Expression and Antibody Preparation of POU Transcription Factor qBrn-1

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Abstract: The transcription factor Brn-1, which belongs to POU-domain family, has been shown to play critical roles in the development of the nervous system. A cDNA clone coding for a quail Brn-1 homologue, qBrn-1, was isolated. To investigate whether this gene plays a role in the development of the quail nervous system, an anti-N-terminal peptide antibody was prepared. The coding region for amino acids 1-79 (the N-terminal domain of qBrn-1) was subcloned into Trx fusion expression vector pET32c and introduced into the *Escherichia coli* Origami(DE3) cells for efficient expression. After purification, Trx-fused polypeptides, called Trx-qBrn-1N, were used to immunize the rabbits to prepare polyclonal antibodies against qBrn-1. The produced and purified antiserum showed specificity not only to the *in vitro* expressed qBrn-1, but also to the natural qBrn-1 in tissues. Immunolabeling on sections by the anti-qBrn-1 serum showed that qBrn-1 was specifically expressed in the developing spinal cord and kidney. This suggests that qBrn-1 may play some roles in the development of avian nervous system and kidney, and the preparation of anti-qBrn-1 polyclonal antibody will facilitate further detection of, and functional study on, qBrn-1 both *in vivo* and *in vitro*.

Keywords: Antibody, development, kidney, nervous system, POU, qBrn-1, quail.

INTRODUCTION

POU domain genes encode transcription factors characterized by a unique bipartite DNA-binding domain referred to as the POU domain. This domain is composed of a classic homeodomain and a highly conserved POU-specific domain of 76-78 amino acids. These two major subdomains are joined together by a short linker region with variable length and sequence [1-3]. The POU domain contributes both to site-specific DNA-binding and to interactions between POU domain proteins and other transcription factors [4]. Experimental and genetic studies have revealed the crucial role of POU family factors in development of multiple tissues, especially in the nervous system [5-8]. Based upon amino acid sequence similarities of the POU domain, POU genes can be grouped into at least six classes [3,9].

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 of particular interest for neuronal development, given that these factors are specifically expressed in the embryonic and adult central nervous system (CNS), and function in cell proliferation and differentiation during the peak period of neurogenesis [10-12]. In this family, *Brn-1* is strongly and widely expressed both in embryonic and adult brain [13] and is implicated in the production and migration of developing cortical neuron [6,12], as well as in the development and function of the nephron in the mammalian kidney [14]. *qBrn-1*, a new *Brn-1* gene member, was shown to be expressed in different tissues of the developing quail embryos by *in situ* hybridization, and exhibited a different expression pattern in comparison with its mammalian counterparts, especially in the embryonic kidney [15], indicating it may possess some species-specific functions in vertebrate development.

To further investigate the developmental role of qBrn-1 factor in embryogenesis, the preparation of a specific antibody for qBrn-1 will be very useful, because the expression detection by specific antibody tells the location of the detected protein more directly than by nucleotide probe, and is more convenient on the application of multiple labeling for the functional analysis at the cellular level. Therefore, we have prepared a polyclonal antibody against qBrn-1 by immunizing a rabbit with the purified bacterially overexpressed N-terminal domain of qBrn-1, corresponding to the deduced amino acid sequence 1-79. This polyclonal antibody was used either in immunoblotting or immunohistochemistry to demonstrate its ability to recognize the qBrn-1 protein in different quail tissues.

MATERIALS AND METHODS

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 [16].

Bacterial Strains and Plasmids

The full-length cDNA of *qBrn-1* was previously cloned from an E5 quail cDNA library in our lab. The *E. coli* Trx

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(thioredoxin) fusion expression vector pET-32c and bacterial strain Origami(DE3) were obtained from Novagen (Madison, WI).

Construction of Plasmid

PCR was employed to amplify the nucleotide fragment encoding the N-terminal polypeptide (1-79) of qBrn-1 protein from the full-length *qBrn-1* cDNA with a forward primer (5'-CAA TAT CCA TGG CCT CCC TGT ACT CG-3', in which the *Nco*I site is underlined) and a reverse primer (5'-CAT GAA TTC TAC CGA GGT CGG CGT GTC-3', in which the *EcoR*I site is underlined). The amplification was performed by using a procedure of 30 cycles of reaction with denaturing at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 30 s. After digestion with the restriction endonucleases, the PCR product was ligated in frame into the *Nco*I-*EcoR*I sites of the expression vector pET-32c as a His-tagged, N-terminal fusion of qBrn-1N to Trx. The positive pET-32c-qBrn1N expression plasmid was identified by restriction endonuclease digestion and further verified by DNA sequencing by Sangon Biotech (Shanghai, China).

Expression and Purification of the Recombinant Protein

For expression of the Trx-qBrn-1N fusion protein, the Origami(DE3) transformants harboring the expression plasmid were cultured in Luria-Bertani (LB) containing 0.1 mg/mL ampicillin and allowed to grow at 37°C until the OD_{600} was above 0.6. Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.8 mM, and allowed to grow for another 4 hours. The cells were then collected by centrifugation (5000 rpm, 10 min, 4°C). The pellet was lysed in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea (pH 8.0) and the lysate was directly applied to SDS-PAGE.

For purification of the Trx-qBrn-1N fusion protein, the bacteria pellet from IPTG-induced culture was washed once with ice-cold phosphate-buffered saline, pH 7.4 (PBS), resuspended in suspension-buffer (25 mM NaH_2PO_4, 75 mM Na_2HPO_4, 0.5 M NaCl, pH 7.4) containing 1% Triton X-100, 10 mM β-mercaptoethanol, 2 μg/mL Aprotinin, 0.7 μg/mL Pepstatin, 0.5 μg/mL Leupeptin and 50 μg/mL lysozyme, and then lysed by sonication on ice with a few bursts. The lysate was centrifuged (13,000 rpm, 4°C, 15 min), and the clarified supernatant was loaded onto pre-equilibrated Ni^{2+}-chelating Sepharose column (Amersham Bioscience, Piscataway, NJ). Rinsing was performed with 50 mM imidazole in suspension-buffer until the absorbance of the flow-through at 280 nm was lower than 0.01, and the protein of interest was eluted with 200 mM imidazole in suspension-buffer.

Immunization

The overexpressed Trx-qBrn-1N fusion protein was recovered in 200 mM imidazole in suspension-buffer. To lower the salt concentration, the eluate was firstly dialyzed against PBS and then against double distilled water. After concentration, the purified fusion protein was used as antigen in immunogenesis. Briefly, New Zealand white rabbits were immunized subcutaneously with 1mg of purified protein emulsified with complete Freund's adjuvant (1:1 v/v). Rabbits were given booster injections of 1mg of protein with Freund incomplete adjuvant every other week. The rabbit was bled prior to immunization (pre-immune serum) and after the last immunization (immune serum). The titers of the immune serum were determined by ELISA.

Purification of Antiserum

To remove the antibody against Trx, the empty vector pET-32c was transformed in Origami(DE3) and induced to express the His-tagged Trx fusion protein. After affinity purification through Ni^{2+}-chelating Sepharose column, the Trx fusion protein was dialyzed against PBS and concentrated. The antiserum was incubated with excess of Ni^{2+}-chelating Sepharose coupled with purified Trx fusion protein in PBS on a rotary at 4°C for 30 min. Then, the supernatant was collected and analyzed by Western blotting.

Preparation of Tissue Extracts and Analysis of Western Blot

Tissue extracts of quail were prepared according to the method described by Liu *et al.* [17], resolved by 15% SDS-PAGE and then transferred onto nitrocellulose membrane. For Western blotting, the transferred filters were saturated overnight at 4°C in PBS containing 5% (w/v) skim milk and 0.2% Tween-20 (PBST), then incubated for 2 h at room temperature with purified antiserum in PBST. The treated filters were subsequently incubated with secondary HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). Detection was performed by using DAB staining. Pre-immune serum was included as negative control.

Immunohistochemistry

Whole-mount immunohistochemistry was performed as described by Lan *et al.* [15]. For immunohistochemistry on sections, embryos were embedded in OCT compound and sectioned on cryostat. Non-specific binding was blocked by incubation with PBS containing 10% goat serum and 0.15% Tween-20. Slides were incubated overnight with antiserum at 4°C and then washed with PBS containing 0.15% Tween-20. As a secondary antibody the alkaline phosphatase conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) was used. The embryos were then washed six times at room temperature and once more overnight in PBS containing 0.15% Tween-20. 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).

In Situ Hybridization

Whole-mount *in situ* hybridization was carried out according to Lan *et al.* [15].

RESULTS AND DISCUSSION

Isolation and Analysis of qBrn-1

The full-length cDNA of *qBrn-1* was cloned from an E5 quail cDNA library and had been submitted to GenBank under the accession number AY781803. The cDNA contains seven open reading frames (more than 60 amino acid residues), in which only one includes a conserved POU domain (Fig. **1**). The homology analysis shows that the predicted

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mBrn-1    MATAASNPYQPGNSLLTAGSIVHSDAAGAGGGGGGGGGGGGAGGGGGGMQPGSAAVTSGAYRGDPSSVKMVQSDFMQGA   80
qBrn-1    ------------------------------------------------------------------------------   0

mBrn-1    ASNGGHMLSHAHQWVTALPHAAAAAAAAAAAAAVEASSPWSGSAVGMAGSPQQPPQPPPPPPQGPDVKGGAGREDLHAGTA  160
qBrn-1    ------------------------------------------------------------------------------   0

mBrn-1    LHHRGPPHLGPPPPPPHQGHPGGWGAAAAAAAAAAAAAAAAAHLPSMAGGQQPPPQSLLYSQPGGFTVNGMLSAPPGPGGG  240
qBrn-1    ----------------------------------------------MAS---------LYSQPGGFTVNGMLS----PAGG   22

mBrn-1    GGGAGGGAQSLVHPGLVRG-DTPELAEHHHHHHHHAHPHPPHPHHAQGPPHHGGGGAGPGLNSHDPHSDEDTPTSDDLEQ  319
qBrn-1    G-------QSLVHPGLVRGGETAELGEHPGHHHHHHHPHP--GHHA---PHHG------AVNSHEAHSDEDTPTSDDLEQ   84

mBrn-1    FAKQFKQRRIKLGFTQADVGLALGTLYGNVFSQTTICRFEALQLSFKNMCKLKPLLNKWLEEADSSTGSPTSIDKIAAQG  399
qBrn-1    FAKQFKQRRIKLGFTQADVGLALGTLYGNVFSQTTICRFEALQLSFKNMCKLKPLLNKWLEEADSSTGSPTSIDKIAAQG  164

mBrn-1    RKRKKRTSIEVSVKGALESHFLKCPKPSAQEITNLADSLQLEKEVVRVWFCNRRQKEKRMTPPGIQQQTPDDVYSQVGTV  479
qBrn-1    RKRKKRTSIEVSVKGALESHFLKCPKPSAQEITNLADSLQLEKEVVRVWFCNRRQKEKRMTPPGIQQQTPDDVYSQVGAV  244

mBrn-1    SADTPPPHHGLQTSVQ  495
qBrn-1    SSDTPPPHHGLQSGVQ  260
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Figure 1. Alignment of qBrn-1 protein sequence with mBrn-1 (M88299). The identical amino acid residues are shadowed, and gaps are represented by '-'. Positions in the amino acid sequence are given by numbers. The POU-box region is boxed, and the underlined sequence was used to generate the antiserum.

amino acid sequence of qBrn-1 exhibits significant high levels of conservation in POU domain (100%) and C-terminal region (97.3%) in comparison with mouse Brn-1 (mBrn-1, GenBank accession number: M88299). However, the N-terminal region of qBrn-1 is considerably divergent from its mammalian counterpart. Thus, we constructed an expression vector encoding N-terminal peptide of qBrn-1 (qBrn-1N) to prepare the antibody (Fig. **2**). The primers were designed to amplify the nucleotide sequence encoding residues 1 to 79 of qBrn-1 by PCR. The PCR product exhibited a single band with an expected length (~237 bp) in agarose gel electrophoresis (Fig. **3A**). It was recovered, digested with *Nco*I and *Eco*RI, and subcloned into pET32c expression vector at the *Nco*I-*Eco*RI site, resulting in an open reading frame of 711 bp containing the *qBrn-1N* fragment fused in frame to Trx and 6xHis tags (Fig. **2**). The sequence of the resultant plasmid was confirmed by DNA sequencing.

Expression and Purification of Trx Fusion Proteins

Based upon the structure of pET32c-qBrn-1N and pET32c plasmids, the fusion proteins have a predicted size of 237 and 207 amino acid residues with a theoretical molecular mass of 26.1 kDa and 22.7 kDa, respectively. The *E. coli* Origami(DE3) host cells harboring the Trx fusion expression plasmids pET32c-qBrn-1N and pET32c were induced by IPTG to produce soluble Trx fusion proteins, which show a distinct band of ~24 kDa and ~20.5 kDa in SDS-PAGE, respectively (Fig. **3B**). By affinity chromatography with a Ni^{2+}-chelating Sepharose column, the purified Trx-qBrn-1N and Trx fusion proteins show a high yield (purity > 95%) as estimated by Coomassie stained SDS-PAGE (Fig. **3B**) and Western blotting using anti-His monoclonal antibody (Novagen) (Fig. **3C**).

Immunization and Antiserum Purification

Consequently, New Zealand rabbits were immunized with the purified Trx-qBrn-1N. As detected by ELISA, the pre-immune serum, used as control, reacted immunologically with neither Trx-qBrn-1N nor Trx, but the avidity of the immune serum towards Trx-qBrn-1N and Trx was $1:5 \times 10^5$ and $1:1 \times 10^5$, respectively. To improve the specificity of the antiserum, we first dialyzed the Trx fusion protein against

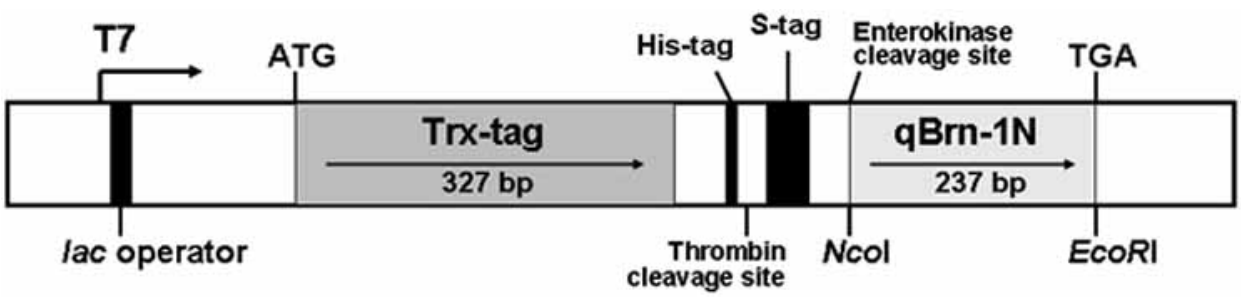

Figure 2. Schematic structure of pET32c-*qBrn-1N* expression plasmid. The coding sequence of the N-terminal region of qBrn-1 was amplified by PCR and subcloned into the *Nco*I and *Eco*RI sites of the pET32c expression vector downstream of Trx, under the control of T7 promoter.

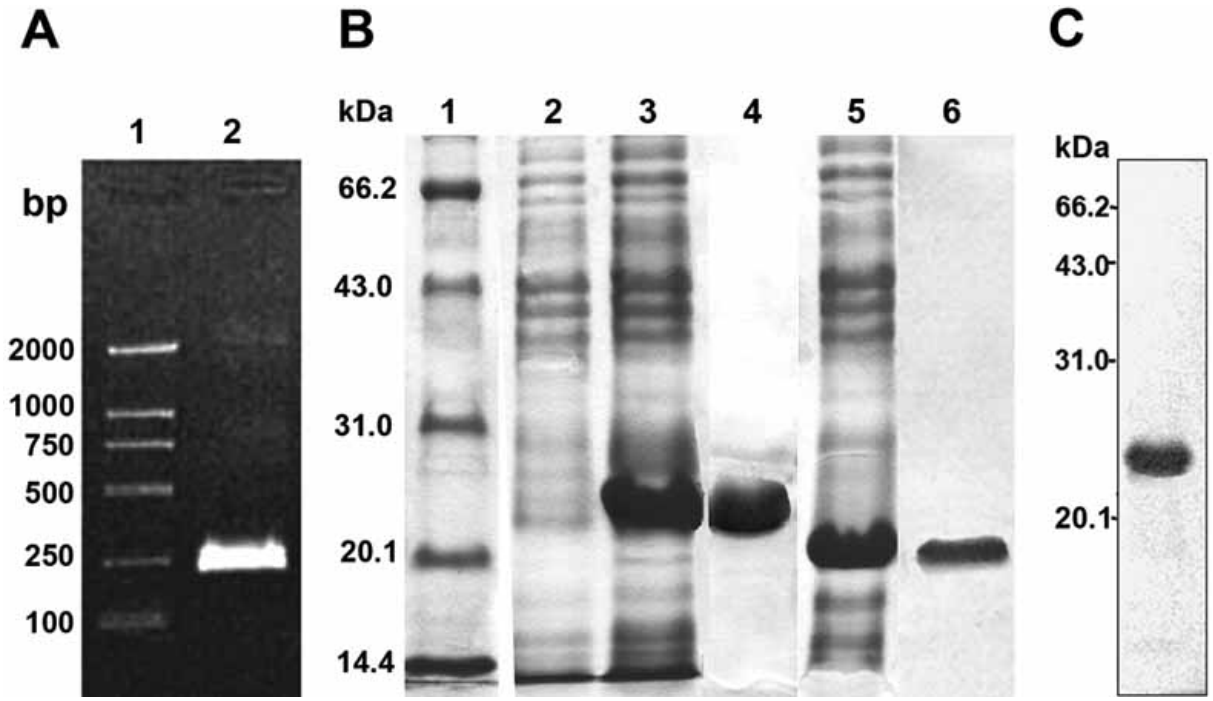

Figure 3. (**A**) Detection of the PCR product on agarose gel. Lanes 1 and 2 are DNA marker and PCR fragment corresponding to the N-terminal region of *qBrn-1*, respectively. (**B**) SDS-PAGE analysis of the expressed and purified Trx-qBrn-1N and Trx fusion proteins. Lanes represent molecular weight marker (lane 1), total bacterial extracts of Origami(DE3) harboring pET32c-qBrn-1N before (lane 2) and after (lane 3) IPTG induction, the eluted fraction of Trx-qBrn-1N (lane 4), total bacterial extract of Origami(DE3) harboring pET32c after IPTG induction (lane 5), and the eluted fraction of Trx (lane 6), respectively. (**C**) Western blot showing the anti-His monoclonal antibody binding to the purified Trx-qBrn-1N fusion protein.

PBS (pH 7.4), and prepared the purification matrix by coupling the resulting Trx with the PBS-equilibrated Ni^{2+}-chelating Sepharose. Then, the antiserum was incubated with the excess of purification matrix to remove the Trx antibody. As shown in Fig. **4**, the purified antibody had a weak immunological reaction with Trx but reacted strongly with Trx-qBrn-1N fusion protein. Measurement of the band density by densitometry showed that about 95% of the Trx antibody had been removed.

Immunodetections by the Purified Antiserum

After removing the anti-Trx antibodies in the rabbit antisera, the produced antiserum showed specificity to bacterially overexpressed full-length qBrn-1 by Western blotting (Fig. **5A**). To determine the capacity and specificity of the antiserum to bind natural qBrn-1, both Western blotting (Fig. **5B**) and immunohistochemistry were performed (Fig. **5C, D,** and **E**). As Fig. **5B** illustrates, the proteins extracted from the brain (or kidney) of quails (2-day after hatching) were detected by western blot immunoassay. The antiserum recognized a single band of approximately 27.8 kDa in both tissue extracts. The molecular weight was in accordance with the predicted size from the *qBrn-1* ORF sequence (28.5 kDa). More convincingly, qBrn-1 immunoreactivity on whole-mount quail embryos also paralleled *qBrn-1* mRNA localization (Fig. **5C**, **D**). Furthermore, the immunolabeling of qBrn-1 in section was confined to the spinal cord and the developing kidney (Fig. **5E**). This result is parallel with that described by He *et al.* and Nakai *et al.* [13,14], Brn-1 is promi-

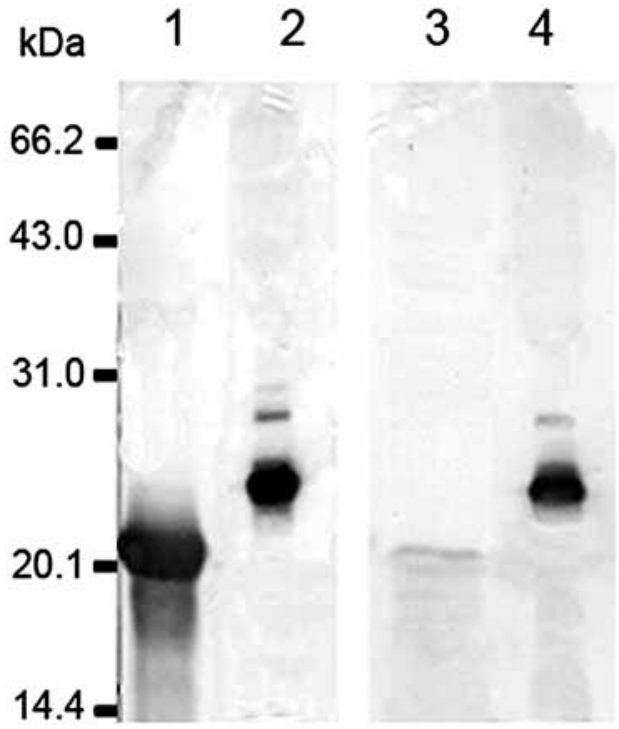

Figure 4. Western blot showing the antiserum to recognize the Trx and Trx-qBrn-1N before (lanes 1 and 2) and after (lanes 3 and 4) purification. Lanes 1 and 3 are purified Trx, and lanes 2 and 4 are purified Trx-qBrn-1N.

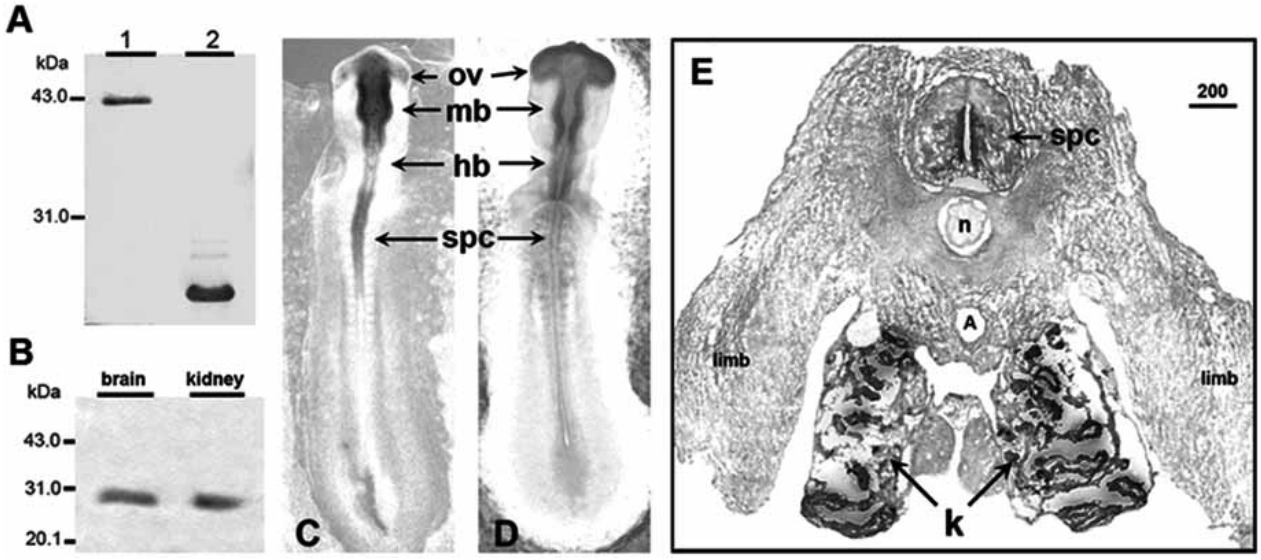

Figure 5. (**A**) Western blot showing that the purified antiserum recognizes the bacterially expressed Trx-fused full-length qBrn-1 protein (lane1) and the antigen (Trx-qBrn-1N, lane2). (**B**) Western blot analyses of qBrn-1 protein in brain and kidney extracts of quail 2-day after hatching (**C**, **D**) The expression of qBrn-1 in neural tube is revealed both by *in situ* hybridization (C) and immunohistochemistry (D). (**E**) Immunohistochemistry of qBrn-1 in transverse section of E9 quail embryo showing the localization of qBrn-1 in the developing spinal cord and kidney (Scale bar: 200 μm). Abbreviations: A, aorta; hb, hindbrain; k, kidney; mb, midbrain; n, notochord; ov, optic vesicle; spc, spinal cord.

nently expressed in the central nervous system and is the only member of POU III class expressed in the kidney of mouse. Therefore, these observations clearly demonstrate the specificity of the antiserum we prepared.

In summary, we herein report the successful expression of the N-terminal portion of the quail POU III family member qBrn-1 and the identification of the polyclonal antibody. As a transcription factor implicated in quail neurogenesis and organogenesis, the preparation of the antibody will be very important in studying the expression and physiological function of qBrn-1.

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