current density. Therefore the peak power is primarily insensitive to size effects (when normalized to the unit area or volume).
26. There is no strong theoretical basis for an "exponential" power increase, in particular because it is based on several different effects and is far-field dependent. Nevertheless, we chose the term "exponential" increase, because it represents the data qualitatively well.
27. The continuity conditions at the boundary cannot be satisfied for real values of the wavevector if there is no incident wave, so one looks for solutions with complex wavevectors. It can be shown that the real part of these solutions gives the wavevector at which scattering resonances would occur for a wave incident from infinity, whereas the imaginary part gives the width (Q value) of the resonance. In a scattering experiment, the measured intensity in the far-field has contributions both from the resonant scattering and the incident beam, whereas in lasing emission only the resonant emission is present. Hence, it is the intrinsic emission pattern of the quasi-bound state that is measured in the experiments reported, above and it is this quantity that we plot in Fig. 3, B and D. See (*13*), chap. 1, for a detailed discussion.
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40. We are grateful to A. Tredicucci for useful discussions. E.E.N., J.U.N., and A.D.S. gratefully acknowledge support from the Aspen Center for Physics for part of this work. The work performed at Bell Laboratories was supported in part by DARPA (Defense Advance Research Project Agency)–U.S. Army Research Office under contract DAAH04-96-C-0026. The work performed at Yale was supported in part by NSF grant PHY9612200.

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Role of the CLOCK Protein in the Mammalian Circadian Mechanism

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The mouse *Clock* gene encodes a bHLH-PAS protein that regulates circadian rhythms and is related to transcription factors that act as heterodimers. Potential partners of CLOCK were isolated in a two-hybrid screen, and one, BMAL1, was coexpressed with CLOCK and PER1 at known circadian clock sites in brain and retina. CLOCK-BMAL1 heterodimers activated transcription from E-box elements, a type of transcription factor–binding site, found adjacent to the mouse *per1* gene and from an identical E-box known to be important for *per* gene expression in *Drosophila.* Mutant CLOCK from the dominant-negative *Clock* allele and BMAL1 formed heterodimers that bound DNA but failed to activate transcription. Thus, CLOCK-BMAL1 heterodimers appear to drive the positive component of *per* transcriptional oscillations, which are thought to underlie circadian rhythmicity.

Circadian clocks are endogenous oscillators that control daily rhythms in physiology and behavior (*1*). Such clocks are phylogenetically widespread (*2*) and are likely to reflect evolutionarily ancient, fundamental mechanisms of timekeeping important for the anticipation of daily variations in environmental conditions (*3*). In mammals, the circadian clock driving metabolic and behavioral rhythms is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (*4*). Mammals and other vertebrates also have an autonomous circadian clock in each retina (*5*) driving rhythms in local physiology that are likely to anticipate the transitions between daytime and nighttime viewing conditions.

The starting point for a molecular analysis of the mammalian circadian mechanism was the identification of a mouse mutant, *Clock*, which has a phenotype affecting both the periodicity and persistence of circadian rhythms (*6*). CLOCK, the predicted protein product of the mutated gene (*7, 8*), is a member of the bHLH-PAS family, some members of which are known to function as transcription factors. The mutant *Clock* allele acts genetically in a dominant-negative fashion (*7, 9*) and encodes a protein with a 51–amino acid deletion in its putative transcriptional regulatory domain (CLOCK-Δ19). How CLOCK controls the periodicity and persistence of circadian rhythms is unknown.

Although not formally demonstrated to encode circadian clock components, three mammalian orthologs of the *Drosophila* clock gene *per*, *mper1* (*10*), *mper2* (*11*), and *mper3* (*12*), have been identified. All three are expressed in the SCN and retina, and, like *Drosophila per*, the levels of their transcripts exhibit a circadian oscillation. Fly and mammalian circadian clocks are thus likely to share a conserved molecular mechanism.

In *Drosophila*, the clock mechanism is constituted in part by a negative feedback loop in which the PER protein directly or indirectly represses transcription of its own gene (*13, 14*). Constitutive *per* mRNA expression has been observed in mutants lacking functional PER protein (*14, 15*), indicating that there is PER-independent positive regulation of *per* transcription. A 69–base pair (bp) "clock control region" located upstream of the *per* gene confers circadian cycling on reporter genes that is dependent on a functional PER protein (*16*). The 69-bp clock control region thus includes sequences sufficient for both PER-dependent negative feedback and PER-independent positive transcriptional regulation. Within this sequence, an E-box element (CACGTG), a binding site for certain transcription factors, is required for the positive component of the transcriptional regulation (*16*).

Precedents for heterodimerization between bHLH-PAS proteins have suggested

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that CLOCK could dimerize with a bHLH-PAS partner to regulate circadian rhythms. Because E-box elements are recognition sites for bHLH DNA-binding domains (*17*), we hypothesized that CLOCK and its predicted partner might drive expression of one or more *per* genes in mammals and that homologous proteins might do the same in *Drosophila*.

Screen for CLOCK-interacting proteins. To identify CLOCK-interacting proteins (CIPs), including the predicted bHLH-PAS partner of CLOCK, we performed a yeast two-hybrid screen (*18*) using a LEXA-CLOCK hybrid as bait (*19*). In our initial control experiments, CLOCK showed clear evidence of interaction with ARNT, a bHLH-PAS protein known to heterodimerize widely (*20*), but no evidence of CLOCK homodimerization was detected (Fig. 1A), implying that CLOCK acts in vivo with a heterodimeric partner. We then carried out a two-hybrid screen of a cDNA library constructed from hamster hypothalamus (*19*). Of 2×10^7 yeast transformants, 154 formed colonies positive for both *HIS3* and *LacZ* reporter genes. After accounting for multiple isolations, we ultimately identified 22 distinct CIPs that gave two-hybrid signals specific for CLOCK, and two proved to be bHLH-PAS proteins (Fig. 1B). One, isolated five times and giving an interaction signal stronger than the ARNT positive control, was BMAL1, a protein of unknown function expressed in brain and muscle (*21*); the other, isolated once and giving a weaker interaction signal, was ARNT2 (*22*), also of unknown function.

Expression at known circadian clock sites. Next we examined *arnt*, *arnt2*, and *bmal1* transcripts for coexpression with *Clock* and *mper1* transcripts in the mouse brain by in situ hybridization (*23*). Of the three potential CLOCK partners, only *bmal1* transcripts showed a clear pattern of expression like *Clock* (*7*) and *mper1* transcripts (*10*) in coronal brain sections (*24*), including robust expression in the SCN (Fig. 2A) and pars tuberalis (*25*). *bmal1* transcripts were coexpressed with both *Clock* and *mper1* transcripts throughout the rostrocaudal extent of the mouse SCN, although the hybridization signal with the *bmal1* riboprobe was somewhat lower than that with *Clock* or *mper1* (Fig. 2A).

We also tested for coexpression in retina, the other known site of an independent circadian clock in mammals. *Clock*, *bmal1*, and *mper1* transcripts showed coexpression in the mouse retina in a characteristic pattern (Fig. 2B), with strong expression in the inner nuclear layer (INL), the outer nuclear (photoreceptor) layer (ONL), and a discrete subset of cells in the ganglion cell layer (GCL). *bmal1* appeared to show somewhat higher expression in the ONL relative to the INL than did *Clock* and *mper1*. Only about 1 in 10 cells in the GCL showed a hybridization signal for the three riboprobes (*25*), and these hybridizing cells could correspond to a subset of ganglion cells or they could be displaced amacrine cells (*26*). Taken together, the coexpression of these transcripts in the SCN and retina strongly suggest that CLOCK, BMAL1, and mPER1 are colocalized within circadian clock cells.

Binding of CLOCK-BMAL1 heterodimer to *per* gene E-box. On the grounds that the circadian mechanism is likely to be conserved between *Drosophila* and mammals (*10–12*), we tested CLOCK-ARNT, CLOCK-ARNT2, and CLOCK-BMAL1 heterodimers for binding to the E-box element within the *Drosophila per* clock control region, known to be important for regulation of *per* gene expression. In effect, we asked the *Drosophila* E-box to select the heterodimer likely to be relevant to the mammalian circadian mechanism.

To test DNA binding, we performed yeast one-hybrid assays (*27*), in which the binding of test proteins to a given DNA fragment is signaled by activation of the *LacZ* gene, resulting in an increase in β-galactosidase (β-Gal) activity. The one-hy-

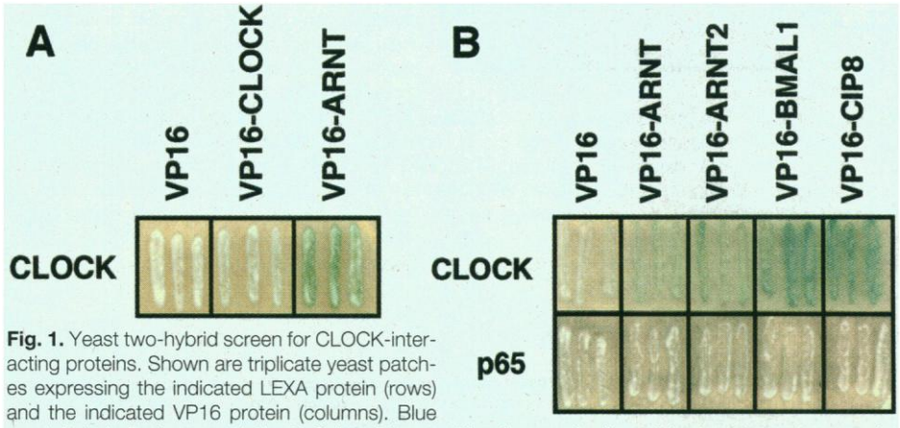

Fig. 1. Yeast two-hybrid screen for CLOCK-interacting proteins. Shown are triplicate yeast patches expressing the indicated LEXA protein (rows) and the indicated VP16 protein (columns). Blue precipitate indicates cumulative β-Gal activity, resulting from activation of the *LacZ* reporter gene by protein-protein interaction. Each triplicate represents three independent transformants. (**A**) Control two-hybrid assays showing interaction of LEXA-CLOCK with VP16-ARNT but not with VP16-CLOCK. Background is defined by the signal from LEXA-CLOCK with VP16. Yeast expressing the LEXA-CLOCK(1-580) bait were transformed with VP16 (negative control), VP16-CLOCK(1-389), or VP16-ARNT(70-474) expression plasmids. The latter two include a complete bHLH-PAS region; VP16-CLOCK(1-389) shows a robust interaction with LEXA-ARNT(70-474) (*25*). (**B**) Two-hybrid assays showing characterization of several CLOCK-interacting proteins obtained in the two-hybrid screen. Yeast expressing the LEXA-CLOCK(1-580) bait or the LEXA-p65 (synaptotagmin) control bait were transformed with the indicated VP16 expression plasmids. VP16 and VP16-ARNT plasmids are the negative and positive controls, respectively, for LEXA-CLOCK, as in (A). VP16-ARNT2, VP16-BMAL1, and VP16-CIP8 are library plasmids isolated from yeast colonies positive in the two-hybrid screen.

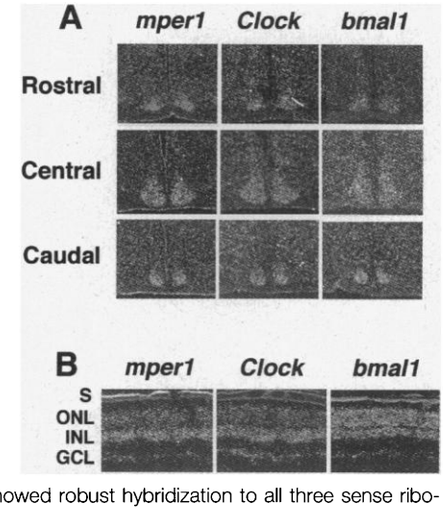

Fig. 2. Localization by in situ hybridization of *bmal1* transcripts as compared with *Clock* and *mper1* transcripts at the two known circadian clock sites in mammals. (**A**) Coexpression throughout the rostrocaudal extent of the mouse SCN. Sets of three neighboring coronal brain sections from a single mouse, each set taken from a different rostrocaudal level. Within each set, the three sections were hybridized to *mper1*, *Clock*, or *bmal1* antisense riboprobes, respectively, as indicated. Rostrocaudal level of each set within the SCN is indicated at left. Only faint background hybridization was observed with sense control riboprobes (*25*). (**B**) Coexpression in the mouse retina. Three neighboring parasaggital eye sections hybridized to *mper1*, *Clock*, or *bmal1* antisense riboprobes, respectively, as indicated. S, sclera; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Only faint background hybridization was observed with sense control riboprobes, except for the sclera, which showed robust hybridization to all three sense riboprobes (*25*).

brid strains we constructed are virtually identical to the two-hybrid strain used above in genotype and reporter gene, differing from the two-hybrid strain in the short DNA sequences inserted adjacent to the *LacZ* reporter gene (*27*).

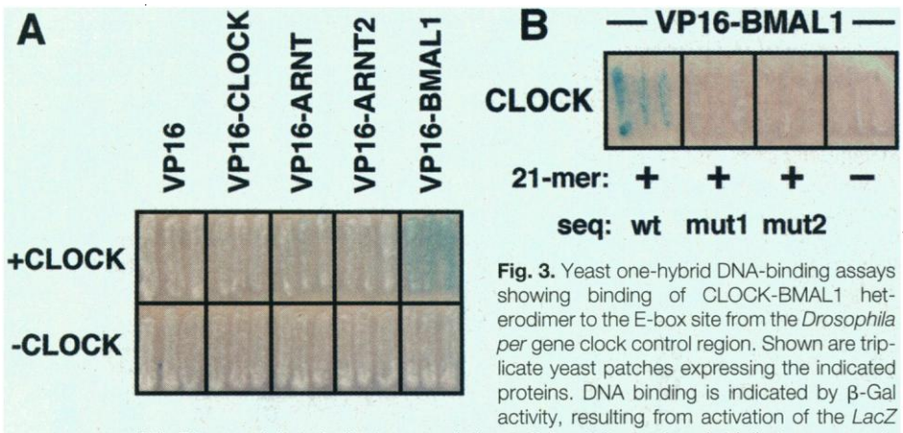

Fig. 3. Yeast one-hybrid DNA-binding assays showing binding of CLOCK-BMAL1 heterodimer to the E-box site from the *Drosophila per* gene clock control region. Shown are triplicate yeast patches expressing the indicated proteins. DNA binding is indicated by β-Gal activity, resulting from activation of the *LacZ* reporter gene (*27*). Blue precipitate indicates cumulative β-Gal activity, and each triplicate represents three independent transformants. (**A**) 69-bp clock control region. Reporter yeast transformed with full-length *Clock* cDNA expression plasmid (+CLOCK) or nonrecombinant expression plasmid (−CLOCK) in combination with the indicated VP16 plasmid. (**B**) Full-length CLOCK and VP16-BMAL1 expressed in four reporter strains. +, reporter gene includes 21-bp sequence. −, reporter gene lacking 21-bp sequence. wt, 21-bp sequence, including E-box, from clock control region. mut1 and mut2, same 21-bp sequence, but with E-box sequence scrambled two different ways (*27*).

CLOCK and BMAL1 together exhibited robust DNA binding to fragments containing the E-box but not to identical fragments with mutated E-box sequences (Fig. 3, A and B). In contrast, CLOCK, ARNT, ARNT2, or BMAL1 alone did not detectably bind to the *per* clock control region, nor did CLOCK and ARNT together or CLOCK and ARNT2 together (Fig. 3A). Failure to detect a signal in the one-hybrid assay could reflect poor binding of the bHLH-PAS dimers to the DNA sequence, poor dimerization (only the dimers bind DNA), or both. Detection of CLOCK-ARNT and CLOCK-ARNT2 heterodimerization in two-hybrid assays with the same reporter gene and the identical expression plasmids (Fig. 1) argues that poor binding of the heterodimers to this particular E-box is the most likely explanation.

Of the candidates tested, only CLOCK and BMAL1 formed heterodimers that robustly bound to the E-box sequence known to mediate positive regulation of the *Drosophila per* gene. In conjunction with the coexpression of *bmal1*, *Clock*, and *mper1* transcripts in the SCN and retina (Fig. 2), these results implicate CLOCK-BMAL1 heterodimers in circadian clock function and suggest that CLOCK-BMAL1 heterodimers drive mammalian *per* gene expression.

Cloning and analysis of *mper1* 5′ flanking region. To test the hypothesis that CLOCK-BMAL1 heterodimers drive expression of one or more mammalian *per* genes, we isolated overlapping genomic clones encompassing ~35 kilobases (kb) from *mper1* and its 5′ flanking region (*28*) on the hypothesis that one or more E-box elements identical to that of the *Drosophila per* clock control region would be found near the transcription start site. Restriction mapping and sequencing indicated that one clone included the 5′ untranslated region, a putative transcription start site, and 3.2 kb of predicted 5′ flanking sequence (*28*). The complete double-stranded sequence of this 3.2-kb region revealed three E-box sites within 1.2 kb of the putative transcription start site, all identical to that from the *Drosophila per* clock control region (Fig. 4A).

Transactivation by CLOCK-BMAL1 heterodimers from *mper1* E-boxes. Our hypothesis that CLOCK-BMAL1 heterodimers drive *per* gene expression requires that they function to activate rather than suppress transcription, suppressor activity having been documented in the bHLH-PAS family (*29*). To test this prediction, we cloned a 2.0-kb fragment of the *mper1* 5′ flanking region that includes all three E-boxes (*30*) for use in luciferase reporter gene assays in mammalian cells (*31*).

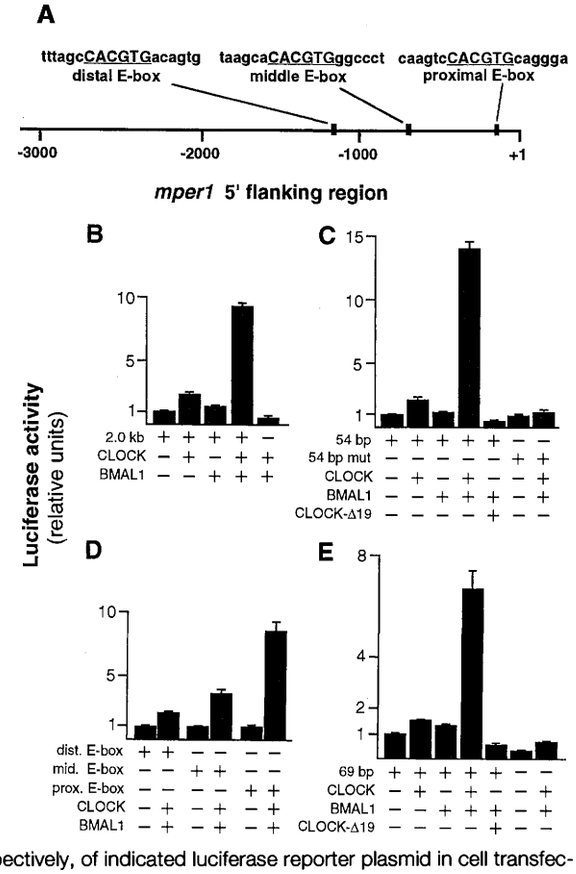

Fig. 4. Analysis of *mper 1* gene 5′ flanking region. (**A**) Location of E-box sites within 5′ flanking region. Numbered axis represents distance in base pairs from the putative transcription start site, marked as +1 (*28*). Filled boxes represent the locations of the three E-boxes; the sequence of each E-box with 6 bp of flanking sequence (lowercase letters) on each side is shown at the top. (**B** to **E**) Transactivation from E-box sites by CLOCK-BMAL1 heterodimer. Transcriptional activation in mammalian cells of luciferase reporter from sequences derived from the 5′ flanking region of the *mper1* gene (B to D) or from the *Drosophila per* gene (E). (B) 2.0-kb fragment including all three E-boxes. (C) 54-bp fragment consisting of the three E-boxes and their immediate flanking sequences linked together (A). 54-bp mut, 54-bp fragment in which all three É-boxes were scrambled (*30*). (D) Each individual E-box with immediate flanking sequences, labeled as in (A). dist., distal; mid., middle; prox., proximal. (E) 69-bp *Drosophila per* clock control region. (B and E) + or −, presence or absence, respectively, of indicated insert in luciferase reporter plasmid. (C and D) + or −, presence or absence, respectively, of indicated luciferase reporter plasmid in cell transfection. (B to E) Expression plasmid with (+) or without (−) the indicated CLOCK, BMAL1, and CLOCK-Δ19 full-length cDNA inserts. Shown are the mean are SEM of ≥6 independent experiments (C and E) or of ≥3 independent experiments (B and D). Some of the standard error bars are too small to be seen at this scale.

CLOCK and BMAL1 together, but not either alone, produced a substantial increase in transcriptional activity (9.3-fold; $P < 0.005$) (Fig. 4B), indicating that recognition sites for the CLOCK-BMAL1 heterodimer are contained within the *mper1* 5′ flanking region. We conclude that CLOCK acts as a transcriptional activator.

To determine if one or more of the *mper1* E-boxes were sites of action of CLOCK-BMAL1 heterodimers, we examined CLOCK and BMAL1 transcriptional activity from a 54-mer in which the three *mper1* E-boxes and their immediate flanking sequences (Fig. 4A) were linked together (*30*). CLOCK alone produced a small increase in transcriptional activity (~twofold), BMAL1 alone produced only a neglible increase in activity, but CLOCK and BMAL1 together produced a substantial transcriptional activation (14.1-fold, $P < 0.0005$) (Fig. 4C). No transcriptional activity of CLOCK and BMAL1 together was detected with a 54-mer in which the three E-box sequences were mutated (*30*) (Fig. 4C), indicating that transcriptional activation by CLOCK-BMAL1 heterodimers requires at least one of the E-box sites.

We next tested each of the three *mper1* E-box sites independently (*30*). CLOCK and BMAL1 together produced a small increase in transcriptional activity from the distal E-box (2.1-fold), a moderate increase from the middle E-box (3.6-fold), and a substantial increase from the proximal E-box (8.5-fold) (Fig. 4D). Thus, all three E-boxes are potential sites of regulation of *mper1* by CLOCK-BMAL1 heterodimers. Together with the coexpression of *Clock*, *bmal1*, and *mper1* transcripts in the SCN and retina, these results strongly suggest that CLOCK-BMAL1 heterodimers drive *mper1* gene expression.

A similar set of results for CLOCK and BMAL1 was obtained with the 69-bp *Drosophila per* clock control region as the DNA target site (Fig. 4E). Thus, *per* gene regulatory mechanisms have been conserved between *Drosophila* and mammals, and a homologous heterodimer is likely to exist in *Drosophila*. We have identified a *Drosophila* homolog of BMAL1 (dBMAL1), and experiments addressing its function in conjunction with a *Drosophila* CLOCK homolog (dCLOCK) are reported separately (*32*).

CLOCK-Δ19 and BMAL1 together showed no transactivation activity from *mper1* E-boxes (Fig. 4C), the amount of activity being significantly below that of BMAL1 alone ($P < 0.0005$). A similar result was observed with CLOCK-Δ19 and BMAL1 together from the *Drosophila per* E-box (Fig. 4E). We conclude that CLOCK-Δ19 is defective in transcriptional activation activity.

Mechanism of the *Clock* dominant-negative mutation. The *Clock* mutant allele acts as a dominant negative in mice (*7*, *9*), and CLOCK-Δ19 and BMAL1 together showed no transcriptional activation from *mper1* E-box elements (Fig. 4C). To determine the mechanism of the dominant-negative *Clock* mutation, we further characterized CLOCK-Δ19 with regard to heterodimerization and DNA binding. In two-hybrid assays, CLOCK-Δ19 showed interactions with ARNT, ARNT2, and BMAL1 that were similar in signal strength and identical in rank order to those shown by wild-type CLOCK (Fig. 5A; compare with Fig. 1B). Thus, CLOCK-Δ19 heterodimerizes, apparently normally, with BMAL1 and other bHLH-PAS proteins. CLOCK-Δ19 is not, however, equivalent to the wild type in regard to all protein-protein interactions, because several CIPs we isolated apparently interacted only with wild-type CLOCK (for example, CIP8 in Fig. 5A; compare with Fig. 1B).

To assess DNA-binding activity, we performed one-hybrid assays using a yeast reporter strain carrying the *per* clock control region. When CLOCK or CLOCK-Δ19 was paired with BMAL1, robust DNA-binding activity was detected, but it was not detected when either was paired with negative controls (Fig. 5B). Thus, the E-box–binding activity of the CLOCK-Δ19–BMAL1 heterodimer is intact. Together these studies indicate that CLOCK-Δ19 acts genetically in a dominant-negative fashion because it forms a DNA-binding heterodimer, its partner thereby sequestered in a protein-DNA complex deficient in transactivation activity. We conclude that the abnormal circadian rhythms of *Clock* mutant mice are caused by this defect, which we predict would lead to decreased transcription of *mper1* and possibly other circadian clock genes. Strong support for this conclusion comes from the observation that in *Clock* mutant mice the peak levels of *mper1* transcript in the SCN are significantly reduced, compared with those of wild-type mice (*33*).

Role of CLOCK and BMAL1 in circadian clock mechanism. We propose that CLOCK-BMAL1 heterodimers directly activate transcription of *mper1* and possibly other circadian clock genes. As in *Drosophila*, it is likely that the mammalian clock mechanism is constituted in part by the direct or indirect repression of *per* genes by PER proteins. Central to the mechanism of circadian oscillations would therefore be the establishment of an alternating regime of *per* gene activation by CLOCK-BMAL1 heterodimers and PER-dependent inhibition of this activation, responsible in turn for the rising and falling phases of the circadian oscillation in the levels of *per* transcripts. In the fungus *Neurospora*, a similar positive role in a circadian transcriptional feedback loop has been proposed for the WC-1 and WC-2 proteins, which are required for normal expression of the circadian clock gene *frequency* (*frq*) and consequently for the circadian oscillation of *frq* expression (*34*). Although shown genetically to be required for this regulation, it is not yet known if WC-1 and WC-2 act directly as transactivators of *frq* expression.

How might PER-dependent negative feedback inhibit or override *per* gene transcriptional activation by the CLOCK-BMAL1 heterodimer? One possibility would be PER-dependent sequestration of CLOCK, BMAL1, or both, leading to a loss of CLOCK-BMAL1 DNA-binding activity, similar to the mechanism by which the Id protein inhibits myogenic differentiation (*35*). Other possibilities would include a PER-dependent repressor that binds either

Fig. 5. Properties of CLOCK-Δ19. (**A**) Two-hybrid assay showing interaction of CLOCK-Δ19 with bHLH-PAS proteins but not with CIP8. Arranged as in Fig. 1. (**B**) One-hybrid assay showing binding of CLOCK-Δ19–BMAL1 heterodimer to E-box site. Arranged as in Fig. 3A. The three proteins indicated at left were LEXA hybrids (*18*) in this experiment, but the LEXA domain is not relevant to the results in the one-hybrid reporter strain, and it does not affect the function of CLOCK in this assay.

to the E-box or to a nearby site within the *per* gene upstream region. It is not known if PER (including any other associated proteins) acts directly to achieve negative feedback, but the observation that coexpression of *Drosophila* PER and its partner TIM is sufficient to inhibit dCLOCK-dependent *per* gene activation in cultured cells suggests a direct mechanism (*32*).

We have identified BMAL1 as a probable component of the circadian clock, defined a biochemical function for CLOCK, and implicated a mammalian *per* homolog as a target gene regulated by CLOCK-BMAL1 heterodimers. These findings place CLOCK in a specific role within the circadian oscillator, and they mechanistically tie together CLOCK, a genetically demonstrated component of the circadian clock, and a mammalian *per* homolog, likely to be a component of the clock. How the circadian transcriptional feedback loop is generated and regulated will provide the basis for understanding how biological clocks evolved, how they operate, and how they control behavioral and physiological programs.

Note added in proof: CLOCK-BMAL1 (MOP3) heterodimerization and transactivation from an E-box site have been reported independently (*36*).

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19. We used LEXA-CLOCK(1-580) as bait, which is lacking most of the COOH-terminal region predicted to be a transcriptional activation domain (*7*) but includes the region deleted from CLOCK-Δ19. The two-hybrid screen was carried out as described (*18*), except library transformants were plated onto X-Gal plates lacking tryptophan, histidine, uracil, lysine, and leucine (*18*) that were supplemented with 30 mM or 50 mM 3-aminotriazole. The cDNA library was generated as follows: 32 male Syrian hamsters (Charles River Laboratories) were maintained on a 14:10 light:dark cycle for ≥3 weeks, transferred to constant dim light (<1 lux) at the time of lights off, and killed by decapitation 24 to 42 hours later, eight at each of four circadian times (CT 1, 7, 13, and 19). Brains were removed and placed in phosphate-buffered saline (4°C) for 30 s, a 1.5-mm coronal slice was cut, and a pyramid of anterior hypothalamus containing the SCNs was dissected out, frozen on dry ice, and stored at −70°C. Purification of mRNA and construction of a randomly primed cDNA library in pVP16 were performed as described (*18*). Care of hamsters and all procedures were in full compliance with institutional guidelines for animal experimentation.
20. J. B. Hoganesch *et al.*, *J. Biol. Chem.* **272**, 8581 (1997).
21. The 2.4-kb clone included a complete coding sequence corresponding to the human BMAL1b isoform [M. Ikeda and M. Nomura, *Biochem. Biophys. Res. Commun.* **233**, 258 (1997)]. BMAL1b is the same as JAP3 (Genbank accession U60415), and it is likely derived from the same gene as the protein MOP3 (*20*), the two being identical except for completely divergent NH_2-terminal regions of 59 and 57 amino acids, respectively. All constructs used in this work corresponded to BMAL1b, but because the existence of the different BMAL1 proteins has not been established, we refer here to the protein generically as BMAL1.
22. The 1.7-kb clone corresponded to codons 1 to 518 of mouse ARNT2 [K. Hirose *et al.*, *Mol. Cell. Biol.* **16**, 1706 (1996)] preceded by 6 bp of apparent 5′ untranslated sequence.
23. Preparation of coronal brain sections from mice (<1 lux; killed at CT 7 or CT 19) and in situ hybridizations were performed as described [M. E. Morris, N. Viswanathan, S. Kuhlman, F. C. Davis, C. J. Weitz, *Science* **279**, 1544 (1998)]. Eyes were removed from perfused mice, the anterior segments were removed by dissection and discarded, and the resulting eye cups were postfixed and stored as described for brains. Parasaggital sections (12-μm) were cut on a cryostat, and in situ hybridizations were performed as for brain sections.
24. With a mouse *arnt* riboprobe (codons 70 to 474), we observed weak or no hybridization to SCN, but hybridization to periventricular nuclei, piriform cortex, subcortical nuclei, and cerebral cortex was consistent. With a hamster *arnt2* riboprobe (6 nucleotides of 5′ untranslated plus codons 1 to 518), we observed very strong hybridization throughout the brain, especially in cerebral cortex, subcortical nuclei, and hypothalamus, likely including the SCN, although it was difficult to distinguish SCN in the context of strong signals from many hypothalamic nuclei. Although they do not show a *Clock*- and *mper1*-like pattern of expression, it is possible that *arnt* and *arnt2* transcripts are localized in the SCN, and we cannot exclude a possible role for either in the circadian clock. Riboprobes were synthesized from polymerase chain reaction (PCR) products that incorporated T3 (for sense strand) and T7 (for antisense strand) RNA polymerase-binding sites. The mouse *Clock* riboprobe corresponded to codons 1 to 389, the mouse *per1* riboprobe corresponded to codons 738 to 835, and the hamster *bmal1* riboprobe corresponded to full-length BMAL1b. No obvious oscillation in the level of *bmal1* transcripts was observed when comparing circadian times 7 and 19.
25. N. Gekakis *et al.*, unpublished data.
26. H. Wassle and B. B. Boycott, *Physiol. Rev.* **71**, 447 (1991).
27. The indicated DNA fragments were ligated into the pBgl-lacZ reporter plasmid [J. J. Li and I. Herskowitz, *Science* **262**, 1870 (1993)], and one-hybrid yeast strains were constructed by integration of recombinant pBgl-lacZ reporter plasmids into the *ura3* locus of YPH 499 [R. S. Sikorsky and P. Hieter, *Genetics* **122**, 19 (1989)]. Negative control strains were constructed identically with nonrecombinant pBgl-lacZ. One-hybrid strains were transformed with the indicated p424-Met25 [D. Mumberg, R. Muller, M. Funk, *Nucleic Acids Res.* **22**, 5767 (1994)] and pVP16 (*18*) expression plasmids, and transformants were patched onto X-Gal plates (*18*) for detection of β-Gal activity. We validated the one-hybrid system by comparing results in electrophoretic mobility shift assays with one-hybrid assays using TFEB, a bHLH protein known to bind the CACGTG E-box as a homodimer [D. E. Fisher, C. S. Carr, L. A. Parent, P. A. Sharp, *Genes Dev.* **5**, 2342 (1991)]. The *Drosophila per* gene 21-mer was derived from the 69-bp clock control region (*16*); it contained the E-box with 8 bp of 5′ and 7 bp of 3′ flanking sequences, respectively. Mutated E-boxes within the 21-mer were generated by scrambling the E-box sequence by means of a random number program: E-box mutant 1, 5′-ATTCGC; E-box mutant 2, 5′-GTAACT.
28. 8×10^5 clones from a mouse genomic lambda phage library (Strategene) were screened with an *mper1* cDNA probe (codons 17 to 84), and 1×10^6 clones were screened with a 200-bp probe derived from a 1.7-kb genomic fragment containing *mper1* 5′ untranslated sequences and an enclosed 1.5-kb intron. Altogether, eight overlapping clones, spanning ~35 kb of sequence, were obtained. Single-stranded sequence of 6.4 kb from one clone revealed part of the *mper1* coding region, including the initiator methionine codon, the 5′ untranslated region and its intron, and a presumptive transcription start site that is consistent with the size and structure of the *mper1* transcript (TSSG program; score, 23.14; threshold, 4.00) [V. Solovyev, A. Salamov, *Intelligent Syst. Mol. Biol.* **5**, 294 (1997)]. On the basis of this putative transcription start site, the clone included 3.2 kb of 5′ flanking sequence, from which the complete double-stranded sequence was obtained. This assignment for the transcription start site has not been confirmed experimentally.
29. P. Moffett, M. Reece, J. Pelletier, *Mol. Cell. Biol.* **17**, 4933 (1997).
30. The 2.0-kb *mper1* upstream fragment (−2122 bp to −129 bp with respect to the putative transcription start site) was generated by PCR using *pfu* and *taq* polymerases together on a cloned lambda phage template (primers: 5′-CATCCGCTCGAGCTCTTTGGTACCTGGCCAGCAACC and 5′-CATCCGCTCGAGACTGAGGTCAGGGCTGTGTCACAC; both primers included Xho I sites added at 5′ ends). Transfections were performed with a pool of 18 independent recombinants derived from different PCRs. The 54-mer derived from the mouse *per1* gene 5′ flanking region consisted of the three 18-bp sequences shown in Fig. 4A; they were linked together in the order shown from 5′ to 3′. The E-box mutant 54-mer was the same except that it had each E-box site independently scrambled by means of a random number program: 5′-GCTAGT (distal), 5′-TTTAGT (middle), and 5′-TCGCTC (proximal). Individual E-box constructs consisted of the 18-bp sequences shown in Fig. 4A.
31. Mouse NIH-3T3 cells were transfected with Lipofectamine-Plus (GibcoBRL). Cells were grown in Dulbecco's minimum essential medium with 10% fetal bovine serum (GibcoBRL) in six-well plates, and cells in each well were transfected with 1 μg (total) of the pcDNA3 expression plasmids (Invitrogen) with the indicated inserts, 10 ng of the pGL3 firefly luciferase reporter plasmid containing the minimal SV40 promoter (Promega) and the indicated inserts, and 0.5 ng of pRL-CMV (*Renilla*) luciferase internal control plasmid (Promega). After 48 hours, cells in each well were extracted with 200 μl of Promega passive lysis buffer, and separate portions of extract were taken for assays of firefly luciferase [A. R. Brasier, J. E. Tate, J. F. Habener, *Biotechniques* **7**, 1117 (1989)] and *Renilla* luciferase [S. Inouye and O. Shinomura, *Biochem. Biophys. Res. Commun.* **233**, 349 (1997)] by luminometry. For each sample, luciferase activity was corrected for transfection efficiency by dividing the measured firefly luciferase activity (from the reporter construct) by the measured *Renilla* luciferase activity (from the constitutively driven construct). A full-length *Clock* cDNA was constructed by fusion at

a Pst I site of clones YZ50 and D1 (7). *Clock*-Δ*19* cDNA was generated by subcloning of full-length *Clock* cDNA into pGEX-4-T3 (Pharmacia Biotech) for oligonucleotide-directed deletion of exon 19 with the U.S.E. Mutagenesis kit (Pharmacia Biotech).
32. T. K. Darlington *et al.*, *Science* **280**, 1599 (1998).
33. M. H. Vitaterna *et al.*, unpublished data.
34. S. K. Crosthwaite, J. C. Dunlap, J. J. Loros, *Science* **276**, 763 (1997).
35. R. Benezra *et al.*, *Cell* **61**, 49 (1990).
36. J. B. Hoganesch, Y.-Z. Gu, S. Jain, C. A. Bradfield, *Proc. Natl. Acad. Sci. USA.* **95**, 5744 (1998).
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