Delay in Feedback Repression by Cryptochrome 1 Is Required for Circadian Clock Function

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SUMMARY

Direct evidence for the requirement of delay in feedback repression in the mammalian circadian clock has been elusive. Cryptochrome 1 (Cry1), an essential clock component, displays evening-time expression and serves as a strong repressor at morning-time elements (E box/E box) within the Cry1-proximal promoter and night-time elements (RREs) within its intronic enhancer gives rise to evening-time expression. A synthetic composite promoter produced evening-time expression, which was further recapitulated by a simple phase-vector model. Of note, coordination of day-time with night-time elements can modulate the extent of phase delay. A genetic complementation assay in Cry1−/−:Cry2−/− cells revealed that substantial delay of Cry1 expression is required to restore circadian rhythmicity, and its prolonged delay slows circadian oscillation. Taken together, our data suggest that phase delay in Cry1 transcription is required for mammalian clock function.

INTRODUCTION

Circadian clocks are thought to consist of autoregulatory loops in which delayed expression of clock components is critical for maintaining circadian rhythmicity (Dunlap, 1999; Reppert and Weaver, 2002; Young and Kay, 2001). However, the underlying molecular mechanism giving rise to such delay remains unknown, hindering formal validation of its biological relevance. In mammalian clocks, circadian transcriptional program is mediated through at least three clock-controlled DNA elements, morning-time (E box/E box, or E/E box: CACGT[G/T]) (Gekakis, 1998; Hognessch et al., 1997; Ueda et al., 2005; Yoo et al., 2005), and night-time elements (Rev-Erβ/ROR-binding element, or RRE: [A/T][A/T]NT[A/G]GGTCA) (Harding and Lazar, 1993; Preitner et al., 2002; Ueda et al., 2002, 2005). The E/E box-mediated transcriptional program has a critical role in the core autoregulatory loop of the mammalian circadian clock (Gekakis, 1998; Sato et al., 2006; Ueda et al., 2005). In this core loop, bHLH-PAS transcription activators such as BMAL1 and CLOCK form heterodimers that bind to E/E box cis-elements in the promoter regions of their target genes, including the Per and Cry genes; CRYS, in turn, form repressor complexes that physiologically associate with the BMAL1/CLOCK complex to inhibit E/E box-mediated transcription (Dunlap, 1999; Griffin et al., 1999; Kume et al., 1999; Reppert and Weaver, 2002; Young and Kay, 2001). Thus, the CRYS play an integral role in the circadian clock by “closing” the core negative feedback loop.

Although Cry1−/− mice and their SCN slices display circadian rhythms at the organisinal and tissue levels, respectively, dissociated Cry1−/− SCN neurons and fibroblasts are largely arrhythmic. The issue of cell autonomy has been carefully examined in several recent studies (Brown et al., 2005; DeBruyne et al., 2007; Liu et al., 2007). By contrast to Cry1-deficient cells, dissociated Cry2−/− SCN neurons and fibroblasts exhibit robust rhythmicity, implying that CRY2 cannot substitute for Cry1 deficiency at the cellular level (Liu et al., 2007). Therefore, we focused on transcriptional regulation of Cry1 gene.

CRY1 and its expression pattern play a pivotal role in the core autoregulatory loop. Either overexpression of Cry1 or interference of Cry1’s repressor activity on E/E box-mediated
transcription can abolish circadian transcriptional oscillations (Sato et al., 2006; Ueda et al., 2005). Remarkably, Cry1 displays delayed gene expression relative to other genes with E/E' box elements (Ueda et al., 2002, 2005). Circadian expression of Cry1 peaks at evening phases in the SCN (~CT12) (Ueda et al., 2002, 2005), which is much later than for typical morning-time E/E' box-regulated genes such as Rev-Erbα, and is intermediate between day-time D box- and night-time RRE-regulated genes such as Per3 and Bmal1, respectively (Ueda et al., 2002, 2005). Dual roles of Cry1 as a strong repressor for E/E' box activity and a time delay mediator fit well with the current model of the circadian clock, i.e., feedback repression with delay may depend on the unique mode of transcriptional regulation of Cry1.

Previous studies identified an E' box and an E box in Cry1's regulatory region (Ueda et al., 2005; Fustin et al., 2009) and two RREs in its first intron (Ueda et al., 2005). In this study, we also identified additional D boxes in the promoter region and confirmed their functionality in conferring day-time expression (delayed phase relative to E box). We further discovered that a combination of the promoter containing E/E' boxes and D boxes with the first intron sequence of Cry1 containing RREs generated delayed-phase expression of Cry1, in which the strength of night-time elements (RREs) can modulate the extent of phase delay. Of note, a simple phase vector model predicts that coordination between day-time and night-time elements can determine the extent of phase delay. Based on this model, we generated an array of Cry1 constructs that display different phases, and these constructs were used in a genetic complementation assay to restore circadian oscillation in Cry1−/−:Cry2−/− cells. These experiments reveal that substantial delay of Cry1 expression is required to restore single-cell level rhythmicity and that prolonged delay of Cry1 expression can slow circadian oscillation. These results suggest that phase delay in transcriptional feedback repression is required for mammalian clock function.

RESULTS

Cry1 Promoter Confers Phase and Amplitude Intermediate between Those Conferred by E/E' Box and D Box Circadian Elements

To examine Cry1 promoter activity, we generated a reporter construct, P(Cry1)-Luc, in which a 1.5 kbp DNA fragment containing the Cry1 promoter was fused to the Luciferase (Luc) gene. Cry1 promoter-driven bioluminescence reached its peak at circadian time (CT) 9.60 ± 0.11 (n = 3, mean ± standard deviation), which was rather close to that of a D box-P(SV40)-Luc reporter harboring three tandem repeats of D boxes fused to an SV40 promoter (CT10.42 ± 0.16), and delayed > 6 hr relative to an E' box-P(SV40)-Luc reporter harboring three tandem repeats of E' boxes fused to an SV40 promoter (CT3.53 ± 0.04) (Figure 1A and Figure S1A and Table S1 available online). On the other hand, the Cry1 promoter produced a higher-amplitude rhythm than that of the D box-P(SV40)-Luc reporter (Figure 1B).

The amplitude of E' box-driven bioluminescence rhythms (Figure 1B, E' box-P(SV40)-Luc) was even higher than those driven by the Cry1 promoter. These data place the Cry1 promoter intermediate between D box and E' box in both phase and amplitude of driven rhythms and suggest that the Cry1 promoter might contain both D box and E' box elements.

D Box in Cry1 Promoter Confers Phase Delay and Day-Time Expression

We investigated the genomic sequences of the Cry1 promoter and found five highly conserved regions, of which two sequences (5'-TTCAGAAA-3' and 5'-AAACGTGA-3') most closely resemble a D box according to position weight matrix analysis. Interestingly, these sequences overlap with the conserved E' box sequences in the promoter region (Figure 1C). We designated this region of the Cry1 promoter as a Cry1proD element and constructed a Cry1proD-P(SV40)-Luc reporter by fusing three tandem repeats of this element to an SV40 promoter. NIH 3T3 cells transiently transfected with this construct showed circadian oscillation of bioluminescence with a peak at day-time (CT12.37 ± 0.05, n = 3; Figure 1D, Figure S1B, and Table S1) and a relative amplitude between those of E' box and D box constructs (Figure 1E). Because the E' box and two putative D boxes in Cry1proD element overlap, it is not practical to isolate each CCE for analysis individually. Instead, we tested whether clock factors involved in E'- or D-box-mediated transcription could activate or repress the Cry1proD element. Cotransfection of E' box activators BMAL1/CLOCK strongly induced not only E' box, but also Cry1proD activity in NIH 3T3 cells (Figure 1F). Cotransfection of a D box repressor E4BP4 inhibited BMAL1/CLOCK induction of Cry1proD activity in a dose-dependent manner (Figure 1F). Interestingly, the E' box repressor CRY1 also inhibited this induction (Figure S1C), and the D box activators DBP, HLF, and TEF (Mitsui et al., 2001) also induced Cry1proD activity (Figure S1D). These results suggest that Cry1proD is regulated by classical transcriptional regulators of both D box and E/E' box activities, consistent with the observation that bioluminescence rhythms driven by the Cry1 promoter display properties intermediate between those driven by D box and E' box constructs.

To confirm that Cry1 expression is delayed relative to E/E' box activity, we measured temporal mRNA profiles of endogenous Per2, Bmal1, and Cry1 mRNA driven by Cry1 in NIH 3T3 cells expressing a P(Cry1)-Luc reporter (Figure 1G, left). As shown, the phase of Luc mRNA driven by the Per2 promoter was almost the same as that of endogenous Per2 mRNA (Figure 1G, right). Furthermore, the phase of Luc mRNA driven by the Cry1 promoter was delayed relative to those of endogenous Per2 mRNA or Luc mRNA driven by the Per2 promoter, with a phase difference of ~1~2 hr. These results further support the notion that functional D boxes in the Cry1 promoter contribute to phase delay of Cry1 expression.

Cry1 Intron Acts as an Enhancer to Confer Phase Delay

In addition to the phase delay caused by D boxes within the Cry1 promoter, we also observed further phase delay of the endogenous Cry1 mRNA by at least 2 hr relative to Luc mRNA driven by the Cry1 promoter (Figure 1G). The endogenous Cry1 mRNA displayed ~3–4 hr phase delay relative to endogenous Per2 mRNA and ~7–8 hr advance relative to Bmal1 mRNA. This observation is consistent with previous reports (Baggs et al., 2009; Etchegaray et al., 2003; Liu et al., 2008; Preitner
et al., 2002; Sato et al., 2006; Ueda et al., 2005), which have speculated that a phase delay would be generated by two functional RREs present in the intron regions of Cry1 (Ueda et al., 2005). To provide experimental evidence for the mechanism of further phase delay of Cry1 expression, we focused on one of the highly conserved regions of the Cry1 gene—the first intron, which contains two RREs, designated here as R1 and R2 (Figure 2A). These RREs are highly conserved and aligned in a head-to-head arrangement, perfectly matched to the consensus RRE sequence ([A/T]A[A/T]NT[A/G]GGTCA). We cloned a 1.03 kbp fragment containing the conserved intronic RREs, designated here as R1 and R2, into the pCry1-Luc reporter plasmid.
to generate a P(Cry1)-Cry1 intron 1.03k-Luc reporter. Cells expressing this reporter displayed a bioluminescence peak at CT14.62 ± 0.20, whereas absence of the 1.03 kbp intron sequence resulted in a peak at CT10.51 ± 0.30, a difference of ~4 hr (Figure 2C, Figure S2B, and Table S1). We next focused on a highly conserved region of 336 bp within the 1.03 kbp intron sequence for further analysis. Whereas cells expressing a P(Cry1)-Cry1 intron Δ336-Luc reporter exhibited a peak at CT10.37 ± 0.24, those expressing P(Cry1)-Cry1 intron 336-Luc peaked at CT14.32 ± 0.23 (Figure 2B, Figure S2A, and Table S1), a 4 hr phase delay. Thus, Cry1 first intron sequences containing RREs likely underlie the delayed phase of Cry1 expression. In addition, their effects appear to be independent of locations (Figure 2C, Figure S2B, and Table S1), suggesting that this sequence functions as a transcriptional enhancer.

Next, we analyzed the regulatory regions for the presence of their corresponding transcription factors in vivo using chromatin immunoprecipitation (ChIP) assays with time series samples from mouse liver. Chromatin from wild-type, Dbp−/− (Lopez-Molina et al., 1997), or Rev-Erbα−/− (Prenter et al., 2002) mice was immunoprecipitated by anti-BMAL1, anti-DBP, or anti-REV-ERBα antibodies (Figure 2D). The levels of BMAL1 and DBP binding to Cry1proD displayed circadian oscillation in wild-type, Dbp−/−, or Rev-Erbα−/− mice, whereas DBP binding in Dbp−/− mice was significantly reduced (p < 0.01 by two-way ANOVA) with residual signals potentially deriving from TEF and/or HLF binding to the same element. On the other hand, no significant reduction was observed for the binding of DBP to this region in Rev-Erbα−/− mice. The level of REV-ERBα binding to the Cry1 first intron region also displayed circadian oscillation in wild-type and Dbp−/− mice, whereas it was significantly reduced in Rev-Erbα−/− mice (p < 0.01 by two-way ANOVA). The levels of BMAL1 binding to the Cdp promoter region displayed circadian oscillation, whereas there was only background binding of DBP and REV-ERBα to this region. This result confirmed that BMAL1 and DBP bind to the Cry1 promoter region, and REV-ERBα binds to the Cry1 first intron region. The peak binding time of each transfactor is consistent with previous reports of its in vivo binding or its nuclear accumulation (Lopez-Molina et al., 1997; Mitsui et al., 2001; Prenter et al., 2002; Ripperger and Schibler, 2006).

In addition to the biochemical interaction between the Cry1 promoter and D box trans-regulators described above, we also examined the role of the D box using genetic approaches; we measured mRNAs expression patterns from time course liver samples of triple-knockout mice of PAR bZips genes (Tef, Hlf, and Dbp) (Gachon et al., 2004). Although these mice displayed normal circadian behavior (possibly due to compensation rendered by posttranslational mechanisms intracellularly and/or intercellular coupling of clock cells in vivo) (Gachon et al., 2004; Lee et al., 2001; Liu et al., 2007), we found that Cry1’s circadian expression level was different from wild-type and its peak of expression delayed (Figure 2E and Figure S2C), whereas those of other measured clock genes (Bmal1, RevErba, and Per1) were not. Importantly, the observed peak delay was reproducible and significant in three independent experiments (p < 0.01 by two-way ANOVA). These results further confirm that PAR bZips genes are important for the proper phase of expression of Cry1.

Strength of Intrinsic RREs Correlates with Phase Delay
To determine whether the strength of RREs in 336 bp of Cry1 first intron sequence correlates with the phase delay, we generated an array of intron sequences harboring mutant RREs, including deletion, mutation, and inversion of the two RREs (Figure 3A). We inserted these mutant intron sequences into P(SV40)-Luc vector to generate an array of P(SV40)-Cry1 intron 336-Luc reporter constructs. As one measurement for the strength of intrinsic RREs, we first examined transcriptional activation of these constructs by RORα, an activator of RRE, in a reporter assay. We found that induced Luciferase activities varied significantly among constructs, ranging from strong induction by wild-type RRE to almost no induction by double-mutant or deleted RREs (R1 and R2) (Figure 3B). These results indicate that the RREs within the intron sequence are functionally responsive to RORα and that intrinsic RREs of various strengths can be obtained from different RRE mutations.

As an independent measurement for the strength of intrinsic RREs, we next examined the amplitude of circadian oscillations expressed by these constructs in reporter rhythm assays (Figure S3A and Table S1). Rhythm amplitude was low when an intron sequence of low RORα responsiveness was used to drive reporter expression and high when an intron sequence of high RORα responsiveness was used (Figures 3B and 3C). Overall, there was a significant positive correlation between the two measurements for the strength of intrinsic RREs: RORα responsiveness and rhythm amplitude among the intrinsic RRE mutants (r² = 0.95, p < 0.01; Figure 3D).

These mutant intron sequences allowed us to analyze quantitatively the role of intrinsic RREs in the phase delay mechanism. Specifically, we examined how intrinsic RRE mutation affects phase delay using a reporter rhythm assay (Figure 3E, Figure S3B, and Table S1). We found that the observed phase delay significantly correlated with the first measurement for the strength of the intrinsic RRE mutants, i.e., RORα responsiveness (r² = 0.82, p < 0.01; Figure 3F, top). Similarly, phase delay also correlated well with the second measurement for the strength of the intrinsic RRE mutants, i.e., the rhythm amplitude (r² = 0.90, p < 0.01; Figure 3F, bottom). Taken together, these data suggest that the strength of RREs correlates with the phase delay, further corroborating our finding that the RREs in the Cry1 intron act as an enhancer to further delay the phase conferred by Cry1 promoter.

Combination of Day- and Night-Time Elements Produces Evening Phase Control
Given that the delayed expression of Cry1 is a combined effect of its promoter and intron, we sought to understand whether this combinatorial effect is a general design principle in the circadian transcriptional network or a mechanism unique to the transcriptional regulation of Cry1. We first asked whether the phase of endogenous Cry1 expression could be synthesized using an artificial promoter in clock cells. We constructed three sets of reporters, with each harboring one of the three CCEs (i.e., E/E’ box, D box, and RRE) in the presence or absence of the RRE-containing intron sequence from the Cry1 gene (Figures 4A and 4B). Real-time bioluminescence recording of transfected NIH 3T3 cells showed that the RRE-containing Cry1 intron sequence, as expected, did not dramatically alter the phase of
Figure 2. Cry1 Intron Acts as an Enhancer to Confer Phase Delay

(A) The first intron of Cry1 contains RRE sequences. The marked 1.03 kbp and 336 bp of Cry1 first intron sequence, which are highly conserved in mammals, were cloned and examined in this study. Two RREs are indicated as R1 and R2, respectively.

(B) Cry1’s first intron confers phase delay. The Cry1 promoter was combined with the Cry1 1.03 kbp intron, 336 bp intron, or Cry1 intron Δ336 deletion mutant to generate composite promoters. The experiment was performed as in Figure 1A.

(C) Cry1’s first intron sequence confers phase delay independently of its location. The 336 bp of Cry1 intron sequence was inserted upstream or downstream of the Cry1 promoter and inside or downstream of the coding sequence. Data are representative of two independent experiments (B and C).

(D) Binding of BMAL1 (an E/E box regulator) and DBP (a D box regulator) to the Cry1 promoter region and REV-ERBs (a RRE regulator) to the Cry1 first intron region in vivo. Chromatin from wild-type (gray), Dbp−/− (orange), or Rev-Erbα−/− (purple) mice was prepared at 4 hr intervals from mice held in a 12 hr light/12 hr dark cycle (LD 12:12). The binding of each regulator to its regulatory region was analyzed by ChIP with the indicated antibodies. Note that DBP binding in Dbp−/− mice was significantly reduced (p < 0.01 by two-way ANOVA), with residual signals potentially deriving from TEF and/or HLF binding. Specific TaqMan probes
RRE-mediated reporter expression, albeit with an increase in amplitude (Figure S4). The intron sequence sometimes caused double peaks for the E-box-driven rhythms (Figure 4A and Figure S4). When these rhythms were fitted to a circadian cosine curve, we observed a reduction of the relative amplitude and a slight but reproducible phase advance (Figure 4A, Figure S4, and Table S1). Importantly, the combination of D box in the promoter and RRE-containing Cry1 intron sequence conferred a substantial phase delay of >5hr (CT14.48±0.21) when compared to the D box alone (CT9.31±0.16) (Figure 4, Figure S4, and Table S1). It is important to note that our result indicates that E/E boxes are dispensable for the generation of delayed-phase expression of Cry1. This is because the synthetic composite “D box + RRE” promoter (i.e., a combination of a synthetic D box-driven promoter and RRE-containing Cry1 intron sequence) lacks functional E boxes, unlike the Cry1 promoter. Thus, the D box and the RRE can combine to generate a distinct intermediate phase.

We were able to recapitulate these experimental measurements in a simple model using “phase vectors.” A phase vector represents phase and amplitude of the oscillation as direction and length of the vector in polar coordinates. In this way, the combination of two oscillations can be represented by the vector sum of two corresponding phase vectors (Extended Experimental Procedures). We plotted measured oscillations (CCE without intron sequence and intron sequence without CCE) and obtained the summed phase vector of the CCE-intron sequence combinations (Figure 4B, left three circles). Interestingly, the summed phase vectors corresponded well with the measured oscillations (Figure 4B, rightmost). These results support the notion that combining two CCEs that otherwise function independently can be a general mechanism for generation of new phases and, more specifically, the combined phase may be predicted, to a first-order approximation, by a vector sum.

Delayed Expression of Cry1 Restores Circadian Rhythmicity in Cry1-/−:Cry2+/− Cells

To address the functional importance of the RRE-mediated phase delay, we employed cell-based genetic complementation, testing for phenotypic rescue in arthrythmic Cry1-/−:Cry2+/− cells. We hypothesized that, if phase delay is an important property of Cry1, its delayed expression, peaking at evening-time, should restore circadian oscillations in these cells. To test this hypothesis, we established mouse embryonic fibroblasts from Cry1+/−:Cry2+/− double-knockout mice (van der Horst et al., 1999). Similar to negative control (Figure 5A, without Cry1), Cry1 expression driven only by the Cry1 promoter, P(Cry1), did not rescue circadian oscillations in these cells (Figure 5A, P(Cry1)). However, when Cry1 expression was regulated by P(Cry1)-Cry1 intron 336, which contains the Cry1 promoter and the RRE-containing 336 bp of Cry1 intron sequence, its exogenous expression restored circadian rhythmicity in these cells, with a period length of 26.73±0.19hr (Figure 5A, Cry1 intron 336). The observed rescue capability was independent of the Cry1 protein level, vector type, or method of DNA delivery (Figures SSA and SSB). Taken together, these results demonstrate that delay of Cry1 expression, conferred by the Cry1 intron, is required for rescue of circadian rhythmicity.

To further assess the contribution of delayed Cry1 expression to the rescued circadian oscillation, we tested the rescue capability of the intronic RRE mutants that possess different RRE strengths, as described above (Figure 3 and Figure 5A, nine panels on the right, and Table S2). The ability of the intronic RRE mutants to rescue rhythmicity, represented as amplitude of circadian oscillations, significantly correlated with the strength of intronic RREs, as measured by bioluminescence levels derived from P(SV40)-Cry1 intron 336-Luc (r²=0.87, p < 0.01; Figure 5B, left). Similarly, the rescue capability also correlated with another measurement of strength of intronic RREs, i.e., RO3x responsiveness of the intronic RRE mutants (r²=0.97, p < 0.01; Figure 5B right). More directly, the rescue capability correlated with the phase delay conferred by the intronic RRE mutants that was measured in bioluminescence rhythms of P(Cry1)-Cry1 intron 336-Luc (r²=0.71, p < 0.01; Figure 5C). It should be noted that the rescue capability in these experiments does not correlate with either amplitude or basal bioluminescence levels of P(Cry1)-Cry1 intron 336-Luc (Figure S5C), suggesting that the rescue capability is most likely attributable to the delayed phase of Cry1 expression conferred by RREs. To directly confirm this, we demonstrated that the pure RREs, when combined with Cry1 promoter, rescued circadian rhythmicity in Cry1-/−:Cry2+/− cells, whereas Cry1 promoter alone could not reliably rescue rhythms (Figure S5D and Figure 5).

The Cry1-rescued Cry1+/−:Cry2+/− cells (a Cry2 knockout, in essence) showed a rather long period length of ∼27hr (Figure 5A), which is consistent with previous reports showing that Cry2+/− single-knockout cells display long periods compared to wild-type cells (∼24–25hr) (Liu et al., 2007). We confirmed that genetic complementation of Cry1 in Cry1+/−:Cry2+/− cells recapitulates the circadian phenotype in Cry2 single-knockout cells, thus phenotypically validating the Cry1 rescue assay (Figure S5E and Table S3).

Cry1 Phase Delay Modulates Circadian Period Length

The genetic complementation assay expressing Cry1 of various phases revealed that delay of Cry1 expression is required to restore circadian rhythmicity, consistent with the proposed design principle for circadian clocks, i.e., transcriptional/translation...
feedback repression with delay. This design principle further predicts that Cry1 expression with a more prolonged delay can slow circadian oscillations. To test this prediction, we first attempted to generate constructs expressing Cry1 with prolonged delays. According to the phase-vector model described above (Figure 4B), we should be able to generate evening-to-night
expression with a more prolonged delay by weakening the day-time promoter but keeping a constant strength of the night-time enhancer of Cry1 intron sequence. Therefore, we generated an array of day-time promoters with various strengths of D boxes, containing 1, 2, or 3 tandem repeats of D boxes or Cry1proD elements; we confirmed that these day-time promoters displayed day-time phased bioluminescence rhythms of various relative amplitudes, as expected (Figure 6A, Figure S6A, and Table S1). We then generated another set of constructs by combining these day-time promoters with the Cry1 intron sequence (marked by asterisk).

Next, we asked whether evening-to-night Cry1 expression with prolonged delay could slow circadian oscillations (Figure 6D and Table S3). Interestingly, the periods of rescued circadian oscillations ranged from 27 to 31 hr. In particular, period length correlated with the delay prolonged by weakening the day-time promoter: the more the Cry1 phase was delayed, the longer the rescued period ($r^2 = 0.81$, $p < 0.01$; Figure 6E and see also Figure S6C). We also confirmed that the period length did not significantly correlate with either amplitude or basal activity of Cry1 expression by using a different constitutive promoter (Figure S6D and Table S3). In addition, CRY1 protein level was not significantly correlated with either amplitude or basal activity of Cry1 expression (see also Table S1).

Figure 4. Combination of Intrinsic RREs with Known Circadian cis-Elements Gives Rise to Emergent Phases that Can Be Predicted by Phase Vectors
(A) Combination of the Cry1 intron sequence with known CCEs gives rise to emergent phases. A promoter was constructed by inserting 3 × E'-box, 3 × D box, or 3 × RRE sequences in the upstream of P(SV40). In the reporter construct, Luciferase expression was under the control of the 3 × CCE-P(SV40) promoter in the absence or presence of the 336 bp of Cry1 intron sequence. The experiment was performed as in Figure 1A. Phases were estimated by fitting a cosine wave with circadian period corresponding to maximum autocorrelation of the time series using detrended bioluminescence data. This method allowed phase estimation of even distorted wave form expressed by E'-box + Cry1 intron sequence (marked by asterisk).

(B) A phase vector model recapitulates the emergent phases. The phase vector of each CCE (E'-box green arrow; D box, orange arrow; RRE, purple arrow) and the vector sum of the two phase vectors (center of colored ellipsoid) are plotted in the polar coordinate (left three circles). The ellipsoidal disk represents 95% confidence region. The phase vectors (colored arrows of black border) represent measured circadian transcriptional activities induced by the combined regulation of Cry1 intron sequence and each CCE (rightmost circle).

Data are representative of two independent experiments. See also Figure S4 and Table S1.
Figure 5. Delayed Expression of Cry1 Restores Circadian Rhythmicity in Cry1/−/−:Cry2/− Cells

(A) Genetic complementation of Cry1 rescues circadian oscillation in Cry1/−/−:Cry2/− cells. A schematic diagram of Cry1 rescue constructs is shown on the left. The composite promoter contains P(Cry1) and the 336 bp Cry1 intron sequence of wild-type or a mutant (deletion, mutation, or inversion) of the R1 and R2 sequences as in Figure 3, which controls Cry1 expression. Cry1 rescue constructs were each cotransfected with a destabilized luciferase reporter construct, P(Per2)-dLuc, into Cry1/−/−:Cry2/− mouse embryonic fibroblast cells (left), followed by bioluminescence recording. Whereas mock-transfected Cry1/−/−:Cry2/− cells were completely arrhythmic and those expressing P(Cry1)-Cry1 were only transiently rhythmic during the first 2 days of recording, P(Cry1)-Cry1 intron 336-Cry1 expression restored circadian oscillation with a period length of 26.73 ± 0.19 hr (bottom in the center column). Rescue effects varied among the intronic RRE mutants (right nine panels). Data are representative of two independent experiments.

(B) Relative amplitude of rescued circadian oscillation correlates with the strength of intronic RREs. The relative amplitudes of rescued oscillation are plotted against two measurements for the strength of intronic RREs, the relative amplitudes of P(SV40)-Cry1 intron 336-Luc expression presented in Figure 3C, and the RORα-responsiveness presented in Figure 3B.

(C) Relative amplitude of rescued circadian oscillation correlates with phase delay. The relative amplitudes of rescued oscillations are plotted against the phase delay of various P(Cry1)-Cry1 intron 336-Luc activities relative to P(Cry1)-Luc activity presented in Figure 3E. Mean and SD (error bar) of two independent experiments are shown (each experiment contains three samples; n = 3 unless otherwise indicated in Table S1). See also Figure S5 and Table S2.

Single-Cell Analysis Confirms the Importance of Cry1 Phase Delay in Feedback Repression

Arrhythmic phenotypes observed in population of cells might be due to rapid damping of individual cells or lack of synchronization among individual cells. To discriminate between these possibilities, we monitored bioluminescence levels in real time at the level of single-cell resolution (Sato et al., 2006; Ukai et al., 2007). As with whole-well assays, single-cell analysis showed that most individual cells expressing Cry1 with a normal delay, driven by the intron sequence containing wild-type RREs, were robustly rhythmic, with a circadian period of 26.77 ± 0.12 hr (Figures 7A and 7B and Table S3), whereas most cells expressing Cry1 without delay, driven by an intron sequence harboring mutated RREs, were arrhythmic (Figure 7B and Movie S1). Moreover, individual cells expressing Cry1 with a prolonged delay driven by the Cry1 intron sequence alone (i.e., in the absence of Cry1 promoter) displayed long circadian periods of up to 32.00 ± 0.58 hr (Figures 7A and 7B and Table S3). The circadian oscillations in Figure 7A with delayed Cry1 expression were statistically significant (p < 0.01 by autocorrelation) and reproducible in different series of experiments. Thus, single-cell analysis confirmed the circadian phenotypes observed in...
Figure 6. Prolonged Delay of Cry1 Expression Slows Circadian Oscillations in Cry1⁻⁻:Cry2⁻⁻ Cells

(A) Promoters harboring various CCEs display different circadian phases. The promoters contain one, two, or three tandem copies of D box or Cry1proD element, which were inserted into the P(SV40)-Luc vector to generate an array of reporter constructs.

(B) The 336 bp of Cry1 intron sequence confers phase delay to D box and Cry1proD element. Reporter constructs were generated similarly as in (A) except that the intron sequence was inserted. The experiment was performed as in Figure 1A (A and B).

(C) The measured phases conferred by the composite promoters are consistent with those predicted by phase vectors. (Left) The phase vectors of oscillations driven by various promoters without the intron sequence (colored arrows) and those driven by the intron sequence (∼CT17.5) or P(Cry1) (∼CT10) (two black arrows) are plotted with summed phase vectors (center of colored ellipsoidal disks). The ellipsoidal disk represents 95% confidence region. (Right) The predicted phases from the simple phase-vector model are plotted against the observed phases. Error bars represent SD (n = 3).

(D) Cry1 rescue of circadian oscillation in Cry1⁻⁻:Cry2⁻⁻ cells using synthetic composite promoters. The composite promoters presented in (B) were used to drive Cry1 expression. Cry1⁻⁻:Cry2⁻⁻ cells were cotransfected with a Cry1 expression construct and a P(Per2)-dLuc reporter.

(E) Prolonged phase delay of Cry1 expression correlates with period length of rescued oscillations. The period lengths of rescued oscillations are plotted against the phase delay of various composite promoters’ activity relative to P(Cry1)-Cry1 intron 336-Luc activity presented in (B). Mean and SD (error bar) of two independent experiments are shown (each experiment contains three samples; n = 3).

Data are representative of two independent experiments (A, B, and D). See also Figure S6, Table S2, and Table S3.
Figure 7. Single-Cell Analysis Confirms the Requirement of Cry1 Phase Delay

(A) P(Per2)-dLuc bioluminescence levels in transfected Cry1<sup>−/−</sup>:Cry2<sup>−/−</sup> cells as recorded. The P(Per2)-dLuc reporter and a Cry1 expression construct as indicated were cotransfected into Cry1<sup>−/−</sup>:Cry2<sup>−/−</sup> cells, and bioluminescence expression was recorded with a PMT. Data from three independent samples are shown.

(B) P(Per2)-dLuc bioluminescence levels in transfected individual Cry1<sup>−/−</sup>:Cry2<sup>−/−</sup> cells as recorded by a luminescence microscope (n = 100). Reporter activities from each cell were normalized so that the maximum and minimum bioluminescence values are 100% and 0%, respectively. The mean reporter activity for all of the analyzed single cells at each time point is indicated by a thick black line (top row). Time series of bioluminescence expression shown in the top row were redrawn as heatmaps (bottom row). Each row in the heatmap represents a time series of P(Per2)-dLuc reporter activities from a single cell. The corresponding p value of rhythmicity at the period of maximum autocorrelation was evaluated for each time series and is depicted on the right. One-hundred cells were randomly selected and individually analyzed. Data are representative of two independent experiments (A and B).

(C) The roles of phase delay in Cry1 expression. Through regulation of Cry1 expression, the promoter and intron primarily affect the amplitude and period of the clock system, respectively.
Cry1-rescued Cry1−/−:Cry2−/− cells at the cell population level, which lends strong support for our finding that delay of Cry1 expression is required for circadian clock function.

**DISCUSSION**

**Cry1 Phase Control Mechanism**

In this report, we provided experimental data, as well as model predictions, for a “combinatorial regulatory mechanism” to explain the delayed expression of Cry1. We newly identified D boxes, which overlap with the E/E′ box and confer phase delay over E/E′ box activity. In addition, we also demonstrated that the previously identified RREs in the first intron (Ueda et al., 2005) can confer the additional phase delay in Cry1 expression. Furthermore, we observed that the synthetic pure RREs, in combination with Cry1 promoter, rescued circadian rhythmicity in Cry1−/−:Cry2−/− cells with statistical significance (p < 0.01; Figure S5D). Together with the observation that the combination of pure D box and Cry1 intron sequence also rescued rhythms (Figure 6D), we conclude that the RRE and D box elements can recapitulate the basic function of Cry1 intron and Cry1 promoter, respectively. It should be noted that the possible contribution from unknown elements in Cry1 intron 336 sequence could not be completely excluded because the relative amplitude of the pure RRE elements (combined with Cry1 promoter) is slightly lower than that of the wild-type Cry1 intron 336 (combined with Cry1 promoter, Figure S5D).

**General Design Principles for New Phases**

As revealed in this study, these multiple distinct regulatory sites (i.e., two RREs in first intron and the E/E′ box and D boxes in the promoter region) function in a coordinated fashion to generate substantial phase delay, leading to evening-time expression. Interestingly, in an effort to study design principles of the circadian clockwork, we employed a simple phase-vector model in which the new evening-time could be predicted by the combination of two component phase vectors. Although the phase-vector model was not used for phase prediction in our previous study (Ukai-Tadenuma et al., 2008), such a model is also applicable to this previous study when we take into account the time delay associated with transcription/translation of regulator proteins and the Luciferase reporter (R² = 0.99, p < 0.01; Figures S7A and S7B and Table S4). These results show that a new phase can be generated through combinatorial synthesis of either two transcriptional regulators or two clock-controlled DNA elements and also can be described, at least to a first-order approximation, by a phase-vector model. Taken together, this combinatorial regulatory mechanism for the generation of new circadian phases of transcription represents a general design principle underpinning the complex system behavior.

Although the phase-vector model predicts the phase of a synthesized oscillation, it is only a first-order approximation. For example, there are some discrepancies between predicted and measured amplitudes. Also, the wave form generated by the combination of E′ box-driven promoter and the Cry1 intron sequence appeared like a “two-peak” wave form (Figure S4, upper-left), indicative of nonlinear effects or involvement of yet unknown factors. In detailed analysis, we found that the ~11 hr period oscillation was actually a significant component in the two-peak wave form (Figure S4, upper-right; p < 0.01). This is the first demonstration of synthesized ultradian rhythms, which may lead to insights into mechanisms of ultradian gene expression with harmonic periods, as recently reported (Hughes et al., 2009).

**Delayed Cry1 Expression Contributes to Clock Robustness**

In this study, we focused on the level of transcriptional regulation and demonstrated the importance of delay in feedback repression at the intracellular level. Constitutive expression of Cry1 abolished circadian rhythmicity in wild-type cells (Ueda et al., 2005) and failed to restore circadian oscillation in arrhythmic Cry1−/−:Cry2−/− cells (Figure S6D and Figure 7A), suggesting that rhythmic expression of Cry1 is important for clock function. We revealed that the transcriptional oscillation of Cry1 with a correct phase with substantial delay was sufficient and required to rescue circadian oscillation in arrhythmic Cry1−/−:Cry2−/− cells (Figure S5) and that transcriptional oscillation of Cry1 with a prolonged delay slows circadian oscillation (Figure 6). Importantly, we also confirmed that the amount (baseline) of CRY1 protein was not responsible for the changes in amplitude (Figure S5A) and period (Figure S6E) of rescued oscillations. These results suggest that the phase of Cry1 expression is responsible for the changes in rescued amplitude and period rather than the amount (baseline) of CRY1 protein. Because we confirmed the significant linear correlation between transcriptional activities and protein levels (p < 0.01), when monitored by firefly Luciferase, and CRY1 protein amounts, when monitored by fusion Renilla Luciferase, irrespective of cell types (R² = 0.93 in NIH3T3 and R² = 0.90 in Cry1−/−:Cry2−/− cells), we speculated that phase of CRY1 protein level would be responsible for the amplitude and period of rescued oscillations.

It should be noted that the CRY1 protein expression levels in our experiments are within a certain range (Figure S5A and Figure S6E), and we do not exclude (and our current results are not in conflict with) the notion that CRY1 protein amounts may affect the parameters of clock function when CRY1 protein levels drastically differ from those in our experimental system, as previously reported (Baggs et al., 2009; Ueda et al., 2005). In addition, we do not exclude the possibility that other regulatory mechanisms such as posttranscriptional modifications (Lee et al., 2001; Liu et al., 2008) play important roles in attaining the robustness of the clock. For example, rhythmic expression of PER2 is recently reported to play a prominent role in Cry1 function (Chen et al.)

(D) A schematic diagram of a minimal circuit for the mammalian circadian transcriptional network. The network can be represented by a simple circuit, consisting of two transcriptional activations (green arrows) and four transcriptional repressions (red arrows) on three regulatory elements (three rectangles).

(E) The minimal circuit envisaged as a composite of two distinct oscillatory network motifs: (1) A repressilator that is composed of three repressions (left) and (2) a delayed negative feedback loop, which is composed of two activations and one repression (right). See also Figure S7, Movie S1, and Table S3.
Cry1 circadian clock function. This qualitatively less-robust clock oscillation even though the rhythms are rather transient (Figure 7B), driven by a constitutive promoter exhibited weak circadian oscillation even though the rhythms are rather transient (Figure 7B), implying that constant Cry1 expression might partially rescue circadian clock function. This qualitatively less-robust clock function is probably attributable to posttranscriptional and post-translational mechanisms (Lee et al., 2001; Liu et al., 2008).

Even in this context, it appears that phase delay in rhythmic Cry1 expression may contribute to the robustness of clock function by ensuring properly timed nuclear translocation of CRY proteins. This idea is strongly supported by our results presented in this study: delayed Cry1 expression via D box-mediated transcription (i.e., from Cry1 promoter) allowed partial rescue, and further delay via the RREs from the Cry1 intron restored circadian rhythmicity with amplitude and persistence comparable to wild-type cells.

Design Principle for a Circadian Transcriptional Network

Delayed feedback repression is one of the most prevailing but as yet unverified design principles for a circadian transcriptional network. This design principle predicts that decreased delay dampens circadian oscillations and that prolonged delay slows down circadian oscillations (Figure 7C, Figures S7C–S7F, and Extended Experimental Procedures) (Bernard et al., 2006; Lewis, 2003; Novak and Tyson, 2008). The results presented in this study are consistent with the two predictions from the delayed feedback repression, suggesting that it is an applicable design principle in the mammalian circadian transcriptional network.

A Minimal Circuit for a Circadian Transcriptional Network

In a previous effort to identify a minimal circuit of the complex autoregulatory transcriptional networks in the mammalian circadian clock, we showed that day-time promoter activity can be reconstructed by combining a morning-time activator and a night-time repres sor and night-time promoter activity by combining a day-time activator and a morning-time repres sor (Ukai-Tadenuma et al., 2008). In this study, we succeeded in synthesizing the evening-time phase control of transcription. Our previous and current results suggest that the complex mammalian transcription network can be reduced to a relatively simple diagram (Figure 7D) that would consist of three regulatory elements and six transcriptional regulations (two activations and four repressions). It is noteworthy that this diagram can be envisaged as a composite of two distinct oscillatory network motifs (Figure 7E). The first oscillatory network motif is composed of three repressions (i.e., E/E′ box to D box and D box to RRE) and one repression (i.e., RRE to E/E′ box), comprising a delayed negative feedback loop. It is interesting to note that oscillatory properties of both network motifs were experimentally suggested by synthetic approaches (Elowitz and Leibler, 2000; Stricker et al., 2008). Therefore, further experimental and theoretical analyses of the composite of these oscillatory network motifs lie ahead.

EXPERIMENTAL PROCEDURES

Preparation of Embryonic Fibroblasts from Cry1−/−:Cry2−/−

Double-Knockout Mice

Cry1−/−:Cry2−/− double-knockout mice (van der Horst et al., 1999) were carefully kept and handled according to the RIKEN Regulations for Animal Experiments. The dissociated cells (mouse embryonic fibroblasts [MEF] from Cry1−/−:Cry2−/− double-knockout mice; Cry1−/−:Cry2−/− cells) were suspended and cultured in DMEM (Invitrogen) supplemented with 10% FBS (URH Biosciences) and antibiotics (see Extended Experimental Procedures for details).

Real-Time Circadian Reporter Assay Using NIH 3T3 Cells and Cry1−/−:Cry2−/− Cells

Real-time circadian assays were performed as previously described (Sato et al., 2006; Ueda et al., 2005) with the following modifications. NIH 3T3 cells were transfected with the Luciferase reporter plasmids. Cry1−/−:Cry2−/− cells were transfected with pGL3-PeVe2-D-Luc reporter plasmid (Sato et al., 2006) and each Cry1 gene expression vector. The cells were stimulated by 10 μM (NIH 3T3) or 30 μM (Cry1−/−:Cry2−/− cells) forskolin (Fermentek), and the bioluminescence was measured at 30˚C (see Extended Experimental Procedures for details).

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H.R.U. and A.C.L. designed the research scheme. M.U.-T. constructed most of materials and performed real-time luminescence assays, R.G.Y. conducted bioinformatic, statistic, and theoretical analyses. H.X. constructed the virus vector and performed the corresponding real-time luminescence assays. J.A.R. performed ChIP analysis and mRNA accumulation analysis. All authors discussed the results and commented on the manuscript text.
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