Biochemical Analysis of the Canonical Model for the Mammalian Circadian Clock

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The current consensus model for the circadian clock in mammals is based on a transcription-translation feedback loop. In this model, CRY and PER proteins repress their own transcription by suppressing the transactivator function of the CLOCK:BMAL1 heterodimer directly (physical model) and by facilitating post-translational modifications (chemical model). Most of the data for this model come from genetic and cell biological experiments. Here, we have purified all of the core clock proteins and performed in vitro and in vivo biochemical experiments to test the physical model. We find that CLOCK:BMAL1 binds to an E-box sequence in DNA and that CRY binds stably to the CLOCK:BMAL1:E-box ternary complex independently of PER. Both CRY and PER bind to CLOCK and BMAL1 off DNA but, in contrast to CRY, PER does not bind to the CLOCK:BMAL1:E-box complex. Unexpectedly, PER actually interferes with the binding of CRY to the CLOCK:BMAL1:E-box ternary complex. CRY likely destabilizes the CLOCK:BMAL1 heterodimer on DNA by a post-translational mechanism after binding to the complex. These findings support some aspects of the canonical model, but also suggest that some key features of the model need to be revised.

The circadian clock is the internal timekeeping system that generates a daily rhythm in physiology and behavior of an organism (1–3). In mice and humans this rhythmic behavior is generated by a molecular clock with a periodicity of about 24 h that consists of a transcription-translation feedback loop (TTFL). The core mammalian molecular clock is made up of 4 genes/proteins and their paralogs: Clock (Npas2), Bmal1, Cry (Cry1 and Cry2), and Per (Per1 and Per2), and according to the current consensus model, operates as follows: the BMAL1 and CLOCK proteins, which are members of the bHLH (basic helix-loop-helix) family of transcription factors, make a heterodimer, which binds to the E-box element in the promoters of Per and Cry genes and activates their transcription (positive arm). The CRY and PER proteins make heterodimeric complexes, which, after a time lag enter the nucleus and inhibit the CLOCK:BMAL1-activated transcription of their own genes (negative arm). This core circuitry is consolidated by additional interlocking transcriptional circuits as well as post-translational modifications and proteolytic degradation of the core clock proteins (1, 3, 4).

The TTFL model is largely based on genetic data from mice with mutations in the so-called core clock genes, on protein-protein interaction data, reporter gene assays, and ChIP analysis (5–10). Various studies have led to several models for how CRY and PER repress their own transcription as well as the transcription of output genes controlled by the transactivation function of CLOCK:BMAL1. These, not necessarily mutually exclusive models, include: 1) physical (steric) models and 2) chemical (catalytic) models. In the steric model, binding of CRY, PER, or the PER:CRY complex to CLOCK and BMAL1 interferes with their transcription function (10). In the catalytic model, the “repressor” is actually a protein that recruits enzymes for post-translational modification of the activator complex and eventually changes its activity (11–13). There are numerous versions of both the chemical and physical models, and quite likely both mechanisms contribute to the generation of robust circadian rhythmity at the transcriptional level.

Here we have used a biochemical approach to investigate the mechanisms by which the two proteins in the negative arm of the mammalian TTFL model, CRY and PER, may interfere with the transactivator function of CLOCK and BMAL1. Specifically, we have addressed the following questions: (a) do CRY or PER interfere with the formation of the CLOCK:BMAL1 complex as has been suggested for zebrafish CRY1a (14) or do they inhibit CLOCK:BMAL1 from binding to its target sequence by shifting the equilibrium to the unbound form as has been suggested for Drosophila PER (15, 16); (b) do both CRY and PER interact with CLOCK and BMAL1 on and off DNA; (c) during repression of CLOCK:BMAL1, does PER bring CRY to the CLOCK:BMAL1:E-box complex (17) or is it the other way around? We find that both CRY and PER bind to CLOCK:BMAL1 off DNA. Unexpectedly, and not predicted by any of the current models, only CRY binds to the CLOCK:BMAL1:DNA ternary complex. Neither the PER:CRY heterodimer nor PER alone can bind to CLOCK:BMAL1:DNA and, in fact, at a sufficiently high concentration PER interferes with the binding of CRY to this complex. These in vitro data were supported by ChIP analysis with various mouse clock gene knock-out cell lines, which revealed that binding of CRY to the cognate promoters is dependent on BMAL1 but independent of PER. In light of these findings we propose a revised TTFL model for the mammalian circadian clock.
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EXPERIMENTAL PROCEDURES

Cells and Antibodies—The Cry1<sup>−/−</sup> Cry2<sup>−/−</sup> mouse skin fibroblasts have been described previously (18). The isogenic Bmal1<sup>+/+</sup> and Bma1<sup>−/−</sup> and Clock<sup>+/+</sup> and Clock<sup>−/−</sup> mouse embryonic fibroblast cell lines (19) were kindly provided by Dr. Marina Antoch (Roswell Park Cancer Institute). The Per1<sup>−/−</sup> Per2<sup>−/−</sup> skin fibroblasts were prepared from a male double knock-out mouse (20) kindly provided by Dr. Cheng Chi Lee (University of Texas, Houston Medical School). Mouse embryonic fibroblasts expressing mCry1-FLAG and mPer2-FLAG were made by retrovirus infection. Briefly, mCry1-Flag-pBABEpuro and mPer2-Flag-pBABEpuro constructs were made and co-transfected together with pVSVG and pCIHPZ into HEK293T to produce retrovirus particles. Then mouse embryonic fibroblast cells were infected with the retrovirus and transfectants were selected in medium containing puromycin for 2 weeks. Single colonies were picked and cultured for subsequent analysis.

Anti-mCRY1 (IgM type monoclonal) and anti-CRY2 antibodies were described previously (18, 21). Anti-CLOCK and anti-BMAL1 antibodies were purchased from Santa Cruz and Bethyl Laboratories. Rabbit anti-CRY2 antibodies were generated by using the N-terminal 200 amino acids of mPER2 protein as immunogen as described previously (22) and immunopurified by antigen that was covalently coupled to Aminolink resin (Pierce). Anti-Myc(9E10), anti-V5, anti-FLAG, and anti-His antibodies were purchased from Santa Cruz, Invitrogen, Sigma, and Abgent, respectively.

Plasmids—Plasmids used in this work were obtained from Addgene. Detailed construct cloning information is described under supplemental “Methods”. The hCry1-His baculovirus construct was described previously (23).

Clock Proteins—Proteins were purified either from baculovirus-infected Sf21 insect cells or from transfected HEK293T cells. Most of the proteins used in our experiments were purified with the baculovirus system, except Myc-CRY1 and Myc-CRY2, which were purified from the mammalian expression system. Proteins purified using the two systems showed similar properties. Detailed purification procedures are described under supplemental “Methods”. MAX (MYC-associated factor X) protein used in some gel mobility shift assays as a control was purchased from ProteinOne, USA. Drosophila CRY was generated as described elsewhere (24).

Electrophoretic Mobility Shift Assay—Two DNA duplexes were used as DNA binding substrates: a 14-bp duplex “M34” (25) and a 60-bp duplex “P2GS.” Sequences of these oligos are listed in supplemental Table S1. To prepare radiolabeled probes, 40 pmol of M34TOP or P2GSGA were labeled with [γ-<sup>32</sup>P]ATP using polynucleotide kinase (New England Biolabs). DNA was then extracted with phenol/chloroform, and the labeled oligonucleotides were annealed with complementary strands (M34BTM and P2GS). Mini-quick spin oligo columns (Roche Applied Science) were used to remove the free ATP from labeled DNA. In EMSA experiments, the binding buffer consisted of 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, 6 mM dithiothreitol, and 20 μg/ml of poly(dI-dC) (Sigma). Radiolabeled DNA at ~1 nM was mixed in 15 μl with clock proteins in the following order depending on the experiments: 1) CLOCK:BMAL1 for 10 min, 2) CLOCK:BMAL1 for 5 min, then CRY, PER, or hCRY1:PER2 protein for 8 min, or 3) CLOCK:BMAL1 for 5 min, then CRY for 8 min, and PER for 8 min. All reactions were incubated at 22 °C followed by 10 min at 4 °C. In antibody supershift experiments, about 1 μg of antibody was added for an additional 5 min after the original incubation at 22 °C. The reactions were loaded onto 4% nondenaturing polyacrylamide gels, and products were resolved at 100 V for 3 h at 4 °C. We note that under these reaction conditions CRY in isolation does not exhibit any DNA binding activity (Fig. 1D). Gels were dried and exposed to x-ray film.

DNase I Footprinting—Protein/DNA mixtures were prepared as in the electrophoretic mobility shift assay using P2GS DNA. Reactions, in addition to DNA, included: 1) no protein, 2) BMAL1:CLOCK (0.2 nM) alone, or 3) CLOCK:BMAL1 (0.2 nM) plus an amount of CRY (1 nM) that binds all of the CLOCK:BMAL1:E-box complexes as determined by gel shift analysis. Reactions in 15 μl were incubated at 22 °C for 20 min. Then 0.5 units of DNase I (Promega) and MgSO<sub>4</sub> (10 mM), CaCl<sub>2</sub> (1 mM) were added to each reaction, and after 5 min at 22 °C, digestion was stopped by adding EDTA to 25 mM. Then the reactions were loaded onto a 4% nondenaturing polyacrylamide gel to separate protein-bound and free DNA. Bands representing free DNA were from reaction 1, the CLOCK:BMAL1:E-box complex from reaction 2, and the CRY:CLOCK:BMAL1:E-box complex from reaction 3 were excised from the gel, and DNA was eluted from the gel slices and run on a 10% denaturing polyacrylamide gel that was visualized by autoradiography.

Pulldown Assay for Protein-Protein Interactions—Purified recombinant proteins at 0.1 nM were mixed in 200 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 100 μg/ml of BSA, 0.05% Nonidet P-40) and incubated at 22 °C for 15 min. Then 20 μl of either FLAG- or V5-agarose (Sigma) beads were added and the mixture was incubated at 4 °C for 90 min. Then, the beads were washed with 1 ml of ice-cold binding buffer 4 times and after the final wash resuspended in 25 μl of SDS loading buffer and heated at 95 °C for 5 min. The proteins were then separated on SDS-PAGE and visualized by immunoblotting.

Chromatin Immunoprecipitation—We used a previously described dual cross-link ChIP procedure (26) with some modifications. Detailed procedures are described under supplemental “Methods”.

siRNA Treatment, RNA Extraction, and Reverse Transcription—siRNAs specific for mCry1, mCry2 (SMARTpool siRNA, L-040485-01-0005, and L-014151-01-0005) and CYCP-B control siRNA (D-001820-02) were purchased from Dharmacon. Transfections were performed with 50 pmol of siRNAs using the transfection reagent RNAi Max (Invitrogen). Following a 48-h transfection the cells were collected and total RNA was extracted using the RNeasy Mini Kit (Qiagen). Total RNA (500 ng) was reverse transcribed with oligo(dT)<sub>20</sub> using SuperScript III (Invitrogen) according to the manufacturer’s instructions.

Quantitative Real-time RT-PCR—Real-time PCR assays were performed by using an ABI 7300 system (Applied Biosystems) and Maxima<sup>TM</sup> SYBR Green/ROX qPCR Master Mix
RESULTS

Purification of Mouse Clock Proteins—To test the conventional TTFL model that posits that the CLOCK:BMAL1 heterodimer drives the transcription of target genes and that the PER:CRY heterodimer binds to and interferes with the transactivator function of CLOCK:BMAL1, we purified these proteins using baculovirus or mammalian expression systems. Fig. 1A shows the purified full-length proteins analyzed by SDS-PAGE and Coomassie Blue staining. We found that BMAL1, CRY1/2, and PER1/2 could be expressed individually and purified in reasonable quantities using immunoadfinity chromatography. However, when CLOCK was expressed alone, it was somewhat unstable and insoluble as reported previously (27). When coexpressed with BMAL1, CLOCK could be purified in a reasonable yield and with one-to-one stoichiometry, as shown in lane 1. We used both mouse CRY1 and CRY2 and mouse PER1 and PER2 in our experiments as dictated by availability and practical considerations. In addition, we used human CRY1 for the CRY1:PER2 heterodimer and human CRY1 alone as a reference cryptochrome in experiments with this heterodimer. Because most of the experiments were performed with mouse clock proteins, we will omit the “m” designation for the sake of simplicity.

Footprint of CLOCK:BMAL1—The data in Fig. 1 show that under appropriate reaction conditions nearly all of the CLOCK:BMAL1:E-box complex is bound to CRY1. In addition, CRY2 protein, but not Drosophila CRY binds to the CLOCK:BMAL1:E-box complex in our assay (supplemental Fig. S1A). The CLOCK:BMAL1:CRY1 complex co-purified from mammalian cells also showed clear binding activity to the E-box as a ternary complex (supplemental Fig. S1, B and C). To ascertain the specificity of CRY1 binding to CLOCK:BMAL1 on an E-box we performed additional experiments to test effects of CRY on other E-box-binding proteins including the BMAL1 homodimer and the MAX homodimer (29, 30). Fig. 1F shows that the association of CRY1 with the CLOCK:BMAL1:E-box complex is specific, because the mobility of the BMAL1:E-box or the MAX:E-box complexes are not affected by CRY1 or CRY1 plus anti-CRY1 antibody under conditions where the entire CRY1:CLOCK:BMAL1:E-box is supershifted by the anti-CRY1 antibody. These data unambiguously show that the CRY protein does not dissociate CLOCK:BMAL1 from the E-box by physical interaction; in contrast, it binds to the CLOCK:BMAL1:E-box complex with high specificity and without the intermediacy of another protein.

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not linear with the size of the binding protein. Indeed, as seen in Fig. 1F the BMAL1 homodimer:DNA complex migrates slower than the CLOCK:BMAL1-DNA complex even though the latter complex possesses a molecular mass ~25 kDa more than the former. Hence, we considered the possibility that the CRY1: CLOCK:BMAL1 complex on an E-box, which is clearly detectable by anti-CRY1 antibody induced supershift in gel retardation experiments, may be more readily detectable by DNase I footprinting, which has higher resolution than the gel retardation assay. For DNase I footprinting, we used a 60-bp fragment P2GS encompassing an E-box in the mouse Per1 promoter, which was shown to be functional in a reporter gene assay (9). In the gel mobility shift assay using this fragment we obtained results similar to those obtained with the 14-bp E-box fragment used in Fig. 1 (Fig. 2B and supplemental Fig. S1D). Importantly, in the presence of anti-CRY1 antibodies, most of the DNA-protein complex was supershifted in the reaction that included CRY1 in the reaction mixture. Interestingly, when these complexes were probed by DNase I footprinting the CLOCK:BMAL1 heterodimer produced a rather small foot-
print of 8-bp centered around the E-box (Fig. 2C), and this footprint was not affected by CRY1 under conditions known to produce the CRY1:CLOCK:BMAL1 complex on the E-box. Even though this result did not provide further insight on the anatomy of the CLOCK:BMAL1:CRY complex, the footprint is consistent with the known properties of interactions of bHLH family protein with DNA (30). In the complex of BMAL1 and CLOCK, both of which are bHLH-PAS domain proteins, contact with DNA is made with the bHLH domains, which are located at the extreme N terminus of each member of the heterodimer. The extended structure of this family of proteins explains the small footprint and also explains the lack of effect of CRY1 on the footprint because CRYs bind to the PAS domains and the C-terminal domains distant from the DNA contact region (31, 32).

Effect of PER on CLOCK:BMAL1:E-box and CRY:CLOCK:BMAL1:E-box Complexes—Although CRY is the dominant repressor of the CLOCK:BMAL1 transactivation function as shown by reporter gene assays (7) and transcription assays with Cry1−/− Cry2−/− knock-out mice (33), numerous studies suggest that CRY works as a heterodimer with PER in the TTFL (3, 22). The coordinated inhibitor function of PER and CRY can be classified by the following different steric models: (a) PER:CRY complex binds to CLOCK:BMAL1 off DNA and disrupts the heterodimer or interferes with its binding to E-boxes in the cognate promoters. (b) PER:CRY binds to CLOCK:BMAL1 through CRY and blocks the transactivation function of the transcription factor (22). (c) The PER:CRY complex binds to CLOCK:BMAL1 by direct binding of PER to the complex, and not the other way around, even though CRY is considered the nominal repressor (17). To differentiate among these models, we investigated the combined effects of PER and CRY on the CLOCK:BMAL1:E-box complex. Because PER2 appears to be more important than PER1 for normal clock function in mice (17, 34), we used PER2 in most of our experiments.

The various models were tested by gel mobility shift assays. Fig. 3A and supplemental Fig. S1E show that, in contrast to CRY1 and CRY2, PER1 and PER2 do not affect the mobility of the CLOCK:BMAL1: E-box complex. However, because even with CRYs the mobility shift is subtle, this finding alone does not prove that PER2 does not associate with the complex. In Fig. 3B we employed the more stringent test of antibody-mediated band supershifting. When Myc-tagged CRY1 and PER2 were added to the CLOCK:BMAL1:E-box reaction mixture, Myc-CRY1 slightly retarded the DNA-protein band, which, importantly, upon the addition of anti-Myc antibodies was nearly completely supershifted. In contrast, Myc-PER2 at the same concentration as Myc-CRY1 had no effect on the mobility of the CLOCK:BMAL1:E-box complex, and most importantly, the addition of anti-Myc antibodies to the Myc-PER2 plus CLOCK: BMAL1:E-box reaction mixture did not affect the mobility of the protein-DNA band. We conclude that under our reaction conditions PER2 does not directly bind to the CLOCK:BMAL1: E-box complex.

Failure of PER2 to bind to the CLOCK:BMAL1:E-box was unexpected. We considered the possibility that solitary PER2 may not be properly folded, and that in vivo PER2 might be mostly associated with CRY and hence the PER:CRY complex may represent the functionally relevant form of the protein. With these considerations, we purified the PER2:hCRY1 complex and the hCRY1 as a control (Fig. 3C and D) and tested them in gel mobility shift assays. The results are shown in Fig. 3D. As expected, CRY1 alone supershifts the CLOCK:BMAL1:E-box complex. Surprisingly, however, the PER2:hCRY1 heterodimer does not affect migration of the CLOCK:BMAL1:E-box even though CRY1 alone at a concentration equivalent to its concentration in the PER2:hCRY1 heterodimer clearly retards the protein-DNA band. The most parsimonious explanation of these data is that PER2 interferes with binding of CRY1 to the CLOCK:BMAL1:E-box complex. However, because such a
conclusion is contrary to the generally held view that PER either mediates the binding of CRY to CLOCK:BMAL1, or is in a complex with CRY that binds to the CLOCK:BMAL1:DNA complex. We were concerned that the lack of binding by PER2:CRY1 might have been caused by an inactive protein preparation. Therefore, to test if indeed PER2 interfered with binding of CRY to the CLOCK:BMAL1:E-box complex we performed gel mobility shift assays to test the effects of PER on a pre-assembled CRY:CLOCK:BMAL1:E-box complex. The results in Fig. 3, E and F, show that supershifting of the CLOCK:BMAL1:E-box complex by CRY is abolished when PER2 is added to the complex as evidenced by the migration of the protein-DNA band without (Fig. 3E) and with (Fig. 3F) antibodies against the affinity tag on CRY1. Based on these findings, we conclude that PER2 does not recruit CRY to CLOCK:BMAL1 bound to the promoter of the cognate genes, but it actually impairs its binding.

In Vitro Interactions Among Purified CLOCK Proteins—Failure to detect an interaction of PER2 with CLOCK:BMAL1 on DNA was surprising because the interactions of PER2 with all clock proteins has been demonstrated by numerous studies (17). Hence, to ascertain whether the PER2 that was used in mobility shift experiments was active, we tested it for protein-protein interactions with the other clock proteins by co-immunoprecipitation of purified proteins. In Fig. 4 we compare the binding of PER2 and CRY1 to each other, and to BMAL1 and CLOCK. As expected, individually purified PER2 and CRY1 interact with one another (Fig. 4A). Significantly, PER2 binds to...
both BMAL1 and CLOCK with higher affinity than CRY1 (Fig. 4, B and C). Although quantitative binding experiments with recombinant proteins must be considered with some caution, these data nevertheless do show that PER2 used in our mobility shift assays is at least as active as CRY1 in protein-protein interactions with CLOCK and BMAL1. Hence, we conclude that the failure to detect binding of either PER2 or the PER2:CRY1 complex to the CLOCK:BMAL1:E-box ternary complex is not due to the misfolding or an otherwise inactive form of PER2; rather, it is a manifestation of an intrinsic property of the core clock system.

**Effect of Mutations in Core Clock Genes on Binding of Cryptochrome to Target Promoters in Vivo**—The in vitro data we have presented indicate that CRY may be recruited to target promoters by direct protein-protein interactions with CLOCK:BMAL1 bound to E-boxes in cognate promoters. To test this prediction in vivo we carried out ChIP assays with CRY and CLOCK antibodies in cell lines from mice in which clock genes have been knocked out (supplemental Fig. S2, B and C). ChIP results are shown in Fig. 5 and supplemental Figs. S3 and S4. Several noteworthy conclusions emerge from ChIP assays with anti-CRY1 antibodies, shown in orange in Fig. 5A. First, in agreement with the in vitro data, CRY1 binds independently of PER1 and PER2 proteins to E-box elements in the promoter regions of both Per1 and Per2 genes. Second, considering that BMAL1 is capable of making heterodimers with other PAS domain proteins such as

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**FIGURE 5.** CRY1 binds E-boxes in Per1 and Per2 promoters in the absence of PER protein and affects CLOCK-BMAL1 dynamics at cognate promoters in mouse fibroblasts. Wild-type or clock mutant mouse fibroblasts were used in ChIP experiments to determine the effects of null mutations of various clock genes on binding of CRY1 and CLOCK to target promoters. ChIP was analyzed by quantitative PCR and the values represent the average of three experiments ± S.E. From left to right, 4 columns of bar graphs show the ChIP data from different knock-out cell lines. From top to bottom, the four rows of graphs show binding to the Per1 Ebox2 or the Per2 Ebox2 (22, 49), as indicated on the left. A, CRY1 ChIP (represented by orange color). Dual cross-linked nuclear extracts were isolated from fibroblasts of the indicated genotypes and subjected to ChIP. The immunoprecipitation was performed with anti-CRY1 or no antibody control. B, CLOCK ChIP (represented by blue color). The immunoprecipitation was performed with anti-CLOCK polyclonal antibodies or IgG control. C, schematic diagram summarizing how CLOCK, BMAL1, and CRY1 occupy E-boxes in Per1 and Per2 promoters in fibroblasts with various genetic backgrounds.
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NPAS2, this was to be expected (35–37). Indeed, ChIP reveals that binding of CRY1 to both Per1 and Per2 E-boxes is absolutely dependent on BMAL1 (Fig. 5A, right column). These data confirm the conclusion from the in vitro experiments with purified proteins that CRY binds directly to CLOCK:BMAL1 situated on an E-box.

Next, we investigated the binding of CLOCK to the same E-boxes (Fig. 5B, blue). The results reveal several interesting patterns regarding the interactions of the core clock proteins with clock gene promoters and with one another. First, the absence of CRY enhances the occupancy by CLOCK of E-boxes in both Per1 and Per2 promoters even though it has been reported that in mouse liver, in the absence of CRY, the level of CLOCK is reduced (22). It is conceivable that CRY promotes post-translational modifications of CLOCK that facilitates its removal from the cognate promoters (11). Second, in contrast to the absence of Cry genes, the absence of Per genes has only a modest stimulatory effect on CLOCK occupancy of Per1 and Per2 E-boxes. This is possibly because in Per1−/−Per2−/− cells, both CRY1 and CRY2 levels are lower in the nucleus (22), resulting in only a modest effect on the CLOCK protein level and its occupancy of E-boxes. Finally, in the Bmal1−/− background, there is no significant binding of CLOCK to the Per1 and Per2 promoters, consistent with the canonical clock model that BMAL1 is an irreplaceable component of the positive arm of the TTFL.

Overall, the ChIP experiments support the conclusions from the gel mobility shift assays with purified proteins that CLOCK:BMAL1 binds to E-boxes in cognate promoters, that recruitment of CRY to the CLOCK:BMAL1:E-box ternary complex is independent of PER and support previous reports that BMAL1, in the absence of CLOCK, can make heterodimers with other PAS domain proteins such as NPAS2 to regulate transcription (38) (Fig. 5C). Hence, in vitro experiments should be considered as essential tools for testing any molecular model for the mammalian circadian clock.

Probing for PER2 Protein Binding in Vivo to E-boxes in Promoters of CLOCK:BMAL1 Controlled Genes—The in vitro experiments showed that PER protein does not bind to the CLOCK:BMAL1:E-box complex. To examine this issue in vivo, we conducted ChIP assays with polyclonal anti-PER2 antibodies and wild-type mouse skin fibroblast cells. However, our experiments failed to reveal any measurable binding of PER2 protein to either Per1 or Per2 promoters (data not shown). Because ChIP assay results may be dramatically affected by the quality of the antibodies, we carried out ChIP assays using anti-FLAG antibodies in fibroblast cell lines that stably express CRY1-FLAG (control) or PER2-FLAG proteins. The immunoprecipitation assay results in Fig. 6A show that PER2-FLAG and CRY1-FLAG were precipitated at comparable levels from the two stable lines. CLOCK and BMAL1 bound to both FLAG-tagged proteins, more strongly to PER2-FLAG. Therefore, both FLAG-tagged proteins are active. However, the ChIP assay results with anti-FLAG antibodies in Fig. 6B show that CRY1-FLAG, but not PER2-FLAG, can bind to Per1 and Per2 promoters. FLAG-tagged PER2 failed to bind these regions when the FLAG tag was at either the C terminus (Fig. 6B) or N terminus (data not shown). These results indicate that at least in skin fibroblast, PER2 does not bind to these E-boxes. The strong interaction of PER2 with CLOCK and BMAL1 probably occurs only when these proteins are off DNA.

Effect of Cryptochrome on Transcription of a Clock-controlled Gene in the Presence and Absence of PER—The data presented so far show that CRY can bind to CLOCK:BMAL1 situated at E-boxes of target promoters without the help of PER. To determine whether this binding affects transcription we used a CLOCK:BMAL1-controlled gene with high amplitude circadian oscillation, Dbp, as a readout (8). First, we confirmed that as in the case of the Per1 and Per2 promoters, CRY1 binds to the E-box in the Dbp gene independently of PER (Fig. 6C). Then to determine the effects of CRY binding on Dbp transcription in the absence and presence of PER, we down-regulated CRY1 and CRY2 in the wild-type and Per mutant background and analyzed the cells for Dbp transcription as measured by quantitative RT-PCR. Clearly, the reduction in CRY level (Fig. 6D) is accompanied by a proportional increase in the level of Dbp mRNA (Fig. 6E), consistent with the consensus clock model whereby the binding of CRY to CLOCK:BMAL1 at the E-boxes of the target genes represses the transcription of these genes. Importantly, both in wild-type and Per1−/−Per2−/− cell lines, the Dbp mRNA level goes up similarly upon down-regulation of CRY. These findings suggest that CRY is the predominant repressor of Dbp, which is regulated with a robust rhythmicity by the CLOCK:BMAL1 transcriptional activator.

DISCUSSION

The TTFL, which was proposed over a decade ago as the mechanistic model for the mammalian circadian clock, remains as the starting point for the more detailed models that have been elaborated subsequently as new levels of regulation were discovered. However, despite the extensive genetic and cell biological data that exist in support of the canonical model, a rigorous biochemical test of the model has been lacking. Clearly, biochemical experiments with purified proteins are necessary to test some key points of the canonical model. Here, we attempted to address some key features of the model by conducting experiments with purified proteins in vitro and ChIP assays with cell lines mutated in various clock genes. Our findings support some aspects of the conventional model, but also suggest that some features of the canonical model need to be revised. A revised model consistent with our data is shown in Fig. 7 and is elaborated below.

Mechanism of CRY Repression of CLOCK:BMAL1 Transcriptional Activation—Our in vitro and in vivo findings are in agreement with previous reports in showing that bHLH-PAS domain proteins CLOCK and BMAL1 make a heterodimer, which binds to E-boxes in the promoters of cognate genes and functions as a transcriptional activator. Furthermore, our reconstituted system and ChIP assays clearly demonstrate that CRY interacts strongly with CLOCK:BMAL1:E-box complexes. Binding was BMAL1 dependent both in vitro and in vivo, indicating that CRY is directly recruited to the E-box by a (CLOCK/NPAS2):BMAL1 heterodimer. Also of considerable importance is our finding that this binding of CRY is independent of PER: ChIP assays clearly demonstrated that CRY binds to regulatory E-boxes even in Per knock-out cells, and in vitro, PER
was not only unnecessary for, but actually impaired CRY binding to the BMAL1:CLOCK:E-box DNA complex. Binding of CRY to the transactivator complex in the absence of PER is biologically meaningful, because we found that in Per1−/− Per2−/− knock-out cells, CRY down-regulated BMAL1:CLOCK-activated transcription of the Dbp gene. All of these results are in agreement with a reporter gene assay that found the inhibition of transactivation by CRY depends on the interaction of CRY with either CLOCK or both CLOCK and BMAL1 bound to DNA in S2 cells, which express no other mammalian clock proteins (5). Thus, independently of PER, CRY directly interacts with the BMAL1:CLOCK:E-box complex in a BMAL1-dependent manner to form a stable complex on DNA in which the transactivator function of CLOCK:BMAL1 is inhibited by CRY.

In addition to blocking transactivation, CRY may mediate an enzymatic function. We found that CLOCK occupied the Per1 E-box2, and to a lesser extent Per2 E-box2, at higher frequency in Cry null cells than wild-type cells, suggesting that CRY may destabilize the CLOCK:BMAL1:E-box complex in vivo (Fig. 7D). Relevant to this is a report showing that CLOCK:BMAL1 binds rhythmically to the Dbp promoter (8). Because under all experimental conditions tested, CRY did not destabilize the ternary complex in vitro, we ascribe the discrepancy between the in vivo and in vitro data to the effect of CRY on the phosphorylation status of other clock proteins including PER, CLOCK, and BMAL1 in vivo as it is known that the phosphorylation status of CLOCK affects its protein stability and DNA binding activity (12, 19, 39).

Regulation of the CRY:CLOCK:BMAL1:E-box Complex by PER—Although it is clear that PER and CRY bind to one another with high affinity, the significance of this interaction for their respective functions has been a matter of considerable debate. Some reports suggest that CRY is required for nuclear transport of PER, and others indicate that, in fact, CRY depends on PER for nuclear entry (40, 41). In addition,
it has been reported that PER and CRY are required for the stability of one another, which somewhat complicates the interpretation of the results obtained with CRY or PER mutant mice and cell lines.

Similarly, even though yeast two-hybrid assays and co-immunoprecipitation experiments have revealed that in pairwise tests both CRY and PER directly interact with both CLOCK and BMAL1, these tests do not address the question of whether these interactions occur both off and on DNA. Some studies have concluded that CRY is the \textit{bona fide} repressor that binds to CLOCK:BMAL1 and brings along PER to E-boxes in target promoters (22). However, a recent study that employed co-immunoprecipitation and behavioral analysis of CRY1- or PER2-overproducing mice suggested that PER2 bridges CRY and CLOCK:BMAL1 bound to DNA to drive the circadian negative feedback loop (17).

In our study, we focused on the question of CRY/PER interactions with CLOCK and BMAL1 both on and off DNA in a defined system. These \textit{in vitro} data were further verified by ChIP experiments in mouse fibroblasts to ascertain that the results we observed with the \textit{in vitro} system were not an artifact of the reaction conditions. Contrary to our expectations, based on numerous reports indicating an essential role for PER in repressing CLOCK:BMAL1 \textit{in vivo}, and based on \textit{in vitro} data, including our own, which show strong PER/CLOCK/BMAL1 interaction off DNA, we were unable to detect the formation of a PER:CLOCK:BMAL1:E-box ternary complex under a variety of experimental conditions. Furthermore, the presence of CRY in the reaction mixture does not promote the recruitment of PER2 to the CLOCK:BMAL1:E-box ternary complex. However, the lack of PER2 binding to cognate promoters in fibroblasts differs from the findings with ChIP using liver nuclei (42). Most likely, liver cells

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{Model for the roles of CRY and PER in the TTFL of the mammalian circadian clock. A and B, during the day (or subjective day) CLOCK:BMAL1 bind to the \textit{Per} and \textit{Cry} promoters and activate their transcription. In addition, REV-ERB/ROR regulates \textit{Bmal1} transcription. The PER and CRY proteins make heterodimers and enter the nucleus. Within the nucleus there is a dynamic equilibrium of the PERCRY complex and solitary PER and CRY (most likely in complexes with other proteins); C and D, during the night (or subjective night), solitary CRY binds to the CLOCK:BMAL1:E-box ternary complex and inhibits the transcription of \textit{Per} and \textit{Cry} as well as other CLOCK:BMAL1 controlled genes both by physically interfering with the transactivation function of CLOCK:BMAL1, as well as by interfering with the post-translational modification of these proteins, which seems to be important for their transactivation functions. Data from mouse liver ChIP shows that PER2 can bind to the promoter of \textit{Bmal1} and control its transcription independently of CRY (42). This is a highly simplified model to emphasize that CRY can directly interact with CLOCK:BMAL1 on DNA to repress its transactivator function and that similarly, based on data from other studies (5, 42), PER2 can regulate the transcription of genes in the “stabilizing loop” of the clock independently of CRY to affect their transcription. NRE, nuclear responsive element.}
\end{figure}
express proteins, which are not expressed in fibroblasts and interact with PER2 and recruit it to the target promoters in a tissue-specific manner.

Most surprisingly, perhaps, was our finding that even though solitary CRY binds to a CLOCK:BMAL1:E-box in vitro, the CRY:PER heterodimer does not, suggesting that PER interferes with binding of CRY to the transactivator on DNA. In support of this conclusion, we found that when PER was added to the CRY:CLOCK:BMAL1:E-box complex, CRY was removed from the complex. This is consistent with a model whereby CRY is in a dynamic equilibrium with CLOCK:BMAL1:E-box, and in the presence of PER at a sufficiently high concentration in the nucleus, all CRY becomes trapped in the form of a PER:CRY heterodimer, which cannot bind to the CLOCK:BMAL1:E-box (Fig. 7B). This conclusion may explain the severely disrupted rhythm in PER2 overexpressing fibroblasts or mice (17), but is counterintuitive to the genetically defined role of PER as a repressor in the core clock circuitry, and calls for re-evaluation of the core molecular clock model or revision of at least some aspects of the model.

Quite conceivably, PER functions as a repressor of CLOCK:BMAL1 not in the conventional sense by physical interaction with the transactivator complex, but by participating in stabilization and nuclear transport of CRY, which is the actual repressor, as well as by regulating transcription through interactions with some nuclear receptors as reported previously (42, 43) (Fig. 7C). We note, however, that this model does not incorporate the well known and rather strong interactions of PER with CLOCK and BMAL1. It is conceivable that PER interacts with these proteins off DNA, and in doing so, prevents their association with DNA although this is not consistent with the reporter gene assay results that show that the PER protein alone is a weak inhibitor of CLOCK:BMAL1 activity. It is, however, possible that PER, in addition to aiding CRY repressor function in its capacity as chaperone. It may, to a limited degree, aid in the formation of the CRY:CLOCK:BMAL1:E-box complex in its capacity as molecular matchmaker by transient interaction with CLOCK:BMAL1 on DNA. Clearly, additional work is needed to clarify the mechanism by which PER participates in the negative arm of the transcription-translation feedback loop, and, if there is some tissue specificity (such as the suprachiasmatic nucleus versus peripheral tissue) in the core clock circuitry.

Enzymatic (Catalytic) Control of Circadian TTFL—It has been shown that the core clock proteins are subject to phosphorylation and ubiquitin-proteasome system-mediated degradation (1). In both Neurospora and Drosophila, the negative elements inhibit the DNA binding of the positive elements by recruiting kinases to phosphorylate the positive elements (44, 45). Undoubtedly, these and other post-translational events contribute significantly to the generation of a robust TTFL with circadian periodicity (21, 39, 46 – 48). Here, we have presented some basic biochemical facts regarding the contributions of the interactions of the proteins in the positive arm (CLOCK and BMAL1) and negative arm (CRY and PER) of the clock with one another on and off DNA in the generation of the circadian TTFL. We believe quantitative in vitro studies of the post-translational modifications of clock proteins are needed for a comprehensive description of the mammalian circadian clock.

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