expression in Sf9 cells was in combination with nontagged mBMAL1. We found that mCLOCK is not readily solubilized because of protein aggregation, an effect significantly decreased by the presence of mBMAL1 (data not shown). Recombinant proteins were first purified by Ni-NTA beads and further purified over FLAG antibody affinity beads. Tandem affinity-purified proteins were subjected to HAT assays as well as SDS-PAGE followed by silver staining. Equivalent levels of soluble His-FLAG-mCLOCK and His-FLAG-mCLOCKΔA proteins were both purified as heterocomplexes with nontagged mBMAL1 (Figure 3B, left). HAT assays showed that His-FLAG-mCLOCK exhibited significant acetyltransferase activity toward free core histones but not bovine

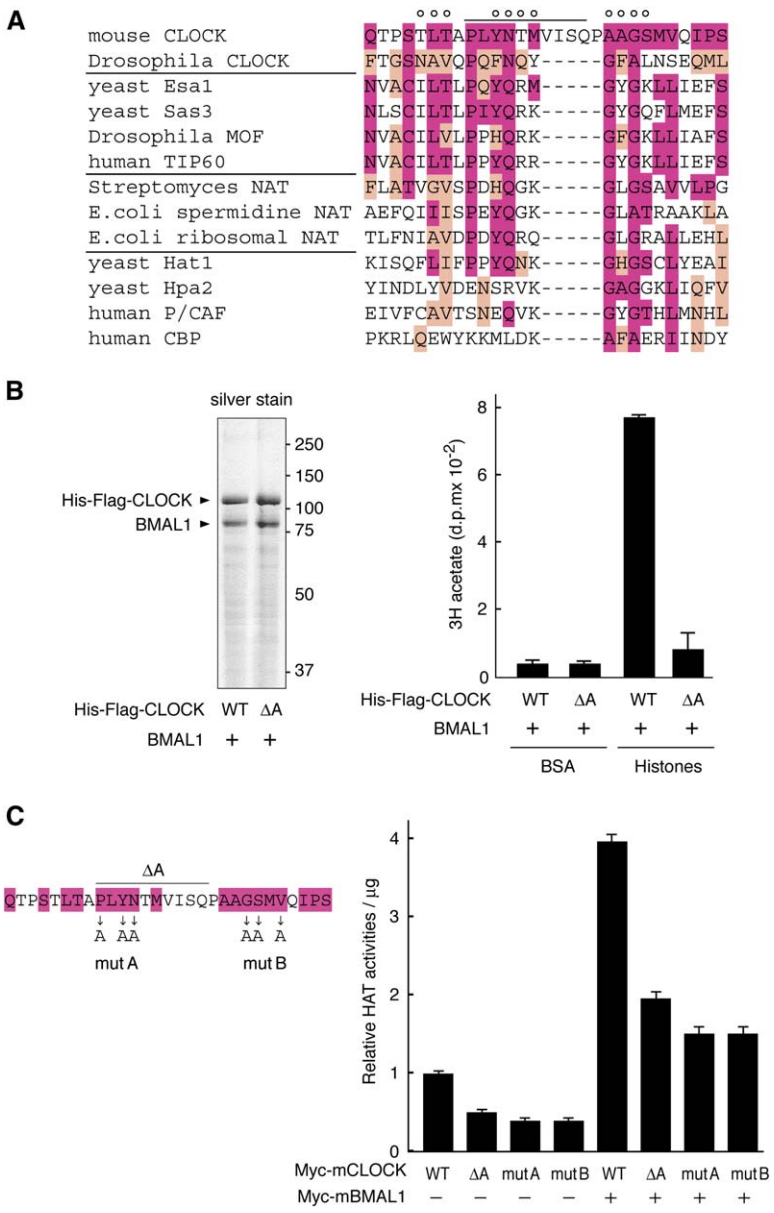

Figure 3. CLOCK Has an Acetyl-CoA Binding Motif Required for HAT Activity

(A) Sequence alignment of CLOCK with acetyl-CoA binding motifs from various acetyltransferases. Identical or chemically similar residues to mouse CLOCK are shown with red backgrounds. Residues shared only with *Drosophila* CLOCK are shown with yellow backgrounds. Both mCLOCK and dCLOCK show sequence similarity to the acetyl-CoA binding motifs of MYST family of HATs (yeast Esa1, yeast Sas3, *Drosophila* MOF, and human TIP60) and some of the *N*-acetyltransferases (*Streptomyces* puromycin NAT, *E. coli* spermidine NAT, *E. coli* ribosomal NAT). Open circles above the sequence indicate the residues interacting with acetyl-CoA in crystallized Esa1 protein (Yan et al., 2000), which shows the highest similarity to mCLOCK. A horizontal line above the sequences indicates the residues deleted in mCLOCKΔA mutant protein.

(B) HAT activity displayed by baculovirus-generated CLOCK protein. Either His-FLAG-mCLOCK or His-FLAG-mCLOCKΔA was expressed in Sf9 cells together with non-tagged mBMAL1. The recombinant proteins were first purified by Ni-NTA beads and further purified over FLAG antibody affinity beads. The tandem affinity-purified proteins were separated on a 13% SDS-PAGE gel and subjected to silver staining (left). The affinity-purified recombinant proteins were incubated with either free core histones or BSA in the presence of [^{3}H] acetyl-CoA. The incorporated [^{3}H] acetate was detected by filter binding assays (right). Data shown are the means ± range of variation from two independent experiments.

(C) HAT activities of motif A mutants. Immunoprecipitation-HAT assays were done with Myc-mCLOCK mutants (mut A and mut B) carrying mutation at the indicated residues in the motif A (left). The HAT activities were determined by filter binding assays as described in Figure 2C (right). Reduced HAT activities were obtained from the mutants (mut A and mut B) as well as Myc-mCLOCKΔA. Data shown are the means ± range of variation from two independent experiments.

serum albumin (Figure 3B, right). In contrast, His-FLAG-mCLOCKΔA displayed a drastically reduced HAT activity, although its heterodimerization capacity with BMAL1 was intact (Figure 3B, left). These results indicate that the integrity of the acetyl-CoA binding motif is required for the HAT enzymatic activity of mCLOCK.

The reduction in HAT activity of mCLOCKΔA mutant was confirmed using proteins immunoprecipitated from cultured cells (Figure 3C). Myc-mCLOCKΔA exhibited a significant reduction of enzymatic activity, either in the presence or absence of Myc-mBMAL1 (Figure 3C). To identify specific residues essential for HAT function, we generated two additional mutants of the motif A. Each mutant carries three single amino acid substitutions into alanine of the highly conserved residues implicated in the acetyl-CoA interaction (mut A and mut B, Figure 3C, left). HAT assays demonstrated that both mutants display drastically reduced enzymatic activity, comparable to the mCLOCKΔA mutant. All together these data show that the integrity of the motif A within the MYST homology stretch of mCLOCK is required for HAT enzymatic activity.

Specificity of CLOCK-Mediated Acetylation

Next, we wished to establish the identity of the histones that are acetylated by mCLOCK. To do so, HAT assays using either free core histones or mononucleosomes were performed and the reaction products analyzed on SDS-PAGE (Figure 4A). The mCLOCK protein acetylated primarily histones H3 and H4 on both free histone and mononucleosomes, demonstrating a significant degree

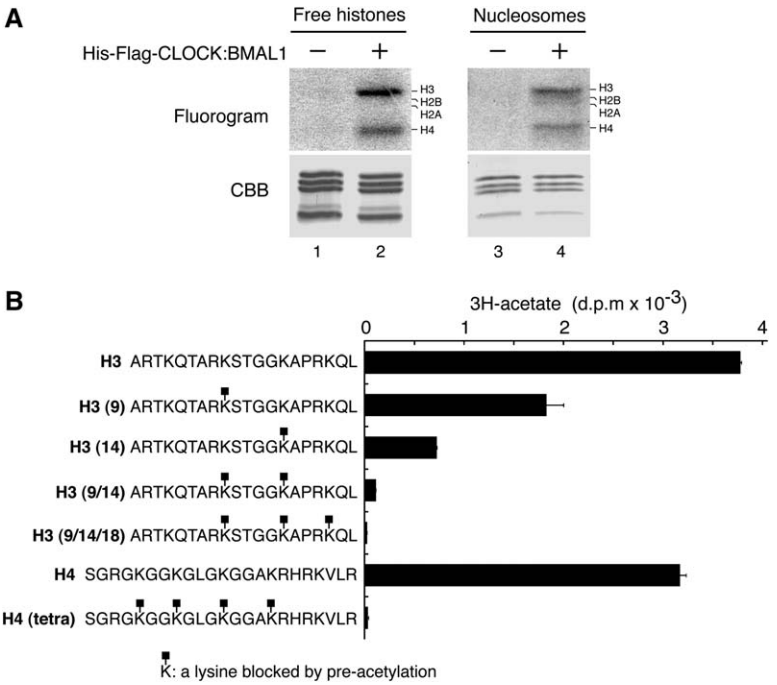

Figure 4. Acetylation Profiles of mCLOCK

(A) Substrate specificity of His-FLAG-mCLOCK:mBMAL1. Free core histones (lanes 1 and 2) or mononucleosomes (lanes 3 and 4) were incubated with BSA (lanes 1 and 3) or recombinant His-FLAG-mCLOCK:mBMAL1 (lanes 2 and 4). [^{14}C]-acetylated histones were resolved by SDS-PAGE, and the gels were stained with Coomassie brilliant blue (CBB) and analyzed by fluorography.

(B) Acetylation site specificity. Filter binding assays were done as described in (A), except that synthetic histone amino-terminal peptides shown on the left were utilized as substrates. Sites where ε-*N*-acetyllysine was incorporated during peptide synthesis in order to mimic sites that are acetylated in vivo are indicated by closed boxes. Data shown are the means ± range of variation from two independent experiments.

of specificity. Interestingly, this substrate specificity is analogous to that of ACTR (Chen et al., 1997), extending the similarity between the two proteins from structural (Figure 1) to functional.

Specificity of CLOCK enzymatic activity was then investigated by using H3 and H4 tails with preacetylated lysines. In this approach, putative HAT substrate sites are occupied, resulting in a block of potential de novo acetylation (Mizzen et al., 1996). Our results determined that histone H3 Lys-14, and in a lesser extent Lys-9, are the major sites acetylated by mCLOCK (Figure 4B). Additional support to this finding is provided by chromatin immunoprecipitation assays described below (Figure 5C). Block of multiple putative lysines in either H3 and H4 results in a complete lack of acetylation, supporting the notion that these histones are natural CLOCK targets. As acetylation at H3 Lys-14 has been intimately coupled to transcriptional activation (Fischle et al., 2003), our findings stress the positive role that the HAT function of CLOCK plays within the clock mechanism.

The HAT Function of CLOCK Is Essential for Circadian Regulation

We next wanted to establish whether the HAT function of CLOCK is required for circadian rhythmicity (Figure 5). As experimental system we used mouse embryonic fibroblast (MEF) cells derived from homozygous *Clock* mutant (*c/c*) mice (Vitaterna et al., 1994). As *Clock* is essential for circadian rhythm (Antoch et al., 1997; King et al., 1997), MEF *c/c* cells show no cyclic expression of clock genes (Pando et al., 2002). We first tested whether ectopic expression of mCLOCK is able to rescue the circadian expression of endogenous target genes. MEF *c/c* cells stably transfected with a mCLOCK expression plasmid were subjected to a serum shock, a stimulus commonly used to trigger circadian gene transcription in a variety of cell lines (Balsalobre et al., 1998; Pando et al., 2002). While the MEF *c/c* cells had no functional circadian clock, ectopic expression of mCLOCK restored circadian expression of the endogenous *mPer1* gene ~20 hr after the serum shock (Figure 5B, left). Similarly, circadian expression of *Dbp*, an E box-regulated circadian output gene, was also rescued (Figure 5B, right). Rescue of circadian transactivation in MEF *c/c* cells was lesser when compared to wild-type MEF cells (see Figure S1 in the Supplemental Data available with this article online). This is most likely due to a semi-dominant-negative effect of the mutant CLOCK protein endogenously expressed in MEF *c/c* cells (Vitaterna et al., 1994; Yoo et al., 2005). As predicted (Gekakis et al., 1998), ectopic expression of a mCLOCK mutant carrying the deletion of exon 19 (Δ19) resulted in no complementation (Figure S2).

These cell-based studies enabled us to analyze the involvement of the HAT function in the chromatin-based transcriptional control of clock genes. We found that the ectopic expression of HAT-deficient mCLOCK (mCLOCK-mut A, Figure 5B, and mCLOCKΔA, Figure S2) failed to restore the circadian transactivation of *mPer1* and *Dbp*. Importantly, the lack of rescuing by mCLOCK-mut A was associated to a significant reduction of histone H3 acetylation on the *mPer1* promoter (Figure 5C). Chromatin immunoprecipitation (ChIP) assays established that H3 acetylation at lysines K9/K14 on the *mPer1* promoter was elevated at the transcription-active time point (16 hr post-serum shock) in the MEF c/c cells ectopically expressing wild-type mCLOCK (Figure 5C, lane 10).

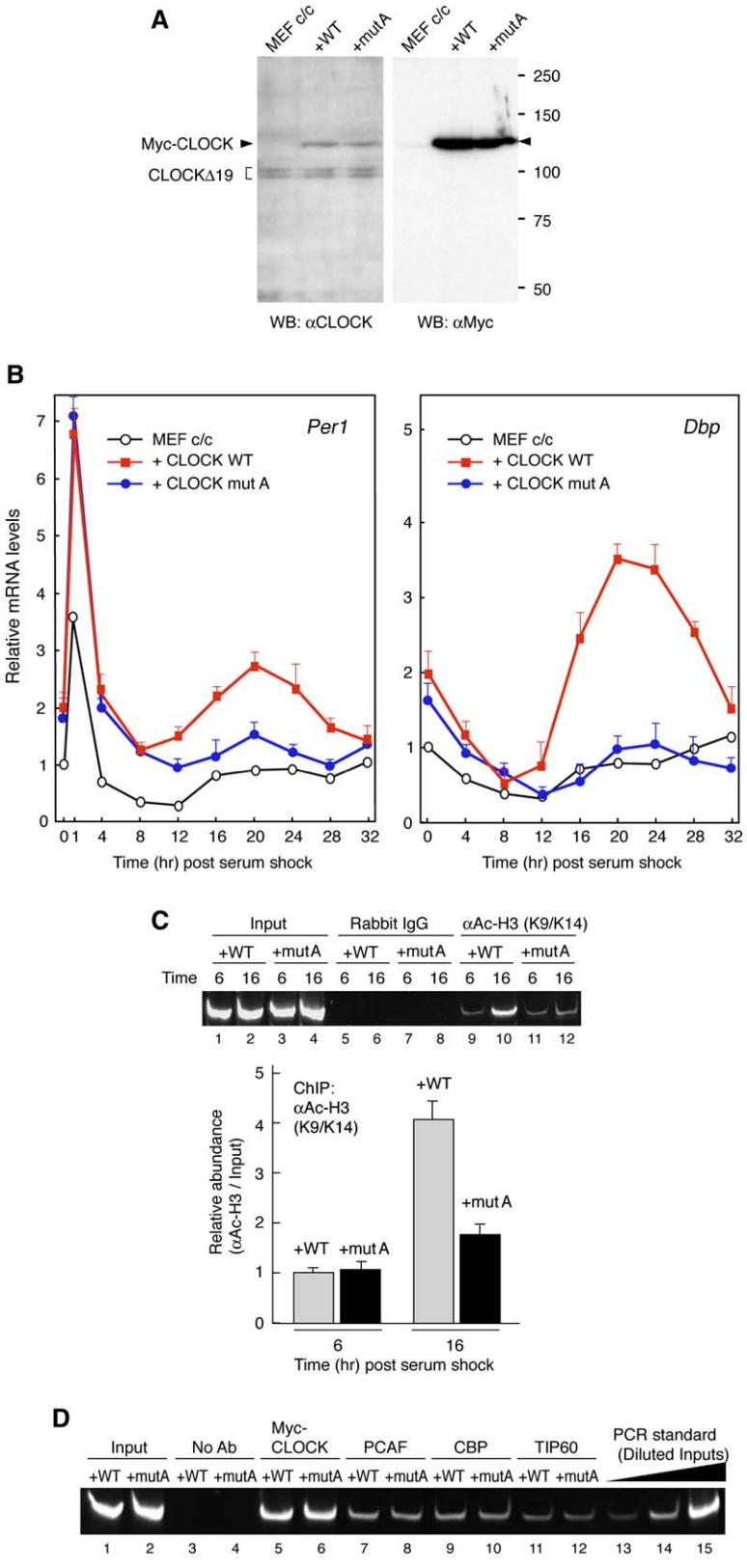

Figure 5. Ectopic Expression of mCLOCK mut A Is Unable to Complement Circadian Expression of *mPer1* and *Dbp* in *c/c* MEFs

(A) Establishment of stable cell lines. MEF c/c cells were stably transfected with either a wild-type (+wt) or mutant (+mutA) form of Myc-mCLOCK. Shown are immunoblot analyses with anti-mCLOCK antibody (left) or anti-Myc 9E10 antibody (right). Arrowheads indicate the ectopically expressed proteins, whose levels were equivalent to the endogenously expressed mCLOCKΔ19 mutant.

(B) Serum shock-dependent circadian transactivation of *mPer1* and *Dbp*. Cells were subjected to a 2 hr exposure to 50% horse serum and then placed in 0.5% serum medium. Total RNA was isolated from the cells harvested at the indicated time points, and expression levels of *mPer1* and *Dbp* were estimated by quantitative real-time PCR. Values were normalized to the expression of *Sumo-3*, a nonoscillating gene (Cardone et al., 2005), and plotted as relative fold of expression at time 0 (set as 1) in *Clock c/c* MEFs. Mean values of four independent experiments are shown, with range variations (SD).

(C) Histone H3 acetylation at *mPer1* promoter. MEF c/c cells stably expressing a wild-type (+wt) or mutant (+mut A) form of myc-mCLOCK were harvested at 6 and 16 hr post-serum shock and subjected to ChIP assays. Shown are representative results from quantitative PCR analysis of inputs (lanes 1–4) and DNA immunoprecipitated with normal rabbit IgG (lanes 5–8) and antiacetylated histone H3 at lysines 9 and/or 14 (lanes 7–12). Relative PCR values of acetyl H3 were normalized to the inputs and the value of time 6 in the +wt cells was set to 1. Plotted values are the mean ± SD from three independent experiments.

(D) Recruitment of CLOCK and coactivators to the E box of *mPer1* promoter. The indicated cells were harvested 16 hr post-serum shock and subjected to ChIP assays. Shown are representative results from quantitative PCR analysis using inputs (lanes 1 and 2) and DNA immunoprecipitated with anti-Myc (lanes 5 and 6), anti-PCAF (lanes 7 and 8), anti-CBP (lanes 9 and 10), and anti-TIP60 (lanes 11 and 12). The samples incubated without antibody (lanes 3 and 4) gave no PCR product. PCR analyses were done with a linear correlation between the amplified products and the starting amounts of template DNA (lanes 13–15).

However, in cells ectopically expressing mCLOCK-mut A, the H3 acetylation was largely reduced (Figure 5C, lane 12). The observed loss of complementation in circadian transactivation and H3 acetylation was not attributed to differences in the protein expression or DNA binding capacity. Indeed, similar levels of wild-type and mutant proteins were detected in the cell lysates (Figure 5A), and also mBMAL1expression levels were comparable in all cells tested (Figure S2). ChIP assays demonstrated that the efficiency of mCLOCK recruiting to the E box of *mPer1* promoter, as compared to the mutated CLOCK proteins, is basically equivalent (Figure 5D, lanes 5 and 6). Finally, the single amino acid substitutions in mCLOCK-mut A, although drastically reducing HAT activity, have no effect on the association with other transcriptional coactivators, including CBP, PCAF, and TIP60. Indeed, coimmunoprecipitation assays demonstrated that mutations in the motif A did not reduce the capacity of CLOCK to form a complex with CBP, PCAF, and TIP60 (Figure S3). Furthermore, unimpaired recruitment of CBP, PCAF, and TIP60 coactivators to the E box of *mPer1* promoter was confirmed by ChIP assays (Figure 5D, lanes 7–12). These data strongly indicate that the HAT activity of CLOCK is essential for circadian control of *mPer1* and *Dbp* genes.

DISCUSSION

Our study establishes that CLOCK, a master controller of circadian rhythms, directly modifies chromatin. By demonstrating that CLOCK possesses intrinsic enzymatic HAT activity, we provide the first evidence that control of chromatin remodeling constitutes a key regulatory step governing the circadian clock machinery.

Paradoxical to the central role played by CLOCK in the rhythmic transcription of the clock-controlled genes, expression of CLOCK is described to be nearly constitutive (Lee et al., 2001; Ripperger and Schibler, 2006). Recent data using chromatin immunoprecipitation assays also show that CLOCK-containing transcriptional complexes bind to E box elements constitutively over the circadian cycles (Lee et al., 2001; Yoo et al., 2005). Our finding of the enzymatic activity intrinsic to mCLOCK challenges a view where the CLOCK:BMAL1 heterodimer functions as a constitutive structural component bound to E box enhancers. Rather, CLOCK may exhibit a regulatable HAT activity that would confer dynamic changes to the local chromatin environment (Figure 6). One intriguing possibility could involve circadian time-specific control of the CLOCK HAT activity by other circadian clock components, such as the heterodimeric partner BMAL1 and/or the PER-CRY negative complex. Indeed, our results indicate that BMAL1 potentiates the HAT function of CLOCK (Figure 2). We have noted that a bacterially expressed GST-CLOCK is prone to aggregation with little HAT activity (data not shown) and that formation of heterocomplexes with BMAL1 drastically increases solubility and in parallel HAT activity (Figures 2 and 3). This would suggest

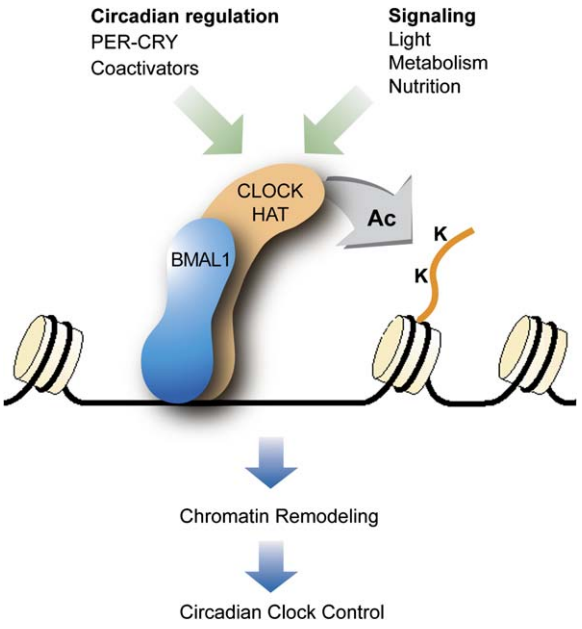

Figure 6. Schematic Model of CLOCK-Mediated Histone Acetylation and Its Role within the Physiological Pathways of Circadian Rhythmicity

The HAT function of CLOCK activity is enhanced by BMAL1, its natural heterodimerization partner with which it binds to E box promoter elements within clock gene promoters (such as *per1*). Acetylation by CLOCK, for example at H3 Lys-14, is thought to elicit chromatin remodeling by inducing a transcription-permissive state. Transcription mediated by the CLOCK:BMAL1 complex has been shown to be stimulated by other coactivators, such as CBP. Thereby we envisage a scenario where circadian control of chromatin remodeling by CLOCK may be influenced by the dynamic assembly of a multiprotein regulatory complex. It is also important to note that metabolic, nutritional, and environmental circadian cues are likely to modulate the HAT function of CLOCK.

that BMAL1 may critically contribute to structural conformational changes of CLOCK, a scenario reminiscent of several other HAT proteins that exhibit full activity only when functioning within a large protein complex in vivo (Sterner and Berger, 2000).

The HAT function of CLOCK is compatible with previous studies supporting the recruitment of other HAT coactivators to the CLOCK:BMAL1 complex (Curtis et al., 2004; Etchegaray et al., 2003). Rather, different types of HATs such as ACTR, CBP, and PCAF have been shown to associate in unique complexes in vivo (Chen et al., 1997; Yang et al., 1996), suggesting that a combination of distinct HAT activities may crucially contribute to orchestrate transcriptional processes in a spatiotemporal specialized manner. Our results indicate that the HAT activity of CLOCK is essential for temporally regulated transactivation (Figure 5B) and histone H3 acetylation (Figure 5C). The concerted contribution of another coactivator(s) is likely to integrate the complex signaling that governs chromatin remodeling and the circadian clock machinery.

CLOCK shares homology to the HAT domain of ACTR, a member of SRC family of HAT proteins (Figure 1). Notably, these two proteins show marked similarity in regions outside of the carboxy-terminal domain, displaying a strikingly analogous organization (Figure 1A). CLOCK and all the members of SRC family (SRC-1, TIF-2, and ACTR) share a highly conserved bHLH-PAS domain at the amino termini. These common features appear to define a specialized class of histone acetylases with a HAT domain conjugated to a bHLH-PAS domain. However, the bHLH-PAS domain of ACTR is thought to have no DNA binding activity (Chen et al., 1997), and a functional contribution of the bHLH-PAS domain to the ACTR HAT activity has not been reported. The possibility of a direct crosstalk between the bHLH-PAS and HAT domains necessitates further investigation within this class of HAT proteins.

Interestingly, the in vitro DNA binding activity of CLOCK:BMAL1 heterodimer is enhanced in the presence of a reduced form of nicotinamide adenine dinucleotide cofactor through a mechanism mediated by the bHLH-PAS domain (Dioum et al., 2002; Rutter et al., 2001). This could suggest a NAD(H)-dependent positive effect on the HAT function of CLOCK:BMAL1. While this possibility still needs to be explored, here it is important to recall the case of transcriptional silencing mediated by NAD(+)-dependent HDACs (histone deacetylases; Imai et al., 2000; Landry et al., 2000). Intriguingly, Sir2, a NAD(+)-dependent HDAC, has been functionally linked to Sas2 (Kimura et al., 2002; Suka et al., 2002), a protein of the MYST family of HATs to which CLOCK belongs.

The molecular and physiological implications of our finding seem numerous. For example, the HAT function of CLOCK could be regulated by intracellular signaling pathways, thereby connecting chromatin remodeling to circadian physiological response (Figure 6). In addition, the compelling links that exist between circadian cycle and metabolism (Rutter et al., 2002; Schibler and Sassone-Corsi, 2002; Turek et al., 2005) suggest that the HAT function of CLOCK may be controlled by changing cell energy levels, or conversely, could regulate them. In this respect, it is notable that CLOCK was found to interact with some nuclear receptors, including RARα and RXRα (McNamara et al., 2001), and that periodic availability of nuclear hormones has been implicated in the resetting of peripheral clocks.

In conclusion, we have identified a novel type of DNA binding HAT protein, CLOCK, whose activity is required for circadian clock function. Future work aimed at deciphering the rules governing the in vivo dynamic change of histone acetylation exerted by CLOCK during the day-night cycle will provide essential new insights into the links between signaling and the circadian clock. Furthermore, our studies may provide additional leads for therapeutic strategies. By controlling its HAT enzymatic activity, CLOCK would make an ideal target for pharmaceutical compounds influencing circadian rhythms, sleep, and jet lag, as well as other physiological and metabolic processes under circadian regulation.

EXPERIMENTAL PROCEDURES

Plasmids

Construction of FLAG-mCLOCK/pSG5 was described (Travnickova-Bendova et al., 2002). Myc-mCLOCK/pSG5 was made by replacing the FLAG epitope in FLAG-mCLOCK/pSG5 with six copies of Myc epitope. Myc-mCLOCKΔN was made by deletion of a DNA fragment encoding the N-terminal part of mCLOCK (residues 1–242) from Myc-mCLOCK/pSG5. To construct a deletion mutant of the motif A (ΔA), a DNA fragment encoding the residues 1–665 of mCLOCK in Myc-mCLOCK/pSG5 was replaced with a PCR-amplified DNA fragment encoding the residues 1–655. Myc-mCLOCK mutants with three residues mutated in the motif A (mut A and mut B) were created by using QuickChange site-directed mutagenesis kit (Stratagene). Construction of Myc-mBMAL1/pCS2 was described (Travnickova-Bendova et al., 2002). All the Myc-tagged proteins contain six copies of Myc epitope at the amino termini.

Antibodies and Immunoblot

Generation of anti-BMAL1 antibody was described (Cardone et al., 2005). The other antibodies were either gifts or commercial products; anti-CLOCK, anti-GCN5, and anti-TIP60 were provided by U. Schibler (University of Geneva, Switzerland), L. Tora (Institut de Génétique et de Biologie Moléculaire et Cellulaire, France), and B. Amati (European Institute of Oncology, Italy), respectively. Purchased were anti-CBP (A-22, Santa Cruz), anti-p300 (N-50, Santa Cruz), anti-PCAF (E-8, Santa Cruz), and anti-Myc (9E10, Transduction Laboratories). Immunoblot analyses were performed as described (Doi et al., 2004). Proteins samples resolved by SDS-PAGE were immunoblotted, and the imunoreactivities were visualized by enhanced chemiluminescence system (NEN) using horseradish peroxidase-conjugatged anti-immunoglobulin (Kirkegaard & Perry Laboratories).

Expression and Immunoprecipitation of Myc-mCLOCK

JEG3 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum. Cells plated in a 10 cm dish were transfected with the indicated combination of plasmids by using Fugene (Roche). The cells were harvested 36 hr posttransfection and lysed in 800 μl of solution-I that contains 10 mM NaCl in IP buffer (15 mM HEPES-NaOH [pH 7.8], 10% glycerol, 2 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 50 mM NaF, 1 mM sodium vanadate, 1 mM sodium phosphate [pH7.8], 2 mM PMSF, 1× protease inhibitor cocktail). After centrifugation of the lysates at 700 × g for 10 min, the insoluble precipitants were subjected to further extraction with 800 μl of solution-II that contains 500 mM NaCl in IP buffer. The soluble protein extracts in solution-I and solution-II were mixed (1:1) and subjected to immunoprecipitation. After preclearing with 30 μl of protein G-Sepharose beads, the extract mixture was incubated with 2.5 μg of anti-Myc 9E10 antibody and 20 μl of protein G-Sepharose beads at 4°C for 3 hr. The beads were then rinsed five times with a washing buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM dithiothreitol, 0.2% Nonidet P-40, 50 mM NaF, 1 mM sodium vanadate, 2 mM PMSF, 1× protease inhibitor cocktail), followed by two washes with HAT buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF). After the final wash, the buffer was aspirated down to 25 μl and subjected to HAT assays as described below. The relative amount of Myc-CLOCK protein added to each reaction was determined by immunoblot analyses with anti-Myc antibody.

Expression and Affinity Purification of His-FLAG-mCLOCK

Construction of His-FLAG-mCLOCK was done by cloning a FLAG-tagged full-length mCLOCK fragment into pAcSG-His NT (Pharmingen), resulting in introduction of double tags (a hexa-histidine tag and a FLAG epitope) at the amino terminus of mCLOCK. Similarly, His-FLAG-mCLOCKΔA/pAcSG was constructed by using a FLAG-tagged mCLOCKΔA fragment. Neither the hexa-His tag nor the

FLAG epitope was introduced into mBMAL1/pAcSG construct. Sf9 cells were cultured in Grace's medium with 10% FBS. Infection was done using either His-FLAG-mCLOCK or His-FLAG-mCLOCKΔA recombinant baculovirus together with mBMAL1 recombinant baculovirus. The cells were harvested 48 hr postinfection. Recombinant proteins extracted as described above were first purified by Ni-NTA beads (Qiagen) and further purified over M2-agarose (Sigma) according to the manufacturer's protocol.

Histone Acetylase Activity Assays

HAT assays were performed as described (Mizzen et al., 1999) by using either 50 μg of calf thymus core histones (type IIA; Sigma), 5 μg of HeLa mononucleosome core particles (Loury and Sassone-Corsi, 2004), or 50 μg of synthetic histone amino-terminal peptides (H3, residues 1–20; H4, residues 1–24), in the presence of 50 pmol of [^{3}H]acetyl-CoA (4.3 Ci/mmol, Amersham Life Science) or 300 pmol of [^{14}C]acetyl-CoA (55 mCi/mmol, Amersham Life Science). The histone amino-terminal peptide derivatives carrying the preacetylated lysine residues were also synthesized. Enzymatic reaction was done using 1 pmol of His-FLAG-mCLOCK protein copurified with mBMAL1, and the HAT activity was determined by liquid scintillation counting of aliquots of the reaction mixture spotted onto P-81 filters (Whatman). For identification of acetylated proteins, aliquots of reaction mixtures (2 μg of calf thymus core histones or 0.5 μg of HeLa mononucleosome) were resolved on 14% SDS-PAGE gels and analyzed by fluorography (Amplify, Amersham Life Science). HAT activity in gel assays were carried out as described (Brownell et al., 1999), except that the electrophoresis of immunoprecipitated proteins was done by using a cathode reservoir buffer supplemented with 0.1 mg/ml calf thymus core histones, and [^{14}C]-acetylated histones were visualized by phosphorimaging with the aide of a bioimaging analyzer BAS2000 (Fuji Film).

Establishment of Stable Cell Lines and mRNA Expression Analysis

MEF cells established from homozygous *Clock* mutant mice were transfected with a linearized plasmid encoding neomycin and Myc-tagged mCLOCK. We used an SV40 promoter for the ectopic expression, as the endogenous *mClock* gene shows a nearly constitutive expression over the circadian cycles. Lipofection was done by using jetPEI (Polyplus-transfection) according to the manufacturer's protocol. Two days later, G-418 (Life Technologies) was added at a final concentration of 350 μg/ml. After 3 weeks of selection, approximately 100 resistant colonies per transfection were visible. Colonies were trypsinized and propagated as a single pool, and then pools of clones were analyzed. Serum-shock experiments were done as described (Pando et al., 2002). Confluent cells were kept for 2 days in medium containing 0.5% serum. At t = 0, 50% horse serum was added to the medium, which was replaced with DMEM containing 0.5% serum after 2 hr. At the indicated times, total RNA was extracted from the cells by using RNA-Solv (Omega Biotek). Relative mRNA levels of *mPer1*, *Dbp*, and *Sumo-3* were evaluated by quantitative real time PCR (RT-PCR) as described (Cardone et al., 2005).

Chromatin Immunoprecipitation (ChIP) Analysis

ChIP assays were performed as described (Hirayama et al., 2005). Immunoprecipitation of the crosslinked chromatin-protein complexes was done with the following antibodies: anti-acetylated H3 histone at lysines 9 and/or 14 (Upstate Biotechnology), anti-CBP (A-22), anti-TIP60 (a gift from B. Amati), anti-PCAF (H-369, Santa Cruz), and anti-Myc (9E10). PCR analyses of the 5′-flanking region of *mPer1* gene were done by using a primer set described previously (Etchegaray et al., 2003; Lee et al., 2001), and SYBR Green I-based quantitative PCR analyses were done as described (Doi et al., 2001). PCR was performed for 23 cycles, a condition optimized for quantitative analyses. The PCR products were subjected to 6% polyacrylamide gel electrophoresis, stained with SYBR Green I (Molecular Probes), and then detected with an image analyzer ChemiGenius XE (SYNGENE). The amounts of the products with the predicted size were quantified with the GeneTool software (SYNGENE).

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.cell.com/cgi/content/full/125/3/497/DC1/.

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