REPEATS program [G. Benson, M. S. Waterman, *Nucleic Acids Res.* **22**, 4828 (1994)]. The minimum length of homopolymeric tracts was set at eight for A and T and at six for G and C; four tandem copies of di- and trinucleotides; and three copies of tetra-, penta-, and hexanucleotides. Heptanucleotides and above were not found in three or more copies, except for the imperfect repeats in SP1772. The ratio of the observed frequency of homopolymeric tracts to their expected frequency was determined by means of Markov chain analysis, as described [N. J. Saunders *et al.*, *Mol. Microbiol.* **37**, 207 (2000)]. It revealed that G or C tracts of 8 bp and A or T tracts of 10 and 11 bp are slightly overrepresented.

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34. The NH_2-terminal regions of all proteins predicted to have signal sequences were collected for clustering and alignment with ClustalW and were scrutinized. A HMM based on an edited alignment of 40-residue segments around the (Y/F)SIRK motif found several hundred hits to a nonredundant amino acid database. A more general motif, based on the larger family of YSIRK proteins, is (Y/F)(S/A)(I/L)(R/K)(R/K)xxxGxxS (*35*).
35. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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38. This method is used to identify genomic differences between the TIGR4 strain and strains R6 and D39. All the predicted genes from the TIGR4 strain were amplified by PCR and arrayed on glass microscope slides as previously described [S. Peterson, R. T. Cline, H. Tettelin, V. Sharov, D. A. Morrison, *J. Bacteriol.* **182**, 6192 (2000)]. Genomic DNA for comparative genome hybridization studies was labeled according to protocols provided by J. DeRisi (www.microarrays.org/pdfs/GenomicDNALabel_B.pdf), except that genomic DNA was not digested or sheared before labeling. Arrays were scanned with a GenePix 4000B scanner from Axon (Union City, CA), and individual hybridization signals were quantitated with TIGR SPOTFINDER [P. Hegde *et al.*, *Biotechniques* **29**, 548 (2000)].
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43. We thank M. Heaney, J. Scott, M. Holmes, V. Sapiro, B. Lee, and B. Vincent for software and database support at TIGR; M. Ermolaeva and M. Pertea for specific computer analyses; the TIGR faculty and sequencing core for expert advice and assistance; I. Aaberge (National Institute of Public Health, Oslo, Norway) for providing the initial clinical isolate labeled JNR.7/87; and G. Zysk and A. Polissi for sharing specific sequence data not deposited in GenBank. Supported in part by the National Institutes of Allergy and Infectious Diseases (grant R01 AI40645-01A1) and the Merck Genome Research Institute (grant MGRI72).

2 April 2001; accepted 4 June 2001

NPAS2: An Analog of Clock Operative in the Mammalian Forebrain

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Neuronal PAS domain protein 2 (NPAS2) is a transcription factor expressed primarily in the mammalian forebrain. NPAS2 is highly related in primary amino acid sequence to Clock, a transcription factor expressed in the suprachiasmatic nucleus that heterodimerizes with BMAL1 and regulates circadian rhythm. To investigate the biological role of NPAS2, we prepared a neuroblastoma cell line capable of conditional induction of the NPAS2:BMAL1 heterodimer and identified putative target genes by representational difference analysis, DNA microarrays, and Northern blotting. Coinduction of NPAS2 and BMAL1 activated transcription of the endogenous *Per1*, *Per2*, and *Cry1* genes, which encode negatively activating components of the circadian regulatory apparatus, and repressed transcription of the endogenous *BMAL1* gene. Analysis of the frontal cortex of wild-type mice kept in a 24-hour light-dark cycle revealed that *Per1*, *Per2*, and *Cry1* mRNA levels were elevated during darkness and reduced during light, whereas *BMAL1* mRNA displayed the opposite pattern. In situ hybridization assays of mice kept in constant darkness revealed that *Per2* mRNA abundance did not oscillate as a function of the circadian cycle in NPAS2-deficient mice. Thus, NPAS2 likely functions as part of a molecular clock operative in the mammalian forebrain.

Locomotor activity, body temperature, endocrine hormones, and metabolic rate fluctuate cyclically with a period of 24 hours. The regulatory apparatus that controls circadian rhythm consists of a transcriptional feedback cycle that is evolutionarily conserved in a wide variety of metazoans (*1*). In mammals, the activating arm of this cycle is executed by a heterodimeric transcription factor composed of the *Clock* and *BMAL1* gene products (*2*). The Clock:BMAL1 heterodimer binds directly to regulatory sequences of the genes comprising the negative arm of the transcriptional feedback cycle. The negative components of the regulatory apparatus include three period (*Per*) genes and two cryptochrome (*Cry*) genes (*3–11*), whose products function in a poorly understood manner to inactivate the Clock:BMAL1 heterodimer. The duration of Per and Cry activity may be modified by a serine-threonine kinase variously termed casein kinase Iε or Tau in mammals and Doubletime in flies (*12–14*). In the absence of entraining influences, this regulatory apparatus oscillates rhythmically at or near the 24-hour light-dark cycle (i.e., 12 hours light, 12 hours dark). Entrainment derived from light, food, temperature, and metabolic activity can advance or delay the central regulatory apparatus such that it is properly adapted to the summation of these external zeitgebers.

The master pacemaker of circadian rhythm resides in the suprachiasmatic nucleus (SCN), a small group of neurons located at the base of the optic chiasma within the central nervous system (*15*). Classical transplantation experiments have demonstrated that the SCN is necessary and sufficient to specify circadian rhythm (*16, 17*). Surprisingly, the same molecular clock is operative in sites peripheral to the SCN (*11, 18*), including cultured mammalian cells of non-neural origin (*19*).

Neuronal PAS domain protein 2 (NPAS2, also termed MOP4) is a member of the basic helix-loop-helix (bHLH)–PAS domain family of transcription factors. The gene encoding NPAS2 is expressed in a stereotypic pattern of brain nuclei located within the mammalian forebrain (*20, 21*). Upon positional cloning of

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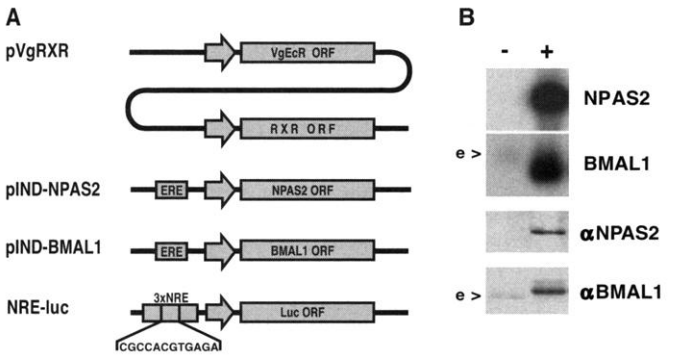

Fig. 1. Conditional expression of NPAS2 and BMAL1 in neuroblastoma cells. (**A**) SHEP neuroblastoma cells were cotransfected (*30*) with ecdysone-inducible expression vectors (pIND) encoding open reading frames for *NPAS2* and *BMAL1*, along with an expression vector (VgRXR) encoding the human retinoid X receptor (RXR) and a modified *Drosophila melanogaster* ecdysone (VgEcR) receptor (*25*). Stable integration of *NPAS2*, *BMAL1*, and *VgRXR* expression vectors was achieved by selecting cells for neomycin, hygromycin, and zeocin resistance, respectively, during two cycles of transfection and selection. A luciferase reporter gene driven by three NPAS2:BMAL1 response elements and a minimal adenovirus E1B promoter (*20*) was cotransfected and selected in the second cycle of DNA transfer. (**B**) Upper panel: Transfected neuroblastoma cells were exposed to ponasterone for 4 hours; mRNA was prepared from vehicle- and ponasterone-treated cells and assayed on Northern blots using probes derived from the *NPAS2* and *BMAL1* genes (*31*). Both *NPAS2* and *BMAL1* mRNA levels were substantially increased by ponasterone treatment. (e) designates the position of a *BMAL1*-positive hybridization signal observed in vehicle-treated cells that may correspond to endogenous *BMAL1* mRNA. Lower panel: Protein lysates were prepared from vehicle- and ponasterone-treated cells and assayed on Western blots using antibodies specific to NPAS2 and BMAL1 (*32*). Both NPAS2 and BMAL1 protein levels were substantially increased by ponasterone treatment. (e) designates the position of a BMAL1-reactive protein observed in vehicle-treated cells that may correspond to endogenous BMAL1.

the mouse *Clock* gene, Takahashi and colleagues noted that the Clock and NPAS2 polypeptides are unusually similar in primary amino acid sequence (*22*), raising the possibility that NPAS2 might function as a molecular clock in the mammalian forebrain.

To investigate this possibility, we studied cultured neuroblastoma cells programmed to conditionally express human NPAS2 along with human BMAL1, its obligate heterodimeric partner (*2, 23*). We cloned *NPAS2* and *BMAL1* cDNAs downstream of an ecdysone-responsive promoter (Fig. 1A) and stably transfected the constructs into the human neuroblastoma cell line SHEP (*24*) together with an expression vector encoding an ecdysone-responsive nuclear hormone receptor (*25*). The cells were also transfected with a luciferase reporter gene driven by a promoter containing three copies of the optimal NPAS2:BMAL1 recognition site (*20, 23*). Exposure of the cells to ponasterone, a synthetic mimic of ecdysone, produced a substantial increase in NPAS2 and BMAL1 at both the mRNA and protein levels (Fig. 1B). In addition, luciferase enzyme activity was conditionally elevated in response to ponasterone, consistent with the interpretation that the induced NPAS2 and BMAL1 polypeptides function as an active, heterodimeric transcription factor (*26*).

To identify potential target genes of the NPAS2:BMAL1 transcription factor, we used mRNA prepared before and after exposure to ponasterone as reagents for representational difference analysis (RDA) and DNA microarrays (*27*). About 90 genes appeared to be activated and about 50 genes repressed in response to ponasterone-induced expression of NPAS2:BMAL1. Among these were several genes encoding known components of the circadian regulatory apparatus. The levels of endogenous *Per1*, *Per2*, and *Cry1* mRNA levels were elevated, whereas *BMAL1* mRNA appeared to be diminished upon conditional activation of NPAS2:BMAL1. [The endogenous and exogenous *BMAL1* transcripts could be distinguished because the oligonucleotide programmed onto the DNA microarray was fortuitously derived from the 3′ untranslated region (3′ UTR) of the human *BMAL1* gene, which was not present in the transfected *BMAL1* gene.]

Northern blot analysis (Fig. 2A) confirmed the microarray and RDA data. Treatment of the SHEP cells with ponasterone produced a rapid increase in the abundance of *Per1*, *Per2*, and *Cry1* mRNAs.

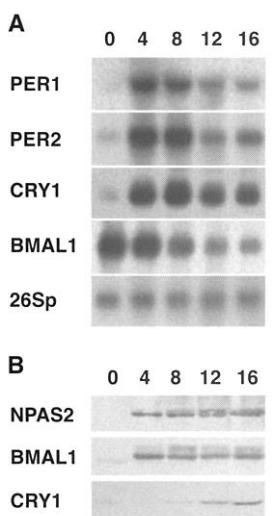

Fig. 2. Effect of conditional NPAS2:BMAL1 induction on *Per1*, *Per2*, *Cry1*, and *BMAL1* mRNA levels. (**A**) Neuroblastoma cells programmed to conditionally express NPAS2 and BMAL1 were exposed to ponasterone for 4, 8, 12, and 16 hours; mRNA was prepared from cells at each time point, as well as a vehicle-alone control (0), and these were used in Northern blot assays to monitor mRNA levels for *Per1*, *Per2*, *Cry1*, *BMAL1*, and a control mRNA corresponding to a subunit of the 26S proteosome (*31*). (**B**) Protein lysates were prepared from cells at the same post-induction time points and used in Western blot assays to monitor protein levels for exogenously expressed NPAS2 and BMAL1, as well as endogenously expressed Cry1 (*32*).

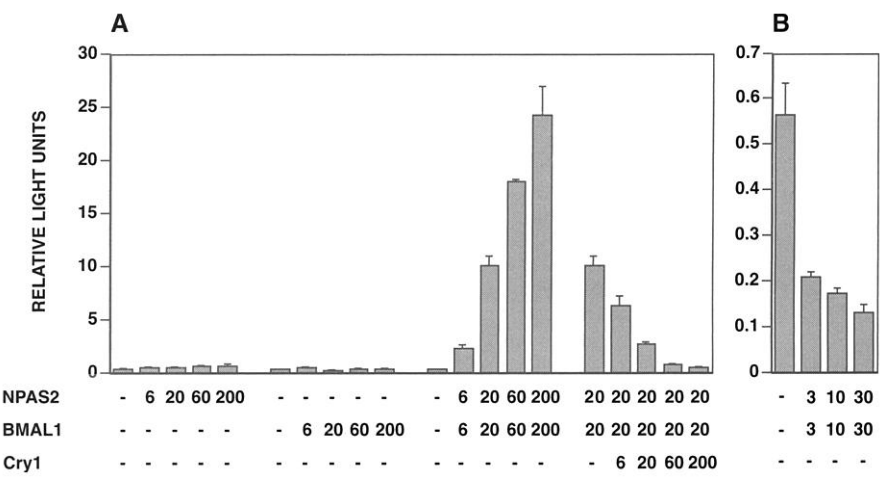

Fig. 3. Transient transfection assays monitoring the effects of NPAS2, BMAL1, and Cry1 on NRE-luc and BMAL1-luc reporters. (**A**) HEK293 cells were transfected with an invariant 20-ng dose of the NRE-luc reporter plasmid (*20*) along with varying amounts of the *NPAS2*, *BMAL1*, and *Cry1* expression vectors, as indicated. Cells were harvested 20 hours after transfection and assayed for luciferase enzyme activity (*30*). (**B**) HEK293 cells were transfected with an invariant 10-ng dose of the BMAL1-luc reporter plasmid (*28*) along with varying amounts of the *NPAS2* and *BMAL1* expression vectors, as indicated, and assayed for luciferase enzyme activity as in (A). Graphs shown are representative of at least three independent experiments.

The levels of *Per 1*, *Per2*, and *Cry1* mRNA remained high 8 hours after induction, but appeared to fall at the 12- and 16-hour time points. Unlike the rise in *Per1*, *Per2*, and *Cry1* mRNA accumulation observed in response to induction of NPAS2:BMAL1, expression of the endogenous *BMAL1* gene diminished in a time-dependent manner. The reduction in *BMAL1* mRNA levels was first observed 8 hours after ponasterone treatment, with further diminution apparent at the 12- and 16-hour time points.

We used Western blotting assays to monitor the effects of ponasterone treatment on expression of exogenous NPAS2 and BMAL1 proteins and of endogenous Cry1 protein (Fig. 2B). Interestingly, although NPAS2:BMAL1 rapidly induced *Cry1* mRNA levels, the increase in Cry1 protein levels was delayed by 4 to 8 hours. It was not until 12 hours after induction that elevated levels of Cry1 protein were observed. Notably, this 12-hour time point coincided with the decline in NPAS2:BMAL1–induced *Per1*, *Per2*, and *Cry1* gene expression. As discussed below, Cry1 is a potent inhibitor of the NPAS2:BMAL1 transcription factor. As such, it is possible that elevated levels of endogenously produced Cry1 are capable of antagonizing the ponasterone-induced NPAS2:BMAL1 heterodimer.

We next evaluated whether Cry1 might antagonize transcriptional activation by NPAS2:BMAL1. Human embryonic kidney (HEK) 293 cells were transiently transfected with a luciferase reporter plasmid driven by a promoter containing three optimal NPAS2:BMAL1 recognition sites (NRE-luc). Cotransfection of the NRE-luc promoter with expression vectors encoding either NPAS2 or BMAL1 alone did not increase luciferase enzyme activity. By contrast, substantive increases in luciferase activity were observed when both expression vectors were cotransfected with the NRE-luc reporter (Fig. 3A). Inclusion of the Cry1 expression vector, however, led to a potent, dose-dependent reduction in NPAS2:BMAL1–induced expression from the NRE-luc reporter (Fig. 3A, right). Similar results have been obtained with luciferase reporter constructs driven by a *Per1* promoter (*5*). Cry1 had no effect on control promoters from genes not involved in circadian rhythm (*26*); thus, Cry1 may be a specific inhibitor of the NPAS2:BMAL1 heterodimer.

To investigate the effect of NPAS2:BMAL1 on the BMAL1 promoter, we transiently transfected HEK293 cells with a BMAL1-luc reporter construct (*28*) along with graded increases of the NPAS2 and BMAL1 expression vectors. BMAL1-luc–driven luciferase activity diminished in a

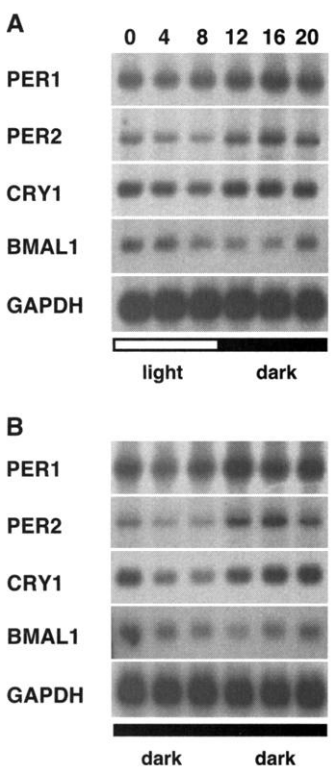

Fig. 4. Rhythmic fluctuations of *Per1*, *Per2*, *Cry1*, and *BMAL1* mRNA levels in the mouse forebrain. C57B6 mice were exposed to a 24-hour light-dark cycle (**A**) or kept in constant darkness (**B**) but otherwise housed under standard conditions. Four animals were killed at each 4-hour time point over a continuous 24-hour period. NPAS2-enriched forebrain tissue was dissected and used for preparation of poly(A)$^+$ mRNA (*33*). Northern blots were probed with radioactive cDNAs derived from the murine *Per1*, *Per2*, *Cry1*, *BMAL1*, and *GAPDH* genes (*31*).

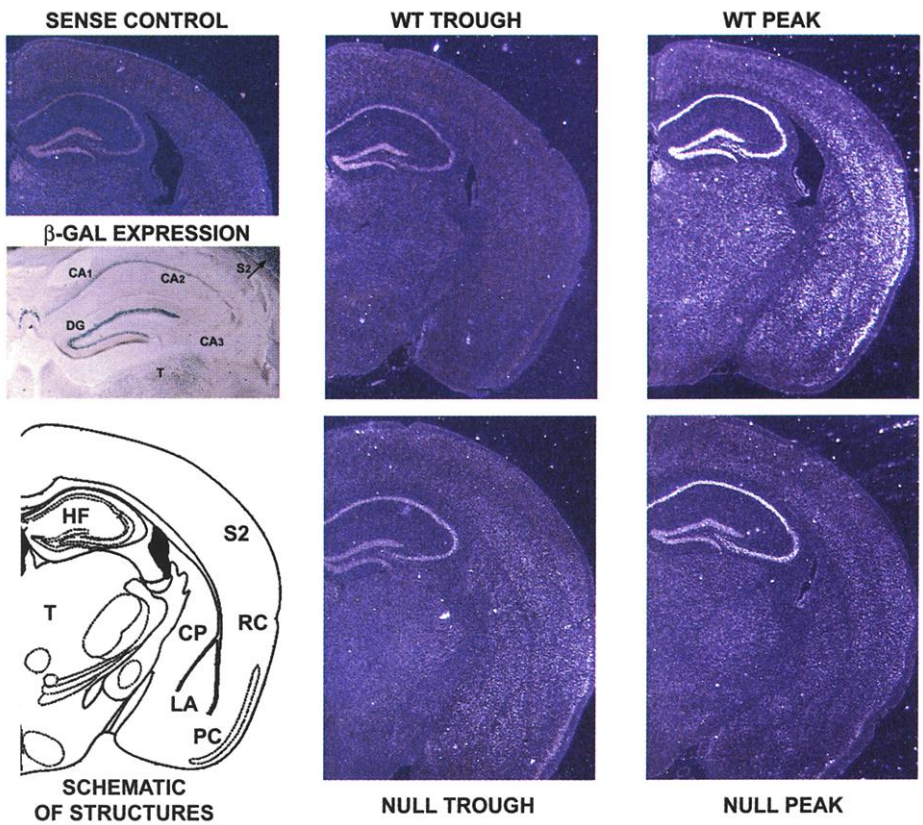

Fig. 5. In situ hybridization assays (*21*) of *Per2* gene expression in wild-type and NPAS2-deficient mice. Wild-type and NPAS2-deficient (null) mice (C57B6 strain) were housed individually in cages equipped with running wheels. Locomotor activity was monitored by recording each revolution of the wheel by computer. After 3 weeks of entrainment in a 24-hour light-dark cycle, animals were placed in conditions of constant darkness for 48 days. Mice were killed 8 hours before (trough) and 4 hours after (peak) the onset of wheel running. Brain tissue was fixed, sectioned, and subjected to in situ hybridization using sense (top left) and antisense (right four panels) probes derived from the murine *Per1* gene (*31*). Inset of upper left panel shows pattern of *NPAS2* gene expression deduced by β-galactosidase staining of material derived from an *NPAS2*:lacZ knock-in mouse (*20*). Coronal sections and schematic correspond to plate 44 of (*34*). Abbreviations: CP, caudate putamen; DG, dentate gyrus; HF, hippocampal formation; LA, lateral amygdala; PC, piriform cortex; RC, rhinal cortex; S2, secondary somatosensory cortex; T, thalamus. CA1, CA2, and CA3 refer to fields of the hippocampus. Relative to wild-type mice, peak levels of *Per2* hybridization signal are diminished in *NPAS2*-expressing regions of the brain section prepared from *NPAS2*-deficient mice (piriform cortex, somatosensory cortex, caudate putamen, dentate gyrus, etc.), but not in brain regions that do not express *NPAS2* (CA1, CA2, and CA3 components of the hippocampus).

dose-dependent manner upon exposure to NPAS2 and BMAL1 (Fig. 3B), precisely the opposite response relative to the effects of the heterodimer on the NRE-luc reporter. As was the case for transcriptional induction of NRE-luc, diminished BMAL1-luc expression was dependent on both NPAS2 and BMAL1 expression vectors (*26*).

To address the physiological relevance of these cell culture observations, we examined *Per1*, *Per2*, *Cry1*, and *BMAL1* mRNA levels as a function of the light-dark cycle in wild-type mice. Forebrain tissue samples were dissected from adult mice at 4-hour intervals, and polyadenylated [poly(A)$^+$] mRNA was evaluated by Northern blotting. *Per1*, *Per2*, and *Cry1* mRNA levels were elevated in the dark period and diminished in the light period, whereas *BMAL1* mRNA levels began to diminish at the light-dark transition and were at their lowest level at the 12- and 16-hour time points, when *Per1*, *Per2*, and *Cry1* levels peaked (Fig. 4A). Northern blots with poly(A)$^+$ mRNA derived from mice kept in constant darkness for 72 hours yielded almost identical results, indicating that rhythmic fluctuation of NPAS2:BMAL1–controlled genes persists under conditions of constant darkness (Fig. 4B).

In situ hybridization assays were used to extend these observations in two ways. Under conditions of constant darkness, wild-type animals were compared with NPAS2-deficient mice using an in situ hybridization probe specific for the mouse *Per2* gene. Circadian locomotor activity was monitored under conditions of constant darkness; mice were killed at time points 8 hours before the onset of wheel running (trough of *Per1*, *Per2*, and *Cry1* gene expression as assayed by Northern blotting) and 4 hours into wheel running (peak of *Per1*, *Per2*, and *Cry1* gene expression). The *Per2* hybridization oscillated in wild-type animals in numerous brain regions, including sites enriched in NPAS2 (somatosensory cortex, piriform cortex, caudate putamen, and dentate gyrus) as well as brain regions that do not express NPAS2 (CA1, CA2, and CA3 fields of the hippocampus). By comparison, the *Per2* hybridization signal did not oscillate in the somatosensory cortex, piriform cortex, caudate putamen, and dentate gyrus of NPAS2-deficient mice (Fig. 5). It is notable that the in situ hybridization signal oscillated in the CA1, CA2, and CA3 regions of the hippocampus in NPAS2-deficient mice, because these regions do not express NPAS2 at high levels. We speculate that a different molecular clock may be operative in the CA1, CA2, and CA3 regions of the hippocampus. If so, this NPAS2-independent clock appears to oscillate with a molecular rhythm grossly similar to that specified by NPAS2. Finally, evidence of oscillating *Per2* hybridization signal in non–NPAS2-expressing brain regions of NPAS2-deficient mice provides a positive control for the in situ hybridization reactions.

Together, our observations indicate that NPAS2 is a functional analog of Clock. NPAS2 is expressed in a stereotypic distribution of forebrain nuclei critical for the processing of touch, pain, temperature, vision, hearing, smell, and certain emotions such as fear and anxiety (*20, 21*). In nocturnal animals such as the laboratory mice evaluated in this study, the NPAS2:BMAL1 heterodimer is likely active at night, as suggested by our light-dark cycle analysis of *Per1*, *Per2*, *Cry1*, and *BMAL1* mRNA levels. It will be important to determine whether the opposite pattern of activity is observed in diurnal animals. Finally, we hypothesize that the NPAS2:BMAL1 heterodimer will control the expression of critical "output" genes in the forebrain, thereby rhythmically changing the physiological properties of brain nuclei expressing this regulatory apparatus. These rhythmic changes in NPAS2-regulated gene expression may underlie light-dark fluctuations in the states of alertness and tiredness.

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27. SHEP cells inducible for NPAS2 and BMAL1 expression were treated with ponasterone or ethanol vehicle for 16 hours. Poly(A)$^+$ mRNA was prepared and reverse-transcribed into cDNA with reagents provided in the PCR-Select kit (Clontech). cDNA tester (induced) and driver (control) populations were digested with Rsa I, and two kinds of adapters were ligated to the two tester populations. Subtractive hybridization of tester and driver populations, as well as selective polymerase chain reaction (PCR)–based amplification of up-regulated sequences, were performed by the PCR-Select method. Genechip analysis was performed by Rosetta Inpharmatics Inc. (Kirkland, WA) (*29*). Total RNA (5 μg) from treated and control cells was reverse-transcribed into cDNAs using an oligo(dT) primer containing a T7 RNA polymerase promoter. cDNA was then transcribed into cRNA and concomitantly labeled with Cy5 and Cy3 dyes (Sigma). cRNAs were hybridized to a pair of DNA microarrays with 49,218 oligonucleotides each. Oligonucleotides were derived from the 3′ end of the longest mRNA transcript of the genes they represent (*30*). Fluorescence intensities were read by a laser scanner.
28. Mouse genomic sequences containing the 5′ untranslated sequences of *BMAL1* mRNA were identified by BLAST (www.ncbi.nlm.nih.gov/BLAST) search. Then, 2.8 kb of 5′-flanking sequences of the *BMAL* 5′ UTR were amplified by PCR (with the primers 5′-GCGGCGGTACCAATGAGGTTCCACCCACAC-3′ and 5′-GCGGCTCGAGGACGGACGCCCGAGACGG-3′) and cloned into pGEMT, and subsequently into the Xho I site of the pGL3-basic vector.
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31. Supplementary data are available at *Science* Online (www.sciencemag.org/cgi/content/full/1060699/DC1).
32. Polyclonal antibodies specific to NPAS2, BMAL1, and Cry1 were prepared by immunizing rabbits with the keyhole limpet hemocyanin (KLH) conjugates of the peptides NH_2- RASRNKSEKKRRDQFNVLIKELSSMLPGNTRKMDKTTVLEKVIGFLQKHN (NPAS2), NH_2-CAQENPGYPYSDSSSIL (BMAL1), and NH_2-CRPSQEEDTQSIPKVQRQSTN (Cry). (Single-letter abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.) For Western blots, about 5×10^6 cells were lysed in 1 ml of 2× SDS sample buffer; 15 μl of sample was loaded and resolved by SDS–polyacrylamide gel electrophoresis (8%) and transferred onto nitrocellulose. All primary antibodies were applied at 1:1000 dilution. Peroxidase-conjugated donkey antibody to rabbit immunoglobulin G (Jackson ImmunoResearch) was used in conjunction with ECL chemiluminescence reagents (Amersham-Pharmacia) to visualize the reactive species on x-ray film.
33. Eight-week-old C57B6 mice on the 24-hour light-dark cycle were killed by cervical dislocation. During the dark period, mice were killed in darkness with safe-light illumination. Brains were excised and the cerebellum, brainstem, and olfactory bulb were removed to avoid tissues enriched in Clock. The remaining brain tissue was homogenized in RNA STAT60 (Tel-Test Inc., Friendswood, TX), and total RNA was isolated by acid phenol extraction and isopropanol precipitation.
34. K. B. J. Franklin, G. Paxinos, *The Mouse Brain in Stereotaxic Coordinates* (Academic Press, New York, 1997).

35. We thank C. Michnoff and L. Wu for technical assistance; P. Lindsley and Rosetta Inpharmatics for extensive help with DNA microarray assays; J. Richardson and J. Stark for processing of tissue sections and execution of in situ hybridizations; and J. Rutter and R. Bruick for intellectual and conceptual advice. Supported by National Institute of Mental Health grant 1RO1MH59388, by unrestricted funds from an anonymous donor (S.L.M.), and by the Donald W. Reynolds Foundation for Cardiovascular Research and the National Alliance for Research on Schizophrenia and Affective Disorders (J.A.G.).

14 March 2001; accepted 11 June 2001
Published online 6 July 2001;
10.1126/science.1060699
Include this information when citing this paper.