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Bidirectional role of orphan nuclear receptor RORα in clock gene transcriptions demonstrated by a novel reporter assay system

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Abstract Circadian rhythms are generated by an extremely complicated transcription–translation feedback loop. To precisely analyze the molecular mechanisms of the circadian clock, it is critical to monitor multiple gene expressions and/or interactions with their transcription factors simultaneously. We have developed a novel reporter assay system, the tricolor reporter in vitro assay system, which consists of green- and red-emitting *Phrixothrix* luciferases as dual reporters and blue-emitting *Renilla* luciferase as internal control. We have successfully employed this system in analyzing the effects of clock gene products on the enhancer elements of *Bmal1* and *Per1* promoters. The results indicate that the orphan nuclear receptor RORα regulates bidirectionally *Bmal1* (positively) and *Per1* (negatively) transcriptions simultaneously.

Keywords: Circadian clock; Reporter assay; Green- and red-emitting luciferases; RORα; BMAL1

1. Introduction

Circadian clock controls a wide variety of physiological and molecular activities in most organisms. In mammals, the core clock lies in the suprachiasmatic nucleus (SCN) of the hypothalamus [1,2]. The molecular core of the biological clock is a transcription–translation autoregulatory feedback loop involving several clock genes and their products [3–5]. CLOCK and BMAL1, both basic helix–loop–helix/PAS-type transcription factors, form a heterodimer and activate the transcription of another clock gene, *Per*, through E-box enhancers (5′-CACGTG-3′) in the 5′ upstream region. The gene product of *Per*, together with the CRY protein, suppresses their own gene transcriptions. The turnover of these inhibitory proteins then leads to a new cycle of activation of CLOCK and BMAL1. This auto-feedback loop is considered to be the molecular machinery of the biological clock. On the other hand, *Bmal1* expression appears to be controlled positively by PER and CRY [6] via an unknown mechanism, demonstrating that there must be another loop interlocked with the core one. Recently, a Rev-Erb and retinoic acid receptor-related orphan receptor (ROR) response element (RORE, 5′-AGGTCA-3′), which is a recognition sequence for members of Rev-Erb and ROR orphan nuclear receptor families, has been identified in the 5′ upstream region of *Bmal1* and may play an important role in the circadian expression of *Bmal1* [7,8]. However, the mechanisms of regulation via this element are poorly understood. Thus, molecular mechanism of the circadian clock seems to be more complex than previously thought. To more precisely analyze the circadian clock mechanism, it is critical to monitor simultaneously at least two gene expressions in the same cell population because the expressions of many genes are regulated concurrently by transcription factors.

To analyze the molecular mechanism of the auto-feedback loop, an in vitro dual reporter assay system using firefly and *Renilla* luciferases is widely employed. Typically, firefly luciferase is used for monitoring gene expression, whereas *Renilla* luciferase is used as an internal control for minimizing experimental variability. However, more than one gene expression cannot be monitored simultaneously by this system because one of the two reporting luciferases must be used as an internal control. To overcome the limitations of the reporter assay system, additional luciferases and a novel measurement technique are required to monitor two gene expressions at once. We have recently succeeded in expressing the green- and red-emitting luciferases (green and red luciferases) of *Phrixothrix* railroad-worms [9] in cultured mammalian cells, and shown that these emitters satisfy all the conditions required for the simultaneous monitoring of two gene expressions [10].

In this study, we developed a tricolor reporter in vitro assay system in which two gene expressions can be monitored simultaneously by splitting the emissions from the green and red luciferases with a long pass filter. Using the system, the synergistic effects of transcription factors CLOCK, BMAL1 and RORα, which is assumed to be a new candidate regulator for *Bmal1*, on the enhancer elements of *Bmal1* and *Per1* promoters were monitored simultaneously and directly compared in the same cells. Furthermore, the effects of RORα on the transcription of the enhancer element and the promoter fragment of *Bmal1* were directly compared.

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2. Materials and methods

2.1. Plasmids

Expression plasmids carrying green and red luciferases, pMK10K-ΔIR-Gr and pMK10K-ΔIR-Red, in which luciferases were expressed under the control of the CAG promoter, which is composed of a CMV immediate early enhancer, a chicken β-actin promoter, and a rabbit β-globin intron II, were constructed as reported previously [10]. To construct the green reporter plasmid carrying RORE, corresponding to the region (−13/+69) in the mouse *Bmal1* (*mBmal1*) promoter [6–8] (5′-GAGCGGATTGGTCGGAAAGTAGGTTAGTGGTGCGACA-TTTAGGGAAGGCAGAAAGTAGGTCAGGGACGGAGGTGC-CTGTTT-3′; RORE consensus sequences are underlined) was PCR-amplified with a Bp/915-Luc reporter vector [6], and the product was ligated into the *Bgl*II/*Hin*dIII site immediately upstream of the β-actin promoter of pMK10K-ΔIR-Gr, resulting in pRORE-Gr. To construct the red reporter plasmid carrying the E54 fragment, mutated red luciferase (mRed), the codons of which were optimized for mammalian expression (unpublished data), was excised with *Nco*I and *Xba*I from SYN325, and ligated into the *Nco*I/*Xba*I site of E54TK [11] firefly reporter plasmid in which the firefly luciferase had been removed, resulting in pE54-mRed. The E54 fragment consisted of three E-boxes within 2.0 kb of the 5′ flanking region of the mouse *Per1* (*mPer1*) gene with their immediate flanking sequences linked together with 5′-TTTAGCCACGTGACAGTGTAAGCACACGTGGGCCCTCAAG TCCACGTGCAGGGA-3′(E-boxes are underlined), as reported previously [11,12]. Bp/915-mRed reporter plasmid carrying the 5′ flanking region (−816/+99) of *mBmal1* was constructed by replacing the *Nco*I/*Xba*I fragment of Bp/915-Luc with mRed from SYN325. cDNA coding for the full open reading frame (ORF) of mouse RORα4 (mRORα4) was amplified by reverse transcription PCR from a mouse testis cDNA library (Clontech, Palo Alto, CA) using 5′-GTAAGCTTCGGCGTAAAGGATGTATTTTGTG-3′ as forward primer and 5′-GCTGTCGACTTACCCGTCAATTTGCATGGCTG-3′ as reverse primer, and cloned into an expression plasmid, pCR3.1 (Invitrogen). The expression constructs consisted of the full ORF cDNAs encoding hBMAL1 (*hBmal1b* [13]) and human CLOCK (hCLOCK), which had been subcloned into an expression plasmid, pcDNA3. The cDNA of hCLOCK (KIAA0334) was kindly provided by Dr. T. Nagase. phRL-TK (Promega), which expresses the *Renilla* luciferase under the control of TK promoter, was used for normalizing transfection efficiency.

2.2. Cell culture and transfection procedures

Mouse NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (ICN Biochemicals, Aurora, OH) at 37 °C. Cells were seeded in 24-well plates at a density of 5×10^4 cells per well, one day before transfection. Transfection was carried out using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. Twenty-four to 48 h after transfection, the cells were washed once with 300 μl of cold phosphate-buffered saline (PBS) and disrupted on ice in 300 μl of cold PBS. Luciferase activity was then measured unless otherwise noted.

2.3. Measurement of bioluminescence spectra

Bioluminescence was measured using an AB-1850S spectrophotometer (ATTO, Tokyo, Japan). Measurement was carried out by injecting 15 μl of PicaGene as a substrate (TOYO Ink, Tokyo, Japan) into 15 μl of the transfected cell extract, and bioluminescence spectra were collected for 2 min. All spectra were corrected for the photosensitivity of the equipment and normalized.

2.4. Measurement of luciferase activity by dual reporter assay system

Briefly, 50 μl of cell lysate in PBS was mixed with 50 μl of PicaGene (for firefly, green and red luciferases) or 50 μl of 250 nM coelenterazine (for *Renilla* luciferase (Sigma)), and then measured using a AB-2200 (ATTO) photometer for 20 s.

2.5. Measurement of luciferase activity by tricolor reporter assay system

Fifty microlitre of cell lysate in PBS was mixed with 50 μl of PicaGene (for green and red luciferases), and then green and red luciferase activities were each measured for 45 s at 6 and 7 min in the absence or presence of a 600 nm long pass filter (R60, HOYA, Tokyo, Japan) using a dish-type luminometer (AB-2500, ATTO). The activities of green and red luciferases were calculated using the simultaneous equation

$$\begin{pmatrix} F(-) \\ F(+) \end{pmatrix} = \begin{pmatrix} 1.0 & 1.0 \\ \kappa G & \kappa R \end{pmatrix} \begin{pmatrix} G \\ R \end{pmatrix}$$

where G and R are green and red luciferase activities, $F(-)$ is the total relative light units (RLU) measured in the absence of the optical filter, $F(+)$ is the RLU that pass through the filter, and κG (0.08) and κR (0.76) are the transmission coefficients of the green and red luciferases, respectively. To normalize green and red luciferase activities, *Renilla* luciferase activity was measured by mixing 50 μl of cell lysate with 50 μl of 250 nM coelenterazine in an AB-2200 luminometer for 20 s.

3. Results

For simultaneous monitoring, luciferases must satisfy the following conditions: (i) the colors must be separable using an optical filter; (ii) the number of substrates must be a minimum; (iii) the cellular half-lives must be very similar; (iv) the basal activities must also be very similar; and (v) the bioluminescence spectra must remain constant even if the intracellular pH changes. As the green and red luciferases of *Phrixothrix* satisfy the above conditions [10], we have developed a tricolor reporter in vitro assay system in which the luciferases are used as emitters for the simultaneous monitoring of two gene expressions (Fig. 1). Briefly, cells are transfected with three reporter plasmids, two of which are green and red reporters harboring clock genes, and one is a *Renilla* reporter harboring a constitutive promoter (e.g., TK or SV40) as an internal control. Green and red luciferase activities are measured in the extracts of the transfected cells after adding Firefly luciferin as a substrate. Each activity is measured simultaneously using a luminometer equipped with an optical filter. *Renilla* luciferase activity is then measured by adding *Renilla* luciferin (coelenterazine) for normalizing green and red luciferase activities.

Fig. 2A shows the bioluminescence spectrum of the co-expressed green and red luciferases in NIH3T3 cells (blue line), the transmission spectrum of the 600 nm long pass filter for

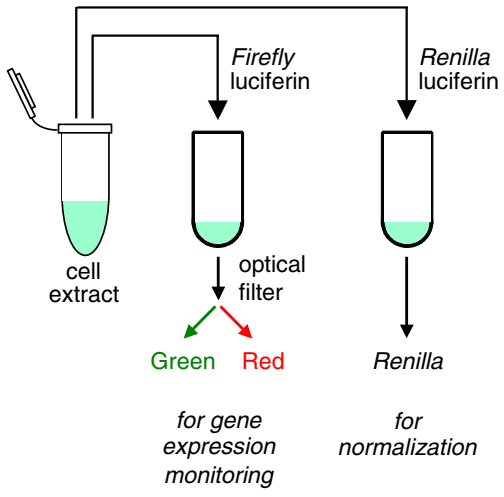

Fig. 1. Schematic of the measurement of luciferase activities by the tricolor reporter in vitro assay system. Activities of green and red luciferases were measured simultaneously by splitting emissions using an optical filter to estimate the transcriptional activities of the two test promoters, and *Renilla* luciferase activity was measured for normalizing green and red luciferase activities.

splitting emissions (orange line), and the simultaneous equation for calculating green (G) and red (R) luciferase activities. Splitting of the emissions using a long pass filter is advantageous in that emission loss is less than that when interference filters are used, thereby improving the signal-to-noise ratio. Fig. 2B shows an example of the reaction kinetics of the green and red luciferases expressed separately in NIH3T3 cells. Both activities increased slowly after the addition of luciferin (PicaGene, time = 0 min) and became constant after 6 min. Therefore, $F(-)$ (open bar) and $F(+)$ (grey bar) were each measured for 45 s at 6 min and 7 min after the addition of luciferin, respectively. It is noted that the kinetics of the green and red luciferases are identical even when the luciferase concentrations used by Fig. 2B are increased to 20-fold or decreased to 50-fold (data not shown). To examine whether the respective activities of the green and red luciferases in a mixture can be measured simultaneously, the activities in mixtures with various volume ratios were measured (Fig. 2C). The measured activity of each luciferase increased in proportion to the amount added and showed good linearity. Thus, this reporter assay system can simply and quantitatively measure two reporter activities by splitting the emissions using an optical filter. We therefore applied this system to the analysis of biological clock mechanisms.

It is well known that the transcription of *Per1* is positively regulated by CLOCK-BMAL1 via E-box elements in its promoter region, whereas *Bmal1* transcription is suggested to be regulated negatively by Rev-Erbα via RORE [7], which has been identified as a response element for orphan nuclear receptor families Rev-Erb and ROR. However, the role of the orphan nuclear receptor RORα in the transcription of *Bmal1* is not known, although the rhythmic expression of RORα mRNA has been clarified in SCN [8,14]. To examine in detail how these transcription factors affect concurrently the transcription of two clock genes, we measured the effects of CLOCK-BMAL1 and RORα4, which is an isoform of RORα, on the RORE of *mBmal1* and the E-box of *mPer1* promoters simultaneously by means of the tricolor reporter in vitro assay system (Fig. 3A). Green luciferase reporter plasmid carrying RORE (pRORE-Gr), red luciferase reporter plasmid carrying E54 (the 5′ flanking region of *mPer1* containing 3 E-boxes, pE54-mRed), and *Renilla* luciferase reporter as internal control were each co-transfected with pairwise combinations of expression plasmids into NIH3T3 cells. The respective activities of the green and red luciferases were measured and calculated as described above, and normalized by *Renilla* luciferase activity.

As shown in Fig. 3A, when RORE- (green bars) and E54- (red bars) mediated transcriptions were monitored simultaneously, RORα4 was found to markedly enhance RORE-mediated transcription, suggesting that RORα4 activates the transcription of *Bmal1* via RORE. On the other hand, RORα4 did not affect E54-mediated transcription. By contrast, CLOCK-BMAL1 enhanced E54-mediated transcription, as reported previously [11,12], but did not affect RORE-mediated transcription. Interestingly, when CLOCK, BMAL1 and RORα4 were co-expressed, CLOCK-BMAL1-induced transcription via E54 element was notably suppressed by RORα4, whereas RORα4-induced transcription via RORE was not markedly affected by CLOCK-BMAL1. These results strongly suggest that RORα4 positively regulates *Bmal1* transcription, whereas it negatively regulates CLOCK-BMAL1-induced transactivation of *Per1*. The identical results were also obtained in an independent measurement by a dual reporter assay system (Fig. 3B), indicating that both RORE- and E54-mediated transcriptions can be monitored simultaneously and quantitatively by the novel reporter assay system.

To examine whether the transcription of the *Bmal1* promoter is activated by RORα4, and to compare directly the effects of RORα4 on the transcription from RORE and the *Bmal1* promoter, both transcriptional activities were measured simultaneously (Fig. 4). Reporter plasmids pRORE-Gr and Bp915-mRed, which carry 915 bp of the 5′ flanking region of *mBmal1* (−816/+99) [6], were co-transfected with various

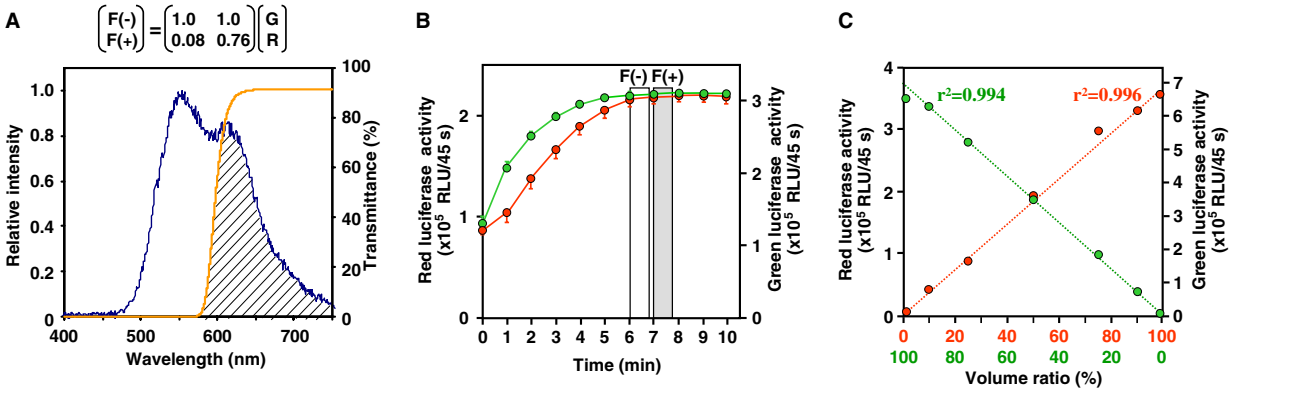

Fig. 2. Estimation of green and red luciferase activities by splitting emissions using a long pass filter. (A) Bioluminescence spectrum of NIH3T3 cells co-expressing green and red luciferases (blue line) and transmission spectrum of a 600 nm long pass filter used (orange line). The activities of green and red luciferases were calculated using a simultaneous equation, where G and R are the activities of green and red luciferases, respectively. $F(-)$ is the RLU measured in the absence of the long pass filter and $F(+)$ is the RLU measured in the presence of the filter (shadowed area). Factors 0.08 and 0.76 are the transmission coefficients of green and red luciferases, respectively. (B) The reaction kinetics of green (green line) and red (red line) luciferases. Expression plasmids pMK10-Δ-IR-Gr and pMK10-Δ-IR-Red were independently transfected into NIH3T3 cells. The cells were disrupted in 300 μl of PBS 48 h after transfection. The kinetics were each measured for 45 s after mixing 50 μl of the extracts and 50 μl of luciferin (PicaGene, time = 0 min) in a 35 mm dish, using a dish-type luminometer (model AB-2500, ATTO). The values are plotted as means ± S.D. ($n = 4$). (C) Quantitative relationship between green (green line) and red (red line) luciferase activities in a mixture of both, measured by the tricolor reporter in vitro assay system. Green and red luciferase activities were each measured for 45 s at 6 and 7 min with a 1 min interval after mixing 50 μl of the cell extract expressing luciferase and 50 μl of PicaGene in a 35 mm dish, using the AB-2500 luminometer.

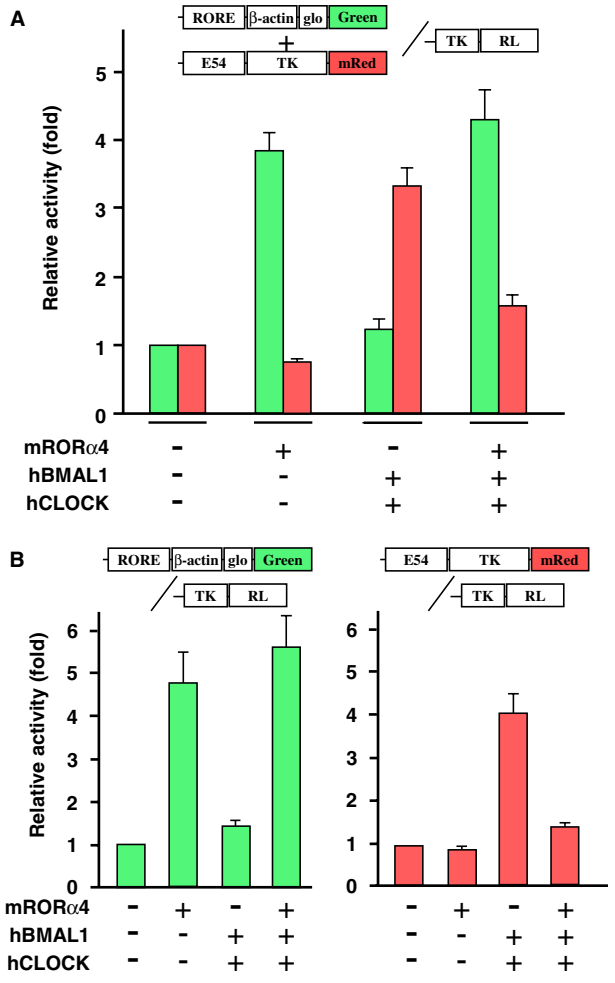

Fig. 3. Synergistic effects of transcription factors mRORα4, hBMAL1 and hCLOCK on RORE- and E-box-mediated transcriptions measured by the tricolor (A) and the dual (B) reporter assay systems. (A) RORE- and E-box-mediated transcriptions were simultaneously measured with green (green bars) and red (red bars) luciferases, respectively, using the tricolor reporter in vitro assay system. Green and red luciferase activities were measured as described above. *Renilla* luciferase activity was measured by mixing 50 μl of 250 nM of coelenterazine using luminometer AB2200 (ATTO). (B) RORE- and E-box-mediated transcriptions were independently measured with green (left panel) and red (right panel) luciferases, respectively, using the dual reporter assay system. Green, red and *Renilla* luciferase activities were measured as described in Section 2. In all transfection experiments, reporter plasmids pRORE-Gr and/or pE54-mRed (30 ng) and 3 ng of *Renilla* reporter plasmid (phRL-TK) were co-transfected with (+) or without (−) the indicated expression plasmids (50 ng) into NIH3T3 cells. The amount of DNA added per well was kept constant (213 ng) by adding pBluescript SK(−). The cells were disrupted in 300 μl of PBS 24 h after transfection. All values are shown as multiples (means ± S.D., $n = 6$) of the control (lacking the expression plasmid). The schematic of the reporter plasmids shows the location of elements. *Key*: β-actin, β-actin promoter; glo, β-globin intron II; Green, green luciferase; Red, red luciferase; TK, TK promoter; and RL, *Renilla* luciferase.

amounts of RORα4 expression plasmid and *Renilla* luciferase reporter plasmid as an internal control into NIH3T3 cells. RORα4 produced substantial increases in the transcriptional activities from both RORE (green bars) and the *Bmal1* promoter (red bars) in a dose-dependent manner, suggesting that RORα4 acts as transcriptional activator of *Bmal1* via RORE. The dose dependence of the RORα4-induced transcription of

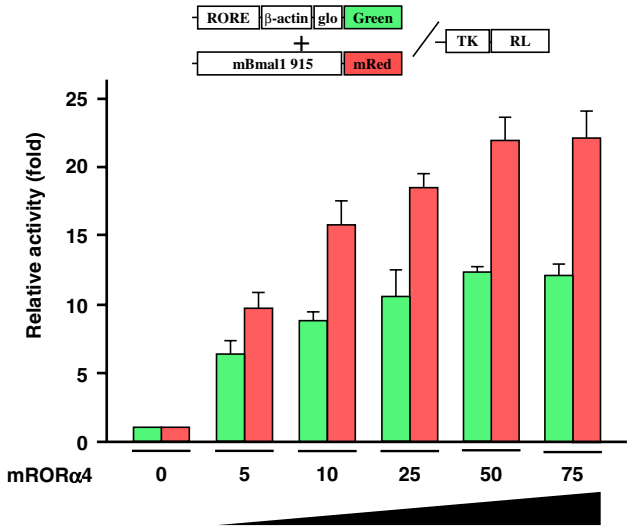

Fig. 4. Simultaneous monitoring of dose-dependent induction of RORE-mediated (green bars) and *mBmal1* promoter fragment-driven (red bars) transcriptions by mRORα4. Reporter plasmids pRORE-Gr and Bp915-mRed (30 ng) and 3 ng of phRL-TK were co-transfected without (0) or with the indicated amounts (in nanograms) of expression plasmids carrying mRORα4 into NIH3T3 cells. The amount of DNA added per well was kept constant (138 ng) by adding pBluescript SK(−). The cells were disrupted in 300 μl of PBS 24 h after transfection. All values are shown as multiples (means ± S.D., $n = 6$) of the control (lacking the expression plasmid). The schematic of the reporter plasmids shows the location of elements. *Key*: β-actin, β-actin promoter; glo, β-globin intron II; Green, green luciferase; Red, red luciferase; TK, TK promoter; and RL, *Renilla* luciferase.

RORE was identical to that of the *Bmal1* promoter, but the response of the *Bmal1* promoter to RORα4 was greater than that of RORE. The result suggests that additional enhancer element(s) that responds to RORα4 may exist in the 5′ flanking region of the *Bmal1* promoter.

4. Discussion

We were able to monitor simultaneously the effects of transcription factors on the expression of two clock genes, *Bmal1* and *Per1*, using the tricolor reporter in vitro assay system. In addition, the effects of RORα on the transcriptions from enhancer element and the promoter fragment of *Bmal1* were directly compared. Results that were identical to those shown in Figs. 3A and 4 were also obtained in an independent measurement by a dual reporter assay system that used green, red and firefly luciferases as reporting luciferases and *Renilla* luciferase as internal control (Fig. 3B and data not shown). This clearly indicates that the tricolor reporter in vitro assay system can accurately monitor respective transcriptional activities even when these are measured simultaneously, and that the simultaneous equation for calculating the activities of green and red luciferases by splitting the emissions (Fig. 2A) is valid.

Using this system, we demonstrated for the first time that the orphan nuclear receptor RORα plays a bidirectional role in RORE-mediated *Bmal1* and E-box-mediated *Per1* transcriptions (Fig. 3A). RORα is critically involved in many physiological functions in several organs [15–17] and regulates a wide spectrum of gene expressions by binding to RORE in the

promoter region as a monomer. In the *mBmal1* promoter, two RORE have been identified in the proximity of the transcription initiation site [8,14]. By electrophoretic mobility shift assay, we confirmed that the in vitro translated-RORα4 protein binds monomerically to a fragment of the *mBmal1* promoter containing two RORE, but the binding is abolished by the mutation of the two RORE (unpublished data). Substantial transactivation by RORα4 through RORE (Fig. 3A) was also confirmed by conducting co-transfection experiments using an *mBmal1* promoter fragment-driven reporter plasmid (Fig. 4), and mutation of RORE in the promoter resulted in failure of the transactivation (unpublished data). From these observations, we conclude that RORα4 positively regulates *Bmal1* transcription by directly binding to RORE in the *Bmal1* promoter.

It is known that Rev-Erbα negatively, rather than positively, regulates many gene expressions by recognizing RORE [15,18,19]. Recently, it has been reported that Rev-Erbα mRNA displays circadian expression in SCN [8], and that knockout of this gene abolishes the circadian expression of *Bmal1* concomitant with the constitutive accumulation of *Bmal1* mRNA at high levels [7]. Rev-Erbα is therefore suggested to play the role of a crucial negative regulator of *Bmal1*. As RORα and Rev-Erbα share their recognition sequence, we assume that RORα and Rev-Erbα may competitively regulate *Bmal1* transcription, although there is no direct evidence that Rev-Erbα suppresses *Bmal1* transcription by directly binding to RORE.

The present study also showed that RORα4 markedly suppressed CLOCK-BMAL1-induced E54-mediated transactivation of *Per1*, concurrent with the transactivation of *Bmal1* (Fig. 3A). Because the RORα orphan nuclear receptor does not bind to E-box, the suppressive effect is not likely due to direct interference with the binding of CLOCK-BMAL1 to the E-box of *Per1*. Although the detailed mechanisms underlying the suppression are not yet known, it may be due to the activation of a negative regulator(s) of *Per1*, such as CRY1 and CRY2, or to the transactivation of *Bmal1* and the subsequent up-regulation of a negative regulator(s). Recently, Yu et al. [6] reported that *Bmal1* transcription is activated by CRY1, CRY2 and PER2, whereas *Per1* transcription is suppressed by these, and proposed that a BMAL1 negative feedback loop interlocks with the CRY and PER2 negative feedback loop inter-activation, forming a third positive forward loop. As the effects of RORα4 on the transcriptions of *Bmal1* and *Per1* are consistent with those of CRYs and PER2, RORα may regulate positively *Bmal1* transcription and negatively *Per1* transcription, in cooperation with CRY1, CRY2 and PER2. This may be supported by the finding that the mRNA of RORα shows rhythmic expression in SCN in phase with *Cry* [8,14], although the precise mechanism remains to be determined.

In conclusion, we have developed a novel tricolor reporter in vitro assay system and have successfully employed it in elucidating the bidirectional role of the orphan nuclear receptor RORα in *Bmal1* and *Per1* transcriptions. This system can be easily developed for monitoring four or more gene expressions using additional luciferases (such as orange-emitting luciferase [20]) and optical filters, and for monitoring real-time rhythmic expression profiles in vivo. Our simultaneous monitoring system will help determine the molecular mechanisms of the circadian clock feedback loop.

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