Transcription factors containing a homeodomain play an important role in the organogenesis of vertebrates. We have isolated a novel homeodomain transcription factor, Otx3, which is structurally and functionally related to Otx1 and Otx2, transcription factors that are critical in brain morphogenesis. Mouse Otx3 is a protein composed of 376 amino acids. Otx3 mRNA was expressed in mouse embryos from 10.5 to 13.5 days postcoitum (dpc) and in adult cerebellum as assessed by Northern blotting. Whole-mount in situ hybridization of mouse embryos from 9.5 to 11.5 dpc revealed strong expression of Otx3 mRNA in the diencephalon, mesencephalon, metencephalon, myelencephalon, and developing eye, indicating an expression pattern largely overlapping but distinct from those of Otx1 and Otx2. In addition, Otx3 was shown by electrophoretic mobility shift assay to bind to the TAATCC motif, the consensus binding sequence for Otx1, Otx2, and Crx. Results of a transcription reporter assay suggest that Otx3 functions as a transcription repressor by binding to this motif. These results suggest that Otx3 is a novel member of the Otx family and may be involved in the development of the central nervous system.

Many signal molecules and transcription factors are required in the control of induction, specification, and regionalization of the CNS in vertebrates (1, 2). Most of them have been identified as genes homologous to those in Drosophila (1, 2) in which the patterning of the neural primordium has been studied extensively. Among them, the Otx family (Otx1, Otx2, and Crx), the vertebrate homologues of orthodenticle (otd), possess a bicoid (Bcd)-like homeodomain and has been shown to play an important role in brain morphogenesis in vertebrates (3). During murine embryogenesis, Otx1 expression is detected first at the early stage of 8.2–8.5 dpc throughout the forebrain and midbrain neuroepithelium and in developing sense organs (4, 5). From birth day onward, Otx1 also is expressed at a relatively low level in the anterior lobe of the pituitary gland (6). Studies of mutant mouse models suggest that Otx1 is involved in corticogenesis, sense organ development, and pituitary function (3). Otx2 is expressed at an earlier developmental stage than Otx1. In mouse, Otx2 already is expressed before the onset of gastrulation in the epiblast and in the visceral endoderm at 5.5 dpc (5) and also in the headfold at 7.5 dpc (4). After 8.5 dpc, the expression pattern of Otx2 largely overlaps that of Otx1 with a posterior border at the mesencephalic side of the isthmic constriction during brain regionalization (3, 4). Crx also was identified as a member of the Otx family (7). The expression of Crx is highly restricted to retina where it is profoundly involved in differentiation and maintenance of retinal neurons. HD proteins are thought to be involved in development and differentiation not only in central and peripheral neurons but also in endocrine and neuroendocrine cells (6, 8). It has been shown that many transcription factors necessary in the development of the CNS also are involved in the development of insulin-secreting pancreatic β cells (9). We hypothesized that known or novel HD transcription factors expressed in pancreatic β cells participate in their development by organizing the regulated expression of the various other transcription factors.

In the present study, we have identified a novel HD transcription factor from a pancreatic β cell line that is highly expressed in brain. It possesses a Bcd-like HD structurally and functionally related to that of Otx1 and Otx2 and has been designated Otx3. Our findings suggest that Otx3 may play an important role in brain morphogenesis.

**EXPERIMENTAL PROCEDURES**

**Screening of cDNA Library**—A partial cDNA fragment was amplified from the mouse pancreatic β cell line MIN6 by reverse transcriptase PCR using fully degenerate primers corresponding to the following amino acid sequences: RS/ER/AT/AFT and QVWFKNR, both of which are highly conserved among most HD proteins. The bands of interest from the PCR were excised from a 2% agarose gel and then subcloned and sequenced. A DNA fragment having a novel HD was identified. This cDNA fragment was used as a probe to screen the MIN6 cDNA library under high stringency hybridization conditions to isolate the full-length cDNA encoding Otx3.

**Northern Blot Analysis**—RNA was extracted from adult Sprague-Dawley rat tissues and cell lines, and 20 μg of total RNA were电phoresed on a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The Northern blot of full stage (4.5–18.5 dpc) mouse embryos was purchased from Seegene (Seoul, Korea), each line containing 20 μg of total RNA. An 802-bp fragment of Otx3 cDNA (nt 1–802), a
full-length coding region of Otx1 cDNA, and a 523-bp fragment of Otx2 cDNA (nt 103–625) were used as probes for the hybridization of Northern blots. Hybridization was performed under standardized conditions. Membranes were washed with 0.1× SSC and 0.1% SDS at room temperature for 2 h and at 50°C for 1 h before autoradiography.

**In Situ Hybridization**—Whole-mount and section RNA in situ hybridization was performed as described previously (10). The cRNA probes for Otx1 and Otx2 were synthesized based on the sequences from GenBank™ accession numbers AF424700 and P80206, respectively. Otx1 and Otx2 cDNAs were amplified by PCR from a mouse brain cDNA library using specific primers and subcloned into pGEM-T easy vector (Promega, Madison, WI). For whole-mount in situ hybridization, digoxigenin-labeled riboprobes were synthesized using linearized DNA templates in pBluescript vector (Otx3) (Stratagene, La Jolla, CA) and pGEM-T easy vector (Otx1 and Otx2). Transcription reactions were carried out according to the manufacturer’s instructions using T3 (for Otx3) and SP6 (for Otx1 and Otx2) RNA polymerase (Promega) in the presence of a digoxigenin-NTP mixture. For in situ hybridization of Otx3 on embryonic sections, 35S-labeled riboprobes were synthesized using T3 RNA polymerase in the presence of 35S-UTP (Amersham Biosciences).

**EMSA**—For EMSA, a partial Otx3 protein (aa 76–148) was expressed as a glutathione S-transferase (GST) fusion protein in Escherichia coli using pGEXT-1 vector (Amersham Biosciences) and purified by glutathione-Sepharose beads (Amersham Biosciences). The HD-containing peptide of Otx2 (aa 34–208) was produced using the same method. Oligonucleotide sequences used as labeled probes in EMSA were as follows: HD consensus sequence, 5′-CAGTAAGCCCTTTAATCCGGCAGGATCCCGTGCTCT-3′ and its exact complement; mutant HD consensus sequence, 5′-CAGTAAAGCCAGATCTACCCTGTCTCT-3′ and its exact complement. The HD consensus sequence contains the binding sequence (TAATCC) for the Otx family, while Otx3 lacks the so-called OTX-tail that is conserved among the other Otx members (7) (Fig. 1B). However, Otx3 lacks the other known domains, including the paired, HOX, and POU domains present in the Pax, HOX, and Pitx families. We also have identified a human full-length Otx3 cDNA (GenBank™ accession number AB037699) from a fetal brain cDNA library (BD Biosciences CLONTECH, Palo Alto, CA). The amino acid identity between mouse and human Otx3 is 94.4% (data not shown).

**Expression of Otx3 mRNA in Adult Rat Tissues, Cell Lines, and Embryos**—To examine the tissue expression pattern of Otx3, total RNA from various adult rat tissues, endocrine cell lines, and mouse embryos at 4.5–15.5 dpc was analyzed by Northern blotting. A single abundant transcript of 4.3 kb was detected in MIN6 and adult cerebellum, but no signal was observed in adult pancreatic islets or the other cells and tissues (Fig. 1C). However, expression in adult pancreatic islets was demonstrated by reverse transcriptase PCR with Otx3-specific primers (data not shown). During embryogenesis, the Otx3 transcript was detected from 10.5 to 13.5 dpc in mouse embryos (Fig. 1D). To compare the expression pattern of Otx3 with Otx1 and Otx2 during mouse development, the expression of Otx1 and Otx2 also was evaluated in the same blot (data not shown). A single Otx1 transcript of about 3.5 kb was detected from 10.5 to 15.5 dpc in mouse embryos. Although a single Otx2 transcript of about 3.3 kb was detected from 7.5 to 18.5 dpc in mouse embryos, most abundant Otx2 mRNA was detected from

**RESULTS**

**Otx3 Is a Novel Member of the HD Gene Family**—Since a partial DNA fragment obtained from degenerate PCR was suggested to be a novel transcription factor, its full-length cDNA was isolated from a mouse MIN6 cDNA library and sequenced (Fig. 1A). Sequence analysis revealed a novel member belonging to the HD gene family that was designated mouse Otx3 (GenBank™ accession number AB037698). The deduced open reading frame encodes 376 amino acids with a predicted mass of 40 kDa. The HD of mouse Otx3 shows homology to HD-containing transcription factors including members of the Pax, HOX, and Pitx families. Among these, Otx3 has the highest amino acid identity with Otx1, Otx2, and Crx (65% for each one). Further, it possesses a lysine at the 50th amino acid residue in the HD, a feature shared by all of the members of the Otx family, while Otx3 lacks the so-called OTX-tail that is conserved among the other Otx members (7) (Fig. 1B). However, Otx3 lacks the other known domains, including the paired, HOX, and POU domains present in the Pax, HOX, and Pitx families. We also have identified a human full-length Otx3 cDNA (GenBank™ accession number AB037699) from a fetal brain cDNA library (BD Biosciences CLONTECH, Palo Alto, CA). The amino acid identity between mouse and human Otx3 is 94.4% (data not shown).

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**Fig. 1.** Structures of the Otx family and Northern blot analysis of Otx3. A, the deduced amino acid sequence of mouse Otx3 cDNA is shown. The boxed amino acid sequence corresponds to the HD. The lysine residue at the 50th position in the HD is indicated by an asterisk. B, structures of members of the Otx family. Schematic structures of Otx3, Otx1, Otx2, and Crx are shown. Hatched, solid, and shaded boxes indicate HD, lysine residue, and OTX-tail, respectively. C, Otx3 mRNA expression in rat adult tissues and endocrine cell lines. Each lane contains 20 μg of total RNA. A single abundant transcript of 4.3 kb (indicated by an arrow) is detected in MIN6 and adult cerebellum. Skeletal m., skeletal muscle; Islets, pancreatic islets. D, Otx3 mRNA expression during mouse development. An arrow indicates a single transcript of about 4.3 kb from 10.5 to 13.5 dpc in mouse embryonic stages.
FIG. 2. In situ hybridization analyses of mouse embryos. A, whole-mount in situ hybridization. Expression of Otx3 (A1–A3), Otx2 (A4–A6), and Otx1 (A7–A9) in 9.5 dpc (A1, A4, and A7), 10.5 dpc (A2, A5, and A8), and 11.5 dpc (A3, A6, and A9) mouse embryos. An arrowhead indicates the midbrain-hindbrain boundary, A10–A12, expression patterns of Otx1 (yellow), Otx2 (red), and Otx3 (blue) in the developing CNS at 9.5, 10.5, and 11.5 dpc mouse embryos. Te, telencephalon; Di, diencephalon; Ms, mesencephalon; Mt, metencephalon; My, myelencephalon; Oc, optic cups. B, in situ hybridization in sections: Otx3 expression of mesencephalon in transverse sections at 10.5 dpc (B1 and B2) and sagittal sections of whole-mount mouse embryos at 13.5 dpc (B3 and B4). The bright field image shown in B2 corresponds to the dark field image shown in B1. B4 is a higher magnification of the boxed region in B3. Scale bar, 200 μm.

10.5 to 13.5 dpc. The temporal expression pattern of Otx3 overlaps that of Otx1 and Otx2 during embryogenesis, especially from 10.5 to 13.5 dpc.

Spatial Expression Pattern of Otx3 in Mouse Embryos—To investigate Otx3 expression at early midgestational stages of mouse development and to obtain more detailed information on the localization of Otx3 mRNA expression in the embryos, we performed whole-mount in situ hybridization of mouse embryos from 8.5 to 11.5 dpc (Fig. 2A, A1–A3). For comparison, whole-mount in situ hybridization of Otx2 (Fig. 2A, A4–A6) and Otx1 (Fig. 2A, A7–A9) also were performed in mouse embryos from 9.5 to 11.5 dpc. At 8.5 dpc, Otx3 transcripts were detected in the prospective midbrain region and preoptic placode (data not shown). From 9.5 to 11.5 dpc, Otx3 expression was craniocaudally delimited to the rostral region of the developing nervous system (Fig. 2A, A1–A3). In 9.5 dpc embryos, the anterior and posterior boundaries of Otx3 expression coincide with those of the diencephalon and mesencephalon, respectively (Fig. 2A, A1). Beginning from 10.5 dpc, Otx3 expression progressively declines in the anterior region, and the most intense hybridization signal coincides with the midbrain-hindbrain boundary (Fig. 2A, A2, arrowhead). In addition, Otx3 expression was observed in the developing eye (Fig. 2A, A2 and A3). At 11.5 dpc, Otx3 expression is enhanced gradually in a craniocaudal direction within the mesencephalon, metencephalon, and myelencephalon (Fig. 2A, A3).

To investigate Otx3 expression along the dorsoventral axis, we performed in situ hybridization on transverse sections through the mesencephalon of 10.5 dpc embryos and found that Otx3 is expressed, but not in the floor plate (Fig. 2B, B1 and B2). In 13.5 dpc embryos, Otx3 expression is present in the mesencephalon and extends posteriorly to regions of the metencephalon and myelencephalon as shown in sagittal sections (Fig. 2B, B3 and B4). Similarly to Otx3, Otx1 and Otx2 are expressed in the restricted regions of the developing forebrain and midbrain in early midgestation of mouse embryos as reported previously (3). In 9.5 dpc embryos, Otx1 is expressed intensely in the telencephalon, diencephalon, mesencephalon, and optic vesicles (Fig. 2A, A7). Otx2 is expressed in the telencephalon, diencephalon, and mesencephalon as well as in optic vesicles at 9.5 dpc (Fig. 2A, A4). At 10.5 dpc, Otx1 expression is still seen in the telencephalon, diencephalon, mesencephalon, and optic cups as is Otx3, but Otx2 is only barely visible in the telencephalon at 10.5 dpc (Fig. 2A, A5). In 11.5 dpc embryos, Otx1 is expressed in the telencephalon as well as the diencephalon, mesencephalon, and developing eye (Fig. 2A, A9), while Otx2 expression is restricted to the mesencephalon and optic cups (Fig. 2A, A6). The expression patterns of Otx3, Otx2, and Otx1 are schematically summarized by a line diagram in Fig. 2A, A10 (9.5 dpc), A11 (10.5 dpc), and A12 (11.5 dpc). All three genes are expressed in the mesencephalon and diencephalon.
throughout these stages, while only Otx3 is expressed in the metencephalon and myelencephalon at 11.5 dpc.

**DNA Binding Activity of Otx3 Protein**—The Otx family has lysine at the 50th position of the HD, which confers DNA binding specificity for the sequence motif TAATC(C/T) (the consensus for Otx1 and Otx2) or TAATC(C/A) (the consensus for Crx) (5, 7, 12). The binding of Otx3 to the motif TAATCC was tested using EMSA. A fusion protein of GST and the HD of Otx3 was incubated with radiolabeled DNA probes. Otx3 HD showed strong binding to the HD consensus sequence in Fig. 3A, lane 3. Fusion protein GST-Otx2 was used as a positive control. Specificity of Otx3 binding to the HD consensus sequence was examined by a competition experiment. The excess amount of unlabeled competitor of the HD consensus sequence resulted in a remarkable inhibition of the DNA binding activity of Otx3. However, binding was not inhibited by competition with the mutant HD consensus sequence (Fig. 3B).

**Transcriptional Activity of Otx3**—To assay the activity of Otx3 as a transcription factor, a transcription reporter assay was performed in the neuroendocrine cell line GH3. Co-transfection of the cells with pP3Ctk-Luc and pCMV-Otx3 resulted in the reversion of Otx3 to an activator. Together these findings suggest that Otx3 functions as a transcription repressor of Otx2 by acting competitively on the target sequence. Interestingly Otx3 lacks the “OTX-tail,” a conserved motif of about 20 amino acids that is generally believed to confer transactivation activity in the Otx family. It should be noted that the spatial and temporal expression patterns of Otx3, Otx1, and Otx2 are similar but not identical. At 9.5 dpc, Otx3 was not expressed in telencephalon, while both Otx1 and Otx2 were expressed in this area. At 10.5 and 11.5 dpc, the expression pattern of Otx3 resembled that of Otx2 but not Otx1 as assessed by whole-mount in situ hybridization. Analysis of in situ hybridization in sections at 13.5 dpc showed that the expression pattern of Otx3 in the myelencephalon was distinct from those of Otx1 and Otx2. Thus, Otx3 expression is cranio-caudally more restricted than Otx1 and Otx2. Furthermore, the phenotypes of Otx1 (Otx1<sup>−/−</sup>) and Otx2 (Otx2<sup>−/−</sup>) knockout mice are different: Otx2<sup>−/−</sup> mice lack forebrain and midbrain, which is embryonically lethal (24, 25); Otx1<sup>−/−</sup> mice are viable but exhibit epilepsy, transient dwarfism, transient hypogonadism, and abnormality in inner ear and eyes (6, 26). Accordingly, although Otx3 is likely involved in brain morphogenesis in concert with Otx1 and Otx2, it may have a functionally distinct role from that of Otx1 and Otx2.

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