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Monitoring circadian time in rat plasma using a secreted *Cypridina* luciferase reporter

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ABSTRACT

A firefly luciferase reporter enabled us to monitor promoter activity *in vivo* as well as *ex vivo*; however, this requires a sufficient supply of the substrate luciferin and specific monitoring devices. To overcome these disadvantages, we developed transgenic rats carrying a secreted enzyme *Cypridina* luciferase (CLuc) reporter under the promoter of clock gene *Per2* (*Per2–CLuc*). *Per2–CLuc* activity in serially sampled blood from freely moving rats exhibited robust circadian rhythms with a peak at early morning. The *Per2–CLuc* bioluminescence could be quantified even with approximately 100 pl of plasma. Plasma *Per2–CLuc* rhythms were phase reversed, and the level was reduced by restricting food access for 2 h during the light phase, suggesting that the plasma *Per2–CLuc* rhythms reflect the phase of peripheral clocks entrained to feeding cues as well as fuel metabolism. Fasting for 2 days did not alter the circadian *Per2–CLuc* rhythms in rats, suggesting that feeding per se did not affect the circadian *Per2–CLuc* rhythms. Tissue-specific *Per2–CLuc* rhythms were observed in culture medium of peripheral tissues. The *Per2–CLuc* reporter is a powerful tool to access gene expression *in vivo* as well as *ex vivo* with ordinary laboratory equipment.

Molecular machinery for circadian rhythm generation is a transcriptional and translational feedback loop consisting of clock genes and their protein products. The CLOCK and BMAL1 heterodimer serves as the positive regulator of the loop and activates the transcription of *Period* (*Per*)[1] genes via binding to the E-box element on the promoter, whereas protein products of *Per1, Per2, Cryptochrome 1* (*Cry1*), and *Cry2* serve as negative regulators of transcription activation [1]. Transgenic animals carrying a clock gene promoter-driven firefly luciferase reporter have been successfully introduced into the field of biological rhythm research. By measuring bioluminescence from cultured tissues of these animals ex vivo [2] or from tissues of interest in living animals [3,4], we are now able to know the circadian time of tissues and animals. However, special attention is needed, especially for *in vivo* analysis, because the quantitative analysis of bioluminescence with firefly reporter requires a sufficient supply of the substrate luciferin [5]. Furthermore, cofactors such as Mg^{2+}, ATP, and oxygen are also indispensable for luciferin–luciferase reaction [5–7]. Thus, hypoxia, ATP deficiency, and acidosis due to insufficient local circulation may affect bioluminescence activity. For the continuous monitoring of firefly luciferase activity from cultured tissues, we need specific and expensive monitoring devices such as a luminometer equipped with an incubator and an electron multiplying charge-coupled device (EMCCD) camera mounted on the microscope installed with a stage-top incubator.

Secreted luciferase overcomes the above-mentioned issues. By simply collecting perfusate or culture medium of living tissues and cells, we can report biological phenomena in the body quantitatively. Collected specimen can either be measured of its bioluminescence in real time or stored until the measurement with a regular luminometer by adding substrate [7,8]. Previously, we monitored promoter activity of growth hormone continuously in the rat pituitary adenoma cell line (GH3) stably transfected with the secreted luciferase, *Vargula hirgendorfii* (VL) reporter [9]. We also demonstrated circadian rhythms of *Per1* expression activity

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[1] *Abbreviations used: Per, Period*; *Cry, Cryptochrome*; VL, *Vargula hirgendorfii*; SCN, suprachiasmatic nucleus; CLuc, *Cypridina* luciferase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; LD, 12 h light:12 h dark; DD, constant darkness; CT, circadian time; RF, restricted feeding; WAT, white adipose tissue; ANOVA, analysis of variance; SD, standard deviation(s); dLuc, destabilized firefly luciferase.

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by means of *VL* reporter in the suprachiasmatic nucleus (SCN) of *Per1–VL* reporter mice [10]. However, the luminescence level of *Per1–VL* mice was not high enough to measure directly with blood plasma. If luciferase is secreted into general circulation without receiving immediate degradation or excretion, gene expression could be assessed with plasma. In the current study, we developed the transgenic rat carrying a *Cypridina* luciferase (CLuc) reporter [11], a brighter secreted luciferase reporter than VL, under the promoter of *Per2* (*Per2–CLuc*), one of the major clock genes of mammals. Here we demonstrate *Per2* expression rhythm *in vivo* by serial blood sampling from freely moving rats. The circadian *Per2–CLuc* rhythm in plasma was not changed by fasting for 2 days but phase-shifted by 180° when the daily meal supply was restricted to 2 h during the light phase for 2 weeks. Tissue-specific *Per2* expression rhythms were also detected with serially collected culture medium of explants of several peripheral tissues. Secreted luciferase provides a powerful tool for monitoring gene expression.

Materials and methods

Plasmids

To construct a reporter vector carrying the mouse *Per2* promoter, the *Per2* promoter fragment (−1198 to +112 bp, where +1 indicates the putative transcription start site) was polymerase chain reaction (PCR) amplified from C57BL/6J mouse genomic DNA using the set of primers, 5'-GCTAGCGTGGATGCTACTAGGCTTCAAG-3' and 5'-CTCGAGTCAACCCGCGCGTCTGTCCCTTG-3', and cloned into the *Nhe*I/*Xho*I site of pGL3–Basic (Promega). The CLuc complementary DNA (cDNA, accession no. AB159608) was replaced with the *Xho*I and *Xba*I fragments of pCL-pBluescript (ATTO, Tokyo, Japan), resulting in *Per2–CLuc*. The expression vectors carrying human (h) BMAL1, hCLOCK, and mouse (m) CRY1 were kindly provided by M. Ikeda (Saitama Medical University). To generate transgenic rats, *Per2–CLuc* was linearized by using *Kpn*I and *Aor*51-HI and injected into a fertilized rat egg.

Cell culture and transfection procedure

NIH3T3 cells (RCB1862) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, ICN Biochemicals, Aurora, OH, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded in 24-well plates at a density of 5×10^4 cells per well 1 day before transfection. Then, 200 ng of *Per2–CLuc* and 10 ng of the *Renilla* luciferase expression vector phRL–TK (Promega, Fitchburg, WI, USA) were cotransfected with or without expression vectors of hBMAL1, hCLOCK, and mCRY1 using Lipofectamine PLUS (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total DNA per well was set to 510 ng by adding pcDNA3. Next, 24 h after the transfection, culture medium was collected and cells were washed once with 300 μl of cold phosphate-buffered saline (PBS) and disrupted in 300 μl of 10 mM Tris–HCl (pH 7.4).

Bioluminescence assay

The standard curve of CLuc bioluminescence was made using CLuc standard enzyme and *Cypridina* luciferin (ATTO) for the range from 0.1 fg/well to 1 ng/well. A serial dilution of CLuc enzyme was prepared according to the manufacturer's instructions and mixed with the same volume of 200 nM *Cypridina* luciferin in 60 mM phosphate buffer (pH 6.4) containing 0.3 M ascorbic acid and 20 mM Na_2SO_4. Bioluminescence was measured using an AB-2250 luminometer (ATTO) for 10 s in rat specimen and for 20 s in transient transfection. For measuring plasma CLuc bioluminescence, 100 μl of diluted plasma (containing 2 μl of plasma) was mixed with 100 μl of 200 nM *Cypridina* luciferin dissolved in the same buffer described above. For measuring bioluminescence of culture medium, 50 μl of culture medium was mixed with 50 μl of *Cypridina* luciferin solution (200 nM luciferin for rat specimens and 280 nM for transient transfection). In the transient transfection experiments, cotransfected *Renilla* luciferase activity was measured separately for 20 s by mixing 50 μl of the cell lysate and 50 μl of 200 μM coelenterazine (Sigma–Aldrich) dissolved in 10 mM Tris–HCl (pH 7.4). The CLuc activity was normalized to the *Renilla* luciferase activity.

Animals

Four lines of transgenic rats of the Wistar strain carrying a reporter gene were developed. Genotyping of the transgene was done by PCR amplification of tail DNA using the following set of primers: 5'-GACAAGACTGGAAGTGGCTGGAGAC-3' and 5'-CAGATCACAGGCTCCTTCAGCATCA-3'. The size of the PCR product was 1015 bp. In addition, bioluminescence of tail samples was examined using 50 μl of 100 nM luciferin dissolved in 10 mM Tris–HCl (pH 7.5) to select transgenic rats. Among offspring of the founder rats, two lines (nos. 28 and 35) were subjected to further experimentation because of higher bioluminescence in the culture medium of various tissues than the rest of the lines. In total, 57 rats were used in the current experiment (wild type, $n = 10$; *Per2–CLuc* no. 28, $n = 11$; *Per2–CLuc* no. 35, $n = 36$).

Transgenic rats were reared in our animal quarters, where the environmental conditions were controlled (12 h light:12 h dark [LD], lights on 6:00–18:00, light intensity ~100 lux) as described elsewhere [12]. Pups were weaned at 21 days of age. They were housed in a cage (30 × 24 × 17.5 cm) with 2 or 3 littermates until the start of the experiments. Only heterozygous adult rats were used for the current experiments. Males were used for behavior recording or blood sampling, and females were used for tissue cultures. Wild-type male littermates were also used as the controls of behavioral rhythm and for measuring the background level of plasma bioluminescence. The current experiments were approved by the animal research committee of Hokkaido University (approval no. 08-0278). Animals were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals at Hokkaido University.

Behavior recording and analyses

Behavior rhythms were measured in three experiments using adult male rats. They were housed individually in a light-tight, soundproof recording box (60 × 60 × 60 cm). Environmental conditions of the recording box were the same as those of the animal quarters except for the light intensity at the surface of the cage (300 lux). Spontaneous locomotor activity was monitored every minute with an infrared thermal sensor, as described elsewhere [12]. Collected data were fed into a PC system (Stanford Chronobiology Kit, Stanford Software Systems) and analyzed by Clock Lab (Actimetrix, Wilmette, IL, USA).

In the first experiment, entrainment to the LD cycle, the free-running period in constant darkness (DD), and phase shifts by brief light pulses were examined in 22 rats (2 months of age at the beginning of experiment; wild type, $n = 8$; no. 28, $n = 7$; no. 35, $n = 7$). After measuring spontaneous activity for 2 weeks under LD (lights on 6:00–18:00), rats were exposed to DD. On the 15th to 21st days in DD and 3 weeks later, rats were exposed to a single light pulse of 300 lux for 30 min at circadian time 23 (CT23) and CT15, respectively, where the activity onset phase was defined as CT12. In the second experiment, the effects of 8 h of phase delay