Circadian Oscillation of 64-kDa Polypeptide in the Rat Suprachiasmatic Nucleus

Rikako NISHI, Noboru MURAKAMI, Nobuyuki MARUMOTO, Tetsuo NASU[1)], Haruto KURODA, and Teiichi ETOH
Departments of Veterinary Physiology and [1)]Veterinary Anatomy, Faculty of Agriculture, Miyazaki University, Miyazaki 899–21, Japan
(Received 18 May 1993/Accepted 6 September 1993)

ABSTRACT. In cell suspensions of suprachiasmatic nucleus harvested every 3 hr from rats kept under 12 hr: 12 hr light-dark cycle and constant darkness, we have detected a Mr 64-kDa protein whose synthesis exhibits two distinct daily peaks in SDS-PAGE. Analysis of densitometer tracings revealed that the synthesis of other proteins was independent of the time of day or not reproducible. Maximum synthesis of the 64-kDa polypeptide occurred at around CT6 and CT21, which are almost coincident with the phase advance regions of circadian activity rhythm induced by anisomycin and light pulses [15], respectively. These results suggest that the 64-kDa protein in SDS-PAGE may be a part of the circadian clock mechanism.—KEY WORDS: circadian rhythm, suprachiasmatic nucleus.
J. Vet. Med. Sci. 56(1): 161–163, 1994

The daily rhythms of physiological and behavioral events in mammals are controlled by a circadian oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus [17, 18]. Although significant progress has been made in our understanding of the cellular and molecular mechanisms of circadian oscillators in microorganisms and invertebrates, very little information is available from mammals [2, 13, 16–18]. It has been well documented that protein synthesis at a specific phase of the circadian cycle is a prerequisite for functioning of the circadian clock in a wide variety of living organisms [6–8, 10]. In the hamster, injection of a protein synthesis inhibitor causes a phase-dependent phase shift with a maximal phase advance around CT9 and maximal phase delay around CT0 [5, 15]. If the specific proteins involved in the circadian clock mechanism could be detected, and if their partial amino acid sequences could be determined, they would be very useful for analysis of the molecular events occurring in the circadian clock. Indeed, a candidate for such proteins has already been reported in unicellular organisms and in the Aplysia eye [4, 12, 19]. Here, we searched for the polypeptide whose rate of synthesis oscillates in the rat suprachiasmatic nucleus.

Adult male Sprague-Dawley rats bred in our laboratory were housed in a temperature-controlled environment (22±1°C) under a 12:12 hr light-dark cycle (LD; lights on 07.00 hr; LD group) and given free access to food and water. One group was kept under reversed light-dark cycle (DL; lights on 19.00 hr; DL group). After 2 weeks adaptation, three of rats in each group were killed by decapitation every 3 hr starting at 07.00 hr during 12 hr. Infrared illumination were used under dark condition. The other group of the rats (DD group) was transferred from the LD room to constant dark room. After 2 days, they were killed by decapitation every 3 hr starting at 07.00 hr or 16.00 hr for 27 hr. In these animals, we assigned CT0 and CT12 to 07.00 hr and 19.00 hr, respectively because two days of free-running does not shift the phase of animal's rhythm longer than 0.5 hr in our rat colony. According to the previous method [9], brain slices of 0.7 mm thick including the SCN were prepared and one area of SCN tissue including the bilateral paired SCN was punched out. To obtain the cells, the tissue was triturated repeatedly through a series of gradually narrowing Pasteur pipettes and centrifuged for 5 min at 500 × g. The resulting cell pellet was resuspended in methionine free-DMEM medium (Dulbecco's modified Eagle medium # 26p1710) containing 10 mM HEPES (Gibco), 1.25 mg/m*l* $NaHCO_3$, 100 μg/m*l* human transferrin (Sigma), 5 μg/m*l* insulin (Sigma), 100 μg/m*l* putrescine, 30 nM selenium, 75 mM nerve growth factor (Sigma, #0513) and 25 μCi/m*l* ^{35}S-methionine (New England Nuclear). Each suspension was placed into three wells (48-well dish, Falcon) at cell density of 10^5/well, and maintained in an incubator at 37°C with 95% air and 5% CO_2 for 3 hr. The time from decapitation of the rats until the start of cell incubation was about 20 min. After 3 hr of incubation, the cells were collected and washed with HEPES buffer. The final cell pellets were sonicated for 2 min by sonicater (UP-209, Tomy Seiko Co., Tokyo) with 0.05 m*l* buffer with the composition of 0.01 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 50 μg/m*l* RNase (Sigma), 25 μg/m*l* DNase (Sigma). The samples were also solubilized in 0.05 m*l* of electrophoresis sample buffer. The sample buffer (pH 6.8) contained 0.0625 M Tris, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.05% (wt/vol) bromphenol blue and 10% (vol/vol) mercaptoethanol. The sample preparation and SDS polyacrylamide gel electrophoresis procedure were as described by Dunba [1]. After staining and drying the gels, they were exposed to X-ray film. The developed films were scanned with an image scanner, and the density of each band was calculated by means of NIH image 1.43 software. Evaluation was performed by relating the area of the rhythmically synthesized polypeptide to that of the total proteins resolved. Then, the quantitative analysis was performed by comparing the candidate protein with the 66-kDa protein whose synthesis is constant during a day.

A fluorograph of electrophoretically separated proteins of SCN from a rat under light-dark cycle and constant dark conditions is shown in Fig. 1. Analysis of densitometer tracings revealed two distinct peaks of a 64-kDa polypeptide produced during a day. The peaks were observed 6–9 hr after lights on and 6–9 hr after lights off in LD-DL samples (Fig. 1A, C) and at around CT6 and CT21 in DD samples (Fig. 1B, D. Fig. 2). The time-dependent changes in the rates of synthesis of some polypeptides with a molecular weight of less than 35 kDa were unclear and not as reproducible as the oscillation of the 64-kDa polypeptide in repeated experiment. We do

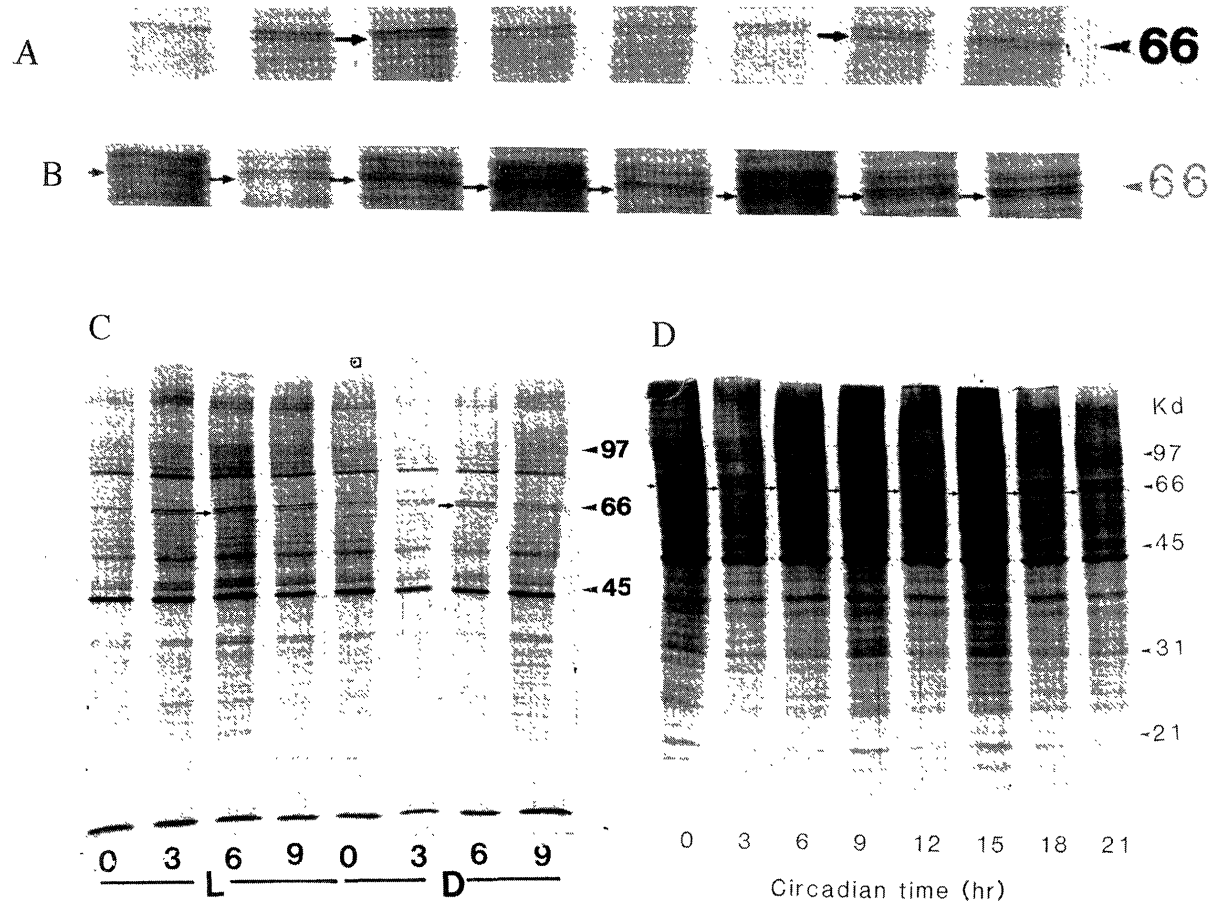

Fig. 1. Fluorograph of labeled proteins synthesized by 3 h cultured SCN cells harvested from a rat kept under LD and DL (A, C), and constant darkness (B, D). The samples indicated by L and D (C) are collected at 3 hr intervals starting at 07.00 hr for 12 hr from LD and DL groups, respectively. The number of abscissa represents the time of decapitation (CT 0 is equal to the time of light on). The arrow indicates the 64-kDa polypeptide. A and B are magnified graph which include the bands of 64-kDa protein observed in C and D, respectively. Gel is 10% and 13% acrylamide for LD and DD samples, respectively.

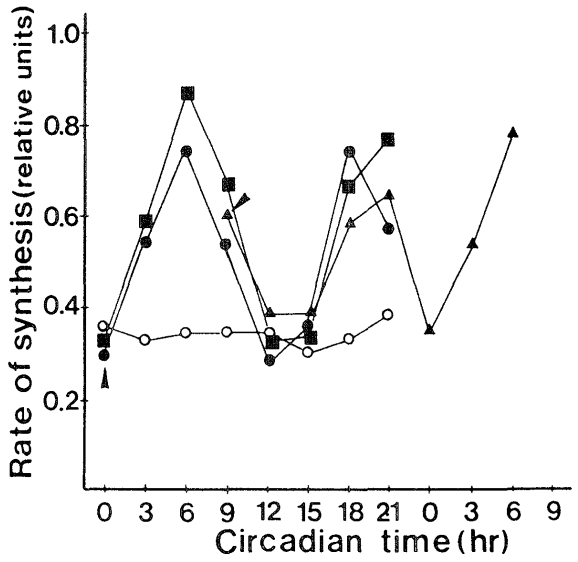

Fig. 2. Oscillation in rate of synthesis of the 64-kDa polypeptide calculated from densitometer tracing of the fluorograph. The closed circle (SCN) and open circle (region other than SCN) represent the samples obtained from LD (CT 0, 3, 6, 9) and DL (CT 12, 15, 18, 21) groups. The closed square and triangle represent the samples obtained from DD group. Arrows indicate the initiation of sample collection.

not think that two distinct peaks of 64-kDa protein are ultradian rhythms but not circadian rhythm, since the peak times are constant at around CT6 and CT21 even if the collection of samples was started at different time. No existence of circadian changes of 64-kDa protein in the region (containing stria terminals, pars precommissualis) other than SCN suggest that the circadian change of 64-kDa protein is specific in SCN.

In a preliminary experiment, we searched for proteins involved in the circadian clock mechanism by incubating SCN tissues rather than cell suspension cultures. The amount of labeled methionine incorporated into the proteins of SCN tissues showed wide variation between individual samples. On the other hand, in cell suspension cultures, the amount of ^{35}S-methionine incorporated did not differ markedly between samples. Therefore, in the case of tissue culture, it was likely that the medium containing ^{35}S-methionine was unable to penetrate into the tissues uniformly.

Absence of a circadian rhythm of protein synthesis in the rat SCN has been reported, since the relative incorporation of labeled leucine into protein did not differ between day and night, as judged from autoradiography of brain slices [3]. On the other hand, microinjection of anisomycin, an inhibitor of protein synthesis, into the SCN region directly induced a phase-dependent phase shift in circadian activity rhythm in hamsters, strongly suggesting that protein synthesis may be involved in the circadian clock mechanism [5]. Although the reason for this discrepancy is unknown, it is possible that autoradiography of brain slices may not be sufficiently sensitive to demonstrate small qualitative circadian changes in proteins, or alternatively, the effect of stimulation and inhibition of SCN protein synthesis may cancel each other. In this study, we do not know whether the 64-kDa polypeptide in the SCN is a part of actual clock mechanism or one of its products. In addition, it is unclear whether the 64-kDa proteins produced at CT6–9 and CT21–0 are identical. Several polypeptide hormones, such as vasopressin, neuropeptide Y (NPY), somatostatin, vasoactive intestinal polypeptide (VIP), bombesin, and cholecystokinin (CCK) are synthesized or secreted rhythmically in the hypothalamus [11, 14]. However, the molecular weight and the circadian oscillations of these hormones are different from those of the 64-kDa polypeptide [11, 14]. For example, the release of hypothalamic bombesin, NPY and VIP increased only in the evening, CCK and vasopressin showed the increase in the morning [11, 14]. Further studies are required to elucidate whether the 64-kDa polypeptide is identical with these hormones.

It is interesting that the peak times of 64-kDa polypeptide synthesis are around CT6 and CT21, which are almost coincident with the regions of phase advance induced by anisomycin and light pulse in hamster, respectively [5, 15]. In addition, a 64-kDa polypeptide has recently been suggested as a candidate of protein involved in the circadian clock mechanism of Chlamydomonas [20]. We do not know whether these two polypeptides observed in SCN and Chlamydomonas are identical. Further study will be needed to clarify the structure and properties of the 64-kDa polypeptide, and its role in the circadian clock mechanism.

ACKNOWLEDGEMENTS. The authors wish to thank Dr. K. Shiota, The University of Tokyo, for his technical advice of densitometer tracing.

REFERENCES

1. Dunba, B. S. 1987. Two Dimensional Electrophoresis and Immunological Techniques. Plenum Press, New York.
2. Edmunds, L. N. Jr. 1988. Cellular and Molecular Bases of Biological Clocks. Springer-Verlag, New York.
3. Glotzbach, S. F., Randall, T. L., Radeke, C. M., and Heller, H. C. 1987. *Neurosci. Lett.* 76: 113–118.
4. Hartwig, R., Schweiger, M., Schweiger, R., and Schweiger, H-G. 1985. *Proc. Natl. Acad. Sci. U.S.A.* 82: 6899–6902.
5. Inouye, S-I. T., Takahashi, J. S., Wollnik, F., and Turek, F. W. 1988. *Am. J. Physiol.* 255: R1055–1058.
6. Jacklet, J. W. 1977. *Science* 198: 69–71.
7. Karakashian, M. W. and Schweiger, H. G. 1976. *Exp. Cell. Res.* 98: 303–312.
8. Murakami, N. and Takahashi, J. S. 1988. *Soc. Neurosci. Abstr.* 14: 385.
9. Murakami, N., Takamure, M., Takahashi, K., Utunomiya, K., Kuroda, H., and Etoh, T. 1991. *Brain Res.* 545: 347–350.
10. Nakashima, H., Perlman, J., and Feldman, J. F. 1981. *Am. J. Physiol.* 241: R31–35.
11. Nicholson, S. A., Adrian, T. E., Bacarese-Hamilton, A. J., Gillham, B., Jones, M. T., and Bloom, S. R. 1983. *Regul. Peptides* 7: 385–397.
12. Raju, U., Yeung, S. J., and Eskin, A. 1990. *Am. J. Physiol.* 258: R256–262.
13. Rosbash, M. and Hall, J. C. 1989. *Neuron* 3: 387–398.
14. Shinohara, K., Isobe, Y., Takeuchi, J., and Inouye, S-I. T. 1991. *Neurosci. Lett.* 129: 59–62.
15. Takahashi, J. S. and Turek, F. W. 1987. *Brain Res.* 405: 199–203.
16. Takahashi, J. S., Murakami, N., Nikaido, S. S., Pratt, B. L., and Robertson, L. M. 1989. *Rec. Prog. Horm. Res.* 45: 279–352.
17. Takahashi, J. S. and Zatz, M. 1982. *Science* 217: 375–378.
18. Turek, F. W. 1985. *Annu. Rev. Physiol.* 47: 49–64.
19. Walla, O. T., de Groot, E. J., and Schweiger, M. 1989. *Eur. J. Cell Biol.* 50: 181–186.
20. Wiedemann, I., de Groot, E. J., and Schweiger, M. 1992. *Planta* 186: 593–599.