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Posttranslational Mechanisms Regulate the Mammalian Circadian Clock

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Summary

We have examined posttranslational regulation of clock proteins in mouse liver in vivo. The mouse PERIOD proteins (mPER1 and mPER2), CLOCK, and BMAL1 undergo robust circadian changes in phosphorylation. These proteins, the cryptochromes (mCRY1 and mCRY2), and casein kinase I epsilon (CKIε) form multimeric complexes that are bound to DNA during negative transcriptional feedback. CLOCK:BMAL1 heterodimers remain bound to DNA over the circadian cycle. The temporal increase in mPER abundance controls the negative feedback interactions. Analysis of clock proteins in mCRY-deficient mice shows that the mCRYs are necessary for stabilizing phosphorylated mPER2 and for the nuclear accumulation of mPER1, mPER2, and CKIε. We also provide in vivo evidence that casein kinase I delta is a second clock relevant kinase.

Introduction

Circadian rhythms are generated by genetically determined biological clocks (Dunlap, 1999). In mammals, the circadian timing system is hierarchical with the main clock located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Reppert and Weaver, 2001). The SCN are entrained (synchronized) to the 24 hr day by the daily light-dark cycle acting through both direct and indirect retina to SCN neural pathways. The master clock, in turn, synchronizes circadian oscillators in peripheral tissues through neural and/or humoral outputs (Ueyama et al., 1999; Balsalobre et al., 2000; McNamara et al., 2001).

The clockwork in the mouse involves interacting positive and negative transcriptional/posttranslational feedback loops (reviewed in Reppert and Weaver, 2001). Key components of this mechanism are two basic helix–loop–helix (bHLH)/PAS-containing transcription factors, CLOCK and BMAL1 (King et al., 1997; Gekakis et al., 1998; Hogenesch et al., 1998; Bunger et al., 2000). CLOCK:BMAL1 heterodimers drive the rhythmic transcription of three *Period* genes (*mPer1–mPer3* in the mouse) and two *Cryptochrome* genes (*mCry1* and *mCry2*). As the mPER and mCRY proteins are translated, they form PER:CRY complexes that are translocated to the nucleus. In the nucleus, the mCRY proteins act as negative regulators by directly interacting with CLOCK and/or BMAL1 to inhibit transcription, forming a negative feedback loop (Griffin et al., 1999; Kume et al., 1999; van der Horst et al., 1999; Vitaterna et al., 1999; Shearman et al., 2000b). *Bmal1* RNA levels are also rhythmic, antiphase to those for the *mPer* and *mCry* genes (see Reppert and Weaver, 2001). Genetic data are consistent with a model in which mPER2 rhythmically stimulates *Bmal1* transcription, forming a positive feedback loop (Zheng et al., 1999; Shearman et al., 2000b). mPER1 appears to influence clock function at a posttranscriptional level through interaction with other circadian regulatory proteins (Bae et al., 2001; Zheng et al., 2001). mPER3 does not have a critical role in the maintenance of the core clock feedback loops, but instead may function as an output signal (Shearman et al., 2000a; Bae et al., 2001). Recent in vitro studies suggest that the transcriptional activity of the CLOCK:BMAL1 heterodimer can be modulated directly by nuclear hormone receptors and redox potential (McNamara et al., 2001; Rutter et al., 2001).

Phosphorylation can determine the cellular location and stability of clock proteins, and is a critical process for building time delays into the 24 hr molecular mechanism (Edery et al., 1994; Dunlap, 1999; Young, 2000; Denault et al., 2001). In *Drosophila*, DOUBLETIME (DBT), which is most closely related to mammalian casein kinase I epsilon (CKIε), phosphorylates PER, thereby influencing PER turnover (Price et al., 1998). Studies of the *tau* mutation in Syrian hamsters (a spontaneous, semidominant mutation leading to marked shortening of the circadian period) reveal that it encodes a missense mutation within CKIε that renders the mutant enzyme deficient in its ability to phosphorylate the mPER proteins (Lowrey et al., 2000). Remarkably, a human genetic disorder characterized by shortened circadian period and advanced sleep phase is associated with a missense mutation in human PER2, and the mutant protein is less effectively phosphorylated by CKIε in vitro (Toh et al., 2001).

Even though genetic studies show that phosphorylation is important for the normal functioning of the mammalian circadian clock, there has been virtually no investigation of the changes in phosphorylation relevant for clock function in vivo. We have developed novel antisera and examined the temporal dynamics of all 7 known clock proteins (mPER1, mPER2, mCRY1, mCRY2, CLOCK, BMAL1, and CKIε). The results show robust circadian patterns of clock protein abundance, phosphorylation, interactions, and subcellular location that appear critical for transcriptional feedback. Collectively, they show that posttranslational alterations in clock proteins regulate their activities and play a vital role in the maintenance of the mammalian clock.

Results

Clock Gene Expression Profiles in Liver

We focused our studies on liver in which temporal changes in clock protein biochemistry can be readily

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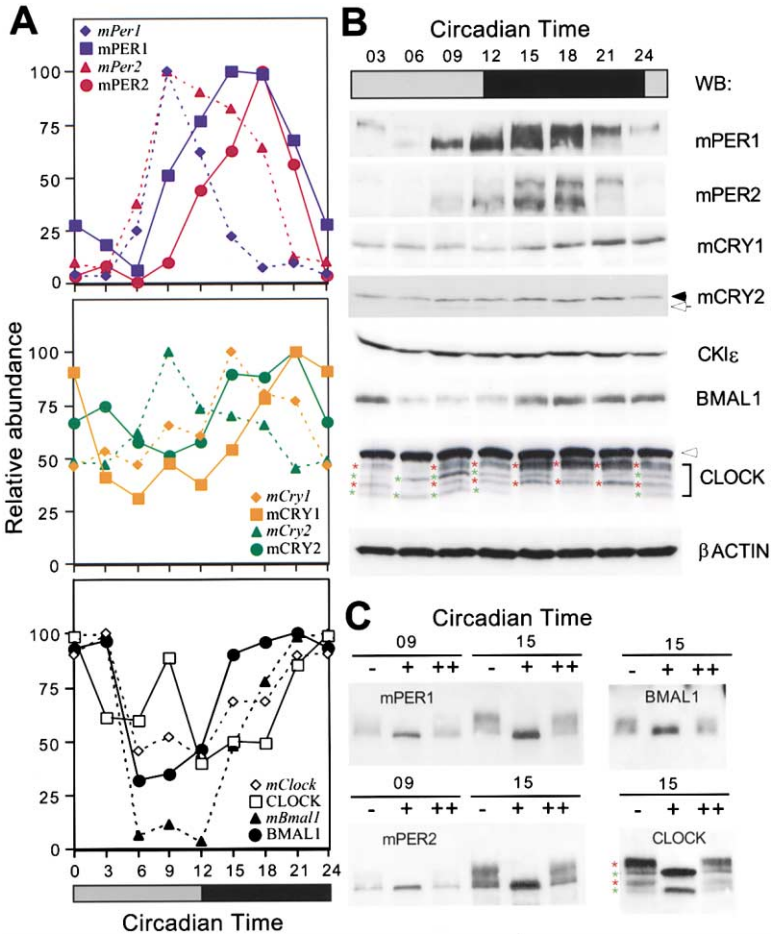

Figure 1. Clock Gene RNA and Protein Rhythms in Mouse Liver

(A) Quantitative analysis of clock gene RNA and protein rhythms. RNA levels (dashed lines) were examined by RNase protection assay at 3 hr intervals over the first day in constant darkness. Each value was normalized to β actin and converted to percentage of maximal level for each gene. Mean values for two separate experiments are shown. Protein abundance (solid lines) was examined by quantitative Western blots of liver. Each value was converted to percentage of maximal level for each protein and is the mean of 3–5 experiments. No value varied from the mean by more than 30%. The values at CT 0/24 are double plotted.

(B) Circadian profiles in clock protein abundance and electrophoretic mobility in liver. Total extracts were examined by Western blots (WB) for clock proteins and βactin on the first day in constant darkness. For CLOCK, the green asterisks indicate nonphosphorylated isoforms, while the red asterisks denote phosphorylated isoforms (see C). The nonspecific band (open arrowhead) is representative of total protein (data not shown) and was used as a loading control. Even loading of proteins across circadian time was verified with β actin.

(C) Changes in clock protein mobility are due to changes in phosphorylation. Immune complexes for mPER1, mPER2, BMAL1, and CLOCK were untreated (−), or treated with either phosphatase alone (+) or both phosphatase and vanadate (40 mM) (++). mPER1 and and mPER2 immune extracts were examined at CT 09 and CT 15, while BMAL1 and CLOCK extracts were examined at CT 15.

monitored in vivo. We first evaluated clock gene RNA levels in liver by RNase protection assay.

mPer1, *mPer2*, *mCry1*, and *mCry2* each exhibited a circadian rhythm in RNA levels during the first day in constant darkness. For *mPer1*, *mPer2,* and *mCry2*, RNA levels peaked at CT 9, whereas *mCry1* RNA levels peaked at CT 15 (Figure 1A, dashed lines). The amplitude of the *mCry* RNA rhythms was <5% that of the *mPer1* and *mPer2* oscillations. *Bmal1* and *Clock* RNA oscillations were antiphase to those of the *mPer* and *mCry2* RNA rhythms, with peak values from CT 21 to CT 3. These clock gene expression patterns are similar to those reported previously in liver and skeletal muscle (Balsalobre et al., 1998; Zylka et al., 1998; Kume et al., 1999; Miyamoto and Sancar, 1999). The coordinated reduction in *mPer* and *mCry2* RNA levels between CT 12 and CT 21 identifies this period as being dominated by transcriptional inhibition.

Clock Proteins Exhibit Circadian Rhythms in Abundance and Phosphorylation

To biochemically characterize clock proteins in vivo, we generated specific antisera against fusion protein fragments derived from mPER1, mPER2, BMAL1, CLOCK, and CKIε. Commercially available antisera were used to examine mCRY1 and mCRY2. The specificity of each antiserum was characterized (see Supplemental Figure S1, available online at http://www.cell.com/cgi/content/full/107/7/855/DC1).

PER1 and mPER2 levels showed striking temporal changes in both abundance and electrophoretic mobility (Figure 1B)(quantitated in Figure 1A, solid lines) over the circadian cycle. Low abundance occurred at CT 06. Both abundance and low mobility forms increased with time, with each reaching a maximum at CT 15 and 18. At these times, the immunoreactive bands for both mPER1 and mPER2 were broad, with each spanning 25 kDa. From CT 21–03, abundance decreased, with the apparent higher molecular weight forms predominating; this was especially apparent for mPER1 (Figure 1B). Similar temporal changes in mPER abundance and electrophoretic mobility were found in kidney, cerebellum, and the SCN (data not shown; Supplemental Figure S2, see above URL).

Both mCRY1 and mCRY2 also showed circadian changes in abundance in liver, but these rhythms were of smaller amplitudes than those exhibited by the mPER proteins (Figures 1A and 1B). The timing of the mCRY2 protein rhythm was similar to that found for the mPER proteins (Figure 1A). For mCRY1, the timing of the rhythm was delayed, with peak abundance occurring 6 hr later (CT 21) than peak abundance for the mPER and mCRY2 rhythms. The delayed timing of the mCRY1 rhythm parallels the delayed phase of the *mCry1* RNA rhythm (Figure 1A). Neither mCRY1 nor mCRY2 showed

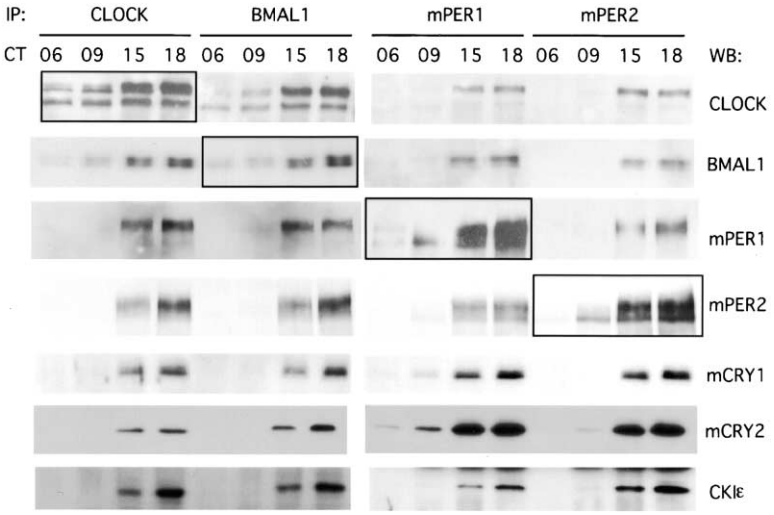

Figure 2. Clock Proteins Form Time-Specific Multimeric Complexes

Liver extracts from CT 06, 09, 15, and 18 were immunoprecipitated (IP) with antibodies against CLOCK, BMAL1, mPER1, or mPER2. Immune complexes generated by each antibody were then Western blotted (WB) and probed for all 7 proteins. Boxed lanes indicate the primary immunoprecipitated proteins.

detectable temporal changes in electrophoretic mobility.

CKIε was moderately expressed in liver, but did not show a circadian rhythm in abundance or detectable changes in electrophoretic mobility (Figure 1B).

BMAL1 exhibited a daily rhythm in both abundance and electrophoretic mobility, with each peaking from CT 15–03 (Figure 1B). At the peak times, the mobility change was due to an increase in abundance of lower molecular weight forms. The increase in abundance of both the lower and higher molecular weight species contributed to the overall increase in BMAL1 abundance. Surprisingly, the lowest level of BMAL1 occurred at CT 06, the time predicted for maximal transcriptional enhancement of *mPer1* and *mPer2* (Figures 1A and 1B). Only the high molecular weight form was apparent at this time (confirmed below).

For CLOCK, there was no consistent circadian change in overall protein abundance (Figures 1A and 1B). There was, however, a clear change in the abundance of individual isoforms of CLOCK separable by their electrophoretic mobility (Figure 1B). At CT 06, bands of 120 kDa and 105 kDa predominated (green asterisks); these bands are specific *Clock* gene products because both were appropriately decreased in size in liver extracts from homozygous *Clock/Clock* mutant mice (Supplemental Figure S1, available online at http://www.cell.com/cgi/content/full/107/7/855/DC1). The top band (120 kDa) runs close to the size of full length, in vitro translated CLOCK, and the lower band (105 kDa) likely represents an alternatively spliced product (see King et al., 1997). At CT 09, two additional CLOCK bands first became apparent (red asterisks), one larger than the 120 kDa band and one between the 120 and 105 kDa bands; these two, newly formed bands became the predominant species from CT 15–21. At CT 24 and 03, all 4 bands were again clearly expressed.

To determine whether the mobility changes in mPER1, mPER2, BMAL1, and CLOCK are due to changes in phosphorylation, liver extracts were incubated with the appropriate antisera and the immune complexes were treated with lambda protein phosphatase. For mPER1, mPER2, and BMAL1, phosphatase treatment changed >90% of the high molecular weight forms to a single lower molecular weight band (Figure 1C) that migrated with a molecular weight similar to the in vitro translated protein. For CLOCK, phosphatase treatment at CT 15 resolved the 4 bands into two—the 120 and 105 kDa species. Treatment of immune complexes with phosphatase and vanadate, a phosphatase inhibitor, prevented the phosphatase-induced mobility changes for all of the clock proteins examined, confirming that they were indeed due to phosphorylation (Figure 1C). It is possible that the mCRY proteins and CKIε are also phosphorylated (e.g., CKIε is autophosphorylated in vitro; Lowrey et al., 2000), but this was not detected as a shift in electrophoretic mobility on Western blots.

Phosphorylated Multimeric Complexes Correlate with Negative Transcriptional Regulation

We examined in vivo protein interactions over the circadian cycle by incubating liver extracts with clock protein antisera and probing the resulting immune complexes for each of the 7 clock proteins by Western blot analysis. These studies focused on the times before (CT 06 and 09) and during (CT 15 and 18) expected peaks in negative feedback.

Immunoprecipitated CLOCK showed daily changes in electrophoretic mobility pattern similar to those found in total extracts (Figure 2). Each of the other proteins (BMAL1, mPER1, mPER2, mCRY1, mCRY2, and CKIε) copurified with CLOCK at CT 15 and CT 18. Both nonphosphorylated and phosphorylated BMAL1 copurified with CLOCK at CT 15 and 18, with reduced amounts of BMAL1 also detected at CT 6 and 9. Predominantly hyperphosphorylated mPER1 was copurified, while both hypo- and hyperphosphorylated mPER2 were coprecipitated. The mPERs, mCRYs, and CKIε were not detected in CLOCK-containing immune complexes at CT 06 and 09.

Immunoprecipitated BMAL1 also showed daily changes in abundance and phosphorylation similar to those described for total extracts (Figure 2). CLOCK, mPER1, mPER2, mCRY1, mCRY2, and CKIε each copurified with BMAL1 in a time-dependent manner, with the mPERs, mCRYs, and CKIε in the immune complexes at

CT 15 and 18, but not detected at CT 6 and 9. Again, predominantly phosphorylated mPER1 and mPER2 were coprecipitated. Both phosphorylated and nonphosphorylated forms of CLOCK coprecipitated with BMAL1.

Immunoprecipitated mPER1 and mPER2 both showed the same temporal pattern as in total extracts, with prominent daily changes in abundance and mobility (Figure 2). The other 6 clock proteins copurified with each of the mPER proteins in a time-dependent manner, with the most prominent interactions, once again, at CT 15 and 18. Interestingly, only the phosphorylated form of full-length CLOCK copurified with the mPER proteins. Both nonphosphorylated and phosphorylated BMAL1 copurified with the mPER proteins, with an apparent preference for phosphorylated BMAL1. Once again, predominantly phosphorylated mPER1 and mPER2 coprecipitated with each other. Immunoprecipitated mCRY1, mCRY2, and CKIε coimmunoprecipitated each of the other 6 proteins at CT 15 and 18, as found for mPER1 and mPER2 (Supplemental Figure S3A, available online at http://www.cell.com/cgi/content/full/107/7/855/DC1; data not shown). The specificity of these multimeric clock proteins interactions in vivo was verified (see Supplemental Figure S3B, see above URL).

The results suggest that all 7 clock proteins associate as multimeric complexes in a time-dependent manner, with complexes most abundant at CT 15 and 18 (Figure 2; also see Supplemental Figure S3A). Both phosphorylated and nonphosphorylated forms of CLOCK and BMAL1 coprecipitate with each other, whereas mainly the phosphorylated forms of the mPER proteins copurify with these transcription factors. But it appears to be mainly the more highly phosphorylated, full-length form of CLOCK that interacts with the mPER and mCRY proteins, and CKIε, since this was the major form that coprecipitated with mPER1, mPER2, mCRY1, mCRY2, and CKIε (Figure 2, S3A; data not shown). Thus, phosphorylation may be important for the formation of protein complexes that inhibit CLOCK:BMAL1-mediated transcription.

Clock Protein Complexes Occur Predominantly in the Nucleus

If multimeric, phosphorylated complexes are important for transcriptional inhibition, they should be found in the nucleus. We therefore used cell fractionation and Western blot analysis to examine the cellular location of the 7 clock proteins over the circadian cycle.

There was a striking circadian rhythm in the nuclear localization of mPER1, mPER2, mCRY1, mCRY2, and CKIε, with the proteins detected in the nucleus predominantly at CT 12, 15, 18, and 21 (Figure 3). Moreover, it was mainly the highly phosphorylated forms of mPER1, mPER2, CLOCK, and BMAL1 that were located in the nucleus at these circadian times (Figure 3, right panel), correlating with the coimmunoprecipitation experiments (Figure 2). These data, along with the results of experiments using gel filtration chromatography (see Supplemental Figure S4), suggest that all 7 clock proteins are complexed together in the nucleus at the time of negative transcriptional regulation.

mCRY1, mCRY2, and CKIε were predominantly expressed in the cytoplasm at all times examined, and mCRY1 and mCRY2 levels in cytoplasm exceeded even the peak levels expressed in the nucleus. These results suggest that the mCRY proteins are highly expressed throughout the circadian cycle and that their rhythmic presence in the nucleus is not determined simply by cytoplasmic availability; that is, nuclear entry is regulated (see below). In contrast, mPER1 abundance in the nucleus was correlated with mPER1 in cytoplasm, and the temporal alteration in mobility suggests that phosphorylation of mPER1 is associated with its nuclear location.

CLOCK: BMAL1 Are Bound to DNA throughout the Circadian Cycle

We next combined subcellular fractionation with immunoprecipitation to obtain a more refined view of the relationship between phosphorylation and the DNA binding activity of CLOCK:BMAL1 heterodimers. Cytoplasmic and nuclear extracts were treated with antibodies against CLOCK or BMAL1 and the immune complexes were probed for interacting proteins. We examined CT 06 and CT 18, times of apparent maximal transcriptional enhancement and inhibition, respectively.

At CT 06, all 4 forms of CLOCK were immunoprecipitated in the nucleus, while only the nonphosphorylated bands were precipitated from the cytosol (Figure 4A). Phosphorylated BMAL1 was immunoprecipitated almost exclusively in the nucleus, and all 4 forms of CLOCK coprecipitated with BMAL1 in the nucleus. At CT 18, only the phosphorylated forms of CLOCK were immunoprecipitated in the nucleus, with the hyperphosphorylated full-length form predominating (Figure 4A). Phosphorylated CLOCK copurified with phosphorylated BMAL1, as well as nonphosphorylated BMAL1.

We next examined whether the phosphorylation of CLOCK alters its ability to bind DNA. For this study, formaldehyde crosslinked chromatin from liver nuclear extracts (collected at CT 06 and CT 15) was Western blotted. Histone H3 served as a positive control for chromatin-bound protein; it was indeed chromatin-bound, and not detected in the remaining soluble fraction (data not shown). UAP56, a nuclear-bound factor involved in pre mRNA splicing by the spliceosome, served as a negative control for nuclear proteins not bound to chromatin; UAP56 was present in total nuclear extracts, but was not detected in the chromatin-bound material (Figure 4B). Both the phosphorylated and nonphosphorylated forms of full-length CLOCK were bound to chromatin at CT 06, with only the phosphorylated forms bound at CT 15 (Figure 4B). Thus, phosphorylation does not substantially alter CLOCK's ability to bind DNA.

To determine whether the multimeric clock protein complexes were associated with DNA-bound CLOCK at the time of negative transcription regulation, crosslinked chromatin from liver extracts obtained at CT 6 and CT 15 was immunoprecipitated with CLOCK and the immune complexes were Western blotted. The mPER and mCRY proteins coprecipitated with chromatin-bound CLOCK at CT 15, but not at CT 06 (Figure 4C). The apparent lower levels of the mPER and mCRY proteins detected in the CLOCK-chromatin complex at CT 15, compared

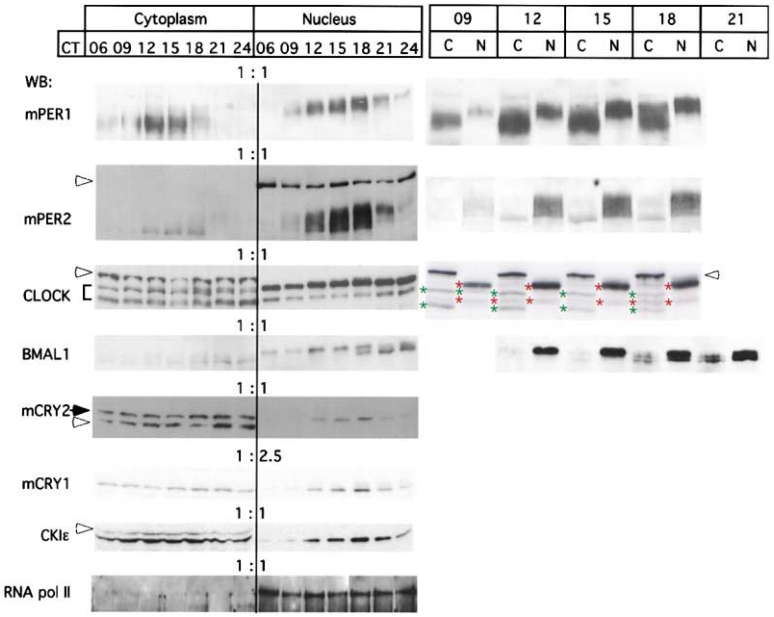

Figure 3. Clock Proteins Exhibit Marked Circadian Changes in Nuclear Abundance

Liver extracts from designated circadian times (CT) were fractionated, and the cytoplasmic (C) and nuclear (N) fractions were Western blotted (WB) and probed for the 7 clock proteins and RNA polymerase II. The amount of nuclear proteins was derived from either the same number of cells (1:1) or 2.5-fold more cells (1:2.5) relative to the corresponding cytoplasmic fraction. Right hand panel shows a side by side comparison of mPER1, mPER2, CLOCK, and BMAL1 in cytoplasm and nucleus at times of maximal nuclear expression. For CLOCK, the green asterisks indicate nonphosphorylated isoforms, while the red asterisks denote phosphorylated isoforms. Open arrowheads, nonspecific bands.

to those complexed with CLOCK in total liver extracts (Figure 4C), probably reflects incomplete protein:protein crosslinking with formaldehyde.

It is important to note that most of the CLOCK or BMAL1 immunoprecipiated from the nucleus at CT 18 copurified the other member of the CLOCK:BMAL1 heterodimer (Figure 4A). This suggests that the CLOCK:BMAL1 heterodimer is intact during negative transcriptional feedback. Thus, transcription inhibition could involve disruption of interactions between the heterodimer and DNA (Lee et al., 1999), rather than disruption of CLOCK:BMAL1 heterodimers. But our experiments with chromatin-bound CLOCK suggested another alternative—CLOCK:BMAL1 heterodimers remain constitutively bound to DNA throughout the circadian cycle, with negative regulation occurring through rhythmic interactions of the DNA-bound heterodimer with negative regulators. The proposed constitutive binding of CLOCK:BMAL1 to DNA was further evaluated by polymerase chain reaction (PCR) analysis of the DNA immunoprecipitated with the anti-CLOCK antibody. For this analysis, we used PCR primers flanking each of the three CACGTG E boxes in the *mPer1* promoter, because CLOCK:BMAL1 heterodimers can bind to each of the three *mPer1* E boxes and activate transcription in vitro, and the first three E boxes are sufficient to generate circadian rhythmicity in reporter gene expression in vivo (Gekakis et al., 1998; Kulman et al., 2000).

PCR analysis of the DNA in the CLOCK-chromatin complex showed that CLOCK is indeed bound specifically to *mPer1* E boxes at both CT 06 and CT 15 (results from 2nd *mPer1* E box shown in Figure 4D, lanes 6 and 7). Using anti-BMAL1 antibodies to immunoprecipitate crosslinked chromatin, we found that BMAL1 was also specifically bound to the E boxes at both circadian times (Figure 4D, lanes 8 and 9). The *mPer1* E box was not amplified when crosslinked chromatin was immunoprecipitated with a nonspecific (HA) antibody (Figure 4D, lanes 10 and 11). In addition, primers that amplify a DNA fragment from a gene not regulated by CLOCK (mammalian *Timeless*) did not amplify DNA from crosslinked chromatin immunoprecipitated by the anti-CLOCK antibody (Figure 4D, lanes 14 and 15). Semiquantitative PCR analysis of *mPer1* E box-bound CLOCK and BMAL1 at CT 03, 09, 15, and 21 showed that both transcription factors are indeed bound constitutively to the E box throughout the circadian cycle, with no large fluctuations in binding (Figure 4E).

mPER Abundance Is Rate Limiting for Initiating Negative Feedback

To further delineate features that might contribute to nuclear translocation of clock protein complexes, we determined the absolute concentrations of the 7 clock proteins in liver (see Experimental Procedures and Supplemental Figure S5, available online at http://www.cell.com/cgi/content/full/107/7/855/DC1). The abundance of mCRY1 and mCRY2 in total extracts was in great molar excess of mPER protein levels throughout the circadian cycle (Figure 5A). Furthermore, the mCRY proteins are present in the cytoplasm throughout the circadian cycle (Figure 3), with only a minority of total mCRY1 and mCRY2 actually found in the nucleus (Figure 5B). Together, these observations suggest that the level of the mPER proteins is rate limiting for PER:CRY heterodimerization and subsequent transport into the nucleus.

To test whether the mPER proteins do regulate the nuclear translocation of the mCRY proteins, we examined the subcellular location of the mCRY proteins in liver extracts from *mPer1/mPer2* double-mutant mice at CT 18. In the mutant animals, which lack mPER1 and functional mPER2 (Bae et al., 2001; Supplemental Figure S1, see above URL), Western analysis of fractionated liver extracts showed that mCRY1 and mCRY2 were prominent in the cytoplasm, but only weakly detected in the nucleus (Figure 5C). Thus, the temporal increase in abundance of the mPER proteins appears to be critical for mCRY:mPER interactions and nuclear translocation.

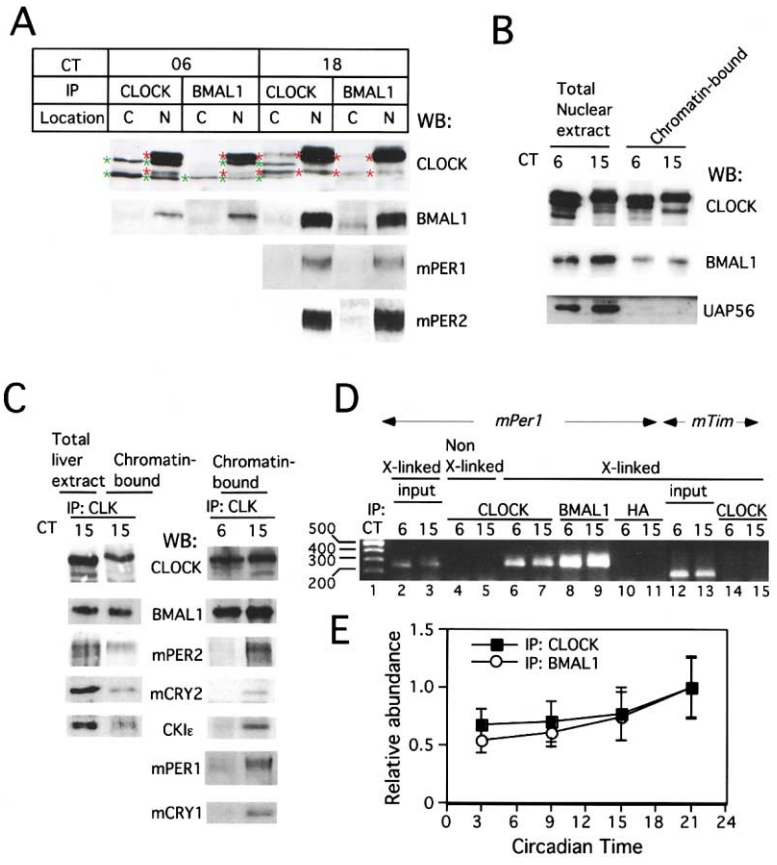

Figure 4. CLOCK:BMAL1 Complexes Are Bound to DNA during Negative Feedback

(A) CLOCK- and BMAL1-associated interactions at times of presumed transcriptional enhancement (CT 06) and transcriptional inhibition (CT 18). Liver extracts were fractioned, and the cytoplasmic (C) and nuclear (N) fractions were immunoprecipitated (IP) with antibodies against CLOCK or BMAL1. The immune complexes were Western blotted (WB) and probed for CLOCK, BMAL1, mPER1, and mPER2.

(B) CLOCK and BMAL1 are bound to chromatin. Nuclear extracts and chromatin from livers collected at CT 06 and CT 15 were Western blotted and probed for CLOCK, BMAL1, and UAP56.

(C) Clock protein complexes are associated with chromatin-bound CLOCK at the time of presumed transcriptional inhibition. Chromatin from livers collected at CT 06 and CT 15 was immunoprecipitated with antibodies against CLOCK. The immune complexes were Western blotted and probed for the 7 clock proteins. At CT 15, the chromatin data (2nd lane) was compared to immunoprecipitation of total liver extracts (1st lane).

(D) CLOCK and BMAL1 are bound to *mPer1* E box at CT 06 and 15. DNA from either crosslinked (X-linked) or noncrosslinked (Non X-linked) chromatin was subjected to PCR using primers flanking the 2nd *mPer1* E box (lanes 2–11) or the *mTim* gene (lanes 12–15). Lane 1: DNA ladder (in nt); lanes 2, 3, 12, and 13: input genomic DNA from crosslinked chromatin. Similar results were found using PCR primers flanking each of the other two E box elements in the *mPer1* promoter (data not shown).

(E) CLOCK and BMAL1 are bound to the *mPer1* E box over the circadian cycle. Crosslinked chromatin was immunoprecipitated (IP) with antibodies against CLOCK or BMAL1. DNA was amplified using primers flanking the 2nd *mPer1* E box using a semiquantitative PCR method. The data were normalized to the input control and plotted as percentages relative to the highest value (100%). The results are mean ± SE of three experiments.

CKIε was also in molar excess of mPER1 and mPER2 in liver (Figure 5A), even though CKIε abundance is much lower in liver than brain (data not shown). This suggests that mPER levels, not CKIε levels, limit mPER phosphorylation by the kinase in the liver.

When Western blots of nuclear protein abundance were quantified, it was evident that mPER1, mPER2, mCRY2, and even mCRY1 and CKIε enter the nucleus synchronously (Figure 5D). This synchrony occurred irrespective of the temporal profiles of protein abundance observed in total cell extracts (e.g., the delayed rhythmic profile of mCRY1 and lack of rhythmicity for CKIε). The only change in protein abundance that correlates with this influx of negative regulators to the nucleus is the rhythmic appearance of mPER (Figures 1 and 3).

We also found that the abundance of CLOCK was in molar excess of BMAL1. Although BMAL1 appears to be rate limiting for heterodimer formation with CLOCK, there are clearly other events, such as phosphorylation and association with other clock proteins that may be important for regulating transcriptional activity of the heterodimer.

Protein Function Deficits in mCRY-Deficient Mice

Having established the time course of clock protein phosphorylation, interactions, and subcellular localization in wild-type mice, we examined these events in mCRY-deficient animals. Previous studies of mCRY-deficient mice have shown that the RNA levels of *mPer1* and *mPer2* are arrhythmic and maintained at moderate levels in the SCN and high levels in liver (Okamura et al., 1999; Vitaterna et al., 1999). Assessment of nuclear mPER protein levels in the SCN by immunocytochemistry (ICC) has revealed differential and reduced abundance of mPER1 and mPER2 in mCRY-deficient mice (Shearman et al., 2000b; Yagita et al., 2000). As detailed below, Western blot analysis of liver extracts provides a more mechanistic explanation for the mPER changes induced by mCRY deficiency.

mPER1 abundance in mCRY-deficient mice was comparable at CT 6 and 18, and similar to the high protein levels seen at CT 18 in wild-type mice (Figure 6A). Moreover, the presence of highly phosphorylated mPER1, normally seen at CT 18 in wild-type mice, was clearly apparent at both clock times in total extracts from the mutant animals. Subcellular fractionation studies showed that mPER1 levels were actually increased in the cytoplasm of mCRY-deficient mice, relative to the amount of cytoplasmic mPER1 normally found at CT 18 (Figure 6B). The cytoplasmic increase in mPER1 in mCRY-deficient livers was likely due to a defect in the nuclear transport of mPER1, because only a very small

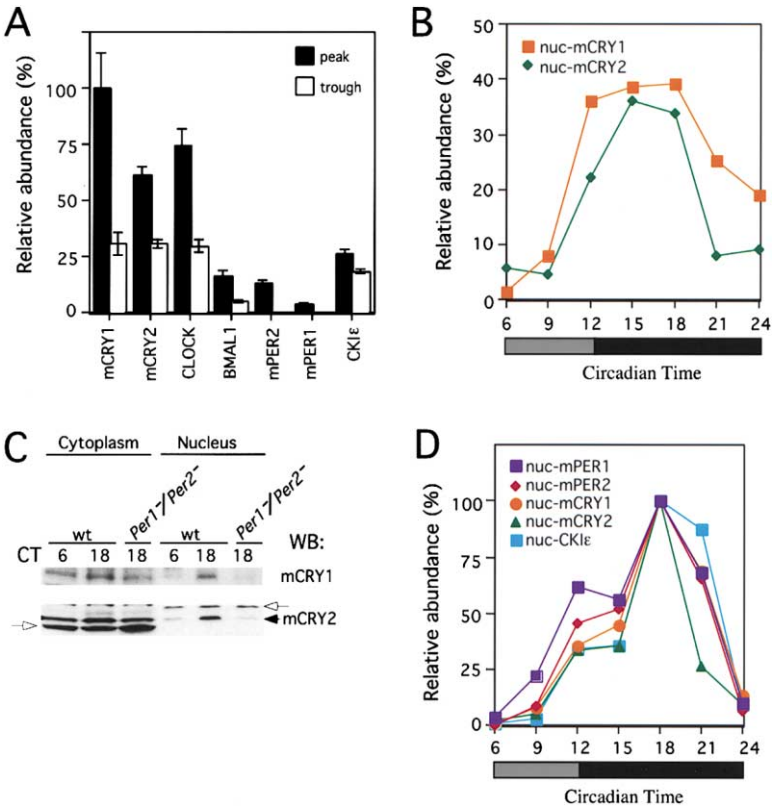

Figure 5. mPER Proteins Are Limiting

(A) Molar concentrations of clock proteins in whole-cell extracts. Peak and trough values of each protein are indicated. The values were obtained from Figure 1 and Figure S5 (see Experimental Procedures). The 100% value is 0.29 fmol. The results are mean ± SE of three experiments.

(B) Relative nuclear concentrations of the mCRY proteins. The Western blots for mCRY in Figure 3 were quantitated by densitometery. The proportion of nuclear mCRY was determined by dividing the nuclear signal by the total (nuclear plus cytoplasmic) signal.

(C) mCRY proteins cannot effectively translocate to the nucleus without the mPER proteins. Liver extracts from wild-type (wt) and *mPer1/mPer2* double mutant mice were fractioned. The cytoplasmic and nuclear fractions were Western blotted (WB) and probed for mCRY1 and mCRY2. Open arrowheads, nonspecific bands.

(D) Nuclear abundance of clock proteins. The Western blots for nuclear fractions of mPER, mCRY, and mCKIε in Figure 3 were quantitated relative to the nonspecific band on the mPER2 blot, which served as a loading control. Each value was converted to percentage of maximal level for each nuclear protein.

portion of hyperphosphorylated protein was found in the nucleus of the mutant mice (Figure 6B). At CT 18, mPER1 abundance in wild-type liver was distributed equally between cytoplasm and nucleus, with the more highly phosphorylated forms localized to the nuclear compartment. Therefore, the mCRY proteins do not appreciably influence mPER1 expression, phosphorylation, or stability per se. What is defective in the absence of mCRY proteins is the ability of mPER1 to assume a nuclear location.

Similar to the ICC findings in the SCN, mPER2 abundance was greatly reduced in the livers of mCRY-deficient mice (Figure 6A). Subcellular fractionation studies showed that the vast majority of mPER2 in the mutant mice was cytoplasmic (Figure 6B). With longer exposures, it was possible to detect some mPER2 in the nucleus (Figure 6B). Importantly, less mobile (hyperphosphorylated) forms were found in both the cytoplasm and nucleus with longer film exposure, showing that mPER2 can be phosphorylated in mCRY-deficient animals. Assuming that mPER2 synthesis is high in the mutant animals because of the high RNA levels, it would appear that mPER2 can be phosphorylated in the mCRY-deficient mice, but that the protein is very unstable in the absence of interaction with the mCRY proteins.

The marked decrease in mPER1 and mPER2 levels in the nucleus of mCRY-deficient mice was also associated with a marked decrease in nuclear CKIϵ abundance (Figure 6B). Like mPER1 abundance, CKIϵ levels were actually increased in the cytoplasm of mCRY-deficient livers (Figure 6B), suggesting that the mCRYs are also important for nuclear translocation of CKIϵ, perhaps by stabilization of an mPER:CKIϵ complex. Immunoprecipitated CKIϵ coprecipitated only hyperphosphorylated mPER1, but both hypo- and hyperphosphorylated forms of mPER2 (Figure 6C). The same associations occurred in the livers of mCRY-deficient mice, but at reduced levels. Thus, the mCRY proteins are not necessary for CKIϵ:mPER associations.

There was also a decrease in mCLOCK levels and phosphorylation in mCRY-deficient animals (Figure 6A). The mCLOCK banding pattern on Western blots of total extracts from mCRY-deficient mice revealed mainly the nonphosphorylated CLOCK isoforms, similar to that seen at CT 6 in wild-types (Figure 6A). In the nucleus, however, both phosphorylated and nonphosphorylated CLOCK were present in mCRY-deficient animals (Figure 6D). This contrasts with CT 18 in which phosphorylated full-length CLOCK is the major form normally found in the nucleus (Figures 3 and 6D). BMAL1 levels were substantially reduced in mCRY-deficient mice, consistent with the low BMAL1 RNA levels reported in these animals (Shearman et al., 2000b). Only phosphorylated BMAL1 was found in the nucleus of CRY-deficient mice (Figure 6D).

We also used immunoprecipitation to increase the signal/noise ratio to examine the importance of the mCRY proteins for formation of PER/CLOCK complexes. When nuclear mPER1 immune complexes were examined for CLOCK in mCRY-deficient mice, the transcription factor was not detected, providing strong evidence against direct PER:CLOCK interactions (Figure 6E). This data provides direct in vivo evidence that CRY proteins are necessary for mPER1, and probably mPER2, associations with CLOCK. This concurs with protein:protein interaction data in yeast in which direct

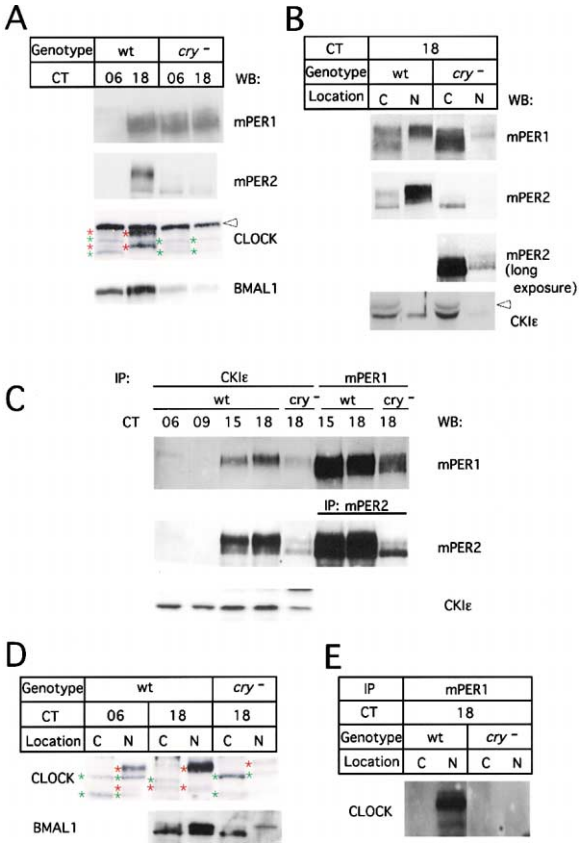

Figure 6. Clock Protein Deficits in mCRY-Deficient Mice

(A) Alterations in mPER1, mPER2, CLOCK, and BMAL1 expression in mCRY-deficient mice. Liver extracts from wild-type (wt) or mCRY-deficient (*cry*−) mice collected at CT 06 and 18 were Western blotted (WB) and probed for mPER1, mPER2, CLOCK, and BMAL1. For CLOCK, the green asterisks indicate nonphosphorylated isoforms, while the red asterisks denote phosphorylated isoforms. Open arrowhead, nonspecific band that served as loading control.

(B) Alterations in the subcellular localization of mPER1, mPER2, and CKIe in mCRY-deficient mice. Liver extracts collected at CT 18 were fractionated, and the cytoplasmic (C) and nuclear (N) fractions were Western blotted (WB) and probed for mPER1, mPER2, and CKIε. For the mPER2 blot from mCRY-deficient mice, two exposures of the same blot are shown: 2 min (above) and 20 min (below).

(C) The mCRYs are not necessary for CKIε:mPER associations. At designated circadian times (CT), total liver extracts from wild-type (wt) and mCRY-deficient (*cry*−) mice were immunoprecipitated (IP) with antibodies against CKIε, mPER1, or mPER2. The immune complexes were Western blotted (WB) and probed for mPER1, mPER2, and CKIε.

(D) Nuclear CLOCK and BMAL1 levels in mCRY-deficient mice. Liver extracts from designated circadian times (CT) were fractionated, and the cytoplasmic (C) and nuclear (N) fractions were Western blotted and probed for CLOCK and BMAL1.

(E) The mCRY proteins are required for mPER1 association with CLOCK. Liver extracts from wild-type (wt) and mCRY-deficient (*cry*−) mice were fractionated, and the cytoplasmic (C) and nuclear (N) fractions were immunoprecipitated (IP) with antibodies against mPER1. The immune complexes were probed for CLOCK. The film was exposed for 20 min.

mCRY:CLOCK interactions have been demonstrated, but consistent mPER:CLOCK interactions have not (Griffin et al., 1999; Shearman et al., 2000b; S.R. et al., unpublished data).

Clock Protein Phosphorylation in *tau* Mutant Hamsters

We examined the temporal profiles of clock protein abundance and phosphorylation in homozygous *tau* mutant Syrian hamsters. Mutant CKIε can bind mPER1 and mPER2 in vitro but has a markedly decreased ability to phosphorylate the mPER proteins (Lowrey et al., 2000). This suggested that CKIε-mediated phosphorylation of the Syrian hamster (sh) PER proteins in vivo might also be severely reduced.

Remarkably, both wild-type and homozygous *tau* mutant animals exhibited robust circadian rhythms in shPER1 and shPER2 abundance and phosphorylation (Figure 7A). Similar shPER protein abundance and phosphorylation were also found in the kidney and cerebellum of *tau* mutant hamsters (data not shown). These results suggest that CKIε is not the only kinase that phosphorylates the shPER proteins (see below). The overall amount of shPER1 and shPER2 in *tau* mutants was less than in wild-types (Figure 7A), which probably reflects decreased synthesis, as the *shPer* RNA rhythms are blunted in *tau* mutant hamsters (Lowrey et al., 2000).

The temporal phosphorylation pattern of CLOCK in *tau* mutant hamsters was also clearly apparent. Using the phosphorylation pattern of CLOCK as an index of the negative transcriptional phase, it appeared that the 4 hr loss in circadian period manifested by the mutant animals at both the behavioral and molecular levels was equally reflected in the positive and negative aspects of the feedback loops (Figure 7A); that is, there was no apparent 4 hr asymmetry in the positive and negative phases of the molecular oscillations that would account for the 4 hr shortening of circadian cycle length in the mutant hamsters. It did appear that the clock protein abundance and phosphorylation oscillations were delayed in *tau* mutant hamsters, relative to wild-type rhythms, but this was hard to firmly establish given the infrequent sampling interval.

CKIε levels were consistently reduced by 50% throughout the circadian cycle in *tau* mutant compared to wild-type hamsters (Figure 7A). The mutant kinase coimmunoprecipitated with shPER1 and shPER2, similar to that found for the wild-type hamster enzyme (data not shown; see below).

We next used immunoprecipitation to determine whether we could detect any qualitative differences in shPER phosphorylation in *tau* mutant hamsters. We did not see a consistent decrease in the degree of hyperphosphorylation attained (Figure 7B). There was a tendency for a delay in the time of appearance of hyperphosphorylated forms, especially shPER2 in *tau* mutants, suggesting less efficient phosphorylation by the mutant enzyme (Figure 7B).

Casein Kinase I Delta Is a Second Clock Relevant Kinase

One explanation for the relatively normal phosphorylation pattern of the PER proteins in *tau* mutant hamsters is that there may be other clock-relevant kinases that normally work together with CKIε to phosphorylate PER. A likely candidate for such a kinase is casein kinase I delta (CKIδ), which is highly homologous to CKIε (76% identical at the amino acid level) and efficiently binds

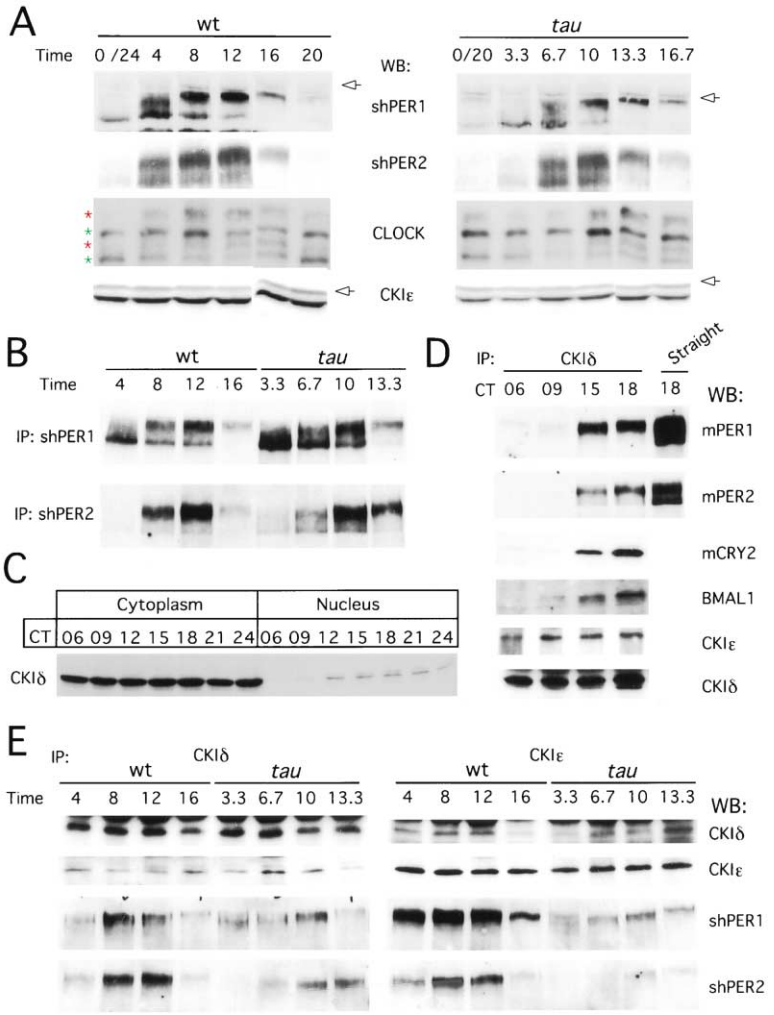

Figure 7. Clock Protein and Kinase Interactions

(A) Temporal patterns of clock protein abundance and phosphorylation in wild-type (wt) and homozygous (*tau*) mutant Syrian (sh) hamsters. Total liver extracts were examined by Western blots (WB) for shPER1, shPER2, CLOCK, and CKIϵ. Open arrowheads, nonspecific bands. For CLOCK, the green asterisks indicate nonphosphorylated isoforms, while the red asterisks denote phosphorylated isoforms. Data is presented as hours after expected activity onset for the first cycle in constant darkness.

(B) Direct comparison of shPER1 and shPER2 phosphorylation profiles between wild-type (wt) and (*tau*) mutant hamsters. Total liver extracts were immunoprecipitated (IP) with antibodies against shPER1 and shPER2. The immune complexes were Western blotted and probed for the same protein.

(C) Circadian variation in the cellular location of mouse CKIδ. Liver extracts from designated circadian times (CT) were fractionated, and the cytoplasmic and nuclear fractions were Western blotted and probed for CKIδ.

(D) Mouse CKIδ coprecipitates phosphorylated mPER1 and mPER2. Liver extracts from CT 06, 09, 15, and 18 were immunoprecipitated (IP) with antibodies against CKIδ. The resultant immune complex was Western blotted (WB) and probed for clock proteins.

(E) CKIδ and mutant CKIϵ complex with shPER1 and shPER2 in wild-type (wt) and *tau* mutant hamsters. At designated times (hrs) after expected activity onset, hamster liver extracts were immunoprecipitated with antibodies against CKIδ or CKIϵ. The immune complexes were Western blotted and probed for CKIδ, CKIϵ, shPER1, and shPER2.

and phosphorylates mPER1and mPER2 in vitro (Camacho et al., 2001; Vielhaber et al., 2000). We generated a specific antiserum to biochemically characterize CKIδ in vivo (see Supplemental Figure S1, available online at http://www.cell.com/cgi/content/full/[107]/7/855/DC1).

The regulation of CKIδ levels and interactions in mouse liver were strikingly similar to those of CKIϵ. CKIδ levels did not vary over the circadian cycle in total liver extracts (Supplemental Figure S6A, see above URL). There was, however, a pronounced circadian oscillation in nuclear localization, with the kinase prominently detected in the nucleus from CT 12–24 (Figure 7C). Moreover, immunoprecipitated CLOCK, BMAL1, mPER1, mPER2, CRY1, and CRY2 each coprecipitated CKIδ in a time-dependent manner, with coprecipitations at CT 15 and 18, but not at CT 06 and 09 (Supplemental Figure S6B, see above URL). Predominantly, hyperphosphorylated mPER1 and mPER2 copurified with CKIδ (Figure 7D). Interestingly, CKIδ coprecipitated with CKIϵ at all circadian times, suggesting that the two kinases are associated in the cytoplasm, perhaps in complexes with proteins involved in nonclock functions (Lee et al., 2001).

Finally, we examined CKIδ levels in the livers of *tau* mutant hamsters and compared CKIϵ and CKIδ interactions with shPER1 and shPER2 in mutant and wild-type hamsters. CKIδ levels were similar over the circadian cycle between wild-type and homozygous *tau* mutant hamsters (Supplemental Figure S6C, see above URL). Immunoprecipitation with antibodies against either CKIδ or CKIϵ coprecipitated the other kinase, as well as shPER1 and shPER2 in both wild-type and *tau* mutant animals (Figure 7E). The major difference between wild-types and mutants was decreased levels of the shPER proteins coprecipitated in *tau* mutants (Figure 7E).

Discussion

We have uncovered several important features of clock protein regulation in mammals. Our results suggest that temporal changes in clock protein phosphorylation, interactions, and subcellular localization play a vital role in the maintenance of the mammalian clock.

The mPER proteins are rate limiting for the mPER:mCRY interactions in cytoplasm that, in turn, are necessary for nuclear translocation of the complex. This finding was unexpected based on previous studies using overexpressed proteins in cultured cells (Kume et al., 1999). In those studies, it appeared that the mCRY proteins were rate limiting, because overexpressed mCRY always translocated to the nucleus, whereas overex-

pressed mPER protein was variable in location without mCRY coexpression. Our in vivo studies of the liver oscillator clearly show that the mCRY proteins are only nuclear when the mPER proteins are increasing in abundance. At other circadian times, the mCRY proteins are highly expressed and yet are cytoplasmic. Moreover, the mCRY proteins are unable to efficiently enter the nucleus in *mPer1/mPer2* double-mutant mice.

These findings provide a novel mechanism by which the mPER proteins control the molecular clockwork; that is, the robust, high-amplitude oscillations in mPER protein abundance are necessary for perpetuating the circadian clock mechanism, as mPER proteins bring clock protein complexes into the nucleus at the proper time for negative transcriptional feedback. With rhythmic accumulation of either mPER1 or mPER2, the clock mechanism persists and drives circadian behavior for a period of time in constant conditions, as occurs when either *mPer* gene is targeted (Zheng et al., 1999, 2001; Bae et al., 2001; Cermakian et al., 2001). Once in the nucleus, mPER2 appears to have the additional function of regulating *Bmal1* transcription (Shearman et al., 2000b; see below), leading to a more severe circadian phenotype with its disruption, compared with *mPer1* disruption. When *mPer1* and *mPer2* are targeted together, the clock immediately ceases to function on placement in constant conditions, because the mPER rhythms are immediately disrupted (Bae et al., 2001; Zheng et al., 2001).

Our data indicate that there is a codependency between the mPER and mCRY proteins for effective nuclear translocation. The critical role of the mCRY proteins in nuclear translocation of clock-relevant protein complexes was shown in mCRY-deficient mice, in which mPER1 and mPER2 are effectively trapped in the cytoplasm. Thus, the presence of either mCRY1 or mCRY2 is sufficient to interact with rising mPER protein levels and translocate them to the nucleus; hence, persistence of robust circadian rhythmicity occurs in either mCRY1- or mCRY2-deficient mice (van der Horst et al., 1999; Vitaterna et al., 1999). In double-knockout mCRY-deficient mice, the clock stops because no mCRY is available to translocate cytoplasmic mPER to the nucleus, and thus the mPER rhythms (the collective driving force for the clockwork) are abolished (van der Horst et al., 1999; Vitaterna et al., 1999). Taken in total, our data demonstrate that the mCRY proteins have supplanted *Drosophila* TIM-like nuclear transport functions for the mPER proteins in the mouse circadian clock. Furthermore, they provide a mechanistic explanation for the partially redundant function and codependence of mPERs and mCRYs.

Our results also define regulatory functions of the mCRY proteins that are dependent on the phosphorylation status of the mPER proteins. The mCRYs are essential for the stability of phosphorylated mPER2, while they are not necessary for stabilization of phosphorylated mPER1. This differential effect of the mCRY proteins underscores the importance of phosphorylation in contributing to distinct mPER functions. For mPER2, phosphorylation appears to enhance the dependency on mCRY for protein stability, and thus on subsequent nuclear translocation, while phosphorylation appears to constrain only mPER1 nuclear translocation. Thus, phosphorylation appears to control critical aspects of mCRY:mPER interactions necessary for normal clock function.

Neither CKIε nor CKIδ levels in total liver extracts vary over the circadian cycle, but there is a striking circadian rhythm in subcellular localization for each kinase, similar to the *Drosophila* CKIε ortholog, DBT (Kloss et al., 2001). The rhythms in kinase subcellular localization are synchronous and appear to be driven by CKIε/CK1δ:mPER:mCRY associations occurring in the cytoplasm, enabling subsequent translocation to the nucleus. These trimeric complexes are possible because CKIε/CK1δ and mCRY have distinct binding sites on the mPER proteins (Vielhaber et al., 2000; A. Gotter and S.R., unpublished data). CKIε and CKIδ appear to bind to the same binding site on the mPER proteins (Camacho et al., 2001). The kinases are in dynamic competition in vivo for the binding site, because both enzymes coprecipitate with mPER1 and mPER2, and thus mPER protein levels are limiting.

Once in the nucleus, the mCRY:mPER:CKIε/CK1δ complex associates with CLOCK and BMAL1 to negatively regulate *mPer* transcription at night (CT 12–21). At the same time, non-CLOCK-containing nuclear complexes containing mPER2 may also be involved in the positive transcription of BMAL1 (see Supplemental Figure S4B, available online at http://www.cell.com/cgi/content/full/107/7/855/DC1), as mentioned above (Shearman et al., 2000b). Based on both in vivo and in vitro studies, it appears that it is the direct association of the mCRY proteins with the CLOCK:BMAL1 heterodimer that leads to transcriptional inhibition (Griffin et al., 1999; Shearman et al., 2000b). Our results are provocative in that they suggest that transcriptional inhibition occurs while CLOCK:BMAL1 heterodimers remain bound to DNA. Thus, mCRY-induced alterations in DNA-bound CLOCK and/or BMAL1 conformation could be the primary mode of negative transcriptional regulation. Although there may also be some disruption of heterodimer-DNA binding during negative feedback, as in the *Drosophila* circadian clock (Lee et al. 1999), our data suggest that such disruption plays a minor regulatory role in mammals. The associated hyperphosphorylated state of mPER1, mPER2, CLOCK, and BMAL1 may target the entire clock protein complex for degradation at the end of the inhibitory phase (CT 21–24/0). The continued existence of CLOCK:BMAL1 heterodimer bound to DNA at the end of negative transcription regulation may reflect a mixed population of newly synthesized and more mature transcriptional complexes.

The mouse clock model of interacting positive and negative transcriptional/posttranslational feedback loops proposes that the *Bmal1* RNA rhythm drives a BMAL1 rhythm after a 4–6 hr delay (Shearman et al., 2000b). In this model, the proposed delay in the protein rhythm is essential because it would provide increasing levels of CLOCK:BMAL1 heterodimers to drive *mPer/mCry* transcription at the proper circadian time to restart the circadian cycle (Shearman et al., 2000b). Our data, however, indicate little delay between *Bmal1* RNA and BMAL1 protein (Figure 1A). An alternative mechanism for the time delay, that does not involve the absolute rhythm in BMAL1 levels, involves association of hyperphosphorylated inhibitory clock protein complexes with BMAL1 to effect transcriptional inhibition (CT 18–24) and

prevent newly made BMAL1 from becoming transcriptionally active at an inappropriate circadian time. This complex-mediated inactivation of nonphosphorylated BMAL1 would provide the necessary time delay between BMAL1 production and the appearance of transcriptionally competent CLOCK:BMAL1 heterodimers to drive *mPer/Cry* transcription at the appropriate circadian time for the next clock cycle (CT 03–06).

Paradoxically, transcriptional activation occurs when CLOCK and BMAL1 levels are at their lowest, suggesting that the phosphorylation status of the transcription factors may be a determining character for the transcriptional competency of the heterodimer. Phosphorylation does regulate the transcriptional activity of other bHLH transcription factors (Neufeld et al., 2000; Park et al., 2000). Phosphorylation may also be important for the formation of protein complexes that inhibit CLOCK:BMAL1-mediated transcription, but it does not appear to alter CLOCK:BMAL1 heterodimerization or binding to DNA.

Our data provide strong in vivo evidence that CKIδ is a second kinase important for the mammalian clock, as CKIδ nuclear localization is under circadian control, and the kinase coprecipitates with the PER proteins. Moreover, CKIδ is abundantly expressed in the SCN and liver (Camacho et al., 2001; Ishida et al., 2001).

Our data in *tau* mutant hamsters are consistent with a dominant-negative function of mutant CKIε. Since mutant CKIε appears to interact with the PER proteins, we propose that the normal equilibrium between CKIε and CKIδ for binding to the shPER proteins is preserved in the *tau* mutant hamster. The decreased ability of mutant CKIε to phosphorylate the shPER proteins could lead to an overall decrease in the efficiency of PER phosphorylation and a modest delay in the time of appearance of hyperphosphorylated forms. The presence of wild-type CKIδ in mutant animals thus ensures that the mPER proteins are fully phosphorylated, and accounts for the lack of an observed quantitative difference in PER phosphorylation between wild-type and *tau* mutant hamsters.

Taken together, the results advance our understanding of the mammalian clockwork by emphasizing its dependency on regulated features of clock protein function. It is apparent that precise regulation of the circadian oscillator requires careful choreography of clock protein production, interactions, and posttranslational modifications that go well beyond the rhythmic dynamics of RNA levels.

Experimental Procedures

Animals and Collections

Mice were entrained in 12 hr light:12 hr dark for at least 10 days before placement in constant darkness. Wild-type and homozygous *tau* mutant hamsters, as described previously (Lucas et al., 1999), were entrained in 12 hr light:12 hr dark or 10 hr light:10 hr dark, respectively, for 2 weeks before placement in constant darkness. At selected times on the first cycle in constant darkness, animals were killed, and livers, kidneys, and brain were dissected and frozen on dry ice.

Antibodies

We generated the anti-mPER1, anti-mPER2, anti-mCLOCK, anti-mBMAL1, anti-mCKIε, and anti-mCKIδ antibodies. Purified proteins were used as immunogen in rats and guinea pigs (Cocalico Biologicals Inc., Reamstown, PA; see Supplemental Methods). Representative antisera to each clock protein were affinity purified and designated CLK-1-R and CLK-1-GP ("R", raised in rats; "GP", raised in guinea pigs) for antibodies against CLOCK; BM1-2-R and BM1-2-GP for BMAL1; PER1-1-R and PER1-1-GP for PER1; CKIε-R and CKIε-GP for CKIε; PER2-1-R and PER2-1-GP for PER2; and CKIδ-R and CKIδ-GP for CKIδ.

We purchased antibodies against mCRY1 and mCRY2 (Alpha Diagnostic International), RNA polymerase II (Santa Cruz Biotechnology), histone H3 (Upstate Bioltechnology), and CREB (New England Biolabs). Antibody against UAP56 was generously provided by Michael R. Green, University of Massachusetts Medical School.

RNase Protection Assay

Total RNA was extracted using Tri-reagent (Sigma), as detailed (Lee et al., 1998). RNase protection assays were performed as described (Lee et al., 1998).

In Vitro Translation

In vitro-translated proteins were produced by coupled transcription/translation rabbit reticulocyte system (TNT, Promega) in the presence of L-[^{35}S]-Methionine according to the manufacturer's protocol.

Western Blotting

Western blotting was performed as described (Shearman et al., 2000a). The relative amounts of the clock proteins between the supernatant and the pellet samples were measured by Western blot analysis to ensure that the conditions used solubilized most of the individual clock proteins. From two independent experiments, we determined that >95% of all clock proteins were in the supernatant fractions (data not shown). For mPER1, mPER2, and mCLOCK, we used 6% SDS-polyacrylamide gels, whereas 8% SDS-polyacrylamide gels were used for BMAL1, CKIε, mCRY1, mCRY2, and CKIδ. As primary antibodies to mPER1, mPER2, CLOCK, CKIε, mBMAL1, and CKIδ we used PER1-1-R, PER2-1-R, CLK-1-GP, CKIε-GP, BM1-2-GP, and CKIδ-GP, respectively, for straight Western blots. Intensities of bands on autoradiographs were quantified with a densitometer from Bio-Rad.

Quantitation of the In Vivo Abundance of Clock Proteins

The absolute concentrations of in vivo clock proteins (mPER1, mPER2, mCRY1, mCRY2, CKIε, CLOCK and BMAL1) were determined as described previously (Bae et al., 2000). Using reticulocyte lysates containing serial dilutions of known amounts of an individual clock protein, standard dose (known amounts of a clock protein)-response (intensities of immunoreactive bands on Western blots) curves were obtained (Figure 2S). The concentrations of the clock proteins in extracts prepared from liver collected at CT 12 and 15 were determined from these standard curves. The peak and trough levels of the in vivo clock proteins were obtained using data above and the relative abundance profiles are shown in Figure 1.

Immunoprecipitation and Phosphatase Treatment

Immunoprecipitation was performed as described (Shearman et al., 2000a). PER1-1-GP, PER2-1-GP, CLK-1-GP, BM1-2-GP, anti-mCRY1 antibody, or anti-mCRY2 antibody was used for immunoprecipitation. To reduce Western blot detection of the guinea pig-derived antibodies from the immunoprecipitates, we used antibodies raised in rats as the primary antibodies for Western blotting (PER1-1-R, PER2-1-R, CLK-1-R, BM1-2-R, and CKIε-R). Phosphatase treatment was performed as described (Lee et al., 1998).

Preparation of Nuclear and Cytoplasmic Extracts from Liver

Fractionation was performed using the procedure of Zhang et al. (2000). Proteins were analyzed by Western blotting as described above.

Preparation of Crosslinked Chromatin

Chromatin was crosslinked using the procedure of Orlando et al. (1997), with minor modifications. Liver nuclei were prepared as described above. To prepare the total nuclear extract, an aliquot of nuclei was resuspended in EB, mixed with same volume of 2×

sample buffer, and sonicated. The remainder of the nuclei was resuspended in 1% formaldehyde-DMEM medium (nuclei from 3 livers in 10 ml medium) and incubated at room temperature for 10 min with gentle shaking. The reaction was stopped by the addition of glycine (final concentration, 0.125 M). Nuclei were collected by centrifugation, washed with PBS three times, then resuspended in EB, and aliquots were stored at 80°C. Aliquots of 300 μl were used for chromatin immunoprecipitation (ChIP) PCR analysis and Western blotting of chromatin-bound proteins.

To isolate crosslinked chromatin, the nuclei were treated with RNase A at room temperature for 30 min and then incubated in EB plus 1% SDS with gentle shaking. NaCl was added (final concentration of 0.5 M) to precipitate the chromatin. The precipitate was isolated with a pair of glass forceps and washed three times in TE. The liquid remaining after chromatin removal served as the soluble fraction of the fixed nuclei. The washed chromatin was transferred to an eppendorf tube, resuspended in 2× sample buffer, and sonicated and served as the chromatin-bound fraction.

ChIP

An aliquot of crosslinked or noncrosslinked nuclei was sonicated (Misonix, XL2020) on ice with the setting at 4 for eight 15 s pulses and centrifuged at 12,000 × g for 15 min. The supernatant was collected and precleared. The average size of the sonicated chromatin was ~500 bp. Prior to ChIP, the protein G Sepharose 4 Fastflow beads were incubated with poly dI-dC (3 μg/μl beads) to reduce nonspecific interactions. Blocked beads (10 μl) and 2 μl of anti-CLOCK, BMAL1, or HA (SantaCruz biotechnology) antibodies were added to the precleared supernatant, incubated at 4°C for 5 hr, and washed as described above, followed by 5 more washes with EB plus 0.3% Triton X-100. For DNA analysis, the immune complexes were eluted with 200 μl of EB plus 1% SDS and shaking. For protein analysis, the immune complexes were eluted with 30 μl of 2× sample buffer. To isolate DNA, the eluted immune complexes were diluted with the same volume of TE, mixed with proteinase K (final concentration of 2 μg/μl), and incubated at 37°C for 1 hr, followed by 65°C for 5 hr. The samples were extracted with phenol/chloroform/isoamylalcohol and precipitated at –20°C overnight. The pellet was resuspended in 30 μl of TE for PCR. Following 32 cycles, amplified products were run on 2% agarose gel and visualized by ethidium bromide staining.

For semiquantitative analysis, PCR was performed for 17 cycles. Amplified products were separated on 4% TBE-polyacrylamide gels and Southern blotted. Blots were hybridized with radiolabeled 25-mer oligonucleotides that were specific for a sequence of the amplified fragment between the PCR primers. The PCR conditions were calibrated with known amounts of genomic DNA to confirm that the amplified products were in the linear range. Band intensity was quantified by densitometry.

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