Nuclear Receptor-mediated Cell-autonomous Oscillatory Expression of the Circadian Transcription Factor, Neuronal PAS Domain Protein 2 (NPAS2)*

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Background: NPAS2 is a circadian transcription factor responsive to a wide range of intra- and extracellular stimuli. Results: Deficiency of RORα caused a damped transcriptional oscillation of Npas2 and functional depletion of a RORE resulted in a complete loss of the oscillation. Conclusion: Nuclear receptors elicit cell-autonomous circadian transcription of Npas2. Significance: Synchronous transcriptional oscillation of Npas2 with Bmal1 provides the foundation for efficient circadian input and robust oscillation.

NPAS2 (MOP4) is a heme-containing sensor transcription factor responsive to a wide range of intra- and extracellular stimuli, which also functions as a circadian transcription factor. This molecule forms a heterodimer with another circadian transcription factor, BMAL1, and activates transcription via E-box elements, indicating that circadian phase synchronization between NPAS2 and BMAL1 expression is important for the efficient transcriptional activation of target genes. However, details of the mechanism of cell-autonomous circadian transcription of Npas2 remain unclear. Here, we show that one of the ROREs (retinoid-related orphan receptor response elements) in the upstream region of the transcription start site is essential for circadian transcription of the Npas2 gene. Furthermore, we also show that endogenous RORα indeed plays an essential role in cell-autonomous circadian transcription of Npas2, because a damped transcriptional oscillation was observed not only by introduction of a dominant negative form or small interfering RNA but also in embryonic fibroblasts obtained from RORα/s and Npas2/s mice. These results indicate that circadian transcription of Npas2 is synchronized with that of Bmal1 in a cell-autonomous nuclear receptor-mediated fashion.

NPAS2, a basic helix-loop-helix PAS protein, has been reported as a heme-containing sensor transcription factor responsive to a wide range of intra- and extracellular stimuli (1–4). NPAS2-deficient mice show altered patterns of sleep, behavioral adaptability, and circadian abnormalities (5–7). Generally, bHLH-PAS transcription factors, including NPAS2, form heterodimers in various combinations, enabling the activation of various sets of genes for appropriate responses to environmental stimuli (8).

The clock components BMAL1 and CLOCK are also known as bHLH-PAS transcription factors. Many previous reports have demonstrated that these two circadian transcription factors form a heterodimer to activate downstream genes such as the clock genes Period (Per) and Cryptochrome (Cry) (9–11). NPAS2 also shows functional interaction with BMAL1, which leads to the activation of clock and clock-controlled genes (3, 12). In the central nervous system, Npas2 may play more important roles than Clock (6). Circadian oscillation in Npas2 transcription is observed in various tissues (13), suggesting that the gene product plays a ubiquitous role in the cell-autonomous core clock.

The circadian phase of Npas2 transcription is synchronized with that of Bmal1 (13), strongly indicating the mechanism for efficient activation of target genes. Although the Bmal1 gene has been well analyzed (14–16), Npas2 remains unclear in light of the mechanism of cell-autonomous circadian oscillation in transcription. A previous report showed that RORα activates and REV-ERBα suppresses Npas2 transcription via two ROREs located in the upstream region of the transcription start site (17). To continue this line of enquiry, we examined whether the RORE-mediated regulation of transcription indeed played a role in driving oscillation in Npas2 transcription.

MATERIALS AND METHODS

Plasmid Construction—The hNpas2 promoter region was isolated and cloned in the pGL3-basic vector (Promega). The hNpas2 region spans from –3063 to +192 (+1 is the putative transcription start site). The coding regions of hRORα and...
mREV-ERBα were ligated into the pcDNA3 expression vector (Invitrogen). To generate a dominant-negative mutant of RORα, the N-terminal fragment of hRORα1 (1–705), which lacks the ligand-binding domain, was subcloned into pcDNA3. To express siRNA, we constructed siRNA expression vectors as reported previously (16).

Transfection and Reporter Assay—NIH3T3 cells were cultured and transfected as described previously (16). Cells were harvested 24 h after transfection, and cell lysates were prepared and then used in the Dual-Luciferase assay system (Promega).

Real-time Monitoring of Luciferase Activity in Living Cells—NIH3T3 cells were cultured, transfected with the promoter-luciferase constructs, and incubated for 24 h. The medium was then exchanged for serum-rich medium (DMEM, supplemented with 50% serum). Two hours later, this medium was replaced with normal culture medium. In the presence of 0.1 mM luciferin, light emission was measured and integrated for 1 min at intervals of 15 min, with a photomultiplier tube (Hamamatsu Photonics). Data sets were detrended by subtracting the 24-h running average from raw data. Relative amplitudes were calculated as trough-to-peak heights. The first amplitude was set to 1.

Pulldown Experiment—NIH3T3 and liver extracts were prepared by homogenizing in ice-cold incubation buffer, and then the extracts were incubated with a double-stranded biotinylated oligonucleotide, including the REV-ERB/ROR response elements, which had been immobilized on streptavidin-Sepharose beads (Amersham Biosciences). After washing with the incubation buffer, the resulting bound protein was subjected to immunoblot analysis with anti-RORα antibody (Santa Cruz Biotechnology, sc-6062).

RESULTS AND DISCUSSION

Circadian Phase of Cell-autonomous Transcriptional Oscillation of Npas2 Is Closely Similar to That of Bmal1—According to previous reports, the circadian pattern of Npas2 mRNA levels is well synchronized with that of Bmal1 mRNA levels in various tissues in vivo (13). To examine whether this synchronization is due to systemic factors or cell-autonomous regulation, we monitored circadian phases of Npas2 (−3.0 kb) promoter-driven luciferase activity in real time using a cell-based system (Fig. 1A) and compared with results with those for Bmal1-luc (−3.5 kb) (Fig. 1B). In a previous study, we found that the murine cell line NIH3T3 possessed a functional circadian clock (18). The promoter-luciferase constructs were therefore introduced into NIH3T3 cells, which were subsequently serum-synchronized for real-time monitoring of circadian gene expression. The data clearly showed that both the Bmal1 and Npas2 genes were expressed in an almost completely synchronized manner, suggesting that the synchronization was due to some cell-autonomous regulation. In addition, the data also indicate that these cloned promoter regions contained elements essential for the synchronization.

It has been reported that RORα and REV-ERBα regulate cell-autonomous circadian transcription of Bmal1 via two ROREs (14–16). Similarly, an in silico study revealed that putative two ROREs (shown as RORE1 and RORE2 in Fig. 1A) are located in the upstream region from the transcription start site of Npas2 (13). Six years after the report, based on a transient reporter assay, another study showed that the Npas2 promoter is activated by RORα and suppressed by REV-ERBα via two ROREs (one is RORE1 mentioned above and the other is a novel one shown as RORE3 in Fig. 1A) (17), indicating that cell-autonomous circadian transcription of Npas2 may be also regulated by the ROR-REV-ERB system. To test the possibility that RORα indeed functions as a contributor to cell-autonomous circadian transcription of Npas2, we introduced a dominant-negative RORα into NIH3T3 cells and found that Npas2 oscillation was severely dampened by overexpression of the protein (Fig. 1C). This result indicates the involvement of endogenous RORα in cell-autonomous circadian transcription of Npas2.

Only One RORE Is Essential for Nuclear Receptor-mediated Regulation of Npas2 Transcription—To investigate whether ROREs located in the upstream region of the transcription start site are functional for circadian transcription of the Npas2 gene, we produced 5′-deleted and RORE site-mutated Npas2 promoters (Fig. 2A) and performed Dual-Luciferase assays with these constructs (Fig. 2, B and C). The data obtained from the 5′-deleted constructs showed that the promoter region from −1.6 kb to −0.8 kb was essential for both RORα-mediated activation and REV-ERBα-mediated suppression of Npas2 transcription. This region contained two RORE consensus elements, RORE1 and RORE2. However, these data demonstrated that RORE3 was not responsive to the nuclear receptors, which is inconsistent with the previous report that showed the binding of in vitro synthesized RORα to an EMSA probe containing RORE3 (17). Moreover, we examined whether the RORα- and REV-ERBα-mediated effects are dependent on both RORE1 and RORE2 by using the site-mutated Npas2 promoter constructs. The results revealed that only one RORE (RORE1) was functional for transcriptional regulation by RORα and REV-ERBα.

Next, we performed Npas2 oligodeoxynucleotide pulldown assays and confirmed that endogenous RORα expressed in NIH3T3 cells and the liver bound to a fragment of the Npas2 promoter containing RORE1 and RORE2 (Fig. 2D). Interestingly, mutation of RORE1 caused a complete loss of endogenous RORα binding to the DNA fragment, indicating that the single RORE site plays a major role as the RORα-responsive element, consistent with the data obtained from our reporter assays in Fig. 2, B and C.

RORE1 Is Indispensable for Cell Autonomic Circadian Transcription of Npas2—These data clearly demonstrate that RORα and REV-ERBα regulate Npas2 transcription via RORE1 and therefore strongly indicate that these nuclear receptors are responsible for cell-autonomous circadian oscillation of Npas2 transcription. To test this hypothesis, we performed real-time monitoring of Npas2 transcription by introducing promoter constructs into NIH3T3 cells. As expected, promoter constructs lacking a region containing RORE1 and RORE2 did not show cell-autonomous circadian transcription of Npas2 (Fig. 3, A and B, deletion mutants). Furthermore, the site-directed mutation of RORE1 caused an almost complete loss of circadian transcription (Fig. 3, A and B, site-directed mutants), whereas that of RORE2 showed no effect on it, which is consistent with the data obtained from the reporter and binding
assays in Fig. 2. As also expected from the data in Fig. 2, RORE3 did not have any effects on circadian transcription of \textit{Npas2}, indicating that EMSA assay may be inadequate for a definitive conclusion. Together, our data demonstrate that only one RORE is responsible for circadian oscillation of \textit{Npas2} transcription. It is well known that cell-autonomous circadian gene expression of \textit{Bmal1} is regulated by two ROREs (16), and this may therefore explain why \textit{Npas2} oscillation is less robust than \textit{Bmal1}, as shown in Fig. 1. Together, these findings show that among ROREs, RORE1 is indispensable for cell-autonomous circadian transcription of \textit{Npas2}.

Endogenous ROR\textalpha Contributes to Robustness of Cell-autonomous Circadian Oscillation of \textit{Npas2} Transcription—Endogenous ROR\textalpha in NIH3T3 cells site-specifically bound to the \textit{Npas2} promoter (Fig. 2D). To clarify whether the protein indeed plays any essential role in cell-autonomous circadian oscillation of \textit{Npas2} transcription, we performed RNAi-induced knockdown of endogenous ROR\textalpha in NIH3T3 cells (Fig. 4A). The data demonstrate that although scrambled small interfering RNA did not show any effect on circadian transcription of \textit{Npas2}, targeted knockdown of ROR\textalpha induced a damped oscillation in circadian expression (Fig. 4B). When combined with the results obtained with a dominant-negative form (Fig. 1C), the idea that endogenous ROR\textalpha contributes to robust cell-autonomous circadian oscillation of \textit{Npas2} transcription is convincing. Furthermore, to exclude the possibility of nonspecific effects of these two experimental approaches, we confirmed the contribution of endogenous ROR\textalpha by using embryonic fibroblasts obtained from staggerer mutant mice (Fig. 4C). The result shows a smaller amplitude of \textit{Npas2} oscillation in ROR\textalpha sg/sg fibroblasts (MEFs sg/sg) than that in wild-type mouse embryonic fibroblasts (MEFs ++/+) (Fig. 4D). Basal tran-
scriptional levels were gradually reduced, consistent with the idea that RORα is a transcriptional activator of Npas2. Taken together, these data provide compelling evidence that endogenous RORα plays an indispensable role in robust cell-autonomous circadian oscillation of Npas2 transcription. However, a functional deficiency in endogenous RORα did not lead to the complete loss of rhythmic expression of Npas2, indicating that REV-ERBα can maintain its transcriptional oscillation to some extent without functional RORα.

Nuclear Receptor-mediated Circadian Transcription of Npas2

RORE-mediated regulation of cell-autonomous circadian transcription may play a significant role in the robust circadian activation of target genes by the circadian positive regulators

![Region of the hNpas2 promoter required for RORα-mediated transactivation and REV-ERBα-mediated suppression.](image)

A. Schematic representation of deletion mutants (upper) and RORE mutants (lower) of the hNpas2 promoter. +1 corresponds to the transcription start site. RORE represents the consensus REV-ERB/ROR response element. B and C, identification of the region of the hNpas2 promoter required for RORα-mediated transactivation (B) and REV-ERBα-mediated suppression (C). Normalized fold suppressions and activations were calculated relative to luciferase activity in the absence of REV-ERBα or RORα. Data represent the mean ± S.D. of triplicate samples. D, NIH3T3-cell and liver extracts were incubated with double-stranded biotinylated oligonucleotides (Npas2 ODN) including RORE1 and RORE2, which was immobilized on streptavidin-Sepharose beads. The negative control samples were treated with streptavidin-Sepharose beads without an oligonucleotide (Unconjugated). The resulting precipitates were subjected to immunoblot analysis with anti-RORα antibody. MYC-RORα represents MYC-tagged RORα protein overexpressed in NIH3T3 cells, which was used as a positive control for Western blotting. Endo RORα represents endogenous RORα.
FIGURE 3. Mapping of the region of the hNpas2 promoter required for transcriptional oscillation. A, the cell culture-based luminescent reporter assay was performed with the mutant constructs indicated. NIH3T3 cells were transfected with hNpas2-luc constructs and then stimulated with a high concentration of serum. After the serum shock, in the presence of luciferin, light emission was measured and integrated for 1 min at intervals of 15 min. Peak values of the curves were set to 1 (horizontal scale, 1440 min = 1 day). For accurate comparison, the light gray line shows the curve for the full-length hNpas2-luc (~3.0 kb) construct. The data are representative raw data from three independent experiments.

FIGURE 4. Effect of endogenous RORα deficiency on the cell-autonomous circadian transcription of hNpas2. A, transcriptional oscillation of hNpas2 was monitored in the presence of a small interfering RNA. NIH3T3 cells were cotransfected with an RNAi vector and the hNpas2-luc construct and then stimulated with a high concentration of serum. For accurate comparison, the light gray line shows the curve for vector. Peak values of the curves were set to 1 (horizontal scale, 1440 min = 1 day). The siRNA expression vectors contain a scrambled-sequence (siCNT, as a negative control) or a 19-nt sequence derived from the Rorα transcript (siRORα). Vector represents cells transfected with empty siRNA expression vector. The data are representative raw data from three independent experiments. B, the data sets obtained from three experiments in A were detrended by subtracting the 24-h running average from raw data. Relative amplitudes were calculated as trough-to-peak heights. The first amplitude was set to 1. The data are represented as means ± S.E. of three independent experiments. C, wild-type (+/+ or RORα mutant mouse embryonic fibroblasts (sg/sg) were transfected with the hNpas2-luc construct and then stimulated with a high concentration of serum. The initial peak values of the curves were set to 1 (horizontal scale, 1440 min = 1 day). Two cell lines shown for each genotype were obtained from the same littermate. The data are representative raw data from three independent experiments. D, the data sets obtained from three experiments in C were detrended, and relative amplitudes were calculated as trough-to-peak heights. The first amplitude was set to 1. The data are represented as means ± S.E. of three independent experiments.
NPAS2 and BMAL1. Because RORα expression levels show a circadian change with relatively low amplitude, REV-ERBα would be a major driver of rhythmic transcription of Bmal1. In fact, Bmal1 transcription does not show circadian oscillation in Rev-erbα-deficient mice (14), indicating that the role of RORα is to enhance circadian amplitude in realizing circadian robustness. When the REV-ERB protein level decreases during the circadian night, RORα effectively activates Bmal1 and Npas2 transcription, which consequently enhances the amplitude of transcriptional oscillation and generates more robust and stable oscillations of the clock. These processes synchronize circadian oscillations between Bmal1 and Npas2 transcription, which enables efficient dimerization of BMAL1 and NPAS2, enhances the amplitude of downstream genes, and leads to robust behavioral and physiological circadian rhythms.

Several lines of evidence have contributed to the idea that RORα displays constitutive transcriptional activity without its ligand (19); however, the possibility remains that endogenous ligands can enhance this transcriptional activity, which might be observed in REV-ERBα-deficient mice (14), indicating that the role of RORα is to enhance circadian amplitude in realizing circadian robustness. When the REV-ERB protein level decreases during the circadian night, RORα effectively activates Bmal1 and Npas2 transcription, which consequently enhances the amplitude of transcriptional oscillation and generates more robust and stable oscillations of the clock. These processes synchronize circadian oscillations between Bmal1 and Npas2 transcription, which enables efficient dimerization of BMAL1 and NPAS2, enhances the amplitude of downstream genes, and leads to robust behavioral and physiological circadian rhythms.

As circadian transcription of Npas2 is detectable in almost all tissues, NPAS2 may ubiquitously and cell-autonomously control the core clock machinery. However, NPAS2 has been reported as a sensor transcription factor responsive to a wide range of intra- and extracellular stimuli, indicating that the protein has distinct functions from its parologue CLOCK. Various circadian input signals could thus phase-shift the cell-autonomous core clock directly via NPAS2.

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