BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **250,** 83–87 (1998)
ARTICLE NO. RC989275

Circadian Oscillation of *BMAL1,* a Partner of a Mammalian Clock Gene *Clock,* in Rat Suprachiasmatic Nucleus[1]

Sato Honma,*[,2] Masaaki Ikeda,† Hiroshi Abe,* Yusuke Tanahashi,* Masakazu Namihira,* Ken-ichi Honma,* and Masahiko Nomura†

**Department of Physiology, Hokkaido University School of Medicine, Sapporo 060-8638, Japan; and †Department of Physiology, Saitama Medical School, Moroyama, Saitama 350-0495, Japan*

Received July 28, 1998

A superfamily gene which encodes a bHLH (basic helix-loop-helix)/PAS transcription factor, *BMAL1,* was cloned and sequenced from rat cDNA. A robust circadian rhythm of rat *BMAL1* expression was detected by *in situ* hybridization in the suprachiasmatic nucleus (SCN), the site of the circadian clock, with the highest level at the subjective night. Less prominent and completely reversed circadian rhythms of *rBMAL1* mRNA were observed in the piriform and parietal cortices. The hybridization signals of *rBMAL1* mRNA were also detected in the olfactory bulb, hippocampus, and cerebellum. Since the product of *rBMAL1* was recently demonstrated to dimerize with the protein of a mammalian clock gene, *Clock,* and the protein complex was shown to bind the E Box in the promoter region of *mPer1* (a mouse homologue to *Drosophila* clock gene, *Per*), *rBMAL1* possibly plays a critical role in the clock mechanism generating the circadian oscillation in rats.

Most organisms from prokaryotes to humans show circadian rhythms in physiology and behavior, which are generated by circadian clock. Circadian clock in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and plays a critical role in adapting bodily functions to cyclic environments. By extensive studies in *Drosophila*, *Neurospora* and *Cyanobacteria*, a molecular feedback loop in which the product of a clock gene(s) regulates its own transcription has been postulated as the intracellular oscillation mechanism of circadian clock (1–5). Although the molecular mechanisms involved in the circadian clock are just beginning to be understood in mammals, substantial progress has been made during the past year by the cloning of a mouse clock gene *Clock* (6, 7). *Clock* was shown to encode a transcription factor which contains a protein-protein interaction sequence known as a PAS domain and a DNA binding sequence, basic helix-loop-helix (bHLH) (7). However, the expression of *Clock* in the SCN did not oscillate (8, 9). In the same year, several members of a PAS family were cloned from mammalian DNA, which includes homologues of human *Arnt* (*BMAL1* or *MOP3*) (10, 11) and of the *Drosophila* clock gene *Per* (*mPer1* and *mPer2*) (8, 9). Although the functions of these genes are not yet clarified, mRNA levels of *mPer1* and *mPer2* showed circadian oscillation in the mouse SCN (8, 9). In addition, the gene transcript was increased in response to a short light pulse (12–14). Because of structural similarities to *Drosophila Per* (*dPer*) and circadian expression in the SCN, they are expected to be components of a molecular feedback loop of circadian oscillation. However, *mPer1* and *mPer2* proteins lack a well-defined bHLH domain which is necessary for DNA binding as a transcription factor (8, 9). By contrast, *BMAL1* is highly homologous to human *Arnt* and the gene product possesses a bHLH as well as a PAS domain (10). Recently, the protein of *mClock* was reported to dimerize with the product of *BMAL1* (15, 16) and the protein complex was shown to bind the E box in the promoter region of *mPer1* (16). In Drosophila, a heterodimer of CLOCK and BMAL1 activates the transcription of *per* and *tim*, and their products block the ability of *Clock* to transactivate their promoter, which could close the feedback loop of the circadian oscillation (17). Furthermore, arrhythmic mutants of *Drosophila*, *Cycle* and *Jrk* were found to be the mutants of *BMAL1* homologue and *Drosophila Clock*, respectively (18, 19). Therefore, BMAL1 was suggested to be a partner of CLOCK and a component

[1] The nucleotide sequence of rat *BMAL1* will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. AB012600.

[2] To whom correspondence and reprint requests should be addressed. Fax: 81-11-706-7871. E-mail: sathonma@med.hokudai.ac.jp.

0006-291X/98 $25.00

of a molecular feedback loop of circadian oscillation in both mammals and *Drosophila* (15–19). Here we cloned and sequenced rat *BMAL1* and examined the temporal as well as spatial patterns of *rBMAL1* expression in the rat brain by means of *in situ* hybridization.

MATERIALS AND METHODS

rBMAL1 cDNAs were isolated by a combination of RACE and RT-PCR. Briefly, 5′ cDNA clone was isolated by the Marathon RACE kit (Clontech) using a degenerate oligonucleotide primer R594-MIXD2 (5′-TC(AGCT)AC(AGCTGCCAT(CT)CT(AGCT) A(AG)(AGCT) AC-3′) for the primary RACE and a nested primer BMAL1RD2 (5′-CAATGATTT-TCTGCTGCTGCTGAT-3′) for the nested PCR. 3′ cDNA clones were isolated by PCR using primers designed from the common sequence among expressed sequence tags of mouse (AA119772 and AA142524) and human *BMAL1*. The sequences of the primer pair for the cloning of 3′ cDNAs were: AA119-207-U1; 5′-CCACAGGATAAGAGGGTCAT-3′ and rBMAL1-2111-D2; 5′-ATGATGAGGAAACACTGGAG-3′. cDNA clones covering the open reading frame were amplified using primers derived from 5′ cDNA sequence and rBMAL1-2111-D2. The primer sequence was: rBMAL1-5U; 5′-ATGGCGGACCAGAGAATGGA-3′. The nucleotide sequence was determined by the fluorescent labeled primer cycle sequencing system (Amersham Pharmacia Biotech) using fluorescein isothiocyanate-labeled primers and an automated DNA sequencer (DSQ1000, Shimazu).

Sixty-two male Wistar rats born and raised under controlled conditions (light: 06:00–18:00 h, temperature 22 ± 2°C) were subjected to hybridization experiments at 8 weeks. Animals were taken care according to the Guideline for the care and use of laboratory animals in Hokkaido University School of Medicine. Sixty rats were decapitated at Zeitgeber Time (ZT) 2, ZT6, ZT10, ZT14, ZT18, and ZT22 either under the condition of 12 h light–12 h dark (LD) or on the 3rd day of constant darkness (DD, the first day starts 12 h after released into constant darkness), where ZT12 was the onset of darkness in the previous LD cycle. Two rats were killed at ZT18 on the third day of DD and spatial distribution of hybridization signals was examined. Decapitation during the dark period was done with the help of a dim red light (<0.1 lux). The brain was quickly removed and frozen. Serial coronal sections of 20 μm thickness were saved throughout the SCN area with a cryostat (Bright), and placed on a slide glass coated with aminopropyltriethoxysilane (Sigma). Slides glasses were air dried and stored in −80°C until hybridization.

In situ hybridization was done according to Watanabe *et al.* (20) with minor modifications. Antisense 45 mer oligonucleotide probe complementary to nucleotide residues 7 to 51 was labeled with ^{35}S-dAMP by using ^{35}S-dATP (NEN) and terminal deoxyribonucleotidyl transferase (BRL). Hybridization was carried out overnight at 42°C. Afterwards, the slide glasses were washed, air dried and apposed to Hyperfilm-β max (Amersham) for 4 weeks. In each cassette, ^{14}C standards (American Radiolabeled Chemicals) were included to quantify the optical density (OD). OD at the structure of interest was normalized by subtracting OD at the corpus callosum in the same section. Hybridization signals were analyzed with a computed image analyzing system (MCID, Imaging Research). Mean densities were calculated for each rat using 14 serial sections.

RESULTS

Entire coding region of *BMAL1* was sequenced in rats. The cDNA sequence of *rBMAL1* contains an open reading frame predicted to encode 626 amino acids. The *rBMAL1* nucleotide identity to *hBMAL1* is 91% and the amino acid sequence identity is 98%. *rBMAL1* protein contains PAS-A, PAS-B and bHLH domains, and the sequence identities to *hBMAL1* for these domains are 100, 100, and 98%, respectively. One amino acid (arginine) in the bHLH motif in hBMAL1 is replaced by lysine in rBMAL1. bHLH region of rBMAL1 has 69 and 36% identity with bHLH region of hARNT and mCLOCK, respectively. For PAS-A and PAS-B, the identity to hARNT is 47 and 44%, to mCLOCK 35 and 46%, to hPER1 24 and 33%, to hPER2 32 and 29%, and to dPER 28 and 37%, respectively. The amino acid sequence of a basic domain in a bHLH is AREAHS-QIEKRRR, and 6 out of 13 amino acids are basic. The *rBMAL1* protein has potential transactivation domains at the C-terminal which contain relatively high concentrations of serine and proline, and a single protein kinase A phosphoacceptor site (Ser 42).

The expression of *rBMAL1* mRNA was examined in the rat brain on the third day of constant darkness (DD). The rats were killed at ZT18, where ZT12 is the time of light-off in the previous light-dark cycle. Although the hybridization signals of the lower level were detected throughout the gray matter of the cerebrum, *rBMAL1* mRNA was strongly expressed in the SCN, olfactory bulb, piriform cortex, hippocampus and cerebellum (Fig. 1). The signals were also detected in the retina and pineal organ (data not shown).

Temporal profiles of *rBMAL1* mRNA expression in the SCN under LD and on the third day of DD were demonstrated in Fig. 2. Under both lighting conditions, strong hybridization signals were detected at ZT14, T18 and ZT22, and weak but still recognizable signals at ZT2, ZT6, and ZT10. The quantification of mRNA level revealed significant circadian rhythms ($p < 0.01$ by one way ANOVA) in the SCN, piriform and parietal cortices under LD and DD conditions (Fig. 3). In the SCN, the circadian rhythm of *rBMAL1* mRNA was robust and showed its peak around ZT14–ZT18. On the other hand, the amplitudes of *rBMAL1* mRNA rhythm in the piriform and parietal cortices were small and the peaks were located around ZT22–ZT2. The level of *rBMAL1* mRNA was not influenced by a short (30 min) light pulse of 300 lx, at least one hour after exposure to light at ZT6 or at ZT18 on the third day of DD (data not shown).

DISCUSSION

A line of evidence suggests that the circadian rhythm is generated by an intracellular molecular feedback loop (1–5) in which a role of cycling transcription factor is of special importance. The present finding of circadian expression of *rBMAL1* in the SCN added the new element sufficient to be a component of an intracellular feedback loop of circadian oscillation in mammals. During the past year, a mouse clock gene, *Clock*, was cloned for the first time in mammals, which encoded a bHLH/PAS transcription factor (7) but the gene expression was not cyclic in the SCN (8, 9). Subsequently, the two

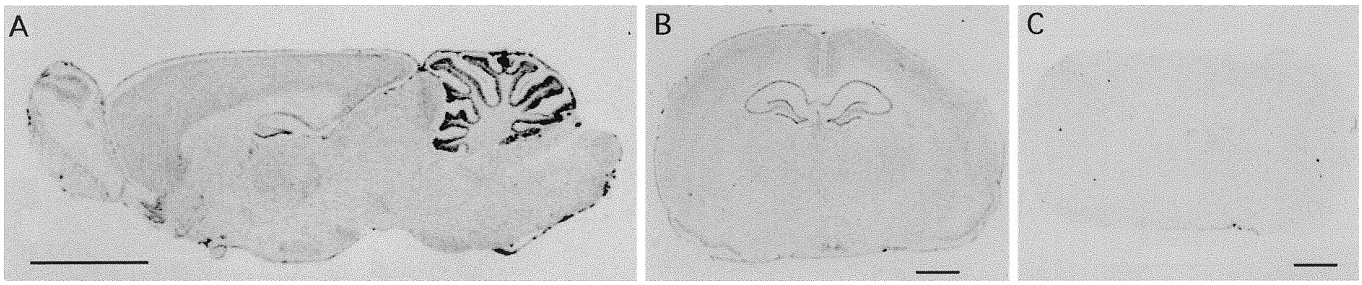

FIG. 1. *rBMAL1* mRNA expression in the rat brain by *in situ* hybridization. Rats were decapitated at ZT18 (A and C) and ZT 10 (B) on the third day of DD. (A) Strong hybridization signals were detected in the olfactory bulb, hippocampus and cerebellum on the sagittal section with a scale bar of 5 mm. (B) Strong hybridization signals were detected in the hippocampus on the coronal section with a scale bar of 2 mm. (C) Hybridization signals were suppressed by an overdose of an unlabelled probe in the SCN on the coronal section with a scale bar of 2 mm.

mammalian homologues of *Drosophila* clock gene *Per* (*mPer1* and *mPer2*) were cloned. They were expressed in the SCN with a circadian fashion but they lack a well-defined DNA binding site (8, 9, 12–14). For DNA binding, the number and type of basic amino acids in the upstream of the HLH domain are of special importance (21, 22). *rBMAL1* and *hArnt* proteins contain 6 basic residues in this region, 5 of which are identical. On the other hand, it is not clear whether *mPer1* and *mPer2* are able to bind DNA, since *mPer1* protein has 3 and *mPer2* has only 2 basic residues in a putative basic domain, respectively (8, 9, 13, 14). In addition, it was demonstrated that replacement of an amino acid sequence such as RHR (AHR) and RRR(ARNT) to RAR extremely reduced the DNA binding capacity (23). The amino acid sequence of mPER1 is RARTQK, which seems to lose a binding capacity to DNA. The *hArnt* protein makes homodimer and binds to DNA at E box (CANNTG) (24). The *rBMAL1* product is able to recognize the same DNA binding sites. Transcription factors in the PAS superfamily are divided into two subgroups; the *Arnt* and *AhR* (25, 26). Those which belong to the *Arnt* groups dimerize not only to each other but also with those in the *AhR* group.

As clearly demonstrated in Figs. 2 and 3, the expression of *BMAL1* undergoes circadian oscillation in the

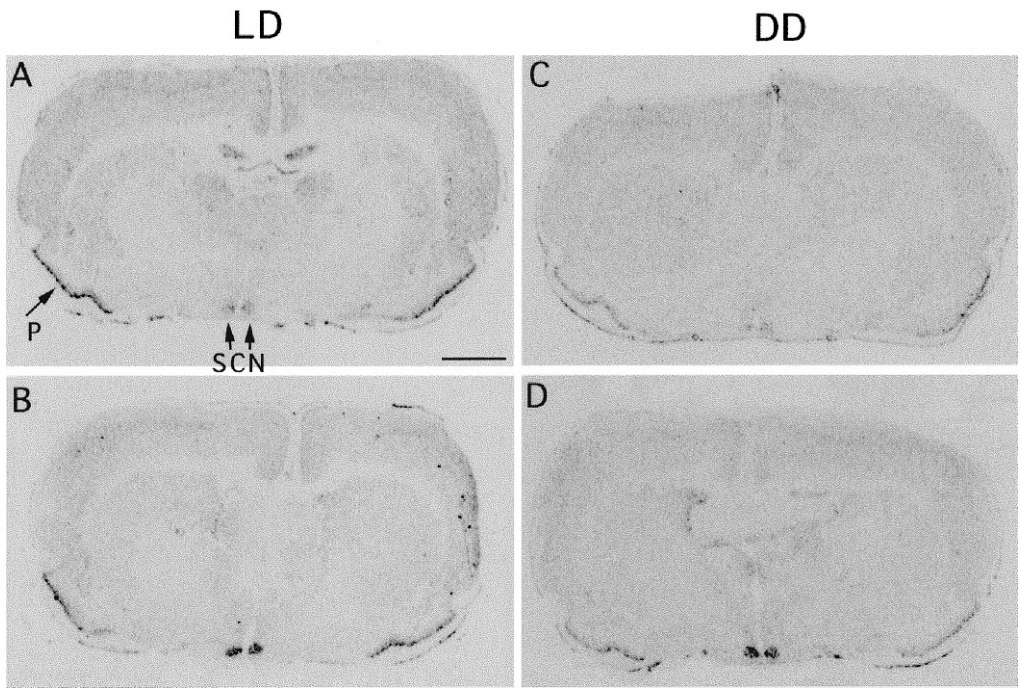

FIG. 2. Circadian expression of *rBMAL1* mRNA in the coronal section of rat brain including the SCN by *in situ* hybridization. A scale bar, 2 mm. (A and B) ZT 6 and ZT 18 under LD, respectively. C and D at ZT 6 and ZT 18 on the third day of DD, respectively. ZT12 is the time of light-off in the previous LD. P, piriform cortex.

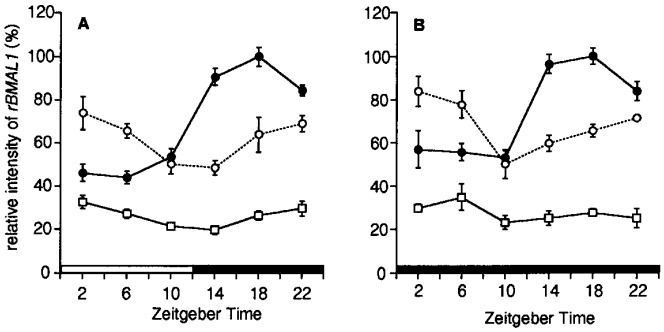

FIG. 3. Circadian oscillation of *rBMAL1*mRNA level in the rat SCN (closed circles with solid line), piriform cortex (open circles with broken line), and parietal cortex (open square with solid line) under LD (A) and on the third day of DD (B). Horizontal black bars on the abscissa indicate the dark period. Data are relative intensity of mRNA (%) to the value at ZT18. Each value is the mean ± SD of five animals. Significant circadian variations were detected in the SCN, piriform and parietal cortices under LD and DD conditions by one way ANOVA ($p < 0.01$). Multiple comparison test (Bonferroni and Dunn) revealed that the level at ZT 6 was significantly different ($p < 0.01$) from those at ZT 14, 18 and 22 in the SCN under DD as well as LD.

rat SCN. The oscillation is robust but almost 180 degree out of phase of circadian rhythm in *mPer1* mRNA level (8, 9, 13, 14). The product of *BMAL1* was demonstrated to make a heterodimer with the *Clock* protein (15, 16) and bind the E box in the promoter region of *mPer1* (16). Therefore, *BMAL1* is thought to be a partner of *Clock*. A nonsense mutation of *Drosophila* homologue of *BMAL1* (*Cycle*) leads to aperiodism in locomotor activity (19). Taking these findings altogether, it is highly possible that a molecular feedback loop of circadian oscillation involves *BMAL1* ,*Clock* and *Per*, and a heterodimer of *Clock* and *BMAL1* proteins regulates the expression of *Per* in the circadian fashion. The *Per* product in turn may feedback to the *BMAL1* or *Clock* activities in mammals, as it does in *Drosophila* (17). Since the expression of *mClock* is not rhythmic (8, 9), the *BMAL1* protein seems to play a critical role in the generation of circadian oscillation in this molecular feedback loop.

The circadian oscillation is reset by light in a phase-response manner. Therefore, the molecular feedback loop is considered to be influenced by a signal of light stimulus. The expression of *mPer1* was enhanced when a light pulse was given at the subjective night where light produces phase-shifts in the circadian oscillation (12–14). On the other hand, *BMAL1* expression was not significantly changed 1 h after a light pulse (30 min, 300 lux), when examined at ZT6 and ZT18 on the third day of DD. These findings suggest that *Per* expression is also regulated by another factor than *BMAL1*, which is mediating a photic signal. At present there is no information about a light responsiveness of *Clock* expression.

The peak of *rBMAL1* expression in the SCN was found in the subjective night. Most endogenous rhythms so far detected in the rat SCN, such as the rhythms in metabolic rate, neuronal activity and secretion of peptide hormones, peaked at the subjective day (27–31). This feature of *rBMAL1* expression strikingly contrasts with that of *mPer1* which was expressed strongly in the subjective day (8, 9). The 12 h difference in the peak phase between *BMAL1* and *Per*, however, is not contradictory to an idea of a reciprocal negative feedback regulation.

The *rBMAL1* expression was also rhythmic in the piriform and parietal cortices. However, the circadian peaks in these structures were located at the subjective day, almost 180 degrees out of phase in the SCN. Similar findings were also reported in *mPer1* gene expression (9).

Other than the SCN, the *rBMAL1* mRNA is expressed strongly in the olfactory bulb, piriform cortex, hippocampus, and cerebellum. Intense hybridization signals were also detected in the retina and pineal gland. It is of interest to note that *rBMAL1* was transcribed where *mClock*, *mPer1*, and *mPer2* mRNA were also expressed (7–9, 12–14). Common functions are highly suggestive to these PAS family genes.

ACKNOWLEDGMENTS

This study was financially supported in part by grants from the Ministry of Education, Science, and Culture of Japan (096970065 and 09877015).

REFERENCES

1. Hardin, P. E., Hall, J. C., and Rosbash, M. (1990) *Nature* **343,** 536–540.
2. Aronson, B. D., Johnson, K. A., Loros, J. J., and Dunlap, J. C. (1994) *Science* **263,** 1578–1584.
3. Kondo, T., Tsinoremas, N. F., Golden, S. S., Johnson, C. H., Kutsuna, S., and Ishiura, M. (1994) *Science* **266,** 1233–1236.
4. Rosbash, M., Allada, R., Dembinska, W. Q., Guo, W. Q., Le, M., Marrus, S., Qian, Z., Rutila, J., Yaglom, J., and Zeng, H. A. (1996) *Cold Spring Harbor Symp. Quant. Biol.* **61,** 265–278.
5. Young, M. W., Wager-Smith, K., Vosshall, L., Saez, L., and Myers, M. P. (1996) *Cold Spring Harbor Symp. Quant. Biol.* **61,** 279–284.
6. Viterterna, M. H., King, D. P., Chang, A. M., and Takahashi, J. S. (1994) *Science* **264,** 719–725.
7. King, D. P., Zhao, Y., Sangoram, A. M., Wilsbacher, L. D., Tanaka, M., Antoch, M. P., Steeves, T. D. L., Vitaterna, M. H., Kornhauser, J. M., Lowrey, P. L., Turek, F. W., and Takahashi, J. S. (1997) *Cell* **89,** 641–653.
8. Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hiros, M., and Sasaki, Y. (1997) *Nature* **389,** 512–516.
9. Sun, Z. S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., and Lee, C. C. (1997) *Cell* **90,** 1003–1011.
10. Ikeda, M., and Nomura, M. (1997) *Biochem. Biophys. Res. Commun.* **233,** 258–264.
11. Hogenesch, J. B., Chan, W. K., Jackiw, V. H., Brown, R. C., Gu,

Y. Z., Pray-Grant, M., Perdew, G. H., and Bradfield, C. A. (1997) *J. Biol. Chem.* **272,** 8581–8593.

12. Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L. Tei, H., Moriya, T., Shibata, S., Loros, J. J., Dunlap, J. C., and Okamura, H. (1997) *Cell* **91,** 1043–1054.
13. Albrecht, U., Sun, Z. S., Eichele, G., and Lee, C. C. (1997) *Cell* **91,** 1055–1064.
14. Shearman, L. P., Zylka, M. J., Weaver, D. R., Kolakowski, L. F., Jr., and Reppert, S. M. (1997) *Neuron* **19,** 1261.
15. Hogenesch, J. B., Gu, Y-Z., Jain, S., and Bradfield, C. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5474–5479.
16. Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., and Weitz, C. J. (1998) *Science* **280,** 1564–1569.
17. Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D. L., Weitz, C. J., Takahashi, J. S., and Kay, S. A. (1998) *Scienc*e **280,** 1599–1603.
18. Allada, R., White, N. E., So, W. V., Hall, J. C., and Rosbash, M. (1998) *Cell* **93,** 791–804.
19. Rutila, J. E., Suri, V., Le, M., So, W. V., Rosbash, M., and Hall, J. C. (1998) *Cell* **93,** 805–814.
20. Watanabe, M., Inoue, Y., Sakimura, K., and Mishina, M. (1993) *J. Comp. Neurol.* **338,** 377–390.
21. Ellis, H. M., Spann, D. R., and Posakony, J. W. (1990) *Cell* **61,** 27–38.
22. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) *Cell* **61,** 49–59.
23. Bacsi, S. G., and Hankinson, O. (1996) *J. Biol. Chem.* **271,** 8843–8850.
24. Sogawa, K., Nakano, R., Kobayashi, A., Kikuchi, Y., Ohe, N., Matsushita, N., and Fujii-Kuriyama, Y. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1936–1940.
25. Hirose, K., Morita, M., Ema, M., Mimura, J., Hamada, H., Fujii, H., Saijo, Y., Gotoh, O., Sogawa, K., and Fujii-Kuriyama, Y. (1996) *Mol. Cell. Biol.* **16,** 1706–1713.
26. Ema, M., Morita, M., Ikawa, S., Tanaka, M., Matsuda, Y., Gotoh, O., Saijoh, Y., Fujii, H., Hamada, H., Kikuchi, Y., and Fujii-Kuriyama, Y. (1996) *Mol. Cell. Biol.* **16,** 5865–5875.
27. Inouye, S. T., and Kawamura, H. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5962–5966.
28. Schwartz, W. B., Davidsen, L. C., and Smith, C. B. (1980) *J. Comp. Neurol.* **189,** 157–167.
29. Green, D. J., and Gillet, R. (1982) *Brain Res.* **245,** 198–200.
30. Schwartz, W. J., and Reppert, S. M. (1985) *J. Neurosci.* **5,** 2771–2778.
31. Reppert, S. M., and Uhl, G. R. (1987) *Endocrinology* **120,** 2483–2487.