Expression of \textit{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, \textit{qBrn-1}, from the cDNA library of embryonic day 5 quail and have made an extensive expression pattern analysis of \textit{qBrn-1} and \textit{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 \textit{qBrn-1} protein was consistent with that of the transcripts in the early developing quail. Our results showed that both \textit{qBrn-1} and \textit{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, \textit{qBrn-1} signal, different from \textit{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 \textit{qBrn-1} in nephric region was earlier and wider than that of mouse \textit{Brn-1}, suggesting the characteristic function of \textit{qBrn-1} in the kidney formation. The distinct dynamic expression patterns of \textit{qBrn-1} and \textit{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.

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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-
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 shading, and the asterisks indicate the identical residues between qBrn-1 and qBrn-2. Gaps are represented by “-” symbols. The POU domain region is underlined.
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; Sugiuchi 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., 2000).

**Fig. 1.** (Continued.)

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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-

![Fig. 2](image-url)
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; Schoenemann 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
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 inset). 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 shown 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. 3c,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 mesodermal 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 immunobssay 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 deoxyribonucleotide (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 MgCl2, 2 mM levamisole, 0.1% Tween-20 [pH 9.5]) was washed performed three times, followed by alkaline phosphatase reaction in BM purple (Roche).
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REFERENCES


