The regulatory factor X (RFX) family of transcription factors is characterized by a unique and highly conserved 76-amino acid residue DNA-binding domain. Mammals have five RFX genes, but the physiological functions of their products are unknown, with the exception of RFX5. Here a mouse RFX4 transcript was identified that encodes a peptide of 735 amino acids, including the DNA-binding domain. Its expression was localized in the suprachiasmatic nucleus, the central pacemaker site of the circadian clock. Also, light exposure was found to induce its gene expression in a subjective night-specific manner. Polyclonal antibodies were prepared, and an 80-kDa band was detected in the suprachiasmatic nucleus by Western hybridization. A histochemical study showed a localization of the products in the nucleus. This is the first report on mouse RFX4, which contains the RFX DNA-binding motif. Our investigation may provide clues to the physiological function of RFX4.

Regulatory factor X (RFX) is a DNA-binding protein that recognizes the X-box sequence in the transcription regulation region of major histocompatibility complex class II genes (1). It has the highly conserved DNA-binding domain (DBD) of 76 amino acids that shows a unique winged helix structure (2–5). RFX is conserved among yeast (6), fungi (7), nematodes (8), fruit flies (9), mice, and humans (3, 10).

In humans and mice, five RFX paralogous genes, RFX1, RFX2, RFX3, RFX4, and RFX5, have been identified (4, 11). The RFX1, RFX2, and RFX3 genes, products of which contain the DBD, the dimerization (DIM) domain, and some other evolutionary conserved sequences (A, B, and C), are similar to each other (4, 11). RFX4 lacks the A region (11) and RFX5 lacks the A, B, and C, and DIM domains (4). Nearly all of the target genes and the physiological roles of mammalian RFX1–4 are unknown (12–15).

Studies using mutants of the RFX orthologues in other organisms have suggested possible functions. Crt1 in Saccharomyces cerevisiae encodes a transcription repressor involved in DNA damage and the replication block checkpoint pathways (6). DAF-19 in Caenorhabditis elegans plays a critical role in ciliated sensory neuron development (8). dRFX in Drosophila melanogaster is also expressed in the sensory neurons during embryonic development (9, 16).

RFX4 was first isolated from human breast cancer cells as a chimeric molecule with the estrogen receptor (17). Two transcripts have been identified in humans (GenBank™ accession numbers AF332192 and AB044245) and one in mice (AK016791), but the mouse transcript fails to encode the RFX-specific DNA-binding domain. This suggests the presence of another RFX4 transcript in mice containing the DBD. Neither target genes nor the physiological role of RFX4 are known, but abundant expression in the testis and some expression in the brain have been shown in humans (11). In this study, a novel mouse RFX4 transcript from the brain was isolated and characterized.

Almost all organisms have an endogenous circadian timekeeper that governs most phenomena in the organism, including its behavior (18). The endogenous oscillator functions on a period of about, but not exactly, 24 h (19–21). This period is synchronized to the day/night environmental cycle by an entrainment mechanism (22). In mammals, the central oscillator resides in the hypothalamic suprachiasmatic nucleus (SCN) (23, 24). Interestingly, the mouse RFX4 transcript was localized to the SCN.

**EXPERIMENTAL PROCEDURES**

**Mice and Tissues**—Male mice (C57BL/6 strain) purchased from Japan SLC, Inc. (Hamamatsu, Japan) were trained in light/dark (LD) cycles (12L:12D) for at least 10 days before being transferred to constant darkness (DD). On the 4th day under DD, the cortex and SCN were isolated by micropunching. For gene expression analysis in response to light exposure, the mice were exposed to light at circadian time (CT)14 and sacrificed 20 or 45 min later. For further RFX4 induction studies, mice exposed to light at CT6, -14, or -22 were sacrificed 45 min later. The light exposure intensity was 200 lux. Control mice were sacrificed at the same CT time points but without light exposure. To determine whether the gene expression was rhythmic, the animals were sacrificed at CT2, -6, -10, -14, -18, and -22. Then, the SCN and cortex tissues were perforated using a puncher with a 400-μm inner diameter. SCN tissues were obtained from the anterior section, and the cortical tissues were obtained near the most dorsal area from the next caudal section. The pineal gland and retina were prepared as described elsewhere (25).

**RNA Preparation, cDNA Synthesis and Sequencing**—Total RNA was prepared from the SCN, cortex, and testis with TRIzol (Invitrogen) and then treated with DNase-I (Invitrogen). First strand cDNA was synthesized with an oligo-(dT)18 primer using the SuperScript II First-Strand

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SCN-specific Light Induction of a Novel RFX4 Transcript

Synthesis System according to the manufacturer’s instructions (Invitrogen). Reverse transcription (RT)-PCR was performed in a 25-μl reaction, which included 2.5 units of KOD DNA polymerase (TOYOBO, Osaka, Japan). The primers were F1 (5’-TCC ACT AGT TCT TTT TCC CCT TTG ATT-3’, R1 (5’-CTA TTT GAG TGA ACC ATC ATC TTT-3’), P2 (5’-ACT TCT TGG TAA TTA CAT CGG CTA AAA AT-3’), and R2 (5’-GTA GAC CAG CAA AAA TTC ACG TTC TCT-3’). RT-PCR conditions were 94 °C for 4 min followed by 35 cycles of 98 °C for 20 s, 60 °C for 10 s, and 74 °C for 1.5 min. The RT-PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and sequenced. To prevent any misreading due to nucleotide misincorporation by the DNA polymerase, RT-PCR was conducted independently three times. For DNA sequencing, three clones were picked from the PCR products obtained from all three independent amplifications. We aligned these 9 sequences, determined the sequence of mNYD-sp10 and hRfx4, and registered the sequences in DDBJ with accession numbers AB088184 and AB086957, respectively.

Messenger RNA Quantification—The amount of mouse RFX4 mRNA was measured by real-time PCR using SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the ABI PRISM 7700 (Applied Biosystems) (26). The amount of mRNA was determined through normalization with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA signal. The primers were mouse RFX4 (forward, 5’-GGG TTC CTC CAG TAA CCC AC-3’, reverse, 5’-GAG GCC CCT GGT GCT GAT GTT C-3’), mPer 2 (forward, 5’-GCT CCA AAT TCC CTA TTT CC-3’, reverse, 5’-GGT GGT GCA GGA TGC ATT GCT G-3’). Pre-heating was performed at 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s, 60 °C for 30 s and 78 °C for 40 s. Five mice were used for the real-time PCR analysis for each sample point, and the analysis was performed twice.

In Situ Hybridization—Two mice were used for the in situ hybridization analysis at each sample point. Whole brain tissue was fixed in a 4% paraformaldehyde/phosphate-buffered saline solution for 24 h followed by immersion in a 20% sucrose/phosphate-buffered saline solution for 24 h. Then, the brains were sliced into 30-μm sections with a Cryostat (Leica, Nussloch, Germany). To prepare the probes for analysis, both tissue-specific regions were amplified by RT-PCR and cloned into pGEM-T Easy (see Fig. 5C). After DNA sequence confirmation, this fragment was utilized as a probe. The primers employed were the following: forward, 5’-ATT ACT GAG TGG CCC CTC GC-3’, reverse, 5’-GGG TTC TCC CTA TAA CCC AC-3’ (see Fig. 5C). Radiolabeled probes were synthesized using [α-32P]UTP (PerkinElmer Life Sciences) with the manufacturer’s protocols for cRNA synthesis. Hybridization was performed as described by Tei et al. (27). The hybridized sections were exposed to BioMax film (Kodak, Rochester, NY). To determine the quantitative number of the transcripts, the optical densities of the SCN from six sections from the rostral to caudal ends were quantified with equal areas of the SCN and the corpus callosum.

Preparation of Antibodies and Western Blot Analysis—To prepare polyclonal anti-RFX4 antibodies, rabbits were immunized with synexpression of mouse RFX4. Real-time PCR was performed using Mouse Rapid-Scan™ Gene Expression Panels (OriGene Technologies, Rockville, MD), and total RNA was prepared from the SCN and cortex. mRNA levels are relative to the amount of RFX4 mRNA in the brain, which is defined as 100. Primers were designed for regions 147–166 and 280–299 (GenBank™ accession number AB101791, see Fig. 5C). The mean ± S.E. (n = 2) is indicated.

RESULTS AND DISCUSSION

A Novel Form of Mouse RFX4 Transcript Is Expressed in the SCN—Recently, the full-length human RFX4 (hereafter referred to as hRFX4) cDNA was isolated, and the interaction of its product with other RFX products was demonstrated. Nevertheless, its physiological function remains unknown. Gene expression analysis of hRFX4 showed that its expression is restricted to the testis (11). In mice, the RFX4 (AK016791) transcript was identified in the testis, but it lacks the RFX-specific DBD (30).

RFX4 expression was examined in several tissues in mice, and its expression was found in the testis, brain, heart, and ovary with expression ratios of 1, 1/36, 1/100, and 1/200, respectively. We failed to detect its expression in other tissues such as kidney, spleen, thymus, liver, muscle, lung, and uterus. These results are consistent with the publicly expressed sequence tag (EST) information. In this study, we focused on the transcript expressed in the brain. Further analyses using the SCN and cortex tissues revealed significant expression in the SCN, which serves as a biorhythm center (Fig. 1).

Two types of alternatively spliced products of RFX4, NYD-sp10 and hRFX4, have been isolated in humans. The hRFX4 product has the DBD motif, which is a characteristic of the RFX transcription factor family (11). NYD-sp10 lacks this domain. In mice, one transcript, AK016791, has been identified, but it lacks the DBD motif (11, 30). To date, these findings suggest the existence of a second RFX4 transcript in mice, which encodes an RFX4 homologue containing the DBD motif. A BLAST search (31) for NYD-sp10, hRFX4, and AK016791 in the UniGene collection of mouse ESTs (www.ncbi.nlm.nih.gov/UniGene/Mm.Home.html) found a cluster (Mm. 32654, build 111) containing 35 3’-ESTs and 15 5’-ESTs. All of the 3’-ESTs and 10 of the 5’-ESTs were identical to the corresponding regions of the NYD-sp10 transcript. The other five 5’-ESTs were all isolated from neural tissues. None of the sequences in this cluster...
were homologous to hRFX4 or AK016791. PCR using total RNA purified from the mouse brain with primers designed in the 5'-ESTs and 3'-ESTs that were registered in Mm. 32654 generated a 3.7-kbp fragment. Sequencing this fragment revealed that the transcript was different from the previously reported transcripts. The novel transcript, which we refer to as brain-specific RFX4 (bRfx4), encodes a peptide consisting of 735 amino acid residues containing the B, C, DIM, and DBD domains (Fig. 2). This is the first reported mouse RFX4, which contains the DBD motif. We also obtained a mouse orthologue of NYD-sp10, mNYD-sp10, using primers F2 and R1, which were designed using the NYD-sp10 5'-untranslated regions and 3'-untranslated regions from the mouse genome (GenBank™ accession number NW_000030) (Fig. 3, A and B, lane F2 + R1 in the testis). A search for bRfx4 in the public genome sequence data base revealed that the 5'-ESTs had been transcribed from novel exons upstream of the first exon of the mNYD-sp10 transcript (Fig. 3A).

Taken together, our findings suggest that bRfx4 expression is SCN-specific and that mNYD-sp10 and AK016791 expression is testis-specific. To confirm this, the expression of these mouse RFX4 transcripts was compared in the SCN and the testis using several primers specific for each transcript. We found that bRfx4 is expressed in the SCN, but mNYD-sp10 and AK016791 are only expressed in the testis (Fig. 3). In situ hybridization was also performed with testis sections using a bRfx4-specific probe (Fig. 5C) but failed to detect any signal (data not shown). As mentioned above, we also searched for a mouse hRFX4 orthologue, but we did not find any ESTs in the public data base.

Induction of the bRfx4 Transcript by Light Exposure—Though the disruption of the RFX5 gene causes immunodefi-
ciency in mice (12, 15), the physiological roles of the other RFX genes in mammals are unknown. The abundant expression of mouse RFX4 in the SCN may indicate that bRFx4 function is related to the circadian clock, because the SCN is the central clock locus for circadian rhythm. After real-time PCR, in situ hybridization was performed using brain sections, and strong signals were found in the SCN with three different probes prepared from the bRFx4 transcript. We also found significant but weak signals in the nucleus accumbens of the cerebellar cortex (data not shown).

The circadian rhythm generated in the SCN is transferred to most of the peripheral tissues in the body. Although peripheral tissues also have their own oscillator, they are thought to be governed by the central clock in the SCN. The circadian gene-related phenotypes were examined in bRFx4 expression. One of these phenotypes is an induction of gene expression by light exposure in a subjective night-specific manner. To date, several such genes have been identified in the SCN (32–40). Another characteristic observed in some of the circadian genes is their rhythmic expression throughout an entire day (41–43).

The expression change of mouse bRFx4 was analyzed as a result of light exposure in the SCN using real-time PCR. We detected a slight and an ~2.5-fold induction at 20 and 45 min after light exposure, respectively (Fig. 4A). No induction was observed in the cortex, pineal gland, and retina (Fig. 4A). Three additional bRFx4 primer sets were utilized, and similar results were obtained (data not shown).

Next, to test whether bRFx4 induction is subjective night-specific, its expression was measured in the SCN at CT6 (subjective day), CT14 (early subjective night), and CT22 (late subjective night) after light exposure. Whereas significant induction was detected at CT14 and CT22, little induction was observed at CT6 (Fig. 4B). No induction was detected in the cortex at any of the three CT points (Fig. 4B).

To determine whether the expression of the gene is rhythmic, gene expression was measured over a period of 1 day at 4-h intervals, CT2, -6, -10, -16, and -22. We did not detect any significant rhythmic expression in the SCN (Fig. 4C). mPer2 expression was also measured, and clear rhythmic expression was detected, confirming the assay reliability (Fig. 4C).

In situ hybridization was performed, and a clear induction of gene expression was detected in the SCN 45 min after light exposure at CT22 (Fig. 5, A and B). To confirm this, three different probes prepared from bRFx4 cDNA were utilized, each of which obtained similar results. Results using the bRFx4-specific probe are shown. Induction of gene expression was detected in all SCN sections: rostral, central, and caudal (Fig. 5A). The sum of the intensities from all six SCN sections covering the entire SCN region is shown in Fig. 5B.

It has been suggested that circadian clock entrainment is regulated at the gene expression level (22). Some light-inducible genes have been isolated in the SCN (32–40), and tremendous efforts have gone into clarifying the role of the products of such light-inducible genes in the entrainment mechanism, but multiple signal pathways in the mechanism make it difficult using the gene-disrupting approach (44–46). Here we report on a novel form of mouse RFX4 that is different from the known RFX4 transcript expressed in the testis. The expression of novel RFX4 is clearly localized in the SCN, and its induction occurs only during subjective night.

Several light-induced genes have been identified in the SCN, and a significant number of them have the cyclic AMP-response element sequence, which functions as a cis-controlling element for light induction, in the upstream region of their initiation codon (47, 48). Bioinformatic analysis revealed the presence of the cyclic AMP-response element sequence at ~6,807 and ~11,500 of the mouse RFX4 gene. Both sequences are conserved between mice and humans (Fig. 3A). This fact is consistent with the bRFx4 induction in response to light exposure. We also searched for the X-box sequence (GTNRCCNNGGYAC) (5, 11) in a 10-kbp region upstream of 19 mouse circadian rhythm genes: per1, per2, per3, bmal1, clock, cry1,2, dbp, dec1,2, e4bp4, rev-erb-a, tef, tim, vasopressin, prokineticin 2, c-fos, fosB, and nr4a2 to find the target gene of RFX4 and identified the X-box consensus sequence in per1 and per3 genomic regions. However, no X-box exists in human PER1 and PER3 genes, suggesting that these genes are not RFX4 targets.

To obtain evidence for the presence of gene products in the SCN, antibodies for mouse bRFx4 were generated using a synthetic peptide. On the other hand, full-length bRFx4 cDNA was cloned, and a FLAG-tagged bRFx4 gene was prepared and introduced into an expression vector. COS7 transfectants with these constructs were used for antibody verification. The anti-bRFx4 antibody detected a band at ~80 kDa, which has the same estimated molecular weight in both COS7 transfectants with bRFx4 or FLAG-bRFx4. The band detected in the FLAG-tagged bRFx4 transfectant was also detected by the anti-FLAG monoclonal antibody. Furthermore, the products immunopre-
The results demonstrated an availability of antibodies and also indicated that the identified \( bRfx4 \) encodes an 80-kDa polypeptide in COS7 transfected cells. Interestingly, the band was comprised of four bands with small molecular mass differences, all of which were also detected by the anti-FLAG monoclonal antibody and polyclonal antibodies (Fig. 6A). Different phosphoryl-

**Fig. 4.** Induction of \( bRfx4 \) transcript expression by light in the SCN in a subjective night-specific manner. A, induction of the \( bRfx4 \) transcript by light exposure in the SCN at CT14. The amount of mouse \( bRfx4 \) transcript was measured using real-time PCR. The \( bRfx4 \) mRNA level in the SCN before light exposure is defined as 100. B, subjective night-specific induction of the \( bRfx4 \) transcript. The amount of mouse \( bRfx4 \) transcript was measured at 0 and 45 min after light exposure at CT6, -14, and -22. The amount of \( bRfx4 \) transcript in the SCN at CT14 before light exposure is defined as 100. C, no rhythmic expression of the \( bRfx4 \) transcript. Expression of \( bRfx4 \) and \( mPer2 \) in the SCN was measured at CT2, -6, -10, -14, -18, and -22. For both genes, the amount of mRNA in the SCN at CT2 is defined as 100. The mean ± S.E. (\( n = 2 \)) is indicated.

**Fig. 5.** Induction of the \( bRfx4 \) transcript observed by in situ hybridization. The arrows indicate the SCN. The results of in situ hybridization with the whole brain section at CT22 with the \( bRfx4 \) transcript probe are shown. A, in situ hybridization with the \( bRfx4 \)-specific probe with rostral, central, and caudal sections. B, sum of the intensities obtained from all six sections prepared from the brain covering the entire SCN region. The \( bRfx4 \) mRNA level in the SCN before light exposure is defined as 100. C, the probe for in situ hybridization, the primers for the real-time PCR, and the locus for the synthetic peptides.
Signals by anti-FLAG detected only in the nucleus (Fig. 6). The results reported in this study suggested that the product encoded by the novel transcript plays a role in the entrainment mechanism.

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Addendum—Since this manuscript was submitted, a paper has appeared (49) identifying the same transcripts in the brain. The authors accidentally obtained a mouse strain in which the bRfx4 gene locus was disrupted by inserting a vector into a transgenic mouse. They could not find any transcripts of bRfx4 in the brain of the transgenic mice. The /mice died within 1 h of birth, and the /mice exhibited marked hydrocephalus of the lateral and third ventricles.
Restricted Expression and Photic Induction of a Novel Mouse Regulatory Factor X4 Transcript in the Suprachiasmatic Nucleus
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