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The Orphan Nuclear Receptor REV-ERBα Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator

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Summary

Mammalian circadian rhythms are generated by a feedback loop in which BMAL1 and CLOCK, players of the positive limb, activate transcription of the cryptochrome and period genes, components of the negative limb. *Bmal1* and *Per* transcription cycles display nearly opposite phases and are thus governed by different mechanisms. Here, we identify the orphan nuclear receptor REV-ERBα as the major regulator of cyclic *Bmal1* transcription. Circadian *Rev-erbα* expression is controlled by components of the general feedback loop. Thus, REV-ERBα constitutes a molecular link through which components of the negative limb drive antiphasic expression of components of the positive limb. While REV-ERBα influences the period length and affects the phase-shifting properties of the clock, it is not required for circadian rhythm generation.

Introduction

In mammals, many aspects of behavior and physiology are subject to daily oscillations. These include sleep-wake cycles, energy homeostasis, blood pressure, body temperature, renal activity, and liver metabolism (Schibler, 1999). These rhythms are driven by a central circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Rusak and Zucker, 1979; Ralph et al., 1990). As the period length of this pacemaker is only approximately 24 hr, the circadian clock has to be reset every day by an input pathway in order to remain in resonance with geophysical time. This synchronization is accomplished by daily variations in light intensity, which adjusts the phase of the SCN oscillator via the retino-hypothalamic tract (Rusak and Zucker, 1979). The oscillations generated in the SCN are translated into overt rhythms in behavior and physiology through output pathways that probably involve both electrical and chemical signals. Although signals sent by the SCN pacemaker are essential for the maintenance of overt circadian rhythms, most peripheral cell types may possess an oscillator with a molecular makeup very similar to that of SCN neurons (Balsalobre et al., 1998; Yamazaki et al., 2000).

Animal circadian rhythms appear to be generated by feedback loops in gene expression that include both transcriptional and posttranscriptional regulatory mechanisms (Allada et al., 2001; Albrecht, 2002). In mammals, the PAS helix-loop-helix transcription factors CLOCK and BMAL1 activate transcription of *Per* and *Cry* genes. Once the PER and CRY proteins have reached a critical concentration, they attenuate the CLOCK/BMAL1-mediated activation of their own genes in a negative feedback loop. A recent study suggests that the PER/CRY complex interacts directly with the CLOCK/BMAL1 complex bound to chromatin. In addition, a number of posttranslational events, such as the control of protein phosphorylation, degradation, and nuclear entry, contribute critically to the generation of daily oscillations in clock gene products (see Lee et al., 2001, and references therein).

Whereas a large body of genetic and biochemical evidence has been collected on the regulation of *Cry* and *Per* gene expression, much less is known about the control of *Bmal1* and *Clock* expression. *Bmal1* mRNA accumulation also follows a robust circadian oscillation, but this cycle is nearly antiphase to that of *Per1* and *Per2* mRNA accumulation (Shearman et al., 2000b). In the liver, *Clock* transcript levels fluctuate during the day with a phase angle similar to that of *Bmal1* mRNA accumulation, albeit with a modest amplitude of only 2- or 3-fold (Lee et al., 2001; this study). Given the large phase difference of cyclic *Bmal1* and *Per* mRNA accumulation, different mechanisms must account for the cyclic transcription of *Bmal1* and *Per* genes. Indeed, in recently published reports, PER and CRY proteins have been suggested to play a positive role in *Bmal1* transcription, thus establishing a positive feed-forward loop (Shearman et al., 2000b; Lee et al., 2001; Yu et al., 2002). However, these studies did not reveal how these negative regulators exert positive effects on *Bmal1* transcription.

Here, we show that REV-ERBα is a major circadian regulator of *Bmal1* expression in the SCN and in the liver. This orphan nuclear receptor also participates in the regulation of *Clock* transcription, albeit to a lesser extent. As *Rev-erbα* itself appears to be negatively regulated by PER proteins, it provides a molecular link through which these proteins can drive circadian tran-

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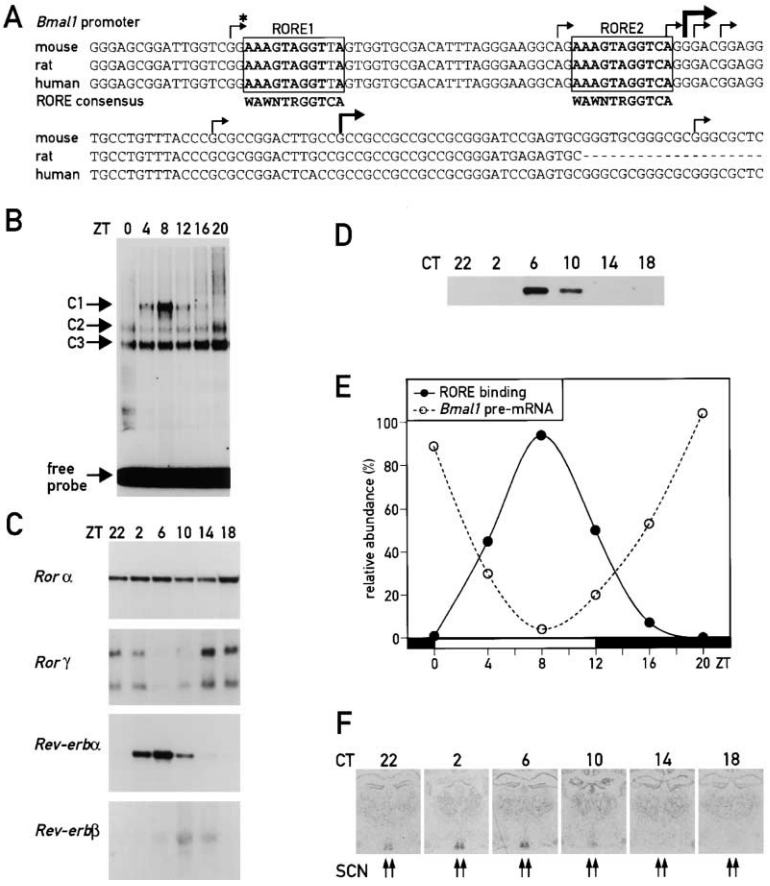

Figure 1. REV-ERBα Is a Putative Regulator of Circadian *Bmal1* Transcription

(A) Sequence comparison of the proximal *Bmal1* promoter regions from mouse, rat, and man. The transcription initiation sites, indicated by bent arrows, have been mapped on the mouse genomic sequence by RACE (rapid amplification of cDNA ends, see Experimental Procedures). Multiple RACE products have been found for the start sites represented by the more prominent arrows (N = 3 for the upstream site and 2 for the downstream site). REs are framed, and the RORE consensus sequence is given below the elements (W = A or T, R = A or G). The arrow marked by an asterisk corresponds to the start site identified by Yu et al. in mouse testis (2002).

(B) Electrophoretic mobility shift assays (EMSA) with liver nuclear proteins harvested at four hour intervals around the clock and a radio-labeled oligonucleotide encompassing RORE 2. The positions of the three most prominent protein:DNA complexes, C1, C2, and C3, are marked by arrows. ZT stands for *Zeitgeber* time. The lights were turned on and off at ZT 0 and ZT 12, respectively.

(C) Temporal accumulation of transcripts encoding RORE binding proteins. Whole-cell liver RNA was prepared from mice sacrificed at the *Zeitgeber* times (ZT, see legend to B) indicated above the panels. The mRNA levels were determined by ribonuclease protection assays (*RORα, Rev-erbα*) or Northern blot hybridization (*RORγ, Rev-erbβ*). For ribonuclease protection, equal loading was verified by including a *Tbp* riboprobe. Methylene blue staining of 28S and 18S ribosomal RNA on the Nylon membrane was used to verify even loading in Northern blot experiments.

(D) Circadian accumulation of REV-ERBα in liver nuclei. Liver nuclear proteins from mice kept for two days in DD were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed by Western blot analysis with a rabbit antiserum raised against a synthetic REV-ERBα peptide (see Experimental Procedures). The circadian times (CT) at which the animals were sacrificed are given on top of the panel. Circadian time is the time under free-running conditions (DD), and the time at which animals start their locomotor activity is set as CT 12.

(E) Anticyclic accumulation of RORE binding activity and *Bmal1* pre-mRNA. The abundance of complex 1 (see B) was quantified in three independent experiments by phosphorimaging, and the maximal value (obtained at ZT 8) was set as 100%. *Bmal1* pre-mRNA levels were determined by Taqman real-time RT-PCR, using an amplicon located in the first intron. In parallel, the levels of *Gapdh* mRNA were estimated by the same method from the same cDNA samples. The values plotted in the diagram correspond to the ratios of *Bmal1* pre-mRNA/*Gapdh* mRNA signals (averaged from four animals). The maximal value, obtained at ZT 20, was set at 100%. The *Zeitgeber* times (ZT) at which the animals were sacrificed are indicated on the abscissa of the diagram.

(F) Temporal *Rev-erbα* mRNA accumulation in the suprachiasmatic nuclei (SCN). Coronal brain sections above the optical chiasma were prepared from mice kept for 2 days in constant darkness, sacrificed at the CT times indicated on top of the panel, and hybridized to a ^{35}S-labeled antisense *Rev-erbα* RNA probe. Only the brain regions containing the hypothalamus, the thalamus, and the hippocampus are shown. The positions of the two SCNs are depicted by arrows.

scription of *Bmal1* and *Clock*. While REV-ERBα is dispensable for basic oscillator function, it participates in determining period length and phase-shifting properties of the mammalian circadian timing system.

Results

The *Bmal1* Promoter Contains Recognition Sequences for ROR and REV-ERB Orphan Nuclear Receptors

We performed RACE (*R*apid *A*mplification of *C*omplementary DNA *E*nds) on whole-cell liver RNA to determine the transcriptional start sites within the *Bmal1* promoter. The results, summarized in Figure 1A, suggest that transcription initiation can occur at multiple cap sites within a region that is highly conserved in mammals. The sequence inspection of this region revealed two REs that match 11 bp and 10 bp, respectively, of the 11 bp consensus sequence WAWNTRGGTCA (where W = A or T and R = A or G). REs are recognition sequences for members of the REV-ERB and ROR orphan nuclear receptor families (Harding and Lazar, 1993; Retnakaran et al., 1994; Dumas et al., 1994; Giguere et al., 1994; Medvedev et al., 1996).

To examine whether this element binds specifically to transcription factors accumulating in liver nuclei, we used electrophoretic mobility shift assays (EMSA) with mouse liver nuclear extracts harvested at four hour intervals around the clock, and a radio-labeled double-stranded oligonucleotide encompassing RORE 2 (see

Figure 1A). As seen in Figure 1B, at least three protein:DNA complexes can be detected with this oligonucleotide probe. Complex 1 represents a strongly circadian RORE binding activity, while complex 3 is formed with a RORE binding protein that accumulates to similar levels throughout the day. Interestingly, the temporal abundance pattern of complex 2 suggests the presence of a circadian RORE binding protein that oscillates in antiphase with the protein responsible for complex 1, albeit with a relatively modest amplitude.

In order to examine whether one or more of the observed RORE binding proteins might correspond to known members of the REV-ERB or ROR families, we used Northern blot experiments or ribonuclease protection assays to determine the temporal mRNA accumulation profiles for all known members of these families. As shown in Figure 1C, transcripts have been detected in liver with probes for *Ror*α, *Ror*γ, *Rev-erb*α, and *Rev-erb*β. Not surprisingly, *Ror*β mRNA could not been detected in liver, as this transcript appears to be expressed exclusively in the brain (Park et al., 1996; Schaeren-Wiemers et al., 1997; Sumi et al., 2002). While *Ror*α mRNA reaches similar levels throughout the day, the accumulation of *Ror*γ mRNA, *Rev-erb*α mRNA, and *Rev-erb*β mRNA follow a diurnal oscillation. As judged from the phase of accumulation, *Rev-erb*α mRNA is the only strong candidate for the gene encoding the complex 1-forming protein revealed by EMSA in Figure 1B. Again, based on the daily accumulation profiles of *Ror*γ and *Ror*α transcripts, it is likely that RORγ and RORα account for the proteins responsible for complex 2 and complex 3, respectively.

We raised a polyclonal antiserum against a peptide within the N-terminal moiety of REV-ERBα and used it in a Western blot assay with liver nuclear extracts harvested at four hour intervals around the clock. The results show that REV-ERBα accumulates with a phase compatible with that of the major circadian RORE binding protein (Figure 1D). Therefore, we tentatively identified the strongly oscillating RORE binding activity revealed by EMSA as REV-ERBα. This hypothesis was unambiguously confirmed by experiments using *Rev-erb*α-deficient mice (see below).

In cotransfection experiments, REV-ERBα acts as a transcriptional repressor rather than as an activator (Zamir et al., 1997; and references therein). Hence, if this orphan nuclear receptor was implicated in the regulation of *Bmal1* transcription, *Bmal1* transcript levels should be lowest at times when REV-ERBα attains peak levels. To record the daily *Bmal1* transcription profile, we performed Taqman real-time RT-PCR experiments with whole-cell RNA, using an amplicon of intronic pre-mRNA sequences. The antiphasic accumulation of REV-ERBα and *Bmal1* pre-mRNA shown in Figure 1E would be consistent with a scenario in which REV-ERBα represses *Bmal1* transcription. As *Rev-erb*α mRNA is expressed in a cyclic fashion in the SCN (Figure 1F), this orphan nuclear receptor could also be implicated in driving circadian *Bmal1* expression in SCN neurons.

Circadian *Bmal1* Expression Is Severely Blunted in *Rev-erb*α−/− Mice

To examine the possible role of REV-ERBα in the control of *Bmal1* transcription genetically, we disrupted the *Rev-erb*α allele in the mouse. The knockout strategy is schematically illustrated in Figure 2A. Briefly, exons 3 and 4 encoding the DNA binding domain of REV-ERBα, and part of exons 2 and 5 were replaced by an in-frame *LacZ* allele and a PGK-*neo* gene using homologous recombination in 129/Sv ES cells. Heterozygous animals with a mixed genetic background (129/Sv × C57BL/6J) were used to produce homozygous F2 *Rev-erb*α knockout mice. The *Rev-erb*α mutant allele segregated at roughly Mendelian ratios into the F2 progeny (23.5% wild-type pups, 48.6% heterozygous pups, 27.9% homozygous mutant pups, n = 383). Homozygous *Rev-erb*α knockout mice are morphologically indistinguishable from wild-type mice. An in-depth histological comparison of fifty tissues from adult knockout and wild-type animals did not reveal conspicuous differences between the two genotypes, and common blood chemistry was also very similar in wild-type and *Rev-erb*α knockout mice (N.P. and U.S., unpublished data). However, in agreement with a previously published report (Chomez et al., 2000), the litter size of −/− females is only about half that observed for wild-type females.

As shown in Figure 2, neither *Rev-erb*α transcripts (B) nor protein (C and D) could be detected in homozygous *Rev-erb*α mutant mice. Hence, the *Rev-erb*α mutant allele we engineered can be considered as a true null allele. These experiments positively identify REV-ERBα as the major cycling RORE binding protein on the proximal *Bmal1* promoter at least in vitro.

To examine whether REV-ERBα is indeed a regulator of *Bmal1* transcription, we recorded the daily accumulation profiles of *Bmal1* mRNA and pre-mRNA. As estimated on the basis of the Northern blot and real-time RT-PCR experiments shown in Figures 3A and 3B, respectively, the amplitudes of *Bmal1* mRNA and pre-mRNA oscillations are at least 20-fold in wild-type animals, but less than 2-fold in *Rev-erb*α mutant mice. The in situ hybridization experiments with coronal brain sections displayed in Figure 3C demonstrate that REV-ERBα is also a major circadian regulator of *Bmal1* transcription in the SCN.

REV-ERBα Also Participates in the Regulation of *Clock* and *Cry1* mRNA Expression

REV-ERBα also controls the activity of clock genes other than *Bmal1*. The temporal mRNA accumulation profiles for *Clock*, *Cry1*, *Cry2*, and *Per2* in wild-type and *Rev-erb*α mutant mice indicate that the disruption of *Rev-erb*α significantly affects the expression of *Clock* and *Cry1*, but has little consequence on *Cry2* or *Per2* mRNA accumulation. In wild-type animals, the accumulation of *Clock* mRNA fluctuates during the day with an amplitude of about 2.5-fold, but is nearly flat in *Rev-erb*α-deficient mice. *Cry1* mRNA levels oscillate with an approximately 7-fold amplitude in wild-type mice and an amplitude of only about 2.5-fold in *Rev-erb*α knockout mice. It is conceivable that the high and nearly constant expression of *Bmal1* contributes to altered *Cry1* and *Clock* transcription in *Rev-erb*α mutant animals.

To assess the effect of altered mRNA accumulation in *Rev-erb*α knockout animals on protein expression, we performed Western blot experiments with liver nuclear extracts and antisera raised against several clock com-

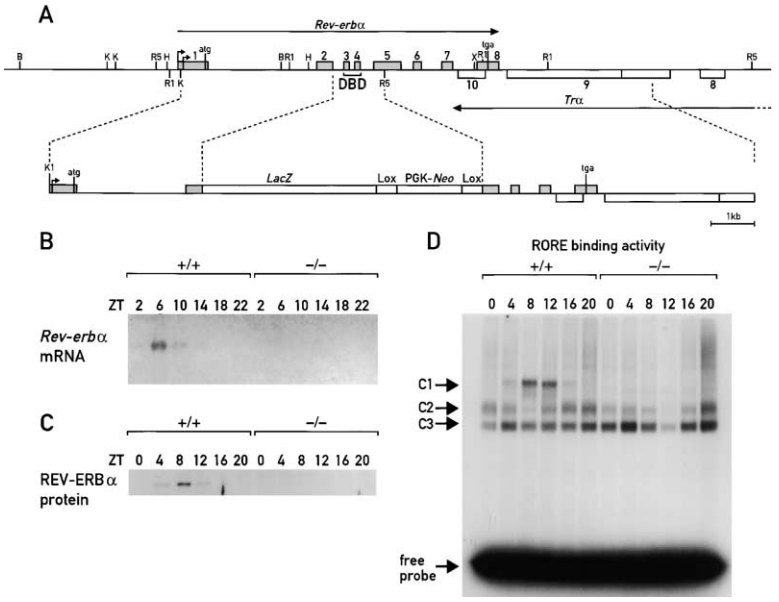

Figure 2. Disruption of the *Rev-erb*α Allele by Homologous Recombination

(A) Strategy used to delete the DNA binding domain (DBD) of the *Rev-erb*α allele. The cartoon displays a map of the eight *Rev-erb*α exons on the top strand and the 3′ terminal *Tr*α (thyroid hormone receptor α) exons on the bottom strand. These two genes are oriented in opposite directions on chromosome 11 and are partially overlapping (Miyajima et al., 1989). The positions of the recognition sites for the following restriction endonucleases are given: BamH1 (B), KpnI (K), HindIII (H), EcoR1 (R1), EcoR5 (R5), and XhoI (X). Atg and tga indicate the positions of the initiation and termination codons, respectively. The structure of the targeting vector, in which part of exon 2, intron 2, exon 3, intron 3, exon 4, intron 4, and part of exon 5 have been replaced by a *LacZ* and a PGK-*neo* gene, is given below the *Rev-erb*α/*Tr*α locus.

(B) *Rev-erb*α mRNA accumulation in the liver of wild-type (+/+) and homozygous *Rev-erb*α mutant mice (−/−). Liver whole-cell RNAs, prepared from animals sacrificed at the indicated *Zeitgeber* times (ZT) were analyzed by Northern blot hybridization using a [^{32}P]-radiolabeled cDNA probe spanning most of the *Rev-erb*α coding sequence.

(C) REV-ERBα protein accumulation in liver nuclei of wild-type (+/+) and homozygous *Rev-erb*α mutant mice (−/−). Liver nuclear proteins, prepared from animals sacrificed at the *Zeitgeber* times (ZT) indicated above the panel, were analyzed by Western blot analysis using an antiserum against the ligand binding domain of REV-ERBα.

(D) RORE binding activity in liver nuclei of wild-type (+/+) and homozygous *Rev-erb*α mutant mice (−/−). EMSA experiments were performed as indicated in the legend to Figure 1B with liver nuclear proteins from animals sacrificed at the indicated *Zeitgeber* times (ZT). Note that complex 1 is not observed in *Rev-erb*α-deficient mice.

ponents (Figure 4B). As anticipated from the expression of their mRNAs, PER2 and CRY2 protein accumulation is very similar in *Rev-erb*α +/+ and −/− mice. In contrast, both BMAL1 and CLOCK levels are elevated in knockout as compared to wild-type mice. The accumulation of these proteins oscillates with a modest amplitude at best in animals of both genotypes, suggesting that the half-life of these proteins is relatively long. CRY1 accumulates in nuclei with large circadian oscillations in both *Rev-erb*α +/+ and −/− mice. Hence, neither the overex-

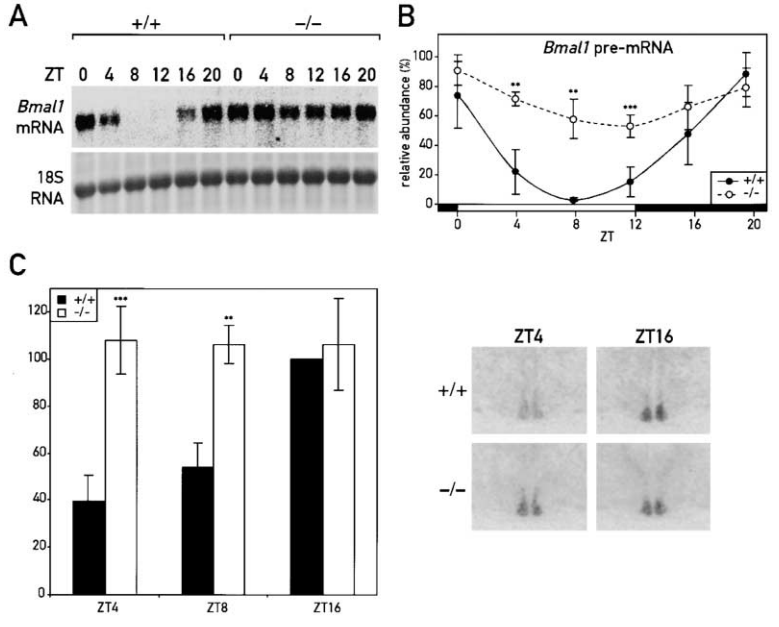

Figure 3. *Bmal1* mRNA and pre-mRNA Accumulation in *Rev-erb*α-Deficient Mice

(A) Temporal accumulation of *Bmal1* mRNA in the liver of *Rev-erb*α-proficient (+/+) and -deficient (−/−) mice. Liver whole-cell RNAs, prepared from animals sacrificed at the indicated *Zeitgeber* times (ZT), were analyzed by Northern blot hybridization using a [^{32}P]-radiolabeled *Bmal1* cDNA probe. After the transfer to the membrane, the RNA was colored with methylene blue and the stained 18S rRNAs are shown as loading references.

(B) Temporal accumulation of *Bmal1* pre-mRNA in the liver of *Rev-erb*α -proficient (+/+) and -deficient (−/−) mice. The relative accumulation of *Bmal1* pre-mRNA was determined as described in the legend to Figure 1E. The values are means +/− standard deviations from four animals. The values indicated by asterisks are statistically highly significant (** p = < 0.005, ***p = < 0.0005)

(C) *Bmal1* mRNA accumulation in the SCN. Coronal brain sections taken above the optical chiasma from *Rev-erb*α +/+ and −/− animals sacrificed at ZT 4, ZT 8, and ZT 16 were hybridized in situ to a ^{35}S-labeled antisense *Bmal1* cRNA probe, and the hybridization signals associated with the SCNs were quantified by phosphorimaging. The values are means +/− standard deviations from three to four animals. The maximal value determined for wild-type animals (obtained at ZT 16) has been set at 100%. Asterisks indicate highly significant differences (** p = < 0.005, ***p = < 0.0005). Representative in situ hybridizations for ZT 4 (minimal accumulation in wild-type mice) and ZT 16 (maximal accumulation in wild-type mice) are depicted at the right of the diagram.

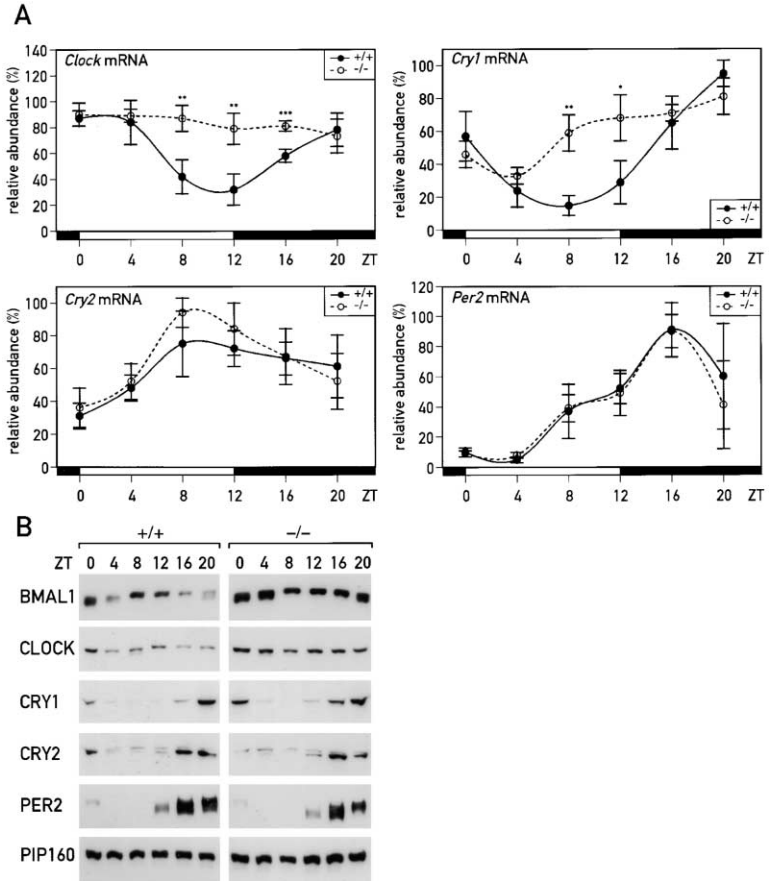

Figure 4. Accumulation of Transcripts and Proteins Encoded by Several Essential Pacemaker Genes in the Liver of *Rev-erb*α-Proficient (+/+) and -Deficient (−/−) Mice

(A) The relative levels of *Clock* mRNA, *Cry1* mRNA, *Cry2* mRNA, and *Per2* mRNA were estimated by Taqman real-time RT-PCR, as described in the Experimental Procedures, and normalized to the corresponding *Gapdh* levels (see legend to Figure 3B). Each value represents the mean of four independent experiments. In each experiment, the maximal value was normalized to 100%. The *Zeitgeber* times at which the animals were sacrificed are given on the abcissae of the diagrams. Solid and dotted lines represent the accumulation curves from wild-type and *Rev-erb*α mutant animals, respectively.

(B) The relative accumulation of BMAL1, CLOCK, CRY1, CRY2, and PER2 in liver nuclei was visualized by Western blot analysis using polyclonal antisera raised against the respective recombinant proteins. The *Zeitgeber* times at which the animals were sacrificed are given on top of the fluorograms. An immunoblot against PIP160, a nucleolar protein whose accumulation does not vary during the day (P. Comte, V. Ossipow, and U.S., unpublished data), is included as a loading reference.

pression of BMAL1 and CLOCK nor the low amplitude of cyclic *Cry1* mRNA accumulation observed in *Rev-erb*α−/− mice have a large impact on nuclear CRY1 protein accumulation. Therefore, *Cry1* mRNA expression does not appear to be the limiting step for the nuclear accumulation of CRY1 protein. The importance of posttranscriptional regulation is even more evident for circadian CRY2 accumulation, given the nearly constant *Cry2* mRNA levels in both wild-type and *Rev-erb*α-deficient mice.

REV-ERBα Regulates Period Length and Phase-Shifting Properties of the Circadian Timing System

In order to examine whether REV-ERBα influences circadian behavior, we compared wheel-running activity of wild-type and *Rev-erb*α-deficient mice in constant darkness (DD, Figure 5A) or light (LL, Figure 5C). Interestingly, the drastic reduction of circadian rhythms in the transcription of *Clock* and *Bmal1* observed in *Rev-erb*α-deficient mice does not result in arrhythmic behavior when mice are placed in a constant environment. This demonstrates that circadian transcription of *Bmal1* and *Clock* is not essential for the basic properties of the circadian system. However, under both DD and LL conditions, the average period length was significantly shorter in *Rev-erb*α−/− animals (Figures 5A–5D), and the distribution of period lengths is much more scattered in *Rev-erb*α knockout mice than in *Rev-erb*α wild-type mice (Figures 5B and 5D).

A strong phenotype in circadian behavior was revealed when we examined the phase-shifting properties of *Rev-erb*α -deficient mice. As shown in Figure 5 (E and F), light pulses delivered at CT 22 provoked dramatic phase advances (5.4 ± 1.8 hr) in *Rev-erb*α knockout mice, compared to wild-type mice (1.1 ± 0.5 hr). Interestingly, the large phase advances of *Rev-erb*α knockout mice were observed only in animals kept in DD for longer than seven days (data not shown). Moreover, the difference in phase-shifting between *Rev-erb*α-proficient and -deficient animals is limited to the second half of the night, when *Rev-erb*α expression is starting to rise in the SCN.

*Rev-erb*α Expression Is Controlled by the Negative Limb of the Oscillator

PER2 has been postulated to be a positive regulator of *Bmal1* expression (Shearman et al., 2000b). Given the dominant role of REV-ERBα in driving circadian *Bmal1* transcription, we considered that PER2 might stimulate *Bmal1* mRNA accumulation indirectly, by repressing *Rev-erb*α expression. The observation that *Rev-erb*α pre-mRNA transcripts show trough levels when PER2 protein attains peak levels in the nucleus is consistent with this hypothesis (Figure 6A). To examine this notion further, we recorded the daily accumulation profiles of *Rev-erb*α mRNA in wild-type mice, *Per2*^{Brdm1} mutant mice, and *Per1*^{Brdm1}/*Per2*^{Brdm1} double mutant mice. The *Per1*^{Brdm1} and *Per2*^{Brdm1} mutant alleles are recessive and

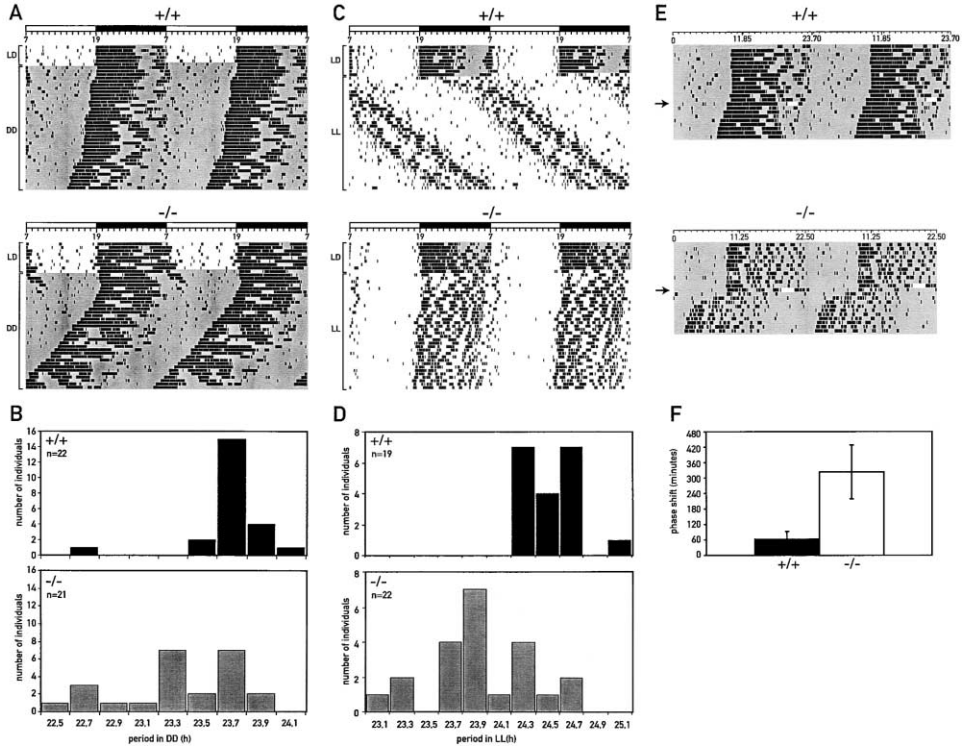

Figure 5. Circadian Locomotor Activity of *Rev-erb*α-Proficient (+/+) and -Deficient (−/−) Mice

(A–D) The voluntary locomotor activity was recorded as wheel-running activity for wild-type mice and *Rev-erb*α mutant mice in constant darkness (DD, A and B) and constant light (LL, C and D). (A) and (C) display typical double-plot actograms obtained for *Rev-erb*α +/+ and −/− animals. In each actogram, the first few days were recorded under LD conditions (lights on at 7:00; lights off at 19:00). Time spans during which the lights were switched off are marked by gray shadowing.

(B) and (D) show histograms for the distribution of period length determined between days 10 and 20 in DD or LL. The number of examined animals (N) is given in each diagram. The lengths of the free-running periods ± standard deviations in hours were 23.76 ± 0.27 (*Rev-erb*α+/+) and 23.38 ± 0.45 (*Rev-erb*α −/−) in DD, and 24.59 ± 0.22 (*Rev-erb*α+/+) and 24.01 ± 0.44 (*Rev-erb*α−/−) in LL. Two different statistical methods (the Student's t test, assuming normal distributions, and the Mann-Whitney as a nonparametric test) have been used to examine the data. The P values for the period length differences are < 0.002 for both methods in (B), and < 0.0001 for both methods in (D).

(E and F) Phase shifts of circadian wheel-running activity induced by a 2 hr light pulse (500 lux) given between CT 22 and CT 24 to mice kept in DD for at least three weeks. (E) shows typical actograms obtained for *Rev-erb*α +/+ and −/− animals. Here, for reasons of clarity, locomotor activity was plotted according to daylengths corresponding to the free-running period of each animal before the light pulse (light pulses are indicated by arrows). In (F), values represent the mean ± standard deviation of four *Rev-erb*α +/+ and eight *Rev-erb*α −/− mice. The difference between genotypes is highly significant (P = 0.0001, using the Student's t test). Large phase advances of 5.7 hr and 7.6 hr were also obtained with two *Rev-erb*α knockout mice exposed to a light pulse of only 15 min (data not shown). The phase shifts produced by light pulses applied at CT 4 and CT 14 were not significantly different in *Rev-erb*α-proficient and -deficient animals (data not shown).

resemble null alleles. The *Per1*^{Brdm1} allele only encodes a small N-terminal fragment of PER1 that does not contain any recognizable sequence motif (Zheng et al., 2001). The *Per2*^{Brdm1} allele contains a deletion corresponding to 87 amino acids in the PAS dimerization domain (Zheng et al., 1999). Wheel-running behavior and clock gene expression are indistinguishable in *Per2*^{Brdm1} mice, in *Per2*^{ldc} mice (Bae et al., 2001), and in mice bearing a true null allele (C.C Lee, personal communication). As shown in Figure 6B, the phase of *Rev-erb*α mRNA accumulation is considerably advanced in *Per2*^{Brdm1}mutant mice. Moreover, *Rev-erb*α transcript levels are constitutively expressed at relatively high levels (approximately half-maximal) throughout the day in *Per1*^{Brdm1}/*Per2*^{Brdm1}double mutant mice. Zenith levels of *Rev-erb*α transcripts are not expected in these mice (see Discussion).

We also recorded *Bmal1* mRNA accumulation in *Per2*^{Brdm1}mutant mice (data not shown). In keeping with previously published results (Shearman et al., 2000b), in *Per2* single mutant mice *Bmal1* mRNA levels peak earlier during the day and reach nadir values precociously between ZT 3 and ZT 9 (as expected from the earlier increase of *Rev-erb*α expression).

In conclusion, the data presented in this section are compatible with a model in which the circadian transcription of *Rev-erb*α—similar to that of cyclic *Per1*, *Per2*, and *Cry1* transcription—is accomplished via a periodic repression by period proteins. In turn, REV-ERBα governs rhythmic *Bmal1* expression by periodically inhibiting the transcription of this target gene.

Discussion

REV-ERBα as a Link between the Negative and Positive Limbs

We demonstrate here that the nuclear receptor REV-ERBα is a major regulator of cyclic transcription within the positive limb of the mammalian circadian oscillator.

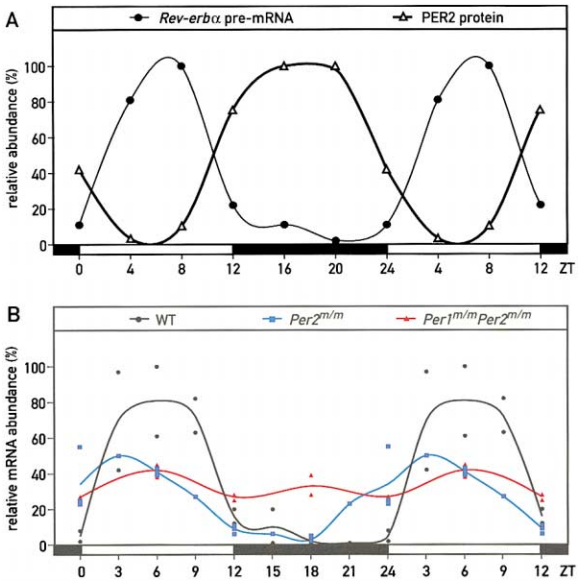

Figure 6. Expression of *Rev-erbα* mRNA in *Per2* and *Per1/Per2* Mutant Mice

(A) Temporal accumulation of nuclear PER2 protein (open triangle) and *Rev-erbα* precursor RNA (closed circles) in the livers of wild-type mice. The relative levels of *Rev-erbα* pre-mRNA from animals sacrificed at the indicated *Zeitgeber* times were determined by Taqman real-time RT-PCR, using an intronic probe. Each value represents the mean of two animals after normalization to *Gapdh* levels, and the highest value (observed at ZT8) was set to 100%. Relative PER2 protein levels were quantified by densitometric analysis of the fluorogram presented in Figure 4, normalized to the PIP160 signals obtained by reprobing the same membrane with a PIP160 antibody, and expressed as percentage of the maximal PER2/PIP160 ratio (determined for ZT 20). The pre-mRNA and protein levels were recorded for 24 hr, but to allow comparisons of phases (with regard to peak and trough levels), some values were double-plotted to cover a 36 hr time span.

(B) Temporal accumulation of *Rev-erbα* mRNA in the liver of wild-type mice (black line, circles), $Per2^{Brdm1}$ single mutant mice (blue line, squares), and *Per1,2* double mutant mice (red line, triangles). The curve is drawn through the estimated mean values (the individual data points are included in the diagram). The mRNA levels were recorded during 24 hr, but to allow comparisons of phases, some values were double-plotted to cover a 36 hr time span.

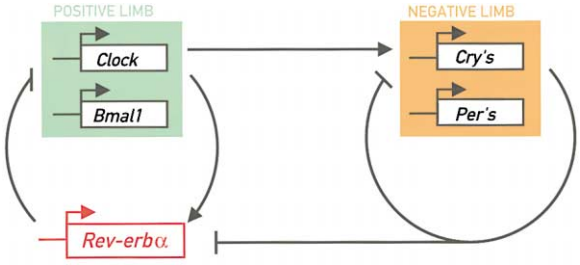

Figure 7. Simplified Model of the Circadian Oscillator

The positive limb consists of the two PAS helix-loop-helix transcription factors CLOCK and BMAL1 that activate transcription of *Cry* and *Per*, which are members of the negative limb. CRY and PER proteins are translocated to the nucleus as multi-subunit protein complexes and, once these complexes have reached a critical concentration, they repress CLOCK:BMAL1-stimulated transcription. This feedback loop generates circadian rhythms of mRNA accumulation for members of the negative limb. In parallel, the same positive and negative elements periodically activate and repress, respectively, the transcription of the orphan receptor *Rev-erbα*. The circadian accumulation of the orphan receptor REV-ERBα then drives cyclic transcription of *Bmal1* and *Clock*.

In previous studies, *Bmal1* transcription has been postulated to be controlled by mechanisms opposite to those involved in *Cry1* and *Per1/Per2* expression (Shearman et al., 2000a; Yu et al., 2002). Thus, circadian *Bmal1* expression appears to be positively controlled by PER and CRY proteins, and transfection studies suggest that *Bmal1* transcription may be negatively autoregulated by BMAL1 and CLOCK (Yu et al., 2002) or Bmal1 and NPAS2 (Reick et al. 2001). Our model, outlined schematically in Figure 7, offers a simple explanation for this regulatory circuit, by proposing that *Rev-erbα* expression is negatively regulated by PER and CRY proteins and positively regulated by BMAL1 and CLOCK. The cyclic accumulation of REV-ERBα then imposes circadian regulation on *Bmal1* transcription (Figure 7). Several observations support this model. (1) *Rev-erbα* transcription appears to be positively regulated by CLOCK and BMAL1, the molecular targets of CRY/PER-mediated repression. Indeed, the *Rev-erbα* promoter contains three evolutionarily conserved E boxes between the major transcriptional start site and position −500 (see Supplemental Figure S1, Part A available at http://www.cell.com/cgi/content/full/110/2/251/DC1), and in cotransfection assays, transcription from the *Bmal1* promoter is activated by CLOCK and BMAL1 (G. Triqueneaux, S. Thenot, and V. Laudet, personal communication). Furthermore *Rev-erbα* mRNA accumulates to constitutively low levels in homozygous *Clock* mutant mice (G. Triqueneaux, S. Thenot, and V. Laudet, personal communication; see also Supplemental Figure S1, Part B available at above URL). (2) The accumulation of *Rev-erbα* mRNA transcripts is lowest at times when PER2 protein reaches high nuclear levels. (3) *Rev-erbα* is constitutively expressed at intermediate levels in $Per1^{Brdm1}/Per2^{Brdm1}$ or *Cry1/Cry2* mutant mice (Figure 6, N.P., F. Tamanini, G.T. van der Horst, and U.S., unpublished data). Zenith levels of *Rev-erbα* transcripts are not expected in these mutant mice. As pointed out above, *Rev-erbα* transcription itself is activated by CLOCK and BMAL1. At a high concentration, REV-ERBα is expected to extinguish the expression of its own activators, which in turn would result in diminished *Rev-erbα* transcription. Hence, *Rev-erbα* mRNA levels should be frozen at intermediate rather than maximal levels in *Per1/Per2* and *Cry1/Cry2* double mutant mice.

Mechanisms similar to the one proposed in Figure 7 for the mouse oscillator might also apply for the circadian timing systems of the zebrafish and the fruit fly. Thus, in zebrafish, *Rev-erbα* (*zfRev-erbα*) also displays circadian expression with a phase opposite to that of *zClock/zBmal1* (Whitmore et al., 1998; Cermakian et al., 2000; Delaunay et al., 2000). It was in *Drosophila* that two interconnected feedback loops driving the nearly antiphasic circadian expression of the positive and negative limb components were first proposed (Glossop et al., 1999). Apparently, the two feedback loops in *Drosophila* are also coupled by an indirect mechanism, in which PER and TIM downregulate the expression of a

repressor that inhibits *clk* transcription in a circadian fashion (Paul Hardin, personal communication).

Mechanisms of Repression by REV-ERBα

REV-ERBα belongs to the large family of transcription factors called "orphan nuclear receptors", nuclear receptors for which no ligand has yet been found (Mangelsdorf et al., 1995). REV-ERBα does not contain the ligand-dependent C-terminal activation domain AF2. Thus, in cotransfection studies with reporter genes carrying RORE sequences, REV-ERBα acts as a repressor rather than an activator (Forman et al., 1994; Harding and Lazar, 1995; Adelmant et al., 1996; Zamir et al., 1996, 1997). This repression might occur by multiple mechanisms. When two REV-ERBα molecules bind either to two closely spaced RORE sequences or to a direct repeat element with the sequence RGGTCANNR GGTCA (DR-2), they can bind the corepressor NCoR1 (Zamir et al., 1997). In turn, NCoR1 might recruit a histone deacetylase, which promotes the conversion of accessible into inaccessible chromatin (Aranda and Pascual, 2001). REV-ERBα might also inhibit transcription more directly by competing with transcriptional activators (e.g., the orphan receptors RORα, RORβ, and/or RORγ) for the occupancy of RORE sequences (e.g., Forman et al., 1994).

In addition to controlling cyclic *Bmal1* transcription, REV-ERBα also participates in the regulation of circadian *Clock* expression. While we did not yet examine whether REV-ERBα interacts directly with *cis*-acting *Clock* regulatory elements, it is noteworthy to mention that the human *Clock* gene harbors a perfect DR-2 element in the first intron, 16,630 bp downstream of the putative transcription initiation site.

REV-ERBα Influences Phase-Shifting and Period Length and Variability

The behavioral analysis of *Rev-erb*α knockout mice revealed three circadian phenotypes.

(1) *Rev-erb*α knockout mice display a significantly shorter period length than wild-type animals under DD and LL conditions. Interestingly, transgenic mice expressing multiple *Clock* gene copies show a similar phenotype (Antoch et al., 1997). The overexpression of components of the positive limb in *Rev-erb*α knockout mice or in mice with additional *Clock* gene copies is expected to result in a more potent activation of *Cry* and *Per* genes, which in turn would reduce the duration required to produce CRY and PER threshold levels sufficient for the downregulation of their own genes.

(2) *Rev-erb*α-deficient mice exhibit a much greater diversity of period lengths than wild-type animals under DD and LL conditions. This high variability between individuals indicates that the action of REV-ERBα on the expression of essential clock genes contributes to the precision of the circadian timing system.

(3) *Rev-erb*α-deficient mice can perform unusually large phase-advances during the second half of the subjective night. Interestingly, the new steady-state phase is reached only a few days after exposure to the light pulse. Conceivably, a light-pulse delivered at the end of the subjective night to *Rev-erb*α knockout mice induces the overexpression of a long-lived clock component (e.g., BMAL1 or CLOCK), which may accelerate the oscillator until its concentration falls back to the initial level. Additional experiments will be required to decipher the precise molecular mechanism by which REV-ERBα restricts the amplitude of phase advances in wild-type animals.

In conclusion, this study suggests that the orphan nuclear receptor REV-ERBα couples the negative limb of the molecular oscillator to its positive limb and provides a molecular basis for a negative feedback within the positive limb. Moreover, REV-ERBα greatly restricts phase-shifting activity during the second half of the night. While transcriptional oscillation of positively acting clock components are not absolutely essential for rhythm generation, the conservation of interconnected feedback loops in all genetically studied animals (Harmer et al., 2001)—and even in the unicellular fungus *Neurospora crassa*—suggests that this mechanism has a selective advantage under natural circumstances.

Experimental Procedures

Rapid Amplification of cDNA Ends (RACE)

Single-stranded cDNA was synthesized from C57BL/6J mouse liver polyA+ RNA, using random hexamers and Superscript reverse transcriptase (Gibco-BRL). Free nucleotides were eliminated using a G-50 spin column, and poly A tails were added to the cDNA 3′ termini by using terminal deoxynucleotidyl-transferase (Gibco/BRL) and dATP. The reaction was stopped with 5 mM EDTA, extracted with phenol-chloroform, and filtered through a G-50 spin column. A first PCR reaction was carried out with 1/200th of the cDNA synthesized from 2 μg of polyA+ RNA, using the Bmal1-specific primer 5′-GGCCTAG GGTTCCACATTTGA-3′ and the anchor primer 5′-ACTCGAGGTGGA GCACGTGTTTTTTTTTTTTTTTTTTTV-3′. 1/100th of the product of this reaction was reamplified in a second (nested) PCR reaction with a different *Bmal1*-specific primer (5′-CATTCACTGCAGCCAACA ACA-3′) and the anchor primer 5′-ACTCGAGGTGGAGCACGTG-3′. The PCR products were separated on an agarose gel, purified (Nucleospin, Macherey-Nagel), cloned into a T-vector, and sequenced.

RNA Expression in the Liver

The antisense *Rev-erb*α RNA probe used in ribonuclease protection assays was transcribed from a pKS+Bluescript vector containing an RT-PCR product of mouse *Rev-erb*α mRNA (+376 to +614). Ribonuclease protection assays of *Ror*α mRNA expression were accomplished as described (Balsalobre et al., 1998).

Northern blot analysis was performed using total liver RNA as described previously (Fonjallaz et al., 1996). The *ROR*γ, *Rev-erb*β, and *Bmal1* radiolabeled cDNA probes were obtained by random priming of the inserts from the plasmids pKS+Bluescript *-ROR*γ, *-Rev-erb*β, and *-Bmal1*, respectively. The cDNA inserts of these plasmids were generated by RT-PCR from mouse liver RNA using the following primers:

5′-CTTCATGGAGCTCTGCCAGAATGACCAG-3′ and 5′-GGTGGA GGTGCTGGAAGATCTGCAG-3′(*ROR*γ); 5′-CCATGATGAACACCCA GTTCAGTGG-3′ and 5′-CGTACCATTAAAACCTCAAAAGTCCC AGC-3′ *(Rev-erb*β*)*; 5′-GTATGGACACAGACAAAGATGACC-3′ and 5′-GTCCCTCCATTTAGAATCTTCTTG-3′ (*Bmal1*).

The Northern blot analysis of *Rev-erb*α mRNA expression in *Rev-erb*α +/+ and −/− mice (Figure 2B) was performed using a cDNA probe encompassing exons 3 to exon 8 (Hind III-EcoR I restriction fragment of a rat *Rev-erb*α cDNA called *Rev-erb*α_{TOT} hereafter). This fragment was generated by RT-PCR from liver RNA using the primers 5′-GTTATCACCTACATTGGCTCCAGCGGATCC-3′ and 5′-CGG GCGGGTCACTGGGCGTCCACCCGGAAGGACA-3′.

For quantification of relative RNA levels by the Taqman real-time PCR technology, 0.5 μg of DNase-treated total RNA were reverse-transcribed using random hexamers and Superscript reverse transcriptase (Gibco). The cDNA equivalent to 20 ng total RNA was

PCR-amplified in an ABI PRISM 7700 detection system (PE-Applied Biosystems; Heid et al., 1996). Forward primer, reverse primer, and probe (purchased from Eurogenetec) were as follows: *Gapdh* forward: 5′-CATGGCCTTCCGTGTTCCTA-3′; *Gapdh* reverse: 5′-CCTGC TTCACCACCTTCTTGA-3′; *Gapdh* probe: 5′-FAM-CCGCCTGGAGA AACCTGCCAAGTATG-TAMRA-3′; *Bmal1* forward: 5′-CCAAGAAAG TATGGACACAGACAAA-3′; *Bmal1* reverse: 5′-GCATTCTTGATCCTT CCTTGGT-3′; *Bmal1* probe: 5′-FAM-TGACCCTCATGGAAGGTTAG AATATGCAGAA-TAMRA-3′; *Bmal1* pre-mRNA forward: 5′-CACCGT TATCATAATGTGTGTGCTT-3′; *Bmal1* pre-mRNA reverse: 5′-TGGA CGTAGAGAGACCGATTCTG-3′; *Bmal1* pre-mRNA probe: 5′-FAM-AGTTGCCCTCTTCCACGTGCACG-TAMRA-3′; *Clock* forward: 5′-TTG CTCCACGGGAATCCTT-3′; *Clock* reverse: 5′-GGAGGGAAAGTGC TCTGTTGTAG-3′; *Clock* probe: 5′-FAM-ACACAGCTCATCCTCTCT GCTGCCTTTC-TAMRA-3′; *Cry1* forward: 5′-CTGGCGTGGAAGTCA TCGT-3′; *Cry1* reverse: 5′-CTGTCCGCCATTGAGTTCTATG-3′; *Cry1* probe: 5′-FAM-CGCATTTCACATACACTGTATGACCTGGACA-TAMRA-3′; *Cry2* forward: 5′-TGTCCCTTCCTGTGTGGAAGA-3′; *Cry2* reverse: 5′-GCTCCCAGCTTGGCTTGA-3′; *Cry2* probe: 5′-FAM-CAGTCACCCTGTGGCAGAGCCTGG-TAMRA-3′; *Per2* forward: 5′-ATGCTCGCCATCCACAAGA-3′; *Per2* reverse: 5′-GCGGAAT CGAATGGGAGAAT-3′; *Per2* probe: 5′-FAM-ATCCTACAGGCCGGT GGACAGCC-TAMRA-3′; *Rev-erbα* forward: 5′-CATGGTGCTACTGT GTAAGGTGTGT-3′; *Rev-erbα* reverse: 5′-CACAGGCGTGCACTCCA TAG-3′; *Rev-erbα* probe: 5′-FAM-ACGTGGCCTCAGGC-MGB-3′; *Rev-erbα* pre-mRNA forward:5′-GGTTGCCCTGCCTGGTTTA-3′; *Rev-erbα* pre-mRNA reverse: 5′-TGCCACCGAGTCGACAGAA-3′; *Rev-erbα* pre-mRNA probe: 5′-FAM-CACATGTCTTGCTCACCCACTGACACA-TAMRA-3′.

The relative levels of each RNA were calculated by 2^{-CT} (CT standing for the cycle number at which the signal reaches the threshold of detection) and normalized to the corresponding *Gapdh* RNA levels. Each CT value used for these calculations is the mean of at least two duplicates of the same reaction. Relative RNA levels are then expressed as percentage of the maximal value obtained for each experiment.

RNA Expression in the SCN

Immediately after dissection, brains were frozen in isopentane (4 min at −20°C) and stored at −70°C until use. Serial coronal brain cryosections of 12 microns above the optical chiasma were prepared using standard procedures. In situ hybridizations with serial sections through the SCN were performed as described previously (Lopez-Molina et al., 1997; Nef et al., 1996). The *Rev-erbα* and *Bmal1* riboprobes, covering most of the coding sequence, were synthesized from the plasmids pKS+Bluescript-*Rev-erbα*$_{TOT}$ and pKS+Bluescript-*Bmal1*, respectively (see above).

Western Blot and Electromobility Shift Assay

Liver nuclear proteins were prepared as described previously (Lavery and Schibler, 1993).

Western blotting was performed as described (Ripperger et al., 2000). The rabbit anti-REV-ERBα antibodies were raised against the peptide CSLQVAMEDSSRVSPSK (Figure 1D) or a recombinant protein encompassing the entire ligand binding domain (Figure 2C). Rabbit antibodies against mouse BMAL1, CLOCK, CRY1, CRY2, PER2, and PIP160 are kind gifts from our colleagues J. Ripperger, S. Brown, and P. Comte.

The *Bma1l*-RORE2 probe used in the electromobility shift assays was prepared by annealing the two complementary oligonucleotides 5′-GAAGGCAGAAAGTAGGTC-3′ and 5′-CGTCCCTGACCTACTTTC TGCCTTC-3′ and by filling in the 5′ overhang with α[^{32}P]dATP and Klenow DNA polymerase. 5 μg of liver nuclear NUN-extract (see above) were incubated with 0.075 pmol of the double-stranded oligonucleotide in a 20 μl reaction containing 150 mM KCl, 5 mM MgCl2, 10 μM ZnSO4, 25 mM HEPES (pH 7.6), 25 ng/μl poly dIdC, and 100 ng/μl salmon sperm DNA. After an incubation of 10 min at room temperature, 2 μl of a 15% Ficoll solution were added, and the protein-DNA complexes were separated on a 6% polyacrylamide gel in 0.25 × TBE. The signals were quantified using a phosphor imager (Bio-Rad).

Disruption of the *Rev-erbα* Allele

Phage lambda clones containing the mouse *Rev-erbα* locus were isolated from a 129/Sv-Lambda FixII genomic library (No. 946305, Stratagene). The targeting vector was constructed in 3 steps. First, the downstream part of the 5′ arm was produced by PCR amplification using the sense primer 5′-TTGCCAGGCCCTCCCCAGGAAT TCA-3′ and the antisense primer 5′-TTGCTGGGGGATATCCGGCTG CTGT-3′. The latter primer was designed to introduce an EcoRV site into exon 2, suitable for in frame junction with LacZ. A 0.5 kb HindIII-EcoRV fragment of the PCR product was subcloned and inserted into the HindIII and SmaI sites of a pLacZNeo vector. Secondly, the 3′ arm, a 6.2 kb EcoRV–NotI fragment spanning from exon 5 to the end of the genomic clone, 2.5 kb downstream of the polyA site, was introduced into pLacZNeo (which had been opened with XbaI, filled with Klenow, and opened with NotI). Finally, the upstream part of the 5′arm, a 2.8 kb fragment spanning from the KpnI site near the start site to the HindIII site in intron 1, was introduced into the construct (digested with the same enzymes). Electroporation of ES cells, selection of neomycin-resistant colonies, and injection of ES cells into blastocysts were accomplished according to standard procedures (Joyner, 1993).

Animal Care and Wheel-Running Activity Monitoring

The mice were housed and their wheel-running activity monitored as described in Lopez-Molina et al., 1997. The *Per2* ^{Brdm1} and *Per1/2* mutant animals used in this study are described in Zheng et al., 1999 and 2001.

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