CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP

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DBP, the founding member of the PAR leucine zipper transcription factor family, is expressed according to a robust daily rhythm in the suprachiasmatic nucleus and several peripheral tissues. Previous studies with mice deleted for the *Dbp* gene have established that DBP participates in the regulation of several clock outputs, including locomotor activity, sleep distribution, and liver gene expression. Here we present evidence that circadian *Dbp* transcription requires the basic helix–loop–helix–PAS protein CLOCK, an essential component of the negative-feedback circuitry generating circadian oscillations in mammals and fruit flies. Genetic and biochemical experiments suggest that CLOCK regulates *Dbp* expression by binding to E-box motifs within putative enhancer regions located in the first and second introns. Similar E-box motifs have been found previously in the promoter sequence of the murine clock gene *mPeriod1*. Hence, the same molecular mechanisms generating circadian oscillations in the expression of clock genes may directly control the rhythmic transcription of clock output regulators such as *Dbp*.

[*Key Words*: PAR factors; circadian transcription; clock; molecular oscillator; output]

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In mammals, many physiological processes are subject to circadian regulation. These include sleep–wake cycles, body temperature, heartbeat, blood pressure, endocrine functions, renal activity, and liver metabolism (for review, see Schibler and Lavery 1999). During the past few years impressive progress has been made in the elucidation of molecular mechanisms generating circadian oscillations in animal systems. In both *Drosophila* and mammals, a feedback loop in gene expression is thought to drive circadian oscillations (Rosbash et al. 1996; Brown and Schibler 1999; Dunlap 1999; Young 1999). In the mouse, the negative limb of this feedback loop is believed to be comprised of *mPer1*, *mPer2*, and *mPer3*, three murine homologs of the *Drosophila period* gene; *Cry1* and *Cry2*, two homologs of the *Drosophila cryptochrome* gene; and perhaps *mTim*, a homolog of the *Drosophila timeless* gene. The positive limb of this feedback loop contains the two basic helix-loop-helix–PAS (bHLH) proteins CLOCK and BMAL1 in both *Drosophila* and mammals. The currently held model for the mammalian feedback loop poses that one or multiple *mPer* and *Cry* genes are activated by a CLOCK/BMAL1 heterodimer via an E-box motif (Kume et al. 1999). The three PER proteins encoded by these genes undergo interactions with themselves and with the two CRY proteins. Once these heteromultimeric complexes reach a critical concentration, they repress transcription of *mPer* genes, probably by attenuating the activation potential of the CLOCK/BMAL1 heterodimer. Upon decay of PER and/or CRY proteins, repression is relieved, and a new wave of *mPer* and *Cry* expression can be initiated. The oscillation of gene expression generated by this feedback loop has a period length of about 24 hr and can thus account for the circadian timing observed in overt rhythms in physiology and behavior. Initially, it was believed that oscillations of clock proteins were present only in specialized pacemaker neurons within the suprachiasmatic nucleus (SCN). Recently, however, molecular clocks similar to those operating in SCN neurons have been uncovered in peripheral cell types (Zylka et al. 1998) and even in immortalized fibroblast cell lines kept in tissue culture (Balsalobre et al. 1998).

An important question to be answered is how the central negative feedback loop drives circadian expression of clock-controlled genes. In this investigation we approached this issue by studying *Dbp*, a clock-controlled gene whose expression oscillates with a very high circadian amplitude (Wuarin and Schibler 1990). DBP (albumin D-element binding protein; Mueller et al. 1990) is the founding member of the PAR family of basic leucine zipper (bZip) transcription factors. Other members of

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this family include TEF (thyroid embryonic factor) (Drolet et al. 1991), its avian ortholog VBP (vitellogenin promoter-binding protein) (Iyer et al. 1991), and HLF (hepatocyte leukemia factor) (Hunger et al. 1992; Inaba et al. 1992). All of these proteins share high amino acid sequence similarities within a amino-terminal activation domain, a PAR domain rich in proline and acidic amino acid residues, and a carboxy-terminal moiety encompassing the bZip region necessary for DNA binding and dimerization. In vitro all PAR bZip proteins avidly bind the consensus DNA recognition sequence 5′-RTTAYGTAAY-3′ as homo- or heterodimers (Falvey et al. 1996).

In rat and mouse liver the expression of all three PAR bZip proteins is subject to strong circadian regulation, peak and trough levels being reached in the early evening and morning, respectively (Wuarin and Schibler 1990; Falvey et al. 1995; Fonjallaz et al. 1996; Lopez-Molina et al. 1997). In the case of *Dbp* the amplitude of circadian mRNA oscillation can largely account for the daily amplitude in protein oscillation (Fonjallaz et al. 1996). The mRNA accumulation oscillates not only in peripheral tissues such as liver, but also in neurons of the SCN, believed to harbor the central circadian pacemaker (Ralph et al. 1990). Moreover, run-on experiments in isolated nuclei (Wuarin and Schibler 1990; Lavery and Schibler 1993) and physical mapping of nascent RNA chains (Wuarin and Schibler 1994) suggest that circadian transcription plays a pivotal role in rhythmic DBP expression.

Genetic loss-of-function experiments have begun to illuminate some physiological roles of DBP (Lopez-Molina et al. 1997). Although this transcription factor is not essential for embryonic development and survival during adulthood, it is involved in the control of several circadian outputs. Mice homozygous for a *Dbp* null allele differ from wild-type mice in the period length and the amplitude of circadian locomotor activity (Lopez-Molina et al. 1997), in several electroencephalogram (EEG) parameters of sleeping behavior (Franken et al. 2000), and in the circadian expression of some liver genes, such as the ones specifying steroid 15α hydroxylase, coumarin 7 hydroxlase, and cholesterol 7α hydroxylase (Lavery et al. 1999; L. Lopez-Molina, D.J. Lavery, and U. Schibler, unpubl.). However, in contrast to mice with mutations in *Clock* (Antoch et al. 1997; King et al. 1997b) or *mPer2* (Zheng et al. 1999) and to *Cry1* and *Cry2* double knockout mice (van der Horst et al. 1999), mice with a deletion of *Dbp* still display rhythmic wheel-running behavior when examined under constant dark conditions. It thus has been concluded that DBP is a participant in output pathways rather a central clock component (Lopez-Molina et al. 1997).

Because the high variation of daily *Dbp* mRNA accumulation was found to be controlled mainly at the transcriptional level (Wuarin and Schibler 1990, 1994; Lavery and Schibler 1993), we wished to use this system to examine how the central oscillator mechanism drives circadian transcription of output genes. To identify upstream regulators of *Dbp* we localized putative circadian regulatory elements within the *Dbp* gene by mapping DNase I hypersensitive sites in mouse liver chromatin. Here we report the identification of E-box motifs within DNase I hypersensitive regions located in the first and second introns and show that these elements can bind CLOCK, an essential component of the molecular oscillator. Moreover, in *Clock* mutant mice circadian *Dbp* expression is abolished in the liver and severely dampened in the SCN. These experiments suggest a direct coupling of *Dbp* expression to the molecular oscillator in both SCN neurons and peripheral cell types.

Results

Intragenic regions are required for circadian Dbp *expression*

To identify possible functions of *Dbp* in the circadian time-keeping system, two different strains of $Dbp^{-/-}$ mice, Dbp^{dim} and Dbp^{null}, have been generated (Lopez-Molina et al. 1997). Strain Dbp^{dim} contains a neomycin-resistance cassette insertion into exon 4 and encodes a truncated and unstable protein devoid of a leucine zipper dimerization domain (Lopez-Molina et al. 1997). Although the protein issued by this mutant allele did not accumulate to detectable levels in liver nuclei, the Dbp^{dim} mRNA displayed a similarly robust circadian accumulation in both SCN neurons and hepatocytes (Lopez-Molina et al. 1997; L. Lopez-Molina and U. Schibler, unpubl.). It thus was concluded that DBP was not critical for the regulation of its own circadian expression. In the second *Dbp* mutant strain, Dbp^{null}, most intragenic sequences that encompass the entire open reading frame (ORF) of *Dbp* were replaced by a bacterial *lacZ* cassette (Fig. 1A) to monitor tissue specific gene expression. However, in situ hybridization revealed that the *Dbp–lacZ* fusion gene, contrary to the wild-type *Dbp* allele, was expressed in only a small subset of brain structures (e.g., hippocampus and dentate gyrus, see Lopez-Molina et al. 1997). Importantly, little if any *Dbp–lacZ* mRNA could be detected in the SCN even at times during which *Dbp* mRNA accumulates to massive amounts in wild-type animals. Taken together, the observations described above strongly suggested that intragenic *Dbp* sequences are essential for high-amplitude circadian expression of *Dbp* mRNA in the SCN.

To examine the importance of intragenic sequences for rhythmic transcription in peripheral organs, the levels of mRNAs issued by either *Dbp* or the *Dbp–lacZ* were compared by RNase protection experiments with whole-cell liver RNA from heterozygous mice containing one each of these alleles. As shown in Figure 1B, the wild-type *Dbp* allele produces at least 100-fold more transcripts than the *Dbp–lacZ* gene. This is unlikely to be due to an intrinsically low stability of *Dbp–lacZ* mRNA, as this transcript accumulates to relatively high levels in certain brain structures (see above) and other fusion mRNAs carrying an identical *lacZ* moiety are readily detected in liver and other peripheral organs (F. Damiola, N. Preitner, and U. Schibler, unpubl.). Hence, intragenic enhancer sequences that have been deleted in

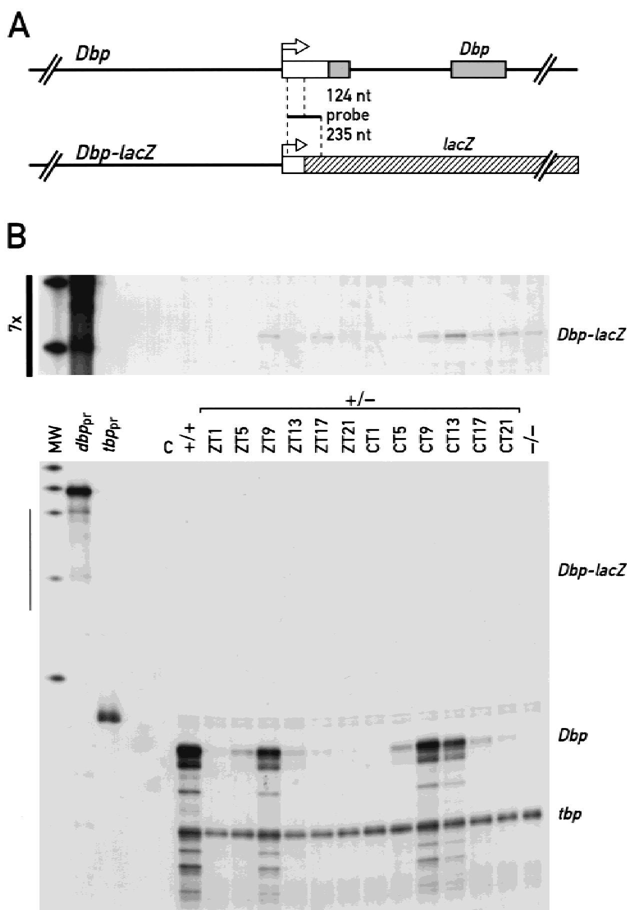

Figure 1. Intragenic regulatory sites are important for high-level expression of *Dbp* in mouse liver. (*A*) Schematic representations of the *Dbp* wild-type and *Dbp–lacZ* fusion alleles. The position of the antisense RNA probe used in the ribonuclease protection experiments shown in *B* is indicated. Upon hybridization with *Dbp* mRNA or *Dbp–lacZ* mRNA 124 nucleotides or 235 nucleotides, respectively, are protected from ribonuclease digestion. (*B*) Ribonuclease protection experiments were performed with whole-cell liver RNA from $Dbp^{+/-}$ mice extracted at the indicated time points from animals kept under 12:12 hr light/dark conditions (ZT) or animals kept for 10 days under constant conditions (CT). The RNA samples were hybridized to specific probes for *Dbp–lacZ* mRNA and *tbp* mRNA. *tbp* mRNA served as an internal control for a transcript that is constitutively expressed throughout the day. Autoradiographs were exposed for 15 hr (*bottom*) or 105 hr (*top*, see bar at *left*), and the resulting bands were quantified with a scanner. (MW) Molecular weight marker; (Dbp_{pr}, tbp_{pr}) undigested probes for *Dbp–lacZ* and *tbp*, respectively; (C) negative control with an equivalent amount of yeast tRNA; (+/+, –/–) whole-cell liver RNAs from wild-type or $Dbp^{-/-}$ mice, respectively, at ZT9.

constructing the *Dbp–lacZ* allele appear to play an important role for high-amplitude circadian *Dbp* transcription in both the SCN and peripheral organs.

The mapping of DNase I hypersensitive chromatin regions within the Dbp *locus*

The binding of transcription factors to specific *cis*-acting DNA sequences frequently causes distortions of the surrounding chromatin, which in turn leads to DNA sites that are particularly sensitive to the attack of nucleases such as DNase I. Hence, nuclease hypersensitive sites are often diagnostic for *cis*-acting regulatory sequences, such as promoter, enhancer, and silencer elements (see Boyes and Felsenfeld 1996, and references therein). DNase I hypersensitive sites within the *Dbp* locus were determined in liver nuclei harvested at intervals of four hr around the clock. As shown in Figure 2A, seven hypersensitive regions can be identified in the *Dbp* gene. Region 1, located at the end of intron 2, and region 3, located at the end of exon 2, seem to be present at all times of day (Fig. 2B). In contrast, regions 4 and 5 around positions +900 and +1, respectively, are much more susceptible to DNase I digestions at times of day when *Dbp* is maximally transcribed [Zeitgeber time (ZT) 7 to ZT 11; Fig. 2C]. The DNase I sensitivity of region 7, around position –900 (Fig. 2C), and the upper band of region 2 (Fig. 2B), located within the intron 2, oscillate in a similar manner, albeit with a lower amplitude. Region 6, around position –400, behaves in a more complex fashion, with some bands being present in phase and others in antiphase to the regulation of *Dbp* transcription (Fig. 2C). Very similar temporal patterns of DNase I hypersensitive sites were observed under light-entrained and constant dark conditions, and the sites were also detected in DNA isolated from nuclei obtained from the kidney (data not shown). No additional hypersensitive sites are detected in the 3′ moiety of *Dbp* or downstream of the polyadenylation site (Fig. 2D). Circadian hypersensitive sites were found to correlate tightly with the daily phase of *Dbp* transcription, and when the same DNA was probed for the constitutively active *C/ebpα* gene (see Wuarin and Schibler 1990), no daytime-related changes in DNase I hypersensitive sites could be detected (Fig. 2E).

As shown in Figure 1, *Dbp–lacZ* is transcriptionally nearly inactive in spite of the fact that it contains all of the *Dbp* 5′-flanking sequences. We thus wanted to determine whether the DNase I hypersensitive sites (5–7) can also been observed within the *Dbp–lacZ* promoter region. Figure 2F shows that a single weak hypersensitive region at position –400 could be observed in this region, whereas the hypersensitive sites 5 (transcriptional start site) and 7 (–900) are lacking. Hence, the formation of these two hypersensitive regions may require interactions with intragenic regulatory sequences.

Intronic DNase I hypersensitive regions encompass E-box motifs that bind CLOCK

Because intragenic enhancer sequences appear to be essential for robust circadian *Dbp* expression (see above), we decided to investigate the hypersensitive regions located downstream of the cap site in greater detail. A precise mapping using internal size markers (data not shown) and subsequent sequence inspection of these regions revealed potential protein–DNA-binding sites located within these sites. We paid particular attention to

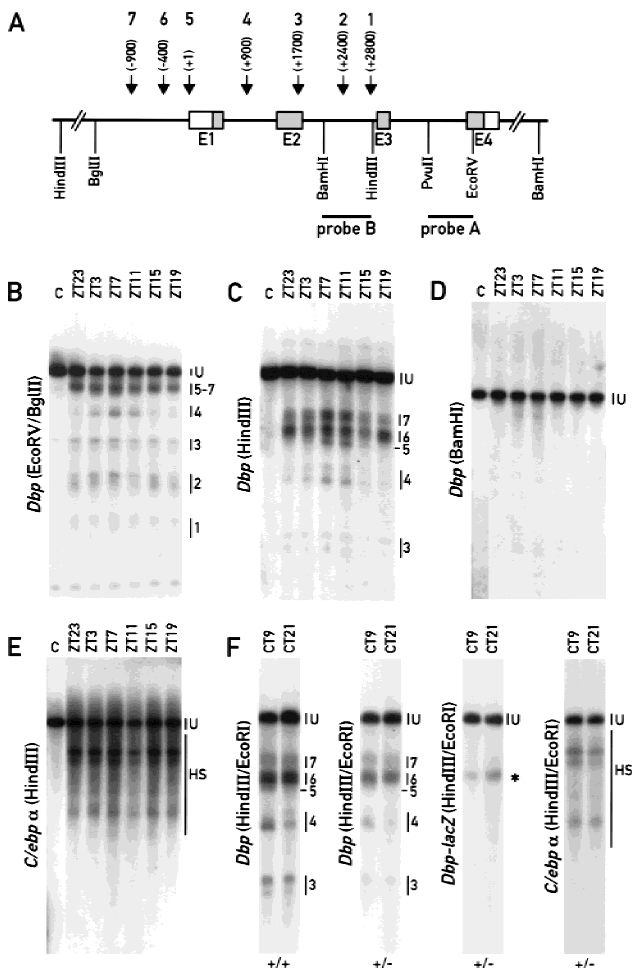

Figure 2. DNase I hypersensitive sites within the *Dbp* locus. (*A*) Schematic representation of the *Dbp* gene with its four exons (E1–E4) and three introns. The positions of the DNA hybridization probes and the restriction fragments used in the indirect end-labeling experiments are indicated. The approximate positions of the seven DNase I hypersensitive regions detected in *B* and *C* are depicted on *top* of the cartoon. (*B*) Mapping of DNase I hypersensitive sites starting from exon 4. Equal aliquots of liver nuclei harvested at the indicated times were treated with DNase I (ZT23 to ZT19) or without DNase I (lane C, derived from ZT7). After exhaustive digestion with *Eco*RV and *Bgl*II, the fragments were visualized using probe A. (*C*) Fine mapping of DNase I hypersensitive sites in the 5′ moiety of the *Dbp* locus. The DNA was digested with *Hin*dIII and probed with probe B. (*D*) Mapping of DNase I hypersensitive sites in the 3′ moiety of the *Dbp* locus. The same DNA was digested to completion with *Bam*HI and processed as described in *C*. A hypersensitive site, mapping to about +500 from the polyadenylation site of *Dbp*, was not reproducibly observed in other experiments. (*E*) DNase I hypersensitive sites within the constitutive *C/ebpα* locus. DNase I/*Hin*dIII-digested DNAs were probed with a *Nco*I/*Hin*dIII fragment encompassing the ORF of the intron-less *C/ebpα* gene. (U) Full-length genomic fragment. (*F*) Comparison of DNase I hypersensitive sites within the *Dbp* wild-type allele (panels 1 and 2) and the *Dbp–lacZ* knockout allele (panel 3). Panel 4 shows DNase I hypersensitive sites within the *C/ebp* locus as a control. (+/+) *Dbp* wild-type mice; (+/−) *Dbp* heterozygous mice. The asterisk marks the position of the hypersensitive region present in the *Dbp–lacZ* allele.

the presence of E-box motifs, as this type of *cis*-acting element has already been shown to drive circadian *period* expression in *Drosophila* (Hao et al. 1997, 1999) and has been proposed to mediate circadian expression of the mouse *period1* (Gekakis et al. 1998) and *vasopressin* genes (Jin et al. 1999). Two E-box motifs resembling the E-box consensus motif CACGTG (Gekakis et al. 1998; Hogenesch et al. 1998) were found to be present at positions +2398 and +2510, mapping very close to the two bands observed within region 2. Another E-box motif with the related core sequence CACATG (located at +857 in the center of the circadian region 4) was identified by an in vitro DNase I footprinting approach (data not shown). A related E-box motif was found in the hypersensitive region 7 at −888. These E-box motifs may be direct targets for CLOCK, an essential pacemaker component (for review, see Schibler 1998). As a large number of bHLH proteins can bind E-box motifs in vitro, simple electromobility shift assays (EMSA) were inadequate to resolve all complexes obtained with these DNA sequences (data not shown). We thus resorted to a two-dimensional high-resolution EMSA technique (Ossipow et al. 1993) and adapted it to the analysis of large DNA-binding proteins (see Materials and Methods). Briefly, after running the protein–DNA complexes in the first dimension, they were cross-linked in the gel and placed on top of a second, denaturing gel. After their final separation these complexes were visualized by autoradiography. To identify potential CLOCK-containing complexes, these two-dimensional EMSA experiments were performed in the presence and absence of CLOCK antiserum.

As shown in Figure 3A, the obtained pattern of protein–DNA complexes was found to be complex. However, two protein–DNA complexes disappear when CLOCK antiserum is included in the first dimension of the EMSA reaction using the E-box motif from DNase I hypersensitive region 4 (+857) as a binding site. As these two complexes migrate at the same position in their first dimension, they most probably formed a heterodimeric complex before denaturation with SDS. The nature of the interaction partner for CLOCK is currently unknown, but it might be related to BMAL1, known to form a complex with CLOCK in vitro (Gekakis et al. 1998; Hogenesch et al. 1998). No attempts have been made to identify the major cross-linked complexes. According to their approximate molecular mass and their high abundance, they may represent protein–DNA complexes containing upstream stimulating factor (USF) isoforms (see Potter et al. 1991; Viollet et al. 1996).

In contrast to the highly circadian hypersensitivity of the +857 binding site in liver chromatin (see above), qualitatively and quantitatively very similar CLOCK/DNA complexes could be formed in vitro with liver nuclear proteins harvested throughout the day (data not shown). Furthermore, a nearly identical pattern of protein–DNA complexes and similar CLOCK-containing complexes could also be observed with either of the two E-box motifs resident in the DNase I hypersensitive region 2 from positions +2398 and +2510 (data not shown).

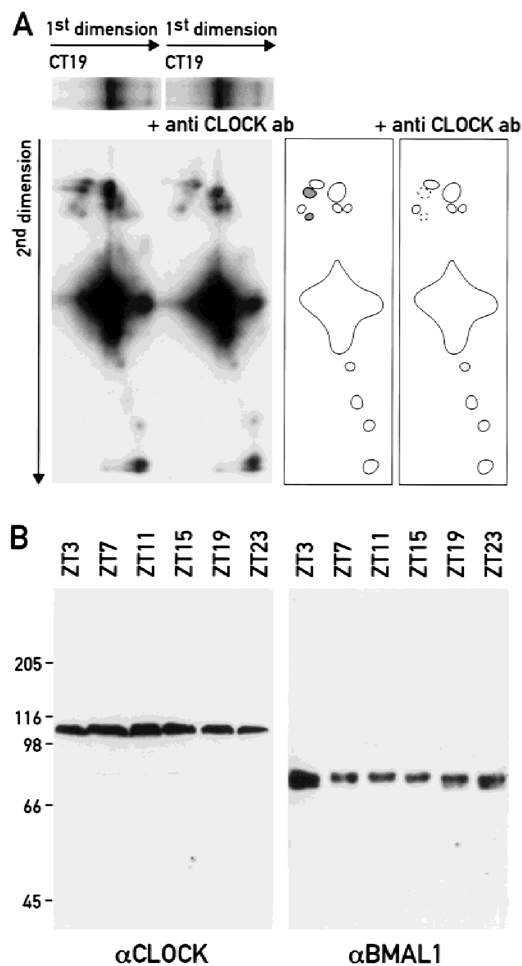

Figure 3. An intronic E-box motif that binds CLOCK in vitro. (*A*) Two-dimensional gel EMSA. Nuclear extracts from mouse livers harvested at CT19 were incubated with an oligonucleotide containing the intronic E-box motif (+857) of *Dbp*. After separation on a 4% polyacrylamide/0.5% agarose composite gel (1st dimension) the protein–DNA complexes were UV cross-linked, size-fractionated by electrophoresis through a 7% SDS–polyacrylamide gel (2nd dimension), blotted onto a membrane, and visualized by autoradiography. In one reaction (+ anti-CLOCK ab), the liver nuclear extract was incubated with a CLOCK-specific antiserum before the two-dimensional EMSA analysis was performed. The contours of the spots corresponding to radiolabeled cross-linked protein–DNA complexes are depicted in the drawings at the right-hand site of the autoradiographs. Two spots are lacking in the experiments with CLOCK-specific antiserum. Their vertical alignment in the second dimension indicates that the two complexes were part of a heterodimeric complex in the first dimension. (*B*) Daily accumulation of CLOCK and BMAL1 in liver nuclei. Nuclear proteins harvested at the indicated time points were separated on a 7% SDS-polyacrylamide gel and probed with CLOCK- or BMAL1-specific antibodies. The migrations of standard proteins according to their molecular mass are indicated on the *left* (in kD).

As judged by Western blot analysis, both CLOCK and BMAL1 were present at nearly invariable levels in liver nuclear extracts at all times of day (Fig. 3B). The molecular basis for the cycling nature of hypersensitive region 4 thus cannot readily be explained solely by a cyclic accumulation of CLOCK protein. Conceivably, however, components of the negative limb of the feedback loop, such as PER and CRY proteins, sequester CLOCK in a complex unable to bind to its cognate E box during times at which *Dbp* is not transcribed. The phase of circadian CRY protein accumulation would be in keeping with such a scenario (J.A. Ripperger and U. Schibler, unpubl.).

In conclusion, the DNase I mapping experiments in conjunction with the two-dimensional EMSA assays presented in this section unveiled six putative *Dbp* regulatory sequences, of which two are located upstream (6 and 7) and four downstream (1–4) of the transcription initiation site. The sensitivity towards DNase I digestion of four of these regions (2, 4, 6, and 7) and that of the promoter region encompassing the transcription initiation site oscillate with the same phase as *Dbp* transcription. Three of the putative regulatory sequences (2, 4, and 7) contain E boxes that are potential binding sites for CLOCK.

The intronic Dbp *E box in DNase I hypersensitive site 4 acts as a CLOCK/BMAL1 target sequence in cotransfection experiments*

All four identified E-box motifs were found to function as *cis*-acting elements when they were linked to a luciferase reporter gene and transfected into murine LTK$^-$ fibroblasts (Fig. 4A). To examine whether CLOCK/BMAL1 can activate transcription of a *cis*-linked luciferase reporter gene via the *Dbp* E-box motif from intron 1, a series of cotransfection experiments were performed. As shown in Figure 4B, CLOCK and BMAL1 increase E box-driven reporter gene activity in a dose-dependent manner. Transfection of either expression vector alone did not significantly influence the basal reporter gene activity (data not shown). In contrast, cotransfection of a mutated form of CLOCK, CLOCKΔ19 (obtained from *Clock/Clock* mutant mice and supposed to act as a dominant-negative inhibitor; Antoch et al. 1997; King et al. 1997a), repressed basal reporter gene activity. Conceivably, this down-regulation is the consequence of a competition of CLOCKΔ19 with endogenous wild-type CLOCK and/or other related bHLH proteins. No effects of CLOCK on luciferase expression have been observed in experiments with reporter genes carrying a mutated version of the E-box motif (Fig. 4B). In conclusion, the cotransfection experiments presented in this section are compatible with the speculation that the intronic *Dbp* E box may serve as a functional CLOCK/BMAL1 recognition sequence. Qualitatively similar experiments were also obtained with either of the two E boxes resident in intron 2 (data not shown) and with E boxes for different clock-related genes (Gekakis et al. 1998; Kume et al. 1999).

The Dbp *mRNA expression is severely down-regulated in the SCN and the liver of* Clock *mutant mice*

On the basis of the observations described above, we

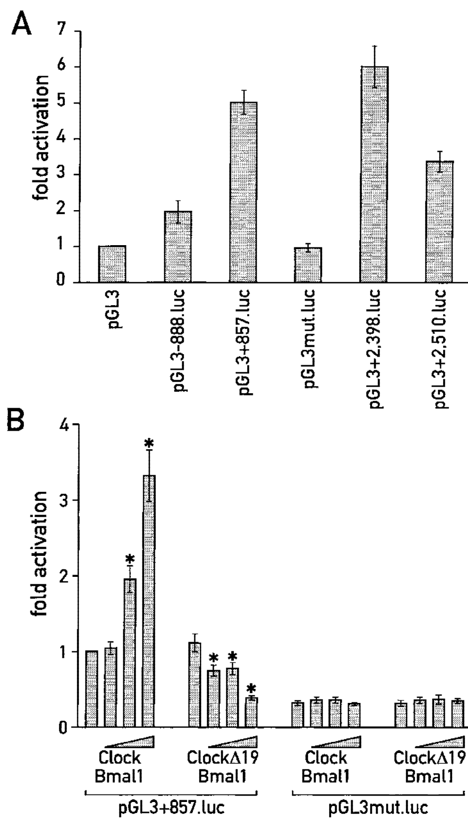

Figure 4. CLOCK activates transcription from the intronic *Dbp* E box. (*A*) Comparison of basal reporter gene activities of luciferase reporter gene constructs containing four different E-box motifs identified in *Dbp*. (*B*) Mouse LTK⁻ fibroblasts were cotransfected with either pGL3+857.luc or pGL3mut.luc and increasing amounts of expression vectors for CLOCK and BMAL1 or CLOCKΔ19 and BMAL1 (80 ng, 400 ng, or 2 µg each). Mean ± S.D. of six experiments. The transfection experiment with pGL3+857.luc and no expression vectors was set to one-fold. An asterisk marks highly significant differences ($P < 0.001$) within the pGL3+857.luc series. There were no such differences within the pGL3mut.luc series.

considered the possibility that circadian *Dbp* expression involves CLOCK. To examine this conjecture, the daily accumulation of *Dbp* mRNA was determined in the SCN of *Clock*/*Clock* mutant mice that express the CLOCKΔ19 protein (King et al. 1998b) by in situ hybridization and in the liver by RNase protection experiments. In the SCN of *Clock*/*Clock* mutant mice *Dbp* mRNA accumulation still appears to be rhythmic (ANOVA, $P = 0.0018$), but the amplitude is significantly reduced as compared with wild-type mice (ANOVA, $P = 0.0001$; Fig. 5). In liver, the difference between mutant and wild-type mice is even more dramatic. As seen in Figure 6A, circadian *Dbp* mRNA accumulation is completely abolished in homozygous *Clock* mutant animals. Surprisingly, the *Dbp* mRNA peak levels are very low even in heterozygous *Clock* mutant mice (Fig 6B). These experiments indicate that the CLOCKΔ19 mutant protein, which lacks a glutamine-rich segment of its supposed transactivation domain, interferes with its wild-type counterpart in a dominant-negative fashion, conceivably by competing for its DNA recognition sequences.

Discussion

Dbp *contains multiple putative enhancer regions*

We have studied the transcriptional regulation of circadian DBP expression by using a variety of genetic and biochemical tools. *Cis*-acting DNA regulatory elements occupied by their cognate transcription factors frequently render the neighboring chromatin hypersensitive to nucleolytic cleavage by DNase I and other nucleases. As a consequence, the mapping of DNase I hypersensitive chromatin regions can be used as a reliable tool to identify transcriptional control elements such as promoters, enhancers, locus control regions, and silencing sequences (Weintraub and Groudine 1976; Thomas and Elgin 1988; Fraser et al. 1990; Felsenfeld et al. 1996;

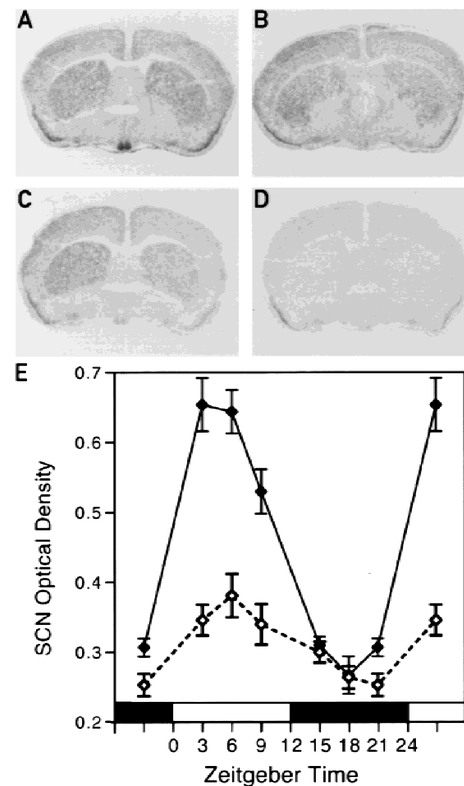

Figure 5. *Dbp* mRNA levels are reduced in the SCN of *Clock*/*Clock* mice. Representative autoradiographs of in situ hybridizations with coronal brain sections containing the SCN from wild-type mice at ZT3 (*A*), wild-type mice at ZT15 (*B*), and *Clock*/*Clock* mutant mice at ZT3 (*C*). A *Dbp* antisense cRNA was used as a hybridization probe to detect *Dbp* RNA. (*D*) A section adjacent to that of the one shown in *A* hybridized with a *Dbp* sense cRNA probe (negative control). (*E*) The temporal accumulation profiles of *Dbp* mRNA in the SCN of wild-type mice (solid line) and *Clock*/*Clock* mutant mice (broken line) in a 12-hr light/dark cycle. Each value is the mean ± S.E.M. of 5–6 animals.

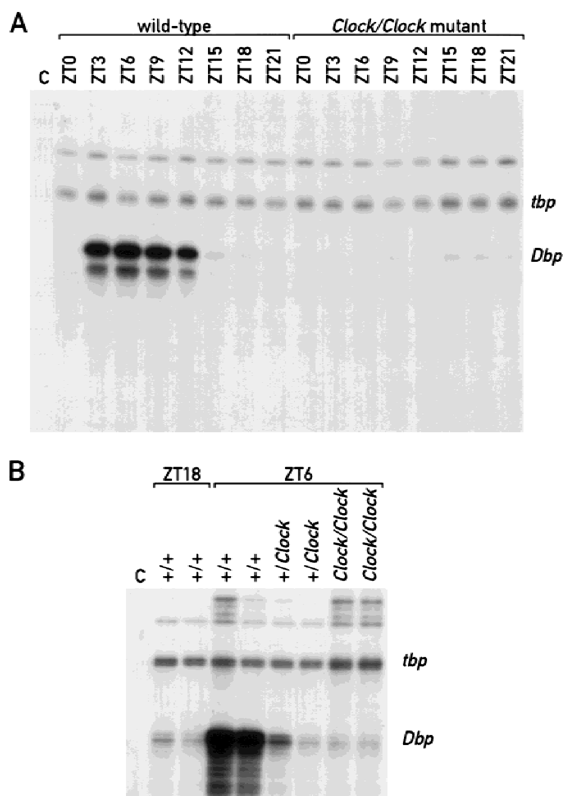

Figure 6. Circadian *Dbp* expression is obliterated in the liver of *Clock* mutant mice. (*A*) RNase protection assays with an antisense *Dbp* cRNA probe and whole-cell liver RNAs prepared from wild-type and *Clock/Clock* mutant mice at the indicated times (ZT0–ZT21). A *tbp* antisense cRNA was included as an internal control for a constitutively expressed mRNA (see Fig. 1). (Lane C) Negative control in which liver RNA was substituted with yeast tRNA. (*B*) RNase protection assays with an antisense *Dbp* cRNA probe and whole-cell liver RNAs prepared from mice of different genotypes. The ZT at which the animals were sacrificed and the genotypes of the mice are indicated at *top*. (Lane C) Negative control in which liver RNA was substituted with yeast tRNA.

Kingston et al. 1996; Sippel et al. 1996). We have employed this technique to locate putative *cis*-acting regulatory regions in the *Dbp* gene. The robust amplitude in circadian *Dbp* transcription makes this search particularly attractive, as it should allow the temporal correlation of hypersensitive sites with the transcription status of *Dbp*. At times of maximal *Dbp* transcription rates [ZT or circadian time (CT) 7 and 11] seven DNase I hypersensitive regions could be identified, of which one maps to the cap site at position +1. The cleavage at the cap site is strictly correlated with transcription efficiency and probably reflects the occupancy of this site by components of the RNA polymerase II transcription machinery. Hence, formation of the transcription preinitiation complex may be limited to the time window during which *Dbp* is actively transcribed. The susceptibility to nucleolytic attack of four of the six remaining DNase I hypersensitive regions (2, 4, 6 and 7, see Fig. 2) also varies significantly with circadian time, albeit with an amplitude lower than that observed for the site mapping to +1. The hypersensitive region 6 displays a somewhat more complex pattern. Whereas some of the cleavages oscillate with the same phase as transcription, others appear to be anticyclic. Conceivably, the DNA-binding proteins responsible for the anticyclic cleavages are transcriptional repressors rather than activators. Interestingly, the hypersensitive sites 5 and 7 (see Fig. 2) were completely absent from the 5′-flanking region of the *Dbp–lacZ* fusion gene, whereas DNase I hypersensitive region 6 appeared as a single band that was constitutively present around the clock (Fig. 2F). This suggests that some of the transcription factors binding to DNA sequences within 5′-flanking sequences can do so only in concert with transcription factors binding to intragenic enhancer elements. Therefore, transcriptional regulatory proteins occupying upstream and intragenic DNA elements are likely to stimulate *Dbp* transcription synergistically, for example by cooperative binding.

The poor transcriptional activity of the *Dbp–lacZ* allele indicates that intragenic regulatory sequences are essential for high-amplitude circadian *Dbp* transcription. However, given the possibly synergistic action of upstream and intragenic regulatory sequences, the same may be true for enhancers located within the 5′-flanking region. In fact, *cis*-acting elements conferring circadian transcription are likely to exist within this region as well, as the few transcripts issued by the *Dbp–lacZ* allele still exhibit circadian accumulation (Fig. 1B). Clearly, the examination of the role that upstream sequences may play in rhythmic *Dbp* transcription will require further studies.

CLOCK is an upstream regulator of Dbp

In *Clock/Clock* mutant mice *Dbp* circadian expression is dramatically dampened in the SCN and nearly extinguished in the liver. However, this genetic analysis does not establish whether CLOCK is a direct or indirect regulator of *Dbp*. Based on two observations we favor a direct interaction between *Clock* and *Dbp*. First, the circadian expression of *Dbp* and *mPer1*, another putative CLOCK target gene (Gekakis et al. 1998; Jin et al. 1999), display peak expression at the same time of day. Secondly, we could identify CLOCK recognition sequences within circadian hypersensitive chromatin regions of *Dbp* that in cotransfection assays are capable of conferring CLOCK/BMAL1-mediated transcriptional stimulation (Fig. 3B; data not shown). Another related E-box motif and potential binding site for CLOCK is located within the circadian DNase I hypersensitive region 7 at position –888. Conceivably, this E box is responsible for the rhythmic accumulation of the few transcripts issued from the *Dbp–lacZ* fusion allele observed in liver (Fig. 1B). In accordance with this speculation, the trace amounts of *Dbp* mRNA detected in the livers of *Clock/Clock* mutant mice, in contrast to those in $Dbp^{+/-}$ mice, are not subject to circadian variation (Figs. 1 and 6A).

Wild-type CLOCK protein and its DNA-binding sites

within extra- and/or intragenic *Dbp* enhancer sequences are likely to participate in both the positive and negative limbs of the circadian feedback loop. This has been suggested for the circadian regulation of both *Drosophila* and mammalian pacemaker genes (Allada et al. 1998; Darlington et al. 1998; Gekakis et al. 1998; Rutila et al. 1998; Jin et al. 1999; Kume et al. 1999). We wish to emphasize, however, that not all E box-containing DNase I hypersensitive regions displayed a strong circadian pattern. In fact, the E box motif centered around +2510 within DNase I hypersensitive region 2 shows less dramatic circadian accessibility than the E boxes at positions +857 and +2398 to nuclease digestion throughout the day, yet binds CLOCK avidly in vitro (data not shown). We can thus only speculate on the molecular mechanisms leading to the different behavior of the various E box-containing motifs in nuclease digestion experiments. One possibility to account for the differential nuclease sensitivity of different E boxes in *Dbp* chromatin would be that only the circadian E-box elements participate in the negative limb of the circadian autoregulatory loop, whereas the other E box acts more as a constitutive enhancer element. Given the multitude of different bHLH proteins present in liver nuclei that bind E-box motifs in vitro (see Fig. 3A), it is conceivable that the circadian and constitutive E-box motifs bind different proteins in vivo. In fact, the occupancy of a given *cis*-acting element in vivo is determined not only by the intrinsic sequence specificity of a transcription factor but also by the cooperative interactions this factor establishes with other proteins binding to nearby or distant promoter and enhancer elements. Therefore, although in vitro binding studies with short DNA recognition sequences can establish a repertoire of possible cognate proteins present in a cellular extract, they are insufficient to positively identify the proteins that occupy these sites in vivo. Nevertheless, together with the genetic evidence obtained with *Clock* and *Dbp* mutant mice, the biochemical experiments presented above suggest that CLOCK protein may interact directly with cognate E boxes in *Dbp* to regulate circadian transcription of this gene.

Dbp *transcription may be linked directly to the circadian clock*

CLOCK was found to be an essential upstream regulator of *Dbp*, and this observation links the expression of this gene directly to the molecular oscillator. Interestingly, we observed some minor differences regarding the effect of CLOCKΔ19 on the expression of *Dbp* in the SCN and the liver. Although in the SCN neurons some rhythmic expression was still observed, in the periphery circadian expression was completely abolished, even in *Clock* heterozygous mice (Figs. 5 and 6). Conceivably, the CLOCKΔ19 protein accumulates to higher levels in liver nuclei as compared with nuclei of SCN neurons.

As a transcription factor expressed in most cell types, DBP can contribute to the cyclic transcription of many target genes both in the SCN and in peripheral tissues. In liver, for example, DBP participates in the circadian expression of coumarin 7 hydroxylase, steroid 15α hydroxylase, and several other cytochrome P-450 enzymes (Lavery et al. 1999; B. Kornmann and U. Schibler, unpubl.). The way by which the molecular feedback loop of cellular pacemakers can drive circadian outputs are illustrated in Figure 7. Whereas some output genes such as the neuropeptide *vasopressin* (Jin et al. 1999) may be directly controlled by the molecular oscillator, others may be regulated by circadian transcription factors that themselves are controlled by the molecular pacemaker.

Although the experimental data on *Dbp* are in strong favor of a model in which the expression of this output gene is directly hardwired to the molecular oscillator, they cannot be regarded as direct proof for such a scenario. In fact, the unequivocal demonstration of a direct functional interaction between a transcription factor and its target gene requires experiments with specificity-shift mutations in both the DNA-binding domain of the transacting regulatory protein and its DNA recognition sequence(s). Owing to the technical difficulty of such an approach in metazoan organisms, this has been accomplished only in very rare cases. One very elegant example is the study by Schier and Gehring (1992) on the negative autoregulatory loop established by the *Drosophila* segmentation gene *fushi tarazu*.

In conclusion, our work has identified CLOCK, a central component of the circadian pacemaker, as a regulator of *Dbp* transcription. The presence of CLOCK-binding sites within DNase I hypersensitive regions, which

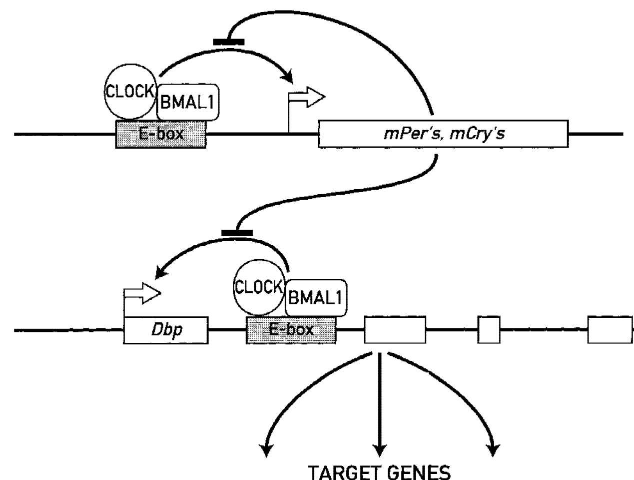

Figure 7. *Dbp* expression may be hardwired to the circadian feedback loop circuitry. The cartoon depicts a speculative model according to which circadian oscillations are generated by a negative-feedback loop of gene expression. The CLOCK/BMAL1 heterodimer activates transcription of essential pacemaker genes such as *mPers* and *mCrys* through an E-box motif. The protein products of these pacemaker genes then form complexes that, once they reach a critical threshold level, attenuate CLOCK/BMAL1-mediated activation. The same mechanism may be employed to regulate circadian transcription of *Dbp*. The cyclic accumulation of DBP may then drive circadian transcription of downstream target genes.

oscillate during the day in phase with *Dbp* transcription, strongly suggests that CLOCK controls rhythmic hepatic *Dbp* expression in a direct manner. Hence, we propose that the regulation of *Dbp* transcription is directly hardwired to the circadian feed back circuitry, as has been suggested for another output gene, *vasopressin* (Jin et al. 1999). In contrast to *vasopressin*, however, *Dbp* is rhythmically expressed in both SCN neurons and most peripheral cell types and, as a transcription factor, can control the circadian expression of many target genes. The observation that the same transcriptional hierarchies can be observed in SCN neurons and hepatocytes supports the idea that central and peripheral circadian systems are similar in their molecular makeup.

Materials and methods

Animal care and handling

The $Dbp^{-/-}$ mouse strain with a substitution of the ORF of the *Dbp* gene by a *lacZ* cassette (Lopez-Molina et al. 1997) and the *Clock/Clock* mutant mouse strain bearing a deletion of exon 19 have been described previously (Antoch et al. 1997; King et al. 1997b). Mice were housed in a strict 12:12 hour light/dark regimen (lights on 7 a.m.; lights off 7 p.m.). Experiments under constant conditions were performed with animals kept in constant darkness for at least one day before the first animal was sacrificed (CT). For animals housed 10 days under constant conditions the period lengths of locomoter activity and their individual time schedules were calculated from their running-wheel activities as described (Lopez-Molina et al. 1997).

Analysis of whole-cell liver RNA

RNA was purified from mouse livers and analyzed by ribonuclease protection assays using probes for *Dbp* and *tata box-binding protein* (*tbp*) described previously (Lopez-Molina et al. 1997). The probe for *Dbp–lacZ*, overlapping the 5′ insertion site of the *lacZ* cassette in the *Dbp* gene, was obtained by PCR using a sense primer from *Dbp*, 5′-TTCTTTGCGAGAAGTGC-3′, an antisense primer from *lacZ*, 5′-AAACCAGGCAAAGCGCAT-3′, and genomic DNA from a $Dbp^{-/-}$ mouse as a template.

Mapping of DNase I hypersensitive sites

Nuclei from mouse liver were isolated as described (Lichtsteiner et al. 1987). The accessibility of the chromatin to limited DNase I digestion was tested as reported by Boyes and Felsenfeld (1996). Briefly, nuclei were treated with 1 U/µl of DNase I for five min on ice. Isolated genomic DNA (20 µg per lane) was digested to completion with the indicated restriction enzyme(s), separated on 0.7% agarose gels, blotted to nylon (Nitran) membranes, and hybridized with a *Bam*HI/*Hin*dIII fragment (2680 to 3447, GenBank accession no. U29762) or a *Pvu*II/*Eco*RV fragment (4333 to 5010, GenBank accession no. U29762) of the mouse *Dbp* gene. For the probing of *C/ebpα* a *Nco*I/*Hin*dIII fragment of the ORF from pMSV-C/EBP (Friedman et al. 1989) was used. The mapping in $Dbp^{+/-}$ animals was performed with *Eco*RI/*Hin*dIII-digested DNA (the *Eco*RI/*Hin*dIII fragment of *Dbp–lacZ* has about the same length as the *Hin*dIII/*Hin*dIII fragment of *Dbp*). The probe for mapping of the *Dbp–lacZ* locus was obtained as an *Eco*RI/*Sac*I fragment from the targeting vector pTK-B.A.-LacZ-NEO-UMS-D (Lopez-Molina et al. 1997). For a fine mapping of hypersensitive sites we obtained restriction fragments of a defined length from the *Dbp* locus. These fragments were adjusted in their quantity to match the hybridization signals derived from DNase I hypersensitive sites, and they were run together with DNase I-digested genomic DNA and detected with the appropriate probes. The resolution of these experiments should be ±100 bp.

Two-dimensional gel mobility shift and Western blot experiments

Briefly, an oligonucleotide encompassing the E box whose thymine residues were substituted with highly photoreactive azido-uracil residues was used as a DNA probe (Ossipow et al. 1993). The protein–DNA complexes formed with liver nuclear extracts were separated by electrophoresis on a composite agarose–polyacrylamide gel. The gel was irradiated with UV light, and the region containing the specific protein–DNA complexes was excised, placed horizontally onto a SDS–polyacrylamide gel, and the photo-cross-linked protein–DNA complexes were size-fractionated by electrophoresis. Because the UV dose chosen for these experiments resulted in the covalent protein–DNA cross-linking of only a small proportion of the complexes, these contain mainly protein monomers.

Proteins from liver nuclei were obtained as described (Ripperger et al. 1995). A double-stranded oligonucleotide encompassing the intronic E-box motif of *Dbp* (+857) was generated according to Ossipow et al. (1993) with Klenow polymerase using the 5′-primer 5′-CCTCGCAGGG-3′, 5-azido-dUTP, [α-^{32}P]-dATP, and the template 5′-CCTAGTTTCCATGTGACCCT-GCGAGG-3′. The gel mobility shift reactions were performed in a 20 µl final volume of 10% glycerol, 50 mM KCl, 10 mM HEPES (pH 7.6), 1 mM dithiothreitol, 0.1 mM EDTA, 5 mM $MgCl_2$, 50 ng/µl poly[d(I-C)], 0.4 µg/µl sheared salmon sperm DNA, 20 µg of protein, and 1×10^6 cpm of the oligonucleotide (1×10^7 cpm/pmole). After an incubation of 15 min at room temperature the protein–DNA complexes were separated on a 4% polyacrylamide/0.5% agarose gel in 36 mM Tris-borate (pH 8.3)/0.8 mM EDTA, cross-linked in situ for 7 min with UV_{302nm}, and layered on top of a 7% SDS-polyacrylamide gel (Laemmli 1970). After electrophoretic separation, the protein–DNA complexes were electroblotted to a nitrocellulose membrane, which was exposed for autoradiography for 92 hr. Rat antibodies against the amino terminus of mCLOCK (amino acids 1–424) and full-length hBMAL1 were raised and purified using standard techniques. Nuclear proteins (30 µg/lane) were separated on a 7% SDS-polyacrylamide gel, transferred to nitrocellulose, and specific antigen/antibody complexes were visualized using a horseradish peroxidase-conjugated secondary antibody and an ECL kit (Pierce).

Cotransfection experiments

Oligomerized E-box motifs ($n = 4$) were ligated into the *Bgl*II restriction site of pGL3 (Promega). The oligonucleotides used were: +888, 5′-GATCCACGTCCCATGTGGCCTTCT-3′; –857, 5′-GATCTAGTTTCCATGTGACCCTGC-3′; mut, 5′-GATC-TAGTTTC<u>AC</u>TG<u>GT</u>ACCCTGC-3′; +2,398, 5′-GATCCCCTC-GCCACGTGAGTCCGC-3′; +2510, 5′-GATCTAGGCCACA-CGTGATGCGGC-3′; and their corresponding complementary sequences containing a 5′-GATC overhang to facilitate cloning. Expression vectors for CLOCK, CLOCKΔ19, and BMAL1 have been described previously (Jin et al. 1999). Mouse LTK^- fibroblasts of 60%–80% confluency were transfected with 150 ng of reporter plasmids and the indicated amounts of either CLOCK expression vector made up to a total of 4 µg with an empty plasmid (pcDNA3.1, Invitrogen), using Superfect (Qiagen). After

48 hr of recovery the reporter gene activity was measured using a luciferase kit (Boehringer), and normalized for the transfection efficiency with a cotransfected *lacZ* plasmid using a β-galactosidase kit (Tropix).

In situ hybridization

The in situ hybridizations of mouse coronal brain sections have been described previously (Jin et al. 1999; Shearman et al. 1999). The cRNA probes were obtained from a 1065-bp fragment of the rat *Dbp* gene (357–1422, GenBank accession no. J03179) in vitro synthesized in sense or antisense orientation.

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