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PATTERNS & PHENOTYPES

Expression of *qBrn-1*, a New Member of the POU Gene Family, in the Early Developing Nervous System and Embryonic Kidney

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It has been shown that POU domain genes play critical roles in the development of the nervous system. We have obtained a new member of the class III POU domain genes, *qBrn-1*, from the cDNA library of embryonic day 5 quail and have made an extensive expression pattern analysis of *qBrn-1* and *qBrn-2* throughout the early embryonic development by in situ hybridization. With a specific antibody we prepared, further analysis by immunohistochemistry showed that the location of qBrn-1 protein was consistent with that of the transcripts in the early developing quail. Our results showed that both *qBrn-1* and *qBrn-2* were preferentially expressed in the developing central nervous system, and their transcripts were initially detected in the neural plate and later in the distinct regions of the neural tube with a stage-dependent pattern. Moreover, their expression was also detected in both notochord and neural crests. However, *qBrn-1* signal, different from *qBrn-2*, was more widely found in the auditory pits, branchial arches, and in the mesodermal components of the developing kidney. And the expression of *qBrn-1* in nephric region was earlier and wider than that of mouse *Brn-1*, suggesting the characteristic function of *qBrn-1* in the kidney formation. The distinct dynamic expression patterns of *qBrn-1* and *qBrn-2* indicate multiple roles of the class III POU genes in quail neurogenesis and organogenesis. *Developmental Dynamics 235:1107–1114, 2006.* © 2006 Wiley-Liss, Inc.

Key words: *qBrn-1*; *qBrn-2*; quail; development; nervous system; kidney; neural crests; notochord; auditory pits; branchial arches

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INTRODUCTION

POU domain genes encode transcription factors characterized by a highly conserved POU-specific domain of 76–78 amino acids, in addition to a POU homeodomain region (Verrijzer and Van der Vliet, 1993; Wegner et al., 1993). Both domains are connected by a linker region of variable length and sequence. The POU domain contributes both to site-specific DNA binding and to protein–protein interactions between POU domain proteins and other transcription factors (Herr and Cleary, 1995). Experimental and genetic studies have revealed the crucial roles of POU family factors in the development and functioning of the nervous system (Latchman, 1999). On the basis of amino acid sequence similarities of the POU domain, POU genes can be grouped into at least six classes (Wegner et al., 1993; Andersen and Rosenfeld, 2001). In the POU family, the POU III class of transcription factors (Brn-1, Brn-2, Brn-4/RHS2, and Oct-6/SCIP/Tst-1) has been demonstrated to function in neuronal development, given that these factors are specifically ex-

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A

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1      TGTGGTCTGCAGATGGCTGAGCCTCTGCATAGGGGAATAGAGGGGACTGCATAGGAAGAA
61     TACCCCAGAACTTGGCAGCTGTTGCGTGCAATGGGTGCAAGCCCCTAACACAGTGTGGAA
121    GCCATTTATCTGTACTTCTCAGTCAGAACAGAGGAGATGCCGGCCAGAAACTTGTCCCAT
181    GCAGCATGAACTTCTGGGGTGAAATCATTGGGATAGCGGGCAGCCACGGAGCACAGGATA
241    CAGTGGGAAAGCAGCTTGAAGTTCACTGGGTCCACCCTAAGGATGTAAGCATGCAGCTCG
301    CTGAGTTTGGCCAAAGCACCTCTAATATCATCGATGTTCTTCACGGCTTCCCCAATGGCA
361    TTCAGGACCTTGGAGCCGTGACCACGAAGCTGAACGGAGCCTTGGCTGAGATCAAAGTGA
421    GGGAAGTAGGTTTTCGTCTGAGGGTAGCTGGCAAAAAGCCTCTCCAGTGATTCCACCCCA
481    ATGGCCTCCCTGTACTCGCAGCCCGGGGGCTTCACGGTGAACGGCATGCTGAGCCCCGCG
1       M  A  S  L  Y  S  Q  P  G  G  F  T  V  N  G  M  L  S  P  A
541    GGCGGCGGACAGAGCCTGGTGCACCCCGGGCTGGTGCGCGGCGGAGAGACGGCGGAGCTG
21      G  G  G  Q  S  L  V  H  P  G  L  V  R  G  G  E  T  A  E  L
601    GGCGAGCACCCCGGGCATCACCATCACCACCACCACCCGCACCCCGGGCACCACGCGCCG
41      G  E  H  P  G  H  H  H  H  H  H  H  P  H  P  G  H  H  A  P
661    CACCACGGCGCCGTCAACAGCCACGAGGCGCACTCGGACGAGGACACGCCGACCTCGGAC
61      H  H  G  A  V  N  S  H  E  A  H  S  D  E  D  T  P  T  S  D
721    GACCTGGAGCAGTTCGCCAAGCAGTTCAAGCAGCGGCGGATTAAGCTGGGCTTCACGCAG
81      D  L  E  Q  F  A  K  Q  F  K  Q  R  R  I  K  L  G  F  T  Q
781    GCCGACGTGGGGCTGGCGCTGGGCACCCTGTACGGCAACGTCTTCTCGCAGACCACCATC
101     A  D  V  G  L  A  L  G  T  L  Y  G  N  V  F  S  Q  T  T  I
841    TGCCGCTTCGAGGCCCTGCAGCTCAGCTTCAAGAACATGTGCAAGCTGAAGCCTTTGTTG
121     C  R  F  E  A  L  Q  L  S  F  K  N  M  C  K  L  K  P  L  L
901    AACAAGTGGCTGGAGGAAGCCGACTCCTCCACCGGCAGCCCCACCAGCATCGACAAGATC
141     N  K  W  L  E  E  A  D  S  S  T  G  S  P  T  S  I  D  K  I
961    GCGGCGCAGGGCAGGAAGAGGAAGAAGCGGACCTCCATCGAGGTGAGTGTCAAGGGGGCC
161     A  A  Q  G  R  K  R  K  K  R  T  S  I  E  V  S  V  K  G  A
1021   TTGGAGAGCCACTTTCTGAAATGCCCCAAGCCCTCCGCCCAGGAGATTACGAACCTAGCG
181     L  E  S  H  F  L  K  C  P  K  P  S  A  Q  E  I  T  N  L  A
1081   GACAGCCTGCAGCTGGAGAAGGAGGTGGTCAGGGTTTGGTTTTGCAATCGGAGGCAGAAA
201     D  S  L  Q  L  E  K  E  V  V  R  V  W  F  C  N  R  R  Q  K
1141   GAGAAACGCATGACCCCGCCGGGGATCCAGCAGCAGACCCCCGACGATGTCTACTCGCAG
221     E  K  R  M  T  P  P  G  I  Q  Q  Q  T  P  D  D  V  Y  S  Q
1201   GTCGGCGCCGTCAGCTCCGACACGCCGCCCCCTCACCACGGACTGCAGAGCGGCGTGCAG
241     V  G  A  V  S  S  D  T  P  P  P  H  H  G  L  Q  S  G  V  Q
1261   TGAGCTGGGCTCCGCGGGGCCGGGCCGGGAGAGCTCCGGCGGCGGCGGCGGCGGCGCCGC
        *
1321   GCTCACACACACGGACACGGACACTCACACTCACACGCACGCACGCACTCACACACACAC
1381   ACACACACTCGCGCGCGGGCCGGGCTCGCTCGGGCCGCCCGTGTCGGTATATGTACGGAT
1441   AGAGGAGGGCGCCTGGATCTATCTGTCTATACGGGATCGATAGAGAGAGGGAGCGCCGGG
1501   GAAGGTCGAGCCGAGCGAGAGCGTTTCCGCTGCGTTGGGAGAACGGGGTGGAGATGCGTA
1561   ATGCACCAAAACTGCAGATGTGCCGGGGGCTGCTGTGGGAAGAGGGCTCGGCCGGGTGGG
1621   TTCTGCCTTGGGTTTCTGTTCGGGTTTTTGCTTTTCGCCCCCCCTCCTCCCCCCTCCCCC
1681   GTGAGCCCCTCTCTCAGAGATGGGGTCTGCATCTCTCCCCTCCTCCCCCCCCCCGTGCTC
1741   TCCCCTATCCCCAAAGGGCTCTTCTCTCAATCACGGAAACTTTATTTTTCCCCCCTTCCT
1801   TTTCCTCTACGCCTTCCCTCTCCCCCCCCCCCCCTTTTTTTTTTTTCCTTATTATTATTA
1861   TTATTATTTCCCCTTTATTTTCTTTATTTATTTTCATTCCTTTTATTTTTATTAGATTTT
1921   TTTTTCTCAATGGGAGAGATCCAAAAGAAAAAAAAAAAAAGAAAAAA
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Fig. 1. **A:** Nucleotide sequence and open reading frame of the *qBrn-1* gene (GenBank accession no. AY781803). The *qBrn-1* cDNA comprises 1,966 nucleotides, containing an open reading frame encoding a protein of 260 amino acid residues. The POU-specific domains and POU homeodomains are underlined, and the start and stop codons are underlined with double line. An asterisk indicates the stop codon. Positions in the nucleotide and the amino acid sequence (in italics) are given by numbers. **B:** Alignment of qBrn-1 protein sequence with orthologs from mouse (M88299), rat (AJ001641), and human (AB001835), as well as *qBrn-2* (AF091043). The identical amino acid residues among Brn-1 members are represented by shadowing, and the asterisks indicate the identical residues between qBrn-1 and qBrn-2. Gaps are represented by "−" symbols. The POU domain region is underlined.

B

```
qBrn-2   MATAASNHY----SLLASGSPMVHAEPP---------------------GGMQPG------GGYR---DA   36
qBrn-1   ----------------------------------------------------------------------    0
mBrn-1   MATAASNPYQPGNSLLTAGS-IVHSDAAGAGGGGGGGGGGGGG-AGGGGGGMQPGSAAVTSGAYRGDPSS   68
rBrn-1   MATAASNPYLPGNSLLAAGS-IVHSDAAGAGGGGGGGGGGGGG-AGGGGGGMQPGSAAVTSGAYRGDPSS   68
HBRN-1   MATAASNPYLPGNSLLAAGS-IVHSDAAGAGGGGGGGGGGGGGGAGGGGGGMQPGSAAVTSGAYRGDPSS   69

qBrn-2   GALVQADYALQ----SNG-HPLSHAHQWIAALSHGGPGGGGGGGGGGGGGGGGGEAPWAAAAAAAAAGAL  101
qBrn-1   ----------------------------------------------------------------------    0
mBrn-1   VKMVQSDFMQG--AASNGGHMLSHAHQWVTALPHAAAAAAAAAAAAVEASSPWSGSAVGMAGSPQQPPQP  136
rBrn-1   VKMVQSDFMQGAMAASNGGHMLSHAHQWVTALPHAAAAAAAAAAAAVEASSPWSGSAVGMAGSPQQPPRP  138
HBRN-1   VKMVQSDFMQGAMAASNGGHMLSHAHQWVTALPHAAAAAAAAAAAAVEASSPWSGSAVGMAGSPQQPPQP  139

qBrn-2   GPPDIKPAAVQAAPRGDELPP-------PPQHPPPPGRAPHLVHHGGGGGGHHAAWRAGG---AAHLPPG  161
qBrn-1   ----------------------------------------------------------------------    0
mBrn-1   PPPPPQGPDVKGGAGREDLHAGTALHHRGPPHLGPPPPPHQGHPGGWGAAAAAAAAAAAAAAAAAHLPS-  205
rBrn-1   PPPPPQGPDVKGGVGREDLHAGTALHHRGPPHLGPPPPPHQGHPGGWGAAAAAAAAAAAAAAAAAHLPS-  207
HBRN-1   PPPPPQGPDVKGGAGRDDLHAGTALHHRGPPHLGPPPPPHQGHPGGWGAAAAAAAAAAAAAAAAAHLPS-  208

qBrn-2   MAAANGAAQAGLLYPQPPGFTVNGMLG--------------AAQPALHHHGLRDAHDEAPGPPAPPHHGA  217
         **          ** ** ********                 *   *    *       *     **
qBrn-1   MAS---------LYSQPGGFTVNGMLS----PAGGG-------QSLVHPGLVRGGETAELGEHPGHHHHH   50
mBrn-1   MAGGQQPPPQSLLYSQPGGFTVNGMLSAPPGPGGGGGAGGGAQSLVHPGLVRG-DTPELAEHHHHHHHH  274
rBrn-1   MAGGQQPPPQSLLYSQPGGFTVNGMLSAPPGPGGGGGAGGGAQSLVHPGLVRG-DTPELAEHHHHHHHH  276
HBRN-1   MAGGQQPPPQSLLYSQPGGFTVNGMLSAPPGPGGGGGAGGGAQSLVHPGLVRG-DTPELAEHHHHHHHH  277

qBrn-2   EHPHGPHPAGGAGP---GGGG-AAAAGGPHHEAHSDEDTPTSDDLEQFAKQFKQRRIKLGFTQADVGLAL  283
          ***         *   *            ****************************************
qBrn-1   HHPHP--GHHA--P-HHG--------AVNSHEAHSDEDTPTSDDLEQFAKQFKQRRIKLGFTQADVGLAL  107
mBrn-1   AHPHPPHPHHAQGPPHHGGGG--AGPGLNSHDPHSDEDTPTSDDLEQFAKQFKQRRIKLGFTQADVGLAL  342
rBrn-1   AHPHPPHPHHAQGPPHHGGGG--AGPGLNSHDPHSDEDTPTSDDLEQFAKQFKQRRIKLGFTQADVGLAL  344
HBRN-1   AHPHPPHPHHAQGPPHHGGGGGAGPGLNSHDPHSDEDTPTSDDLEQFAKQFKQRRIKLGFTQADVGLAL  347

qBrn-2   GTLYGNVFSQTTICRFEALQLSFKNMCKLKPLLNKWLEEADSSSGSPTSIDKIAAQGRKRKKRTSIEVSV  353
         ******************************************* **************************
qBrn-1   GTLYGNVFSQTTICRFEALQLSFKNMCKLKPLLNKWLEEADSSTGSPTSIDKIAAQGRKRKKRTSIEVSV  177
mBrn-1   GTLYGNVFSQTTICRFEALQLSFKNMCKLKPLLNKWLEEADSSTGSPTSIDKIAAQGRKRKKRTSIEVSV  412
rBrn-1   GTLYGNVFSQTTICRFEALQLSFKNMCKLKPLLNKWLEEADSSTGSPTSIDKIAAQGRKRKKRTSIEVSV  414
HBRN-1   GTLYGNVFSQTTICRFEALQLSFKNMCKLKPLLNKWLEEADSSTGSPTSIDKIAAQGRKRKKRTSIEVSV  417

qBrn-2   KGALESHFLKCPKPSAQEITSLADSLQLEKEVVRVWFCNRRQKEKRMTPPGGTLPGAEDVYGP----SRD  419
         ******************** ******************************       ***      * *
qBrn-1   KGALESHFLKCPKPSAQEITNLADSLQLEKEVVRVWFCNRRQKEKRMTPPGIQQQTPDDVYSQVGAVSSD  247
mBrn-1   KGALESHFLKCPKPSAQEITNLADSLQLEKEVVRVWFCNRRQKEKRMTPPGIQQQTPDDVYSQVGTVSAD  482
rBrn-1   KGALESHFLKCPKPSAQEITNLADSLQLEKEVVRVWFCNRRQKEKRMTPPGIQQQTPDDVYSQVGTVSAD  484
HBRN-1   KGALESHFLKCPKPSAQEITNLADSLQLEKEVVRVWFCNRRQKEKRMTPPGIQQQTPDDVYSQVGTVSAD  487

qBrn-2   TPP-HHGVQTPVQ                                                          431
         *** *** *  **
qBrn-1   TPPPHHGLQSGVQ                                                          260
mBrn-1   TPPPHHGLQTSVQ                                                          495
rBrn-1   TPPPHHGLQTSVQ                                                          497
HBRN-1   TPPPHHGLQTSVQ                                                          500
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Fig. 1. (Continued.)

pressed in the embryonic and adult central nervous system (CNS) and function in the proliferation and specification of neuronal cell types during the peak period of neurogenesis (Alvarez-Bolado et al., 1995; Schreiber et al., 1997; McEvilly et al., 2002; Sugitani et al., 2002). Particularly, *Brn-1* and *Brn-2* are named for their strong and wide expression in the brain, and their POU domains share extremely high sequence homology (He et al.,

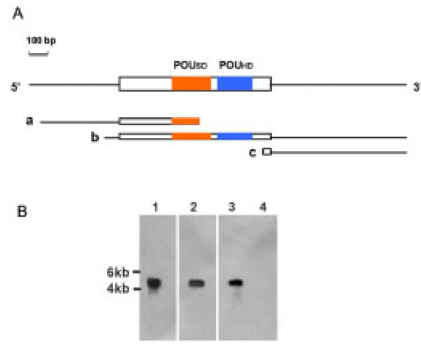

Fig. 2. Fig. 2. **A:** Schematic diagram of the *qBrn-1* cDNA and probes (a–c) used in Northern blot hybridization and in situ hybridization. The open reading frame is boxed, and the POU-specific domain (POU_{SD}) and POU homeodomain (POU_{HD}) are indicated by colored boxes. **B:** Northern blot analyses of *qBrn-1* mRNA in embryonic day 5 (E5) quail embryos (lanes 1–3) and in hearts of quails (2 days after hatching, lane 4). Expression of *qBrn-1* in E5 embryos was detected by probe a, b, and c, respectively. Total mRNA from hearts in lane 4 was used as the negative control. Molecular weight markers are indicated.

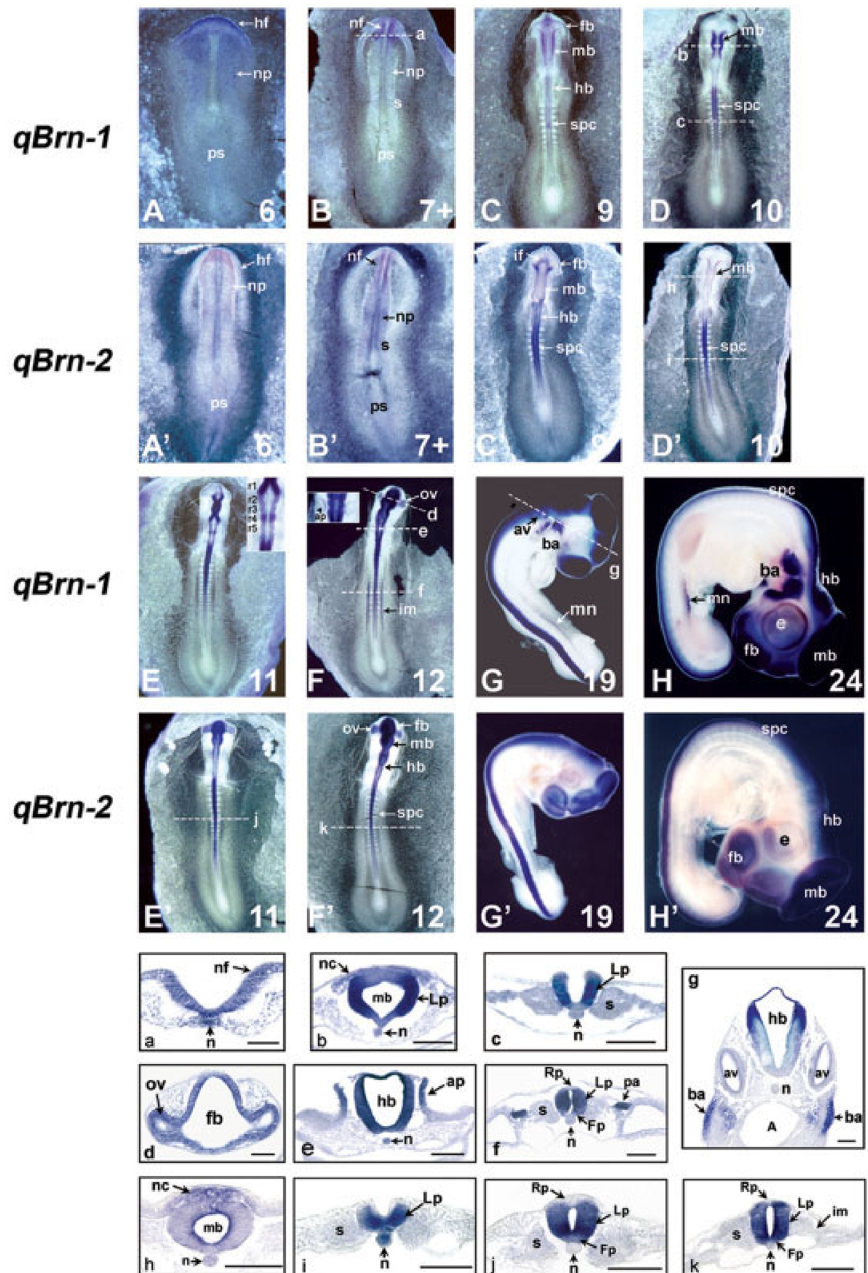

Fig. 3.

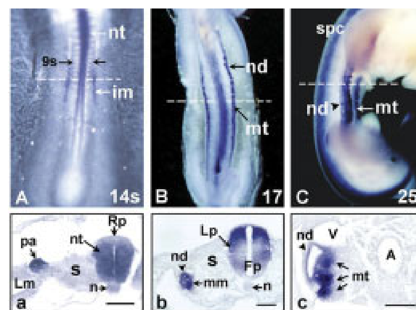

Fig. 4.

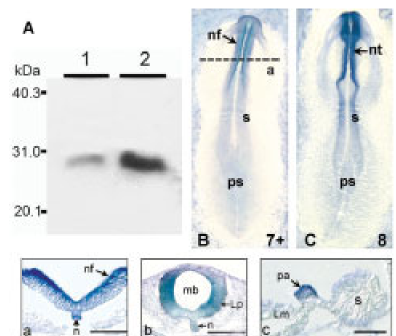

Fig. 5.

1989). Targeted mutagenesis of either *Brn-1* or *Brn-2* in mouse suggests that they have complementary roles in brain development (Sugitani et al., 2002).

Although several class III POU homeobox genes have been found in nematodes, *Drosophila*, amphibians, and mammals (Ryan and Rosenfeld, 1997; Anderson and Rosenfeld, 2001), there is no description of *Brn-1* homologue in birds. In comparison with *Brn-2*, much less is known about the expression and function of *Brn-1* genes, especially in nonmammalian vertebrates. In this study, we report a new cDNA corresponding to a quail ortholog of *Brn-1*, referred to as *qBrn-1* (GenBank accession no. AY781803), and we also present the comparative expression analysis of *qBrn-1* with *qBrn-2*, another member of class III POU genes previously cloned from quail in this lab (Liu et al., 2000).

RESULTS AND DISCUSSION

Isolation of *qBrn-1* cDNA

A cDNA was isolated from an embryonic day 5 (E5) quail cDNA library. The cDNA comprises 1,966 nucleotides, and the predicted full-length protein contains 260 amino acid residues including a conserved POU do-

main (Fig. 1A). The sequence comparison shows that the predicted POU domain is identical to those of mammalian Brn-1 proteins, such as HBRN-1 (human), rBrn-1 (rat), and mBrn-1 (mouse; Fig. 1B). The significant high levels of homology are also observed in the region immediately before the POU domain (a 14-amino acid residue stretch, 85.7%) and in the C-terminus (97.3%) between our predicted protein and other Brn-1 members. Hence, we named this gene as *qBrn-1*, a *Brn-1* orthologue in quail. However, the N-terminal region of this putative protein is considerably shorter than its mammalian counterparts and lacks the homopolymeric amino acid repeats, including alanine, glycine, and proline residues (Fig. 1B). These repeats, considered to form transactivation domain in POU proteins, are well conserved in both the position and the repeat number (Meijer et al., 1992; Verrijzer et al., 1993, Nakachi et al., 1997). The lack of such repeats indicates qBrn-1 might have a transactivation domain different from the mammalian Brn-1, which had been revealed to contain proline and alanine homopolymeric amino acid stretches (Schreiber et al., 1997).

Northern Blot Analysis

The *qBrn-1* transcripts were detected by three different antisense RNA probes in the total RNA from E5 embryos and in the total RNA from hearts of quails 2 days after hatching, since no expression of mammalian *Brn-1* was found in the heart before that time (He et al., 1989; Fig. 2A). All of the probes revealed a single band of an approximately 5-kb transcript in the E5 embryos, whereas no signal was found in the hearts (Fig. 2B). The hybridization results not only indicate one *Brn-1* homologue in quail but also demonstrate the specificity of the probes.

Expression of *qBrn-1* and *qBrn-2* in the CNS

Expression patterns of *qBrn-1* and *qBrn-2* were examined extensively by whole-mount in situ hybridization on quail embryos from Hamburger and Hamilton (HH) stage 3 to HH stage 25 (Hamburger and Hamilton, 1951).

No *qBrn-2* transcripts were detected markedly on quail embryos before HH stage 6, whereas extremely faint signal of *qBrn-1* came out in the anterior neural plate at HH stage 5 with the beginning of neurulation (data not shown). At HH stage 6, as the neural plate had just shaped, strong expression of *qBrn-1* was found in the head fold and neural plate, with weak labeling in the primitive streak (Fig. 3A). At this stage, *qBrn-2* transcripts could also be detected clearly in the neural plate, but were still absent from the head fold and faint in the primitive streak (Fig. 3A′). At HH stage 7+, when the neural tube was not yet closed at head fold, both *qBrn-1* and *qBrn-2* signals were clearly found in the neural folds, and *qBrn-2* was additionally observed in the posterior neural plate and somites (Fig. 3B,B′). After the fusion of neural folds, the expression of *qBrn-1* and *qBrn-2* was extended more caudally, and covered the entire neural tube by HH stage 9 (Fig. 3C,C′). And the signal of *qBrn-1* was intensified in forebrain and midbrain (Fig. 3C), whereas the expression level of *qBrn-2* exhibited in an anterior low and posterior high manner, despite a clear labeling in the future infundibulum (Fig. 3C′). The expression of *qBrn-2* in infundibular region suggests its role in neuroendocrine development as its *Brn-2* homologues (Nakai et al., 1995; Schonemann et al., 1995). At HH stage 10, a dramatic increase of *qBrn-1* expression was observed in the spinal cord, while the anterior high level of *qBrn-1* signal was clearly restricted in the midbrain and the adjacent forebrain and hindbrain regions. In the other regions of forebrain and hindbrain, no significant expression was detected (Fig. 3D). However, except for the midbrain, *qBrn-2* expression in the anterior neural tube was decreased to an almost undetectable level (Fig. 3D′). From HH stage 11 onward, the expression of *qBrn-1* and *qBrn-2* was

Fig. 3. A–H′: Whole-mount in situ hybridization of *qBrn-1* (A–H) and *qBrn-2* (A′–H′) on early developing quail embryos. The developmental stages are indicated in each panel. A–H and A′–H′ show embryos viewed dorsally (A–F,B–F), ventrally (A), and laterally (G,G′,H,H′), anterior is to the top. Sections of the stained embryos in B, D, F, G, D′, E′, and F′ are shown in a–k, respectively, with the dash lines showing the approximate levels of sections. Insets in E and F are the high-magnification images of the hindbrain region. The inset in E shows the unequal expression of *qBrn-1* in hindbrain with the stronger signals in the first and the fourth rhombomeres, and the inset in F shows the expression of *qBrn-1* in the auditory pit. A, aorta; ap, auditory pit; av, auditory vesicle; ba, branchial arches; e, eye; fb, forebrain; Fp, floor plate; hb, hindbrain; hf, head fold; Hn, Hensen's node; if, infundibulum; im, intermediate mesoderm; Lp, lateral plate; mb, midbrain; mn, mesonephros; n, notochord; nf, neural fold; nc, neural crest cells; nt, neural tube; ov, optic vesicle; pa, pronephric anlage; ps, primitive streak; r, rhombomere; Rp, roof plate; s, somite; spc, spinal cord. Scale bars = 100 μm.

Fig. 4. *qBrn-1* expression in the developing quail kidney and the neural tube. **A:** In kidney, the expression of *qBrn-1* was first detected in the intermediate mesoderm at the 14-somite stage (14s). Transverse section shows the signals in the intermediate mesoderm-derived pronephric anlage (a). **B,C:** Mesonephric *qBrn-1* expression was continuously detected in the nephric duct and condensed mesonephrogenic mesenchyme at Hamburger and Hamilton (HH) stage 17 (b) and persisted in both tubular and nephric duct-derived structures at HH stage 25 (c). A temporal and spatial change of *qBrn-1* expression in the neural tube is shown in a and b. An accumulation of *qBrn-1* mRNAs was found in the neural tube but clearly was excluded from the roof plate at stage 14s (a) and disappeared late from the floor plate and became progressively restricted in the lateral plate (b). A, aorta; Fp, floor plate; im, intermediate mesoderm; Lm, lateral plate mesoderm; mt, mesonephric tubule; n, notochord; nd, nephric duct; nt, neural tube; pa, pronephric anlage; Rp, roof plate; s, somite; spc, spinal cord; V, postcardinal vein. Scale bars =100 μm.

Fig. 5. A: Western blot analyses of qBrn-1 protein in tissues of quails 2 days after hatching. A 27.8-kDa band can be seen both in kidney (lane 1) and brain (lane 2). **B,C:** Locations of qBrn-1 quail embryos as determined by whole-mount immunohistochemistry. The developmental stages are indicated in each panel, and embryos are viewed dorsally; anterior is to the top. **a–c:** Sections of the embryos after whole-mount immunochemistry to show the locations of qBrn-1 in the neuroepithelium, notochord, neural tube, and in the pronephric anlage. Lm, lateral plate mesoderm; Lp, lateral plate; mb, midbrain; n, notochord; nf, neural fold; nt, neural tube; pa, pronephric anlage; s, somite. Scale bars = 100 μm.

markedly increased, and then maintained at a high level throughout the neural tube, including optic vesicles (Fig. 3E,F,E′,F′,d). Of interest, at HH stage 11, *qBrn-1* transcripts obviously showed a disequilibrium distribution in hindbrain, where they were more abundant in the first and the fourth rhombomeres in a transient manner (Fig. 3E, and insert). These expression patterns of *qBrn-1* and *qBrn-2* persist to late stages at least until HH stage 24 (Fig. 3H,H′).

Further analysis on transverse sections revealed the temporal and spatial changes of *qBrn-1* expression during the development of CNS. At an early stage (HH stage 7+), a high expression of *qBrn-1* was detected in the entire neuroepithelium from the neural groove to neural folds (Fig. 3a). During the fusion of neural folds, the expression of *qBrn-1* became enhanced in the lateral plate than in the floor and roof plates at both the anterior and posterior neural tube (HH stage 10 to 12, Fig. 3b–f). However, unlike *qBrn-1*, *qBrn-2* transcripts maintained a uniform distribution in neuroepithelium at HH stage 10 (Fig. 3h,i). At later stages, such as HH stage 17 (Fig. 4b) and 19 (Fig. 3g), the signal of *qBrn-1* showed a dorsal high and ventral low gradient in the lateral plate, while the expression of *qBrn-2* exhibited a reverse pattern before the end of neurulation (Fig. 3j,k).

The above results have showed that *qBrn-1* and *qBrn-2* are expressed predominantly in the CNS, suggesting an evolutionarily conserved role of class III POU genes during vertebrate neurogenesis. We have also found that both genes have a widespread and distinct dynamic expression pattern in the developing quail embryo, which is different from the situation in mice (He et al., 1989).

Expression of *qBrn-1* and *qBrn-2* Outside the CNS

Besides the predominant expression of *qBrn-1* and *qBrn-2* in the central nervous system, both genes were also found to be expressed in some other regions. During the early stages, the expression of *qBrn-1* and *qBrn-2* was observed in the neural crests and notochord (Fig. 3b,h). Within the notochord, both *qBrn-1* and *qBrn-2* displayed a dynamic expression. *qBrn-1* expression was first observed in the notochord at HH stage 7+ (Fig. 3a), maintained to express only at the cephalic region during HH stage 10 to 12 (Fig. 3b,c,e,f), and was no longer detectable at HH stage 19 (Fig. 3g). In contrast, the expression of *qBrn-2* in the notochord was visible before the neural folds closed (HH stage 10, Fig. 3i) and was no longer detectable when the neural tube fused (HH stage 12, Fig. 3j). The expression of *qBrn-2* was also transiently found in the somites at HH stage 7+ (Fig. 3B′) but disappeared thereafter. In addition, *qBrn-1* was found in the branchial arches and auditory pits (Fig. 3F,G,e,g), whereas the corresponding expression sites have not been reported for mammalian *Brn-1*.

The expression of *qBrn-1* in branchial arches and auditory pits was consistent with a possibility that it was involved in the differentiation of the neural crest cells. In cranial regions where neural tube fused, the hybridization signal of *qBrn-1* localized to the neural crest cells that was determined through comparison with the HNK-1 labeling (data not shown), a well-characterized antibody marker that recognizes the neural crest cells (Kuratani, 1991). Then, *qBrn-1* was expressed in the auditory pit, which was populated from the neural crest (Gilbert, 2000), suggesting that *qBrn-1*-positive cells represent a crest-derived component of the ear. Furthermore, the expression domain of *qBrn-1* extended to branchial arches at HH stage 16. It has been previously shown that the cranial neural crest cells emigrate from rhombomeres into the pharyngeal arches (Lumsden et al., 1991). These observations suggest that *qBrn-1* is involved in specifying the cranial neural crest cells and branchial arch derivatives. Although *qBrn-2* was also expressed in the neural crests, its transcripts were excluded from the above sites where *qBrn-1* was expressed, indicating that *qBrn-2* has different roles in specifying the neural crests.

Another characteristic expression region of *qBrn-1*, in comparison with *qBrn-2*, was the mesodermal components of embryonic kidney. The expression of *qBrn-1* in kidney was first detected in the intermediate mesoderm caudal to the ninth somite at the 14-somite stage (HH stage 11+, Fig. 4A). Transverse sections through this level showed that *qBrn-1* expression was confined to the intermediate mesoderm-derived pronephric anlage situated between the lateral plate mesoderm and somites (Fig. 4a). By HH stage 17, *qBrn-1* transcripts were observed not only in the nephric duct but also in the adjacent mesonephrogenic mesenchyme, which would form the tubules of the initial kidney (Fig. 4B,b). Furthermore, *qBrn-1* expression was maintained at a high expression level in the nephric duct and mesenchymally derived tubules by HH stage 25 (Fig. 4C,c).

It is interesting to compare the expression of *qBrn-1* with that of *mBrn-1*, a mouse *Brn-1* orthologue, in embryonic kidney. The expression of *mBrn-1* in kidney was found initially in the renal vesicle and then in a restricted manner during nephron formation, with its transcripts detected only in the tubule-derived components (Nakai et al., 2003). In contrast, the expression of *qBrn-1* in pronephric kidney was first detected in the intermediate mesoderm at the 14-somite stage and was later found in both the tubular and nephric duct-derived structures. Thus, it seems that the onset of nephric expression of *qBrn-1* is earlier than that of *mBrn-1*, and the expression domain of *qBrn-1* is wider in the embryonic kidney than is that of *mBrn-1*.

Western Blotting and Immunohistochemistry

To confirm the distribution of *qBrn-1* transcripts, the location of qBrn-1 protein needs to be investigated. We prepared a polyclonal antiserum against the N-terminus of qBrn-1 and performed immunohistochemical staining on quail embryos. To determine the capacity and specificity of the antiserum to bind qBrn-1, Western blot immunoassay was carried out by using protein samples extracted from the brain and kidney of quails (2 days after hatching). The antiserum recognized a single band of approximately 27.8 kDa in tissue extracts (Fig. 5A). The molecular weight was in accordance with the predicted size from the *qBrn-1* ORF sequence (28.5 kDa),

which also demonstrated the predicted difference in molecular weight between qBrn-1 and mammalian Brn-1.

The results of immunohistochemistry on whole-mount quail embryos are consistent with those of in situ hybridization. The first visible immunolabeling appeared at HH stage 6, when the neural plate had just shaped. At the 2-somite stage, when the neural plate began to fold, the presumptive cephalic level of neural folds was immunoreactive (Fig. 5B). From HH stage 8 onward, qBrn-1 was expressed strongly in the neural tube, including optic vesicles (Fig. 5C, and data not shown). Sections showed that qBrn-1 was expressed in the neuroepithelium and notochord at the 2-somite stage (Fig. 5a) and strongly in the lateral plate at the level of midbrain at HH stage 10 (Fig. 5b). Not until HH stage 13 was the nephric expression of qBrn-1 clearly observed in the intermediate mesoderm-derived pronephric anlage (Fig. 5c), although obscure immunolabeling was present already in the intermediate mesoderm at 14-somite stage.

CONCLUSION

In this report, we have described the isolation of a new member of class III POU domain genes, *qBrn-1*, from the cDNA library of embryonic day 5 quail. We also have presented the distinct dynamic expression patterns of *qBrn-1* and *qBrn-2* throughout the early embryonic development. Our results showed that both *qBrn-1* and *qBrn-2* are expressed predominantly in the CNS from the beginning of neurulation to the specification of the nervous system. However, their temporal and spatial locations are quite different during the entire neurula period, suggesting a different role of *qBrn-1* during quail neurogenesis comparing with that of *qBrn-2*. Moreover, the observations of *qBrn-1* expression in neural crests, notochord, and branchial arches, as well as the characteristic expression manner in the kidney, indicate that *qBrn-1* has multiple functions in quail organogenesis, including kidney formation.

EXPERIMENTAL PROCEDURES

Embryos

Embryos of quail (*Coturnix coturnix japonica*) were used. The fertilized eggs were incubated at 38 ± 1°C under a humidified atmosphere (70%). The embryos were staged according to Hamburger and Hamilton (1951).

Isolation of *qBrn-1* cDNA

A cDNA library of embryonic day 5 quail was screened using the double-stranded DNA probe containing almost full sequence of POU box (Liu et al., 2000). Several clones containing quail POU domain cDNA were obtained, in which one was identified as *qBrn-1* by sequence homology analysis under the GenBank accession number AY781803.

Northern Blot Analysis

Total RNA was isolated from quail embryos (E5) and hearts (2 days after hatching) using RNeasy kit (Qiagen), respectively. The 10 μg of total RNA was separated on a 1.5% agarose/1.5% formaldehyde gel and subsequently transferred onto nylon membrane (Hybond-N$^+$, Invitrogen) in 20× standard saline citrate (SSC). Three different digoxigenin (DIG) -labeled single-stranded antisense RNA probes were transcribed from linearized cDNA templates: a fragment containing the 5′-untranslated region and the partial open reading frame (ORF) of *qBrn-1* (a, corresponding to nucleotides 11–860), a fragment containing the entire ORF of *qBrn-1* and 3′ untranslated region (b, corresponding to nucleotides 331–1966), and a fragment containing the 3′ flanking region of cDNA that lies outside the POU domain (c, corresponding to nucleotides 1163–1966), respectively (Fig. 2A). The membranes were hybridized in DIG Easy Hyb (Roche) at 65°C overnight then washed two times with 2× SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature and two times with 0.1× SSC/0.1% SDS at 65°C. The hybridization signal was detected with Chemiluminescent substrate (CDP-Star, Roche) according to the manufacturer's protocol.

In Situ Hybridization

Expression patterns for *qBrn-1* and *qBrn-2* were visualized in different stages of embryos using whole-mount in situ hybridization. Three different antisense riboprobes described above were used in hybridization. All those probes gave the identical patterns of expression. The probe used for *qBrn-2* was described previously (Liu et al., 2000). Whole-mount in situ hybridization was carried out following the modified method of Riddle et al. (1993).

Antibody Preparation and Western Blot

A polyclonal anti-qBrn1 antibody was generated by immunizing a rabbit with the purified bacterially overexpressed N-terminal domain of qBrn-1, corresponding to amino acids 1–79 (unpublished data). The specificity of the qBrn-1 antibody was confirmed by Western blotting (Fig. 5A).

Immunohistochemistry

Embryos were fixed for 1 hr in 4% paraformaldehyde–phosphate buffered saline (PBS) at room temperature. After rinsed with PBS containing 1% Triton X-100, blocking was achieved by overnight incubation with PBS containing 2% blocking reagent (Roche), 10% goat serum, and 1% Triton X-100. The primary antibody was incubated with the embryos for at least 8 hr at 4°C. The washing step was performed at room temperature with PBS containing 1% Triton X-100. Before incubation with secondary antibody, nonspecific binding was blocked in MABT (100 mM maleic acid, 150 mM NaCl, 2 mM levamisole, 0.1% Tween-20 [pH 7.5]) containing 10% goat serum and 2% blocking reagent. The alkaline phosphatase–conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) was used as the secondary antibody. The embryos were then washed six times at room temperature and once more overnight in MABT. NTMT (100 mM NaCl, 100 mM Tris-HCl, 50 mM $MgCl_2$, 2 mM levamisole, 0.1% Tween-20 [pH 9.5]) washes were performed three times, followed by alkaline phosphatase reaction in BM purple (Roche).

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