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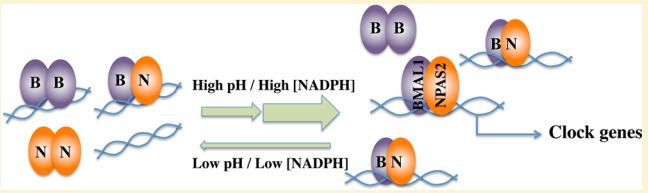
Changes in pH and NADPH Regulate the DNA Binding Activity of Neuronal PAS Domain Protein 2, a Mammalian Circadian Transcription Factor

Katsuhiro Yoshii, Fumihisa Tajima, Sumio Ishijima, and Ikuko Sagami*

Graduate School of Life and Environmental Science, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan

Supporting Information

ABSTRACT: Neuronal PAS domain protein 2 (NPAS2) is a core clock transcription factor that forms a heterodimer with BMAL1 to bind the E-box in the promoter of clock genes and is regulated by various environmental stimuli such as heme, carbon monoxide, and NAD(P)H. In this study, we investigated the effects of pH and NADPH on the DNA binding activity of NPAS2. In an electrophoretic mobility shift (EMS) assay, the pH of the reaction mixture affected the DNA binding activity of the NPAS2/BMAL1 heterodimer but not that of the BMAL1/BMAL1 homodimer. A change in pH from 7.0 to 7.5 resulted in a 1.7-fold increase in activity in the absence of NADPH, and NADPH additively enhanced the activity up to 2.7-fold at pH 7.5. The experiments using truncated mutants revealed that N-terminal amino acids 1−61 of NPAS2 were sufficient to sense the change in both pH and NADPH. We further analyzed the kinetics of formation and DNA binding of the NPAS2/BMAL1 heterodimer at various pH values. In the absence of NADPH, a change in pH from 6.5 to 8.0 decreased the K_D^{app} value of the E-box from 125 to 22 nM, with an 8-fold increase in the maximal level of DNA binding for the NPAS2/BMAL1 heterodimer. The addition of NADPH resulted in a further decrease in K_D^{app} to 9 nM at pH 8.0. Furthermore, NPAS2-dependent transcriptional activity in a luciferase assay using NIH3T3 cells also increased with the pH of the culture medium. These results suggest that NPAS2 has a role as a pH and metabolite sensor in regulating circadian rhythms.



In mammals, physiological and behavioral rhythms are controlled by the circadian molecular clock system.[1−3] The central clock, which exists in the hypothalamic suprachiasmatic nucleus (SCN), is entrained mainly by cycles of light and darkness, whereas the peripheral clocks in various tissues are synchronized by the central clock. At the molecular level, the circadian clock consists of transcriptional and translational feedback loops of clock genes and proteins.[1−3] As positive components, CLOCK and BMAL1 activate the transcription of various genes, including *Pers* and *Crys*. PERs and CRYs proteins eventually inhibit the transcriptional activity of CLOCK and BMAL1 as the negative components of the clock machinery.[4,5] In addition to this core loop, the orphan nuclear receptors RORs and Rev-erbs play important roles in the transcription of *Clock* and *Bmal1* genes, enhancing and inhibiting the transcription of these genes, respectively, via RORE sites to establish rhythmic expression.[6−9] The circadian clock generated by these transcriptional feedback loops is affected by various environmental stimuli, including light, foods, and hormones.[10−12] The clock proteins are also regulated at the post-transcriptional level, such as phosphorylation, sumoylation, and ubiquitylation, to ensure the maintenance of circadian rhythms.[13−18] Thus, various types of clock genes and proteins have been reported to date, and the understanding of their rigorous and concerted regulatory mechanisms for circadian clock is accelerating.[19]

Neuronal PAS domain protein 2 (NPAS2), a homologue of CLOCK, was originally discovered in the mammalian forebrain[20,21] but is also expressed in the periphery.[22,23] Similar to CLOCK, NPAS2 forms a heterodimer with BMAL1 that binds to E-box or E-box-like elements in the regulatory regions of numerous clock genes to activate the expression of these genes.[5,21,24] Although the analysis of knockout mice revealed an overlapping role of CLOCK and NPAS2 in the circadian clock,[22,23] *Npas2*-deficient mice, not *Clock*-deficient mice, are less able to adapt to restricted feeding, suggesting a specific role of NPAS2 in metabolism.[25] This was supported by the observations that single-nucleotide polymorphisms (SNPs) in *Npas2* have been linked to increased risks of cancer, metabolic syndrome, and hypertension.[26−28]

NPAS2 consists of a bHLH domain, two PAS domains (PASA and PASB), and a transactivation domain. The bHLH domain of NPAS2 serves as a basic DNA binding domain and a dimerization domain with other bHLH proteins. It was reported that the DNA binding activity of the NPAS2/BMAL1 heterodimer is enhanced by NADH and NADPH.[29] Our previous study revealed that the bHLH domain of NPAS2, containing N-terminal amino acids 1−61, was sufficient to form a heterodimer with BMAL1, to bind to E-box, and to sense

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NAD(P)H.[30] The PAS domains of NPAS2, however, serve as protein−protein interaction sites for dimerization and heme-containing gas sensor domains.[31,32] Although the two PAS domains bind heme, the main gas sensor is the PASA domain because the CO binding affinity of PASB-heme is <10% of that of PASA-heme.[31] The binding of CO to the heme of the PASA domain inhibits the DNA binding activity of the NPAS2/BMAL1 heterodimer. During these studies, we also found that the formation of the complex of the NPAS2/BMAL1 heterodimer with DNA is affected by pH.

Recently, two research groups reported that extracellular pH levels affect the expression rhythms of clock genes in cultured cells, indicating that pH could be a cue for the molecular clock.[33,34] However, little is known about the molecular mechanism of the regulation of circadian rhythm by pH. Nonetheless, it is known that pH plays a critical role in many cellular functions, such as the cell cycle, proliferation, metabolism, synthesis of cellular macromolecules, and signal transduction.[35] Therefore, the intracellular pH of mammalian cells is strictly maintained within a narrow range, even though it varies among different organelles. For example, the cytoplasmic and nuclear pH values are regulated at approximately 7.2, while those of mitochondria and lysosomes are approximately 8 and 4.7, respectively.[36] Additionally, the extracellular fluid has various pH values depending on the tissue and organ: the pH of plasma is slightly alkaline (7.3−7.4), while the digestive organs have a very low pH. Furthermore, extracellular acidification has been observed to be as low as pH 6.5 in tumor tissues in comparison to normal tissues.[37] Considering that NPAS2 is a multifunctional sensor responsive to various environmental stimuli, in this study, we examined the effects of pH on the DNA binding activity of NPAS2 *in vitro* and also NPAS2-dependent transcriptional activity in mammalian cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction. For a luciferase assay, plasmids *Npas2*/pCGN and *Bmal1*/pcDNA for expressing full-length murine NPAS2 (amino acids 1−816) and murine BMAL1 (amino acids 1−626), respectively, and a reporter plasmid, m*Per1p*/pSLO, containing the murine *Per1* enhancer/promoter region (nucleotides −1803 to +40) that has three canonical E-boxes (CACGTG) were constructed as previously described.[38] Another reporter plasmid, m*Per1p*-E3M/pSLO, was also prepared by a PCR-based method for mutation. In this reporter plasmid, all three E-boxes in the m*Per1* enhancer/promoter region were mutated to TCTAGA. The desired mutations were confirmed by sequencing. The plasmids for the EMS assay, NPAS2 bHLH-PASA/pET28a(+), NPAS2 bHLH/pET28a(+), and BMAL1 bHLH-PASA-PASB/pMAL-c2X, for expressing His-tagged NPAS2 bHLH-PASA (amino acids 1−240), NPAS2 bHLH (amino acids 1−61), and MBP-tagged BMAL1 bHLH-PASA-PASB (amino acids 1−447), respectively, were also constructed as previously described.[30,38] To create a truncated mutant of BMAL1, BMAL1 bHLH (amino acids 74−128), PCR was performed using pMAL-c2X containing BMAL1 bHLH-PASA-PASB cDNA as the template. The primers used for the PCR were 5′-GGGATCCCATATGAGGGAGGCCCACAGTC-3′ as the sense primer and 5′-CGAATTCGTCGACTAGGCACCTCTCAAAG-3′ as the antisense primer. The resulting product was subcloned into the *Nde*I and *Sal*I sites of pET28a(+). The desired construct was confirmed by sequencing.

Expression and Purification of Truncated NPAS2 and BMAL1 Proteins. The expression and purification of NPAS2 proteins (the isolated bHLH-PASA and bHLH domains of NPAS2) and BMAL1 proteins (the isolated bHLH-PASA-PASB and bHLH domains of BMAL1) were conducted basically as previously described,[30,39] except that *Escherichia coli* BL21-CodonPlus (DE3)-RIPL (Stratagene) was used as the host cell.

Briefly, the cell extract of *E. coli* BL21-CodonPlus (DE3)-RIPL cells expressing each NPAS2 protein was prepared by sonication in buffer A [50 mM sodium phosphate (pH 7.8), 50 mM NaCl, 10% glycerol, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, 1 mM PMSF, and 0.2 mM DTT]. After ultracentrifugation, the supernatant was applied to a Ni-NTA column (Qiagen) pre-equilibrated with buffer A. The column was washed with buffer A containing 20 and 70 mM imidazole for His-NPAS2 bHLH-PASA or 50 and 100 mM imidazole for His-NPAS2 bHLH. His-NPAS2 bHLH-PASA and His-NPAS2 bHLH were then eluted with buffer A containing 150 and 250 mM imidazole, respectively. His-NPAS2 bHLH-PASA was prepared as the holoprotein by reconstitution with hemin and the apoprotein.[38] As the final step in the purification, the proteins were applied to a Sephadex G-25 (GE Healthcare) column pre-equilibrated with buffer B [50 mM HEPES (pH 7.5), 10% glycerol, and 1 mM DTT] for buffer exchange.

The MBP (maltose binding protein)-tagged bHLH-PASA-PASB domain of BMAL1 was purified using an amylose resin column (New England Biolabs), as described previously.[38] The His-tagged bHLH domain of BMAL1 was purified like His-NPAS2 bHLH. The purified proteins were applied to a Sephadex G-25 column pre-equilibrated with buffer B to exchange the buffer with the appropriate one.

EMS Assay. An oligonucleotide pair containing a canonical E-box sequence (5′-GGGGCGCCACGTGAGAGG-3′ and 5′-GGCCTCTCACGTGGCGCC-3′) was used as an EMS probe after end labeling with [^{32}P]dCTP (Japan Radioisotope Association) by the Klenow enzyme (Takara) and purification with a gel-filtration column. The concentration of labeled probe was determined in a competition analysis of the DNA binding activity with unlabeled DNA probe, the concentration of which was spectrophotometrically estimated and confirmed on a 15% polyacrylamide gel by comparison with a double-stranded DNA standard.

The DNA binding reactions were typically performed for 30 min on ice in a 6 μL reaction mixture [0.3 μM His-NPAS2, 0.6 μM MBP-BMAL1, 0.1 μM ^{32}P-labeled E-box, 50 mM HEPES (pH 7.5), 50 mM NaCl, 1.2 mM $MgCl_2$, 10% glycerol, 0.5% *n*-octyl glycoside, 0.12 mg/mL BSA, 0.05 mg/mL poly dI-dC (Sigma), and 2 mM DTT] in the presence or absence of 5 mM NADPH (Oriental yeast). To analyze the effects of pH on the DNA binding activity of the NPAS2/BMAL1 heterodimer or BMAL1 homodimer, the pH of the reaction mixture was varied from 6.5 to 8.5. In assays of the DNA binding rate and affinity of the NPAS2/BMAL1 heterodimer or BMAL1 homodimer, the concentrations of proteins or DNA probe in the mixture and the reaction time were variously altered for analysis, as described in detail in each figure legend.

The reaction mixtures were separated by electrophoresis on 5 or 7.5% (w/v) nondenaturing acrylamide gels in buffer C (20 mM Tris-acetate and 0.5 mM EDTA) for 2 h at 100 V and 4 °C. After electrophoresis, the gels were dried and analyzed using a BAS-1800 II Image Analyzer (Fujifilm) with Multi

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Gauge version 2.1. Igor Pro was used to analyze the binding data.

Cell Culture and Luciferase Assay. NIH3T3 cells were seeded in 24-well plates (2×10^4 cells/well) (Iwaki) with DMEM (catalog no. 08456, Nacalai) containing 5% FBS (Equitech-bio) and cultured for 16 h at 37 °C under 5% CO_2 prior to transfection. The NPAS2 expression plasmid *Npas2*/pCGN (0.01 μg), the BMAL1 expression plasmid *Bmal1*/pcDNA (0.025 μg), a reporter plasmid, m*Per1p*/pSLO or m*Per1p*-E3M/pSLO (0.2 μg), and an internal control plasmid, pSLG-SV40 (0.025 μg) (Toyobo), were cotransfected into cells using Trans First Reagent (Promega). The total amount of DNA was brought to 0.3 μg by the addition of the empty vector. After a 3 h treatment with the transfection mixture at 5% CO_2 and 37 °C, the medium was replaced with DMEM/5% FBS medium at pH 6.8, 7.2, or 7.7. Before use, the pH of the medium preincubated for 2 h at 37 °C and 5% CO_2 was adjusted and then the mixture further incubated for 1 h at 37 °C and 5% CO_2; there was little change in the pH of each medium (pH 6.8, 7.2, or 7.7, respectively) after the cells had been cultured for an additional 24 h. The collected cells were then lysed to analyze luciferase activities using MultiReporter Assay System-Tripluc-Detection Reagents (Toyobo) according to the manufacturer's protocols. The relative luciferase activities were normalized to the activity obtained for the internal control, pSLG-SV40.

RESULTS

DNA Binding Activities of NPAS2 at Various pH Values *in Vitro*. We overexpressed and purified several truncated proteins of mNPAS2 and mBMAL1 to examine the effects of pH on their DNA binding activities. The domain structures of the proteins are shown in Figure S1 of the Supporting Information. All of the purified proteins were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), showing >90% homogeneity (Figure S2 of the Supporting Information). The UV−vis absorption spectra of the ferric, ferrous, and CO-bound forms of His-NPAS2 bHLH-PASA were obtained as previously reported, confirming the structure of the PASA domains.[40]

We examined the DNA binding activity of His-NPAS2 bHLH-PASA at various pH values by an EMS assay. As shown in Figure 1A, two bands with different mobilities were observed when the DNA probe containing an E-box was incubated with both His-NPAS2 bHLH-PASA and MBP-BMAL1 bHLH-PASA-PASB proteins. Our previous study revealed that the upper and lower bands corresponded to the complexes of the BMAL1/BMAL1 homodimer and the NPAS2/BMAL1 heterodimer with DNA.[38] The homodimer of either holo-NPAS2 or apo-NPAS2 bound to the DNA probe was not observed under the conditions used in this study,[38] though weak binding of holo-NPAS2 to the E-box DNA was observed by quartz crystal microbalance analyses.[39] As shown in panels A and B of Figure 1, the DNA binding activity of the NPAS2/BMAL1 heterodimer increased with pH. Indeed, the activity at pH 8.5 was approximately 2.0- and 4.1-fold greater than that at pH 7.5 and 6.5, respectively, in the absence of NADPH. The addition of NADPH further increased the DNA binding activity at each pH value. The DNA binding activity increased up to 2.7-fold at pH 7.5 and 3.2-fold at pH 6.5 in the presence of NADPH (Figure 1B). These results indicate that the effects of pH and NADPH were additive in enhancing NPAS2/BMAL1/DNA complex formation. As previously reported,[30] the 61 N-terminal

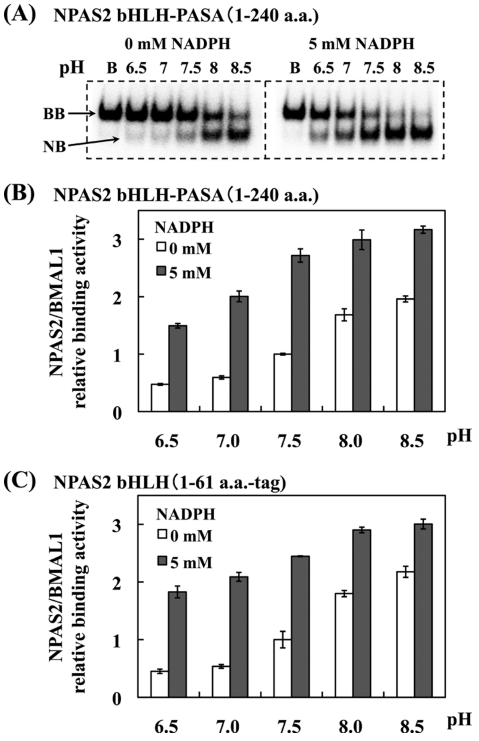

Figure 1. Effects of pH on the DNA binding activity of NPAS2 bHLH-PASA (amino acids 1−240) and NPAS2 bHLH (amino acids 1−61). (A) EMS assay using His-NPAS2 bHLH-PASA (amino acids 1−240) and MBP-BMAL1 bHLH-PASA-PASB at various pH values in the absence or presence of 5 mM NADPH. The label B at the top of the gel indicates an EMS assay in which the BMAL1 protein was incubated with DNA in the absence of NPAS2. Abbreviations: BB, BMAL1/BMAL1 homodimer with a ^{32}P-labeled E-box DNA probe; NB, NPAS2/BMAL1 heterodimer with a ^{32}P-labeled E-box DNA probe. (B) Relative DNA binding activity of the NPAS2/BMAL1 heterodimer at various pH values in the absence (white) or presence (gray) of NADPH. The intensity of the bands shown in panel A was quantified by Multi Gauge version 2.1 and is represented as a relative value normalized to the value obtained at pH 7.5 in the absence of NADPH. Each value is the mean of at least three independent experiments ± SD. (C) Relative DNA binding activity of His-NPAS2 bHLH (amino acids 1−61) with MBP-BMAL1 bHLH-PASA-PASB at various pH values in the absence or presence of 5 mM NADPH. The conditions for the EMS assay were as described in Experimental Procedures and are the same as those for panel A. Each value is the mean of at least three independent experiments ± SD.

amino acids of NPAS2, NPAS2 bHLH (amino acids 1−61), are sufficient to form a heterodimer with BMAL1, bind to the E-box, and sense NADPH. Effects of pH on NPAS2 bHLH (amino acids 1−61) were also examined after the elimination of the His tag by thrombin (Figure 1C). Similar to His-NPAS2 bHLH-PASA, the DNA binding activity of NPAS2 bHLH was increased by pH and NADPH, indicating that N-terminal amino acids 1−61 of NPAS2 are sufficient to sense pH and that these effects are independent of the His tag (Figure 1C). Moreover, the DNA binding activity of His-NPAS2 bHLH-PASA (amino acids 1−240) with BMAL1 bHLH (amino acids 74−128) without the MBP tag was also affected by pH, as was MBP-BMAL1 bHLH-PASA-PASB, indicating that the PASA and PASB domains of BMAL1 and MBP tag were not responsible for the observed effects of pH alteration (Figure S3 of the Supporting Information). We also found similar effects of pH and NADPH on the formation of the complex of the

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CLOCK/BMAL1 heterodimer with E-box DNA, as shown in Figure S4 of the Supporting Information. In the case of CLOCK, the bHLH domain containing amino acids 10−86 was essential and sufficient to sense pH and NADPH, similar to NPAS2.

DNA Binding Activity of the BMAL1 Homodimer at Various pH Values. Because the DNA binding activity of the NPAS2/BMAL1 heterodimer was affected by pH, we tested the effects of pH on the BMAL1 homodimer by an EMS assay in the absence of NPAS2. As shown in Figure 2, the DNA binding activity of the MBP-BMAL1 bHLH-PASA-PASB homodimer was not significantly affected by pH in the absence or presence of NADPH, though the addition of 5 mM NADPH rather inhibited the activity, as previously reported.[30] These results indicate that NPAS2, but not BMAL1, is responsible for the regulation of the DNA binding activity of the NPAS2/BMAL1 heterodimer by pH. Therefore, further studies focused on the effects of NADPH and pH on the affinity of DNA for the NPAS2/BMAL1 heterodimer.

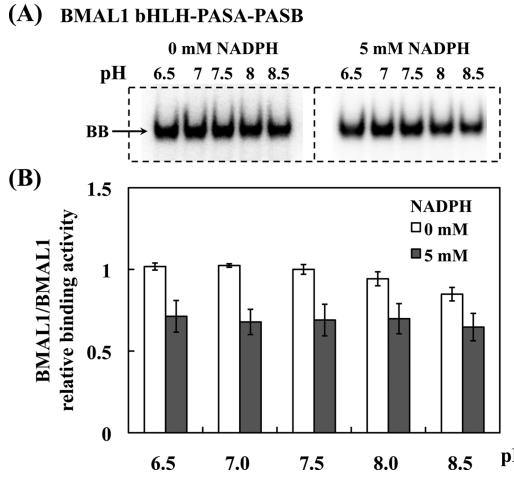

Figure 2. DNA binding activity of BMAL1 at various pH values in the absence of NPAS2. (A) EMS assay using MBP-BMAL1 bHLH-PASA-PASB at various pH values in the absence or presence of NADPH. (B) DNA binding activity of the BMAL1/BMAL1 homodimer in the absence (white) or presence (gray) of NADPH. The intensities of the bands shown in panel A were quantified and are represented as a relative value, as described in the legend of Figure 1. Each value is the mean of at least three independent experiments ± SD.

Kinetics of DNA Binding of the BMAL1 Homodimer and NPAS2/BMAL1 Heterodimer. To further elucidate the effects of pH and NADPH on the DNA binding of the NPAS2/BMAL1 heterodimer, we analyzed their kinetics by an EMS assay with several modifications of the standard protocol, as described in Experimental Procedures. Initially, we examined the DNA binding rate of the MBP-BMAL1 bHLH-PASA-PASB homodimer at pH 7.5 in the absence of NPAS2. As shown in Figure 3A, the DNA binding activity of the BMAL1 homodimer reached a plateau within 10 min in a reaction mixture containing 20 nM BMAL1 (monomer concentration) and 50 nM E-box DNA. Figure 3B shows the binding affinity of the E-box DNA for the BMAL1 homodimer, with an apparent dissociation constant (K_D^{app}) of 17 nM.

After equilibration between DNA (50 nM) and BMAL1 (20 nM monomer) during a 20 min incubation, an excess amount of His-NPAS2 bHLH-PASA (400 nM) was added to the reaction mixture to follow formation of the complex between

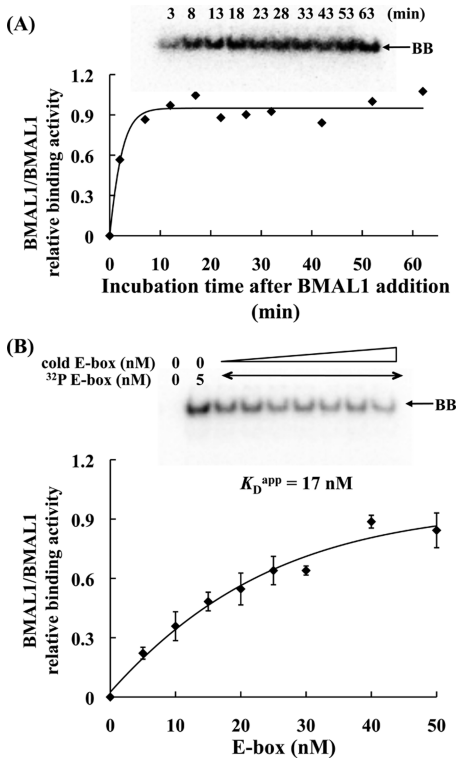

Figure 3. (A) Time course of formation of the complex of the BMAL/BMAL1 homodimer with DNA at pH 7.5 in an EMS assay. The reaction mixture contained 20 nM MBP-BMAL1 bHLH-PASA-PASB and 50 nM E-box DNA in the absence of NPAS2, with the other conditions described in the legend of Figure 2, and was incubated on ice. (B) Effects of E-box DNA concentration on the formation of the complex of the homodimer of MBP-BMAL1 bHLH-PASA-PASB with DNA in an EMS assay. The reaction mixtures contained 20 nM MBP-BMAL1 bHLH-PASA-PASB and various amounts of E-box DNA, as indicated; incubation was for 30 min on ice. The activity is represented as a relative value of the BMAL/BMAL1 homodimer complex with DNA quantified by Multi Gauge version 2.1. Each value is the mean of at least three independent experiments ± SD.

the NPAS2/BMAL1 heterodimer and DNA. As shown in Figure 4, the dissociation of the BMAL1/BMAL1 homodimer from the E-box DNA was complete within 5 min and was immediately followed by the binding of the NPAS2/BMAL1 heterodimer to the E-box DNA in the absence of NADPH. The addition of NADPH to the system accelerated the dissociation of the BMAL1/BMAL1 homodimer and the binding of the NPAS2/BMAL1 heterodimer. It is noteworthy that the maximal amount of the NPAS2/BMAL1/DNA complex was 0.6-fold of that of the BMAL1/BMAL1/DNA complex in the absence of NADPH, demonstrating that the affinity of DNA for the NPAS2/BMAL1 heterodimer was lower than that for the BMAL1/BMAL1 homodimer under these conditions, consistent with the K_D^{app} values for the NPAS2/BMAL1 heterodimer described below. In addition, the maximal amount of the NPAS2/BMAL1/DNA complex was 3.7-fold greater than that of the BMAL1/BMAL1/DNA complex in the presence of NADPH, indicating that NADPH significantly increased the extent of NPAS2/BMAL1/DNA complex formation.

We next examined the effects of the amount of NPAS2 relative to that of BMAL1 on the formation of the NPAS2/BMAL1/DNA complex (Figure 5). The extent of formation of the NPAS2/BMAL1/DNA complex was increased with an

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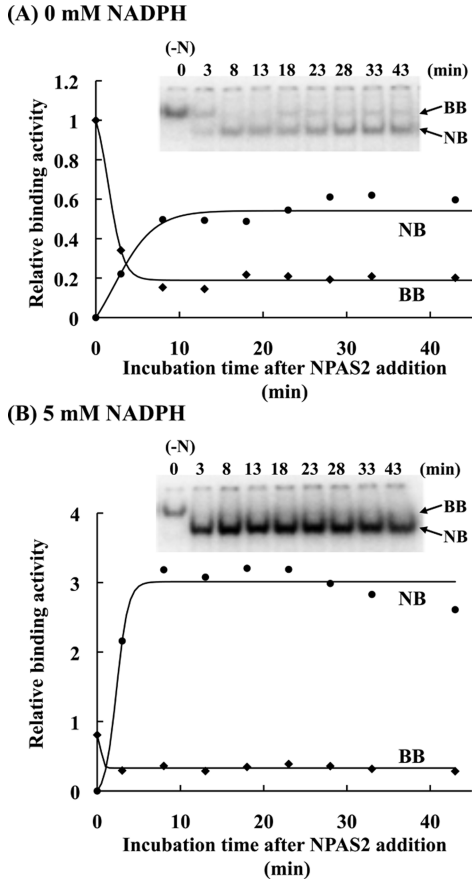

Figure 4. Time course of complex formation of the NPAS2/BMAL1 heterodimer and decrease in the level of the BMAL/BMAL1 homodimer complex with DNA at pH 7.5 in the absence (A) or presence (B) of NADPH in an EMS assay. Reaction mixtures contained 50 nM E-box DNA, 20 nM MBP-BMAL1 bHLH-PASA-PASB, and 400 nM His-NPAS2 bHLH-PASA. After incubation on ice for 20 min without NPAS2, NPAS2 was added to the reaction mixture and incubated on ice. The complexes with DNA were quantified by Multi Gauge version 2.1 and are represented as a relative value normalized to the value of the BMAL/BMAL1 homodimer complex with DNA in the absence of NPAS2 and NADPH.

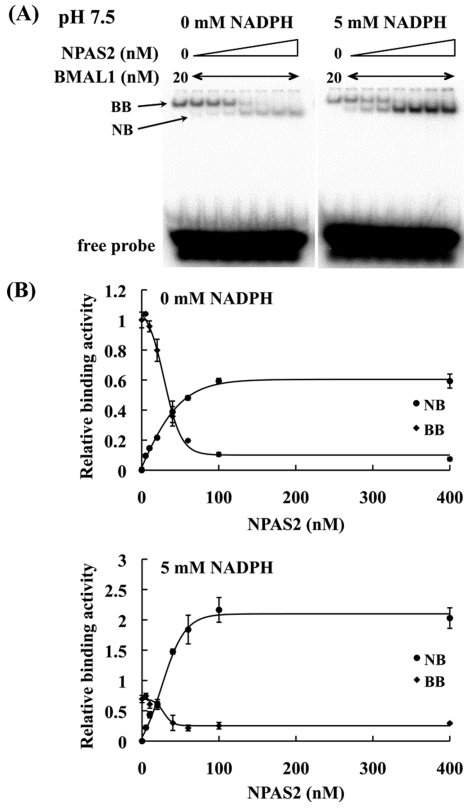

Figure 5. (A) Effects of NPAS2 concentration on the formation of the complex of the NPAS2/BMAL1 heterodimer with DNA. The reaction mixtures contained 50 nM E-box DNA, 20 nM MBP-BMAL1 bHLH-PASA-PASB, and various concentrations of His-NPAS2 bHLH-PASA (0, 5, 10, 20, 40, 60, 100, and 400 nM). (B and C) The complexes with DNA in panel A were quantified by Multi Gauge version 2.1 in the absence and presence of 5 mM NADPH, respectively, and are represented as a relative value normalized to the value of the BMAL/BMAL1 homodimer complex with DNA in the absence of NPAS2 and NADPH.

increasing concentration of NPAS2 in the reaction mixture, whereas the level of formation of the BMAL1/BMAL1/DNA complex was decreased to an undetectable level in the EMS assay. In the absence of NADPH, the maximal DNA binding activity of the NPAS2/BMAL1 heterodimer was again lower than that of the BMAL1/BMAL1 homodimer, even though the free probe was still abundant (>90% of the total amount) in the reaction mixture (Figure 5A). The addition of 5 mM NADPH resulted in a great increase in the level of NPAS2/BMAL1/DNA complex formation (Figure 5B). The amount of DNA used for NPAS2/BMAL1/DNA and BMAL1/BMAL1/DNA complex formation was maximally 5 and 10% of the total amount in the absence and presence of NADPH, respectively, indicating that enough free DNA remained in the reaction mixture. These results also indicate that ~25% of BMAL1 protein bound DNA in the absence of NADPH. BMAL1 exists in equilibrium between the BMAL1 dimer and oligomers in solution as shown in the results of gel filtration (Figure S5 of the Supporting Information), and addition of DNA, NPAS2, and/or NADPH changes the equilibrium. In the presence of NADPH, the maximal amount of NPAS2/BMAL1/DNA was 2.7-fold greater than that of BMAL1/BMAL1/DNA (Figure 5B), indicating that more BMAL1 than that contained in the BMAL1/BMAL1/DNA complex could form a heterodimer with NPAS2 and bound DNA under our conditions. We also performed a similar experiment at pH 7.0 (data not shown). Although the shift in pH of the reaction mixture from pH 7.5 to 7.0 resulted in the decreased DNA binding activity of the NPAS2/BMAL1 heterodimer, NADPH again increased the level of formation of the NPAS2/BMAL1/DNA complex. Taken together, these results demonstrated that 400 nM NPAS2, which was in 20-fold excess compared to BMAL1 (20 nM), was sufficient to saturate the formation of the NPAS2/BMAL1/DNA complex, with a background level of BMAL1/BMAL1/DNA complex formation.

Effects of pH and NADPH on the DNA Binding Affinity of NPAS2. To evaluate the effects of pH and NADPH on the DNA binding activity of NPAS2 quantitatively, we determined the apparent dissociation constant (K_D^{app}) of E-box DNA to the NPAS2/BMAL1 heterodimer in a reaction mixture containing 400 nM NPAS2 bHLH-PASA and 20 nM BMAL1 bHLH-PASA-PASB with various concentrations of E-box DNA in the absence or presence of NADPH. High concentrations of E-box DNA were achieved by the addition of cold E-box DNA to the labeled probe. As expected, we detected only bands corresponding to the NPAS2/BMAL1 heterodimer under these

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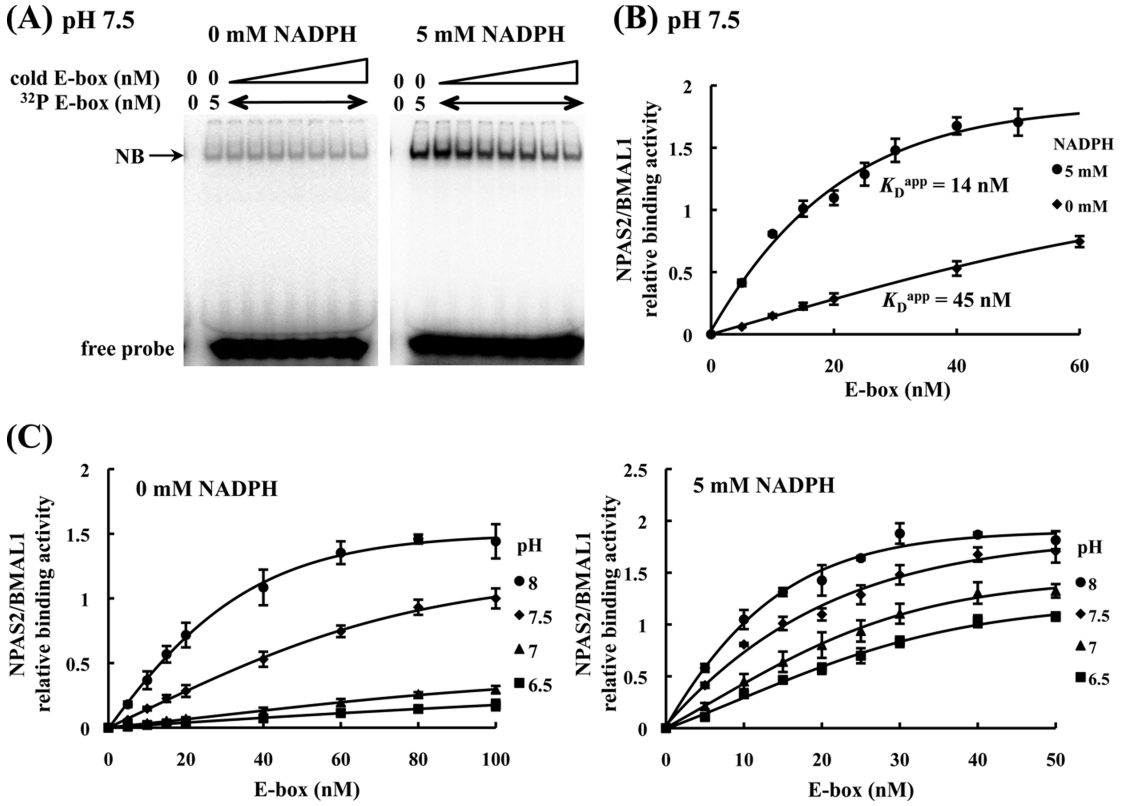

Figure 6. Effects of pH and NADPH on the DNA binding affinity of the NPAS2/BMAL1 heterodimer. (A) EMS assays with various amounts of DNA at pH 7.5. The reaction mixtures contained 20 nM MBP-BMAL1 bHLH-PASA-PASB, 400 nM His-NPAS2 bHLH-PASA, and various concentrations of E-box DNA (total of 0, 5, 10, 15, 20, 40, 60, 80, and 100 nM DNA in the absence of NADPH or total of 0, 5, 10, 15, 20, 25, 30, 40, and 50 nM DNA in the presence of 5 mM NADPH). Each DNA contained 5 nM ^{32}P-labeled probe. (B) DNA binding activity of the NPAS2/BMAL1 heterodimer at pH 7.5 shown in panel A quantified and represented as a relative value normalized to the maximal value obtained from the fitting curve in the absence of NADPH. Each dot is the mean of at least three independent experiments ± SD. The K_D^{app} values were estimated from a sigmoidal plot fit to the data. (C) DNA binding affinity of the NPAS2/BMAL1 heterodimer at various pH values in the absence (left) or presence (right) of NADPH. All obtained data were analyzed as described for panel B. Each dot is the mean of at least three independent experiments ± SD. The K_D^{app} values estimated from a sigmoidal plot fit to the data are summarized in Table 1.

conditions (Figure 6A). The signals for the NPAS2/BMAL1/DNA complex were quantified and corrected to draw fitting curves (Figure 6B). At pH 7.5, the K_D^{app} values determined from the fitting curve were 45 and 14 nM in the absence and presence of NADPH, respectively. Similarly, we determined the K_D^{app} values of the E-box DNA with the NPAS2/BMAL1 heterodimer under various pH conditions in the absence (Figure 6C, left panel) or presence (Figure 6C, right panel) of NADPH, as summarized in Table 1. In the absence of NADPH, a change in pH from 6.5 to 8.0 resulted in a decrease in the K_D^{app} value from 125 to 22 nM, with an 8-fold increase in the maximal level of DNA binding for the NPAS2/BMAL1 heterodimer (B_{max}). The K_D^{app} value was also decreased from 125 to 21 nM by the addition of NADPH at pH 6.5. In the absence and presence of NADPH, the DNA binding affinity of the NPAS2/BMAL1 heterodimer increased with pH and was further increased by the addition of NADPH, indicating that the effects of pH and NADPH were additive. Furthermore, the DNA binding activity of the NPAS2/BMAL1 heterodimer was more sensitive to pH in the absence of NADPH.

Table 1. Effects of pH and NADPH on the K_D^{app} Value of DNA Binding to the NPAS2/BMAL1 Heterodimer and the Maximal Value of Relative Binding Activity (B_{max})

0 mM NADPH				
pH	6.5	7.0	7.5	8.0
K_D^{app} (nM)	125	61	45	22
B_{max}	0.18	0.29	1.00	1.44
5 mM NADPH				
pH	6.5	7.0	7.5	8.0
K_D^{app} (nM)	21	18	14	9
B_{max}	1.08	1.32	1.71	1.81

Effects of pH on the Transcriptional Activity of NPAS2 in Cells. We conducted a luciferase assay in mouse NIH3T3 cells to investigate whether the transcriptional activity of NPAS2 was affected by extracellular pH. We used two reporter plasmids, m*Per1p*/pSLO containing the enhancer/promoter region of mouse *Per1* (nucleotides −1803 to +40) harboring three canonical E-boxes (CACGTG) and m*Per1p*-E3M/pSLO containing three mutated E-boxes (M; TCTAGA) instead of canonical E-boxes in the m*Per1* enhancer/promoter region (Figure 7A). The luciferase activities for both m*Per1p*/pSLO and m*Per1p*-E3M/pSLO without transfection of the NPAS2 expression plasmid were not much changed with an increase in pH (6.8, 7.2, and 7.7). By the transfection of the full-length NPAS2 expression plasmid, the activities for m*Per1p*/pSLO significantly increased, whereas the activities for m*Per1p*-E3M/pSLO were not much affected (Figure 7B). We evaluated the NPAS2-dependent transcriptional activity at each pH by subtracting the activity without NPAS2 expression from that

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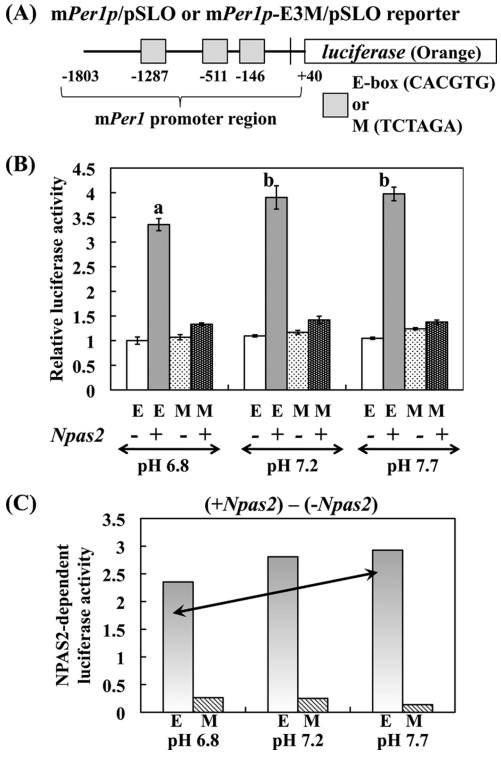

Figure 7. Effects of pH on the transcriptional activity of full-length NPAS2 in NIH3T3 cells. (A) Construction of m*Per1p* and m*Per1p*-E3M reporter plasmids. The m*Per1* promoter region contains three canonical E-boxes (CACGTG). In the m*Per1p*-E3M reporter plasmid, all three E-boxes were mutrated to TCTAGA. (B) Relative luciferase activity for m*Per1p* (E) and m*Per1p*-E3M (M) reporter plasmids in cells cultured at pH 6.8, 7.2, and 7.7 for 24 h in the absence (−) or presence (+) of the NPAS2 expression plasmid, *Npas2*/pCGN. Luciferase activity is represented as a value relative to that obtained with m*Per1p* reporter and *Npas2* empty vector, pCGN, at pH 6.8. Each value is the mean of at least three independent experiments ± SD. Significant differences between a and b were evaluated by the *t* test ($p < 0.02$). (C) NPAS2-dependent transcriptional activity via E-boxes increased with pH. The NPAS2-dependent luciferase activities for m*Per1p* (E) and m*Per1p*-E3M (M) reporter plasmids were calculated by subtracting the activities without *Npas2* from the activities with *Npas2* at each pH, as shown in panel B.

with NPAS2 expression. As shown in Figure 7C, the NPAS2-dependent transcriptional activity for m*Per1p*/pSLO increased with pH, whereas that for m*Per1p*-E3M/pSLO rather decreased. These results suggested that the extracellular pH affected the expression of the *mPer1* gene via E-box/NPAS2 in cells.

DISCUSSION

Molecular rhythms in mammalian cells and tissues can be entrained by various extra- and intracellular stimuli, such as light, temperature, hormone, gaseous signaling molecules, and metabolites. These stimuli simultaneously and concertedly act on clock machinery to reset behavioral and physiological rhythms. In this study, we demonstrated the effects of pH as well as NADPH on the DNA binding and transcriptional activity of NPAS2, a mammalian circadian transcription factor acting as a positive limb of core loops in the clock.

Using the EMS assay, we showed that the DNA binding activity of the NPAS2/BMAL1 heterodimer increased with increasing pH. The addition of NADPH to the system further increased the level of formation of the complex of the NPAS2/BMAL1 heterodimer with E-box DNA at each pH value, indicating that the observed pH and NADPH effects were additive (Figure 1). As shown in previous papers, NADPH itself enhances the DNA binding activity of the NPAS2/BMAL1 heterodimer.[29,30] Conversely, the formation of the DNA complex of the BMAL1/BMAL1 homodimer, which is not functional as a transcription factor, was not affected by pH and NADPH (Figure 2). In addition, N-terminal amino acids 1−61 of NPAS2, which are adequate for heterodimer formation with BMAL1 and binding to E-box DNA, were sufficient for the effects of pH on its DNA binding activity. The bHLH domain (amino acids 1−61) of NPAS2 was also responsible for the NADPH effect, as reported in our previous paper,[30] suggesting that pH and NADPH are sensed by a similar region of NPAS2.

We obtained K_D^{app} values of the E-box DNA for the NPAS2 bHLH-PASA/BMAL1 bHLH-PASA-PASB heterodimer at various pH values in the presence or absence of NADPH (Table 1), and changes in pH and NADPH concertedly decreased the K_D^{app} value. Under our experimental conditions, the K_D^{app} values were between 9 and 125 nM. These values for the NPAS2/BMAL1 heterodimer were comparable to those for other bHLH-PAS family proteins, such as human HIF-1α/HIF-1β (ARNT) and mouse-DR/human-ARNT heterodimers.[41] Gel filtration analysis suggested that in our system, BMALI bHLH-PASA-PASB formed an oligomer that was higher-order than a dimer, and NPAS2 bHLH-PASA formed mainly dimers, with a minor monomer fraction, indicating that all species of those exist in a dynamic equilibrium in solution. In the absence of NADPH, the mixture of NPAS2, BMAL1, and DNA revealed only the partial formation of a heterodimer complex with DNA, and the addition of NADPH increased the level of formation of the heterodimer complex, with a decrease in the amount of BMAL1 oligomers (Figure S5 of the Supporting Information). Therefore, under our EMS conditions, we postulated formation of the complexes of DNA with the BMAL1/BMAL1 homodimer and the NPAS2/BMAL1 heterodimer, though the BMAL1 oligomer, NPAS2 oligomer, and NPAS2/NPAS2 dimer did not form a detectable complex with DNA. Figure 4 demonstrates that the NPAS2/BMAL1/DNA complex was preferentially formed, with a decrease in the level of the BMAL1/BMAL1/DNA complex, even though the K_D^{app} value of DNA for the BMAL1/BMAL1 homodimer was smaller than that for the NPAS2/BMAL1 heterodimer in the absence of NADPH and even in the presence of NADPH at pH <7.5. These results suggest that the formation of the NPAS2/BMAL1 heterodimer was preferred over that of the BMAL1/BMAL1 homodimer.

Recently, the K_D^{app} value of fluorescein-labeled E′-box DNA (CACGTT) for the murine CLOCK bHLH-PASA-PASB/BMAL1 bHLH-PASA-PASB heterodimer was reported to be 59 ± 7.3 nM by fluorescence anisotropy.[42] It is similar to the values of the NPAS2 bHLH-PASA/BMAL1 bHLH-PASA-PASB heterodimer that we obtained in this study, suggesting that the ability of the murine NPAS2/BMAL1 heterodimer to bind to the E-box DNA was comparable to that of the murine CLOCK/BMAL1 heterodimer. In contrast, the K_D value of E-box DNA for the human CLOCK bHLH/BMAL1 bHLH heterodimer was also reported to be 1.52 ± 0.10 μM by ITC measurement at pH 7.8,[43] which is 30 and 70 times lower than our results at pH 7.5 and 8.0, respectively (Table 1). These authors also reported that none of the NAD(P)H cofactors tested significantly affected the K_D value of the E-box DNA for

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the human CLOCK bHLH/BMAL1 bHLH heterodimer.[43] Although the reasons for these differences are not clear, each experiment used different protein expression systems and analysis methods. Because the PAS domain(s) is known to play an important role in dimer formation for various PAS proteins, the PAS domains contained in our NPAS2 and BMAL1 constructs used to evaluate K_D^{app} values must have contributed to the stabilization of the NPAS2/BMAL1 heterodimer, resulting in an increase in its DNA binding activity.

Interestingly, the effects of pH were observed only for DNA complex formation of the heterodimer NPAS2/BMAL1 or CLOCK/BMAL1 bHLH domains but not for that of the BMAL1/BMAL1 homodimer. Therefore, we should focus on the differences between the bHLH domains of CLOCK/NPAS2 and BMAL1 (Figure S6 of the Supporting Information). Two groups recently reported the crystal structures of the bHLH-PASA-PASB domains of the murine (m) CLOCK/BMAL1 heterodimer[42] and the bHLH domains of the human (h) CLOCK/BMAL1 heterodimer bound to E-box DNA.[43] On the basis of the structure, Wang et al. demonstrated that His84 of hCLOCK and Leu125 of hBMAL1 are critical residues involved in mutual recognition for heterodimer formation. His59, which is contained in our construct of the bHLH domain of mNPAS2 (amino acids 1−61) as the only His, corresponds to His84 of hCLOCK. His has also been reported to be a residue responsible for pH sensing among many pH sensor proteins, such as KcsA, ASIC1a, and epithelial Na^+ channel.[44−46] Therefore, His59 in NPAS2 is a possible candidate for sensing pH and for the formation of a stable heterodimer with BMAL1. Ser38 and Ser42 of hCLOCK, corresponding to Ser13 and Ser17 of mNPAS2, respectively, have been reported to reduce transcriptional activity *in vivo* when phosphorylated,[13] but neither the S38E mutation nor the S42E mutation of CLOCK has a significant influence on DNA binding *in vitro*.[43] At the interface between the CLOCK (NPAS2) and BMAL1 bHLH domains, Ser70 of hCLOCK (Thr46 of mNPAS2), which is not conserved in the BMAL1-bHLH domain, appears to interact with Lys87 of hBMAL1 and mBMAL1 (Figure S6 of the Supporting Information). Ser70 also possibly affects heterodimer formation depending on pH. Further studies are required to elucidate the residues responsible for the effects of pH on CLOCK and NPAS2.

In NIH3T3 cells, we demonstrated that the NPAS2-dependent transcriptional activity of m*Per1* increased with extracellular pH (from pH 6.8 to 7.7), whereas that for the reporter containing all mutated E-boxes decreased (Figure 7). These results suggested that the transcriptional activity depending on E-box/NPAS2 is significantly affected by a change in pH even within the physiological pH range, although it is not clear whether pH effect is direct and, if so, how extracellular pH affects the intracellular and nuclear pH under these conditions. Although intracellular pH is generally strictly regulated within a narrow neutral region, it is known that the pH decreases in muscles after hard exercise because of the accumulation of pyruvate and lactate.[47] In cancer cells, extracellular acidification is caused by the enhancement of glycolysis, which is followed by an increase in the intracellular level of lactate and H^+, the induction of some of ion channels and receptors, and the efflux of these acids.[37] It is also demonstrated by measuring the autofluorescence lifetime of NADH that alteration of the cytosol pH induced a change in pH in nuclei in culture cells.[48] Interestingly, our results are consistent with the results of two other reports indicating the effects of extracellular pH on circadian rhythm.[33,34] Kon et al. reported that alkali signals triggered a resetting of the molecular clock in cultured Rat-1 fibroblasts.[33] A shift in the pH of the culture medium in a range of 0.1−0.4 resulted in an increase in the level of *Dec1* mRNA and consequently the phase-shift of the cellular rhythm. It was also reported that a change in the pH of the culture medium from 6.7 to 7.2 affected both the amplitude and the phase of the circadian expression of the human *BMAL1* reporter in human primary fibroblasts.[34] Taken together, the balance between NAD(P)H and pH appears to be a cue for the regulation of clocks via NPAS2 (CLOCK) under the various physiological conditions of tissues and cells.

■ CONCLUSIONS

The data presented here demonstrate that the binding affinity of NPAS2 for the E-box was considerably increased with pH changes *in vitro* and additively by the presence of NADPH. Using a truncated mutant of NPAS2, the 61 N-terminal amino acids were found to be sufficient to sense pH and NADPH. The NPAS2-dependent transcriptional activity regulating the m*Per1* gene was also increased with extracellular pH in cultured cells. These results suggest that NPAS2 has a role as a pH and metabolite sensor in regulating circadian rhythms.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Domain structures of truncated NPAS2 (A), BMAL1 (B), and CLOCK (C) mutants used in this study (Figure S1), SDS−PAGE of purified NPAS2 (A), BMAL1 (B), and CLOCK (C) mutants (Figure S2), effects of pH on the DNA binding activity of His-NPAS2 bHLH-PASA (amino acids 1−240) and BMAL1 bHLH (amino acids 74−128) without the MBP tag (Figure S3), effects of pH on the DNA binding activity of His-CLOCK and MBP-BMAL1 (Figure S4), gel-filtration column chromatography of the mixture containing His-NPAS2 bHLH-PASA and MBP-BMAL1 bHLH-PASA-PASB in the absence or presence of NADPH (Figure S5), and multiple-sequence and -structure alignments of the bHLH domains of CLOCK, NPAS2, and BMAL1 (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Graduate School of Life and Environmental Science, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan. Telephone and fax: +81-75-703-5672. E-mail: sagami@kpu.ac.jp.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

NPAS2, neuronal PAS domain protein 2; BMAL1, brain and muscle ARNT-like 1; CLOCK, circadian locomotor output cycles kaput; bHLH, basic helix−loop−helix; EMS, electrophoretic mobility shift; MBP, maltose binding protein; SD, standard deviation; PCR, polymerase chain reaction.

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