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-
Results

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

We performed RACE (Rapid Amplification of Complementary DNA Ends) 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 ROREs that match 11 bp and 10 bp, respectively, of the 11 bp RORE consensus sequence given below. For the upstream site and 2 for the downstream site. ROREs are framed, and the 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 tests (2002).

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 human. 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). ROREs are framed, and the 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 tests (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 32P-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.
As shown in Figure 2, neither Rev-erbα transcripts (B) nor protein (C and D) could be detected in homozygous Rev-erbα mutant mice. Hence, the Rev-erbα 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-erbα 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-erbα mutant mice indicate that the disruption of REV-ERBα 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-erbα-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-erbα knockout mice. It is conceivable that the high and nearly constant expression of Bmal1 contributes to altered Cry1 and Clock transcription in Rev-erbα mutant animals.

To assess the effect of altered mRNA accumulation in Rev-erbα knockout animals on protein expression, we performed Western blot experiments with liver nuclear extracts and antisera raised against several clock com-
antiserum against the ligand binding domain of REV-ERB complex 1 is not observed in Zeitgeber as indicated in the legend to Figure 1B with liver nuclear proteins from animals sacrificed at the indicated times (ZT). Note that complex 1 is not observed in Rev-erb- deficient mice.

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

(A) Strategy used to delete the DNA binding domain (DBD) of the Rev-erb allele. The cartoon displays a map of the eight Rev-erb exons on the top strand and the 3’ terminal Trα (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 Xhol (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/Trα locus.

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

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

(A) Temporal accumulation of Bmal1 mRNA in the liver of Rev-erb-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 [32P]-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-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+/+ and −/− animals sacrificed at ZT 4, ZT 8, and ZT 16 were hybridized in situ to a 35S-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.
expression of BMAL1 and CLOCK nor the low amplitude of cyclic Cry1 mRNA accumulation observed in Rev-erbα−/− 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-erbα-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-erbα-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-erbα-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-erbα−/− animals (Figures 5A–5D), and the distribution of period lengths is much more scattered in Rev-erbα knockout mice than in Rev-erbα wild-type mice (Figures 5B and 5D).

A strong phenotype in circadian behavior was revealed when we examined the phase-shifting properties of Rev-erbα−/− 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-erbα knockout mice, compared to wild-type mice (1.1 ± 0.5 hr). Interestingly, the large phase advances of Rev-erbα 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-erbα-proficient and -deficient animals is limited to the second half of the night, when Rev-erbα expression is starting to rise in the SCN.

Rev-erbα 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-erbα expression. The observation that Rev-erbα 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-erbα mRNA in wild-type mice, Per2βdmt mutant mice, and Per1βdmt/Per2βdmt double mutant mice. The Per1βdmt and Per2βdmt mutant alleles are recessive and...
Figure 5. Circadian Locomotor Activity of Rev-erbα-Proficient (+/+) and -Deficient (−/−) Mice

(A–D) The voluntary locomotor activity was recorded as wheel-running activity for wild-type mice and Rev-erbα 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-erbα +/+ 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-erbα +/+ ) and 23.38 ± 0.46 (Rev-erbα −/− ) in DD, and 24.59 ± 0.22 (Rev-erbα +/+ ) and 24.01 ± 0.44 (Rev-erbα −/− ) 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-erbα +/+ 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-erbα +/+ and eight Rev-erbα −/− 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-erbα 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-erbα-proficient and -deficient animals (data not shown).

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.
REV-ERBα and Circadian Rhythms

In previous studies, Bmαf1 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 Bmαf1 expression appears to be positively controlled by PER and CRY proteins, and transfection studies suggest that Bmαf1 transcription may be negatively autoregulated by BMAL1 and CLOCK (Yu et al., 2002) or Bmαf1 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 Bmαf1 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 Bmαf1 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/Per2−/− 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 also displays circadian expression with a phase opposite to that of zClock/zBmαf1 (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

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, 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−/− 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 Bmαf1 and Clock.
repressor that inhibits ciki 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 known as “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; Adelman 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 RGTTCAANR 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, 16,830 bp downstream of the putative transcription initiation site.

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

The behavioral analysis of Rev-erbα knockout mice revealed three circadian phenotypes.

1. Rev-erbα 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-erbα 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-erbα-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-erbα-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-erbα 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 deci-
PCR-amplified in an ABI PRISM 7700 detection system (PE-Applied Biosystems; Heid et al., 1996). Forward primer, reverse primer, and probe were designed from the published sequences as follows: Gapdh forward: 5'-CTAGGCTCTTCCGTCTCTA-3'; Gapdh reverse: 5'-CTCGCTTTCCACCTTGAGA-3'; Gapdh probe: 5'-FAM-CACCGGAGGGCTCTGGG-MGB-3'. Probe was designed to have a TAMRA reporter at the 3' end and a Quasar 705 quencher at the 5' end. Semiquantitative RT-PCR was performed using the ABI PRISM 7700 detection system (PE-Applied Biosystems; Heid et al., 1996). Forward primer, reverse primer, and the downstream part of the 5' UTR sequence were as follows: forward: 5'-TTGCCAGGCGCTTCCGGAGAT-3'; reverse: 5'-CCAGAAAGTATTGGACACAGACAA-3'; probe: 5'-GCTGCCCTTTC-TAMRA-3'.

Immediately after dissection, brains were frozen in isopentane (4°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 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-5'linker 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 CSLOVAMEDSRVSPSK (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, Wilsbacher, L.D., Sangoram, A.M., King, D.P., Pinto, L.H., and Takahashi, J.S. (1997). Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. Cell 89, 655–667.

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 Per2BM and Per1/2 mutant animals used in this study were described in Zheng et al., 1999 and 2001.

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