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A novel Brn3-like POU transcription factor expressed in subsets of rat sensory and spinal cord neurons

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ABSTRACT

Brn3a and Brn3b are mammalian members of the POU class of transcription factors. They are closely related to each other and to Unc86, which determines the normal development of certain cells, including mechanoreceptive sensory neurons in *Caenorhabditis elegans*. We screened a rat dorsal root ganglion (DRG) cDNA library at moderate stringency with a Brn3a POU-domain probe and identified a novel transcript encoding a POU protein that we have named Brn3c. Brn3c closely resembles Brn3a and Brn3b in its POU-domain and thus helps define a family of Unc86-related mammalian POU factors. Both Brn3a and Brn3c are expressed only in the central and peripheral nervous systems. In the neonatal rat, northern blots revealed a 3.6 kb Brn3a transcript in DRG, spinal cord and hindbrain, and a 2.6 kb Brn3c transcript in DRG and spinal cord. *In situ* hybridization showed that most DRG neurons express Brn3a whereas only a small subset of neurons expresses Brn3c. In the spinal cord, Brn3a is expressed by many dorsal horn neurons. In contrast, Brn3c is expressed by a very small number of cells in laminae 4/5 of the dorsal horn. These data suggest that Brn3-related POU factors may be involved in the development or function of particular subclasses of sensory and spinal cord neurons.

INTRODUCTION

POU domain proteins are a class of transcriptional regulators that appear to have particularly important roles in tissue specific gene regulation (1–3). They are named after the first three proteins recognised to share the POU-domain structural motif of a homeobox preceded by a POU-specific domain (POU box), both of which are required for high affinity DNA binding (1, 2). Pit1 (also known as GHF1) plays a critical role in the development of the pituitary and the regulation of prolactin and growth hormone synthesis (1); Oct1 is a ubiquitous transcription factor and Oct2 regulates immunoglobulin synthesis in B lymphocytes (1,4); Unc86 was defined in a series of *C. elegans* behavioural mutants, and determines the developmental fate of some cell types including mechanoreceptive sensory neurons (5). Brn3, which is a rat homologue of Unc86, has been reported to be expressed at high levels in sensory neurons, as well as in discrete areas of the central nervous system (2). A full length human Brn3 cDNA (RDC1) has also been cloned (6). Evidence that more than one Brn3-related sequence exists has come from PCR amplification of RNA extracted from a DRG-derived neuronal cell line (7); a novel Brn3-related isoform was identified, which was named Brn3b to distinguish it from the original isoform, which was renamed Brn3a (8). This suggested that other Brn3-related POU proteins might remain to be identified, and raised the question of what regulatory function(s) such factors might perform in sensory neurons. Because a great deal is known about the development and properties of sensory neurons, they provide an attractive system for examining the role of POU proteins in establishing and maintaining neuronal phenotype.

We therefore screened a neonatal rat DRG cDNA library at moderate stringency with a Brn3a POU domain probe, and isolated rat cDNAs encoding two Brn3-related proteins. The amino acid sequence of one of these was identical to the published sequence of rat Brn3a (2) while the other differed from both Brn3a and Brn3b and was hence designated Brn3c. In addition, using the polymerase chain reaction (PCR), we found a third transcript present in neonatal rat brain which corresponds at the amino acid level to Brn3b, which was first identified in a rat-mouse hybrid neuronal cell line (7,8). Thus, there are at least three rat POU domain sequences that together define a Brn3-related subgroup of POU proteins.

Using probes from the 3′ non-coding sequences of Brn3a and Brn3c for northern and Southern analysis and *in situ* hybridization, we have shown that Brn3a and Brn3c are encoded by distict RNA transcripts that are differentially expressed in the developing peripheral and central nervous systems (PNS and CNS). Brn3a transcripts are present in most or all DRG neurons, as well as in many cells in the dorsal horn of the spinal cord. Brn3c is expressed in a subset of DRG neurons and a very small number of cells in the dorsal horn of the spinal cord.

The isolated POU domain of Brn3a has been shown to activate transcription from the corticotrophin releasing factor gene promoter (9), perhaps by binding to a cellular trans-activating

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factor. The amino acid sequences of the homeodomains of Brn3a and Brn3c differ at a residue in the first helix that determines the ability of the POU protein Oct1 to interact with the Herpes simplex virus (HSV) transactivating protein VP16 (10, 11). This raises the possibility that analogous interactions with cellular trans-activating factors could specify distinct functions for the related Brn3 isoforms.

MATERIALS AND METHODS

Library construction and screening

Poly A+ RNA from neonatal Sprague Dawley rat DRG was isolated using CsCl centrifugation of guanidinium isothiocyanate disrupted tissue (14). This was used to generate an oligo-dT primed directional cDNA library in lambda zap II (Stratagene). A POU-domain specific probe was generated using an *Eco*RI-*Hin*dIII fragment from the POU domain of murine Brn3a (13), labelled by random priming (Promega) and used to screen the library. Filters were hybridized at 65°C for 16 hours in 4×SSC, 5×Denhardt's solution, 0.2% (w/v) SDS, 5 mM EDTA and 100 μg/ml salmon sperm DNA. Positive clones were identified by radioautography and plaque purifed by 3 further rounds of screening. They were then sequenced using Sequenase II (USB).

Production of specific probes

Specific 3′ probes for Brn3a and Brn3c were generated by PCR amplification of fragments located downstream of the respective POU domains. A 1 kb fragment for Brn3a was generated using the following oligonucleotides:
5′-GCA CAC TGG GGA GCT GAG
5′-GTG AGA TTG TAT TCA GTG GAG
A 1.3 kb fragment specific for Brn3c was generated using primers:
5′-GGG ATC AAG GAA AGA AAC AC
5′-GGT TCT GTA ACC AGA GAC AG
These fragments were gel purified and labelled with ^{32}P-dCTP using random oligonucleotide primers (Promega), and used for northern and Southern blot analysis and colony hybridization.

RNA extraction and Northern hybridization

Total RNA was extracted from neonatal Sprague Dawley rat tissues frozen in liquid nitrogen by guanidium thiocyanate extraction and ultracentrifugation through 5.7 M CsCl (14). Approximately 15 μg of RNA from each tissue was denatured in formamide and electrophoresed on a 1.5% agarose formaldehyde gel (12). Gels were blotted with Hybond-N (Amersham). Northern hybridization was carried out at 42°C in 50% formamide, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 2 mM EDTA 0.25 mg/ml denatured salmon testis DNA and washed in 0.5×SSC, 0.5% SDS at 65°C.

For control hybridization of northern blots, a probe for the constitutively-expressed L27 ribosomal protein was used (15). A 300 bp fragment of this gene was generated from DRG total RNA using PCR and the following primers (16):
5′-ATC GCT CCT CAA ACT TGA CC
5′-AAA GCC GTC ATC GTA AAG AAC

Southern blots

High molecular weight DNA was isolated from rat kidney (17). 20 μg of DNA was digested with *Eco*RI, *Bam*HI, *Hin*dIII or *Xho*I enzymes (Promega) and separated on a 1% agarose gel. The DNA fragments were Southern blotted on Hybond-N (Amersham) and hybridized with POU-specific or Brn3a- and Brn3c-specific probes using the same conditions described above for northern blots.

Isolation of POU factor cDNAs by polymerase chain reaction

We purified total cellular RNA from postnatal day 7 (P7) rat brain by the guanidinium isothiocyanate method (18). First-strand cDNA synthesis was performed using 1 μg RNA with 0.5 μg oligo-dT(15) primer and 200 units of Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL). The RNA and primers were heated together at 65°C for 2 min in 13 μl water, then added to 7 μl of (50 mM Tris−HCl pH 8.3, 75 mM KCl, 5 mM $MgCl_2$) containing 10 mM of each deoxyribonucleotide (dNTPs) and 20 units of human placental ribonuclease inhibitor (Promega). The reverse transcriptase was added and the reaction incubated at 37°C for 2 hr. 5 μl of this reaction was added to a polymerase chain reaction (PCR) mix containing 10% (v/v) dimethyl sulfoxide (DMSO), 45 mM Tris−HCl pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM β-mercaptoethanol, 4.5 mM ethylenediamine-tetraacetic acid (EDTA), 110 μg/ml bovine serum albumin (fraction V, Sigma), 200 μm dNTPs and 1 μg of each of two degenerate oligonucleotide primers flanking the POU domain in a final volume of 100 μl. The first primer pair was:
5′-TT(T/C)AA(A/G)(A/C)GN(A/C)GNAT(A/C/T)AA(A/G)(C/T)NGG
5′-(T/C)TGNC(T/G)N(T/C)(T/G)(A/G)TT(A/G)CA(A/G)AACCANACNC
The reaction was heated to 95°C for 5 min under paraffin oil before the addition of 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The reaction was then cycled 30 times on a Hybaid programmable heating block at 55°C (2 min), 72°C (1 min) and 92°C (0.5 min), ending with a 10 min incubation at 72°C. One-tenth (10 μl) of this reaction was added to a second

Table 1. Alignment of Brn3a, Brn3b and Brn3c POU homeodomains with related POU proteins

	POU specific box	Linker		POU homeodomain Helix 1		Helix 2		Helix 3
unc86	SQTTICRFESLTLSHNKMVALKPILHSWLEKAE	EAMKQKDTIGDINGILPNT	DKKRKRTSIA	APEKRELEQFFKQ	QPRPS	GERIASIADRL	DLK	KNVVRVWFCNQRQKQKRDF..
Ipou	SQSTICRFESLTLSHNKMIALKPILQAWLEEAE	AQAKNKRRDPAPSVLPAG	EKK--RTSIA	APEKRSLEAYFAV	QPRPS	GEKIAAIAEKL	DLK	KNVVRVWFCNQRQKQKRIVS..
RDC1	SQSTICRFESLTLSHNKMIALKPILQAWLEEAE	GPSEKMNKPELFNGG	EKKRKRTSIA	APEKRSLEAYFAV	QPRES	SEKIAAIAEKL	DLK	KNVVRVWFCNQRQKQKRMKF..
Brn3A	SQSTICRFESLTLSHNNMIALKPILQAWLEEAE	GAQREKMNKPELFNGG	EKKRKRTSIA	APEKRSLEAYFAV	QPRPS	SEKIAAIAEKL	DLK	KNVVRVWFCNQRQKQKRMKFSAT
Brn3B	SQSTICRFESLMLSHNNMIALKPILQAWLEEAE	KSHREKLTKPELFNGA	EKKRKRTSIA	APEKRSLEAYFAI	QPRPS	SEKIAAIAEKL	DLK	.....
Brn3C	SQSTICRFESLTLSHNNMIALKPVLQAWLEEAE	AAYREKNSKPELFNGS	ERKRKRTSIA	APEKRSLEAYFAI	QPRPS	SEKIAAIAEKL	DLK	KNVVRVWFCNQRQKQKRMKYSAVH
OCT1	SQTTISRFEALNLSFKNMCKLKPLLEDWINDAENL	SSDSSLSSPSALNSPL	SRRKKRTSIE	TNIRVALEKSFLE	NQKPT	SEEITMIADQL	NME	KEVIRVWFCNRRQKEKR IN..
OCT2	LQTTISRFEALNSFKNMCKLKPLLEKWLNDAETM	SVDSSLPSPNQLSSPSLGFDGL	PRRKKRTSIE	TNVRFALEKSFLA	NQKPT	SEEILLIAEQL	HME	KEVIRVWFCNRRQKEKR IN..

Alignment of class IV POU domain proteins from drosophila I-POU (3) and C.elegans unc86 (2) human RDC-1 (6), and rat Brn3a (2), Brn3b (9) and Brn3c with Oct1 and Oct 2 (1). Brn3a and Brn3c were sequenced from partial rat DRG cDNA clones and brain PCR products. Brn3b , apparently not represented in DRG libraries, was sequenced only from brain PCR products and hence the complete 3′ sequence has yet to be determined, symbolised by the dotted lines. Highlighted residues differ between Brn3a, Brn3b and Brn3c.

PCR reaction mix containing a different pair of primers, and the PCR amplification repeated. The second primer pair was:
5′-[GTCGACAAGCTT] GGN(G/T)(A/T)(C/T)ACNGA(A/G)(A/G)(G/C)N(A/G)A(C/T)GTNGG
5′-[GTCGACGGATCC] CCANACNC(G/T)NA(C/T)NACNT(T/C)(T/C)TT(T/C)T(T/C)
where square brackets contain restriction sites that were included to aid subsequent cloning. The products of this reaction were visualized on a 2% agarose DNA gel, purified directly from the PCR reaction with GeneClean (Bio101 Inc.), digested with *Bam*HI and *Sal*I, cloned into Bluescript (Stratagene) and sequenced using the T7 DNA polymerase sequencing kit (Pharmacia) on a double-stranded template (19).

In situ hybridization

Sprague-Dawley rats were used for in situ hybridization. Whole bodies of embryonic day 12 (E12), E14, E16, E18 and newborn rats were fixed in paraformaldehyde, infused with sucrose, and frozen in OCT embedding compound (Tissue-Tek; Miles Inc.) as described previously (20). Cryosections (10−20 μm) were cut and subjected to *in situ* hybridization by the procedure of Lawrence and Singer (21), modified as described (22, 23). ^{35}S-labelled RNA probes were generated by *in vitro* transcription of 1.5 kb (Brn3a) and 1.35 kb (Brn3c) partial cDNA clones corresponding to the 3′ end of the coding sequences (including ~100 bp of the homeobox) and the entire 3′ untranslated regions of the mRNAs. These fragments were isolated by PCR-amplification from the corresponding cDNA clones in lambda phage (see below), and subcloned directly into plasmid pCR II (Invitrogen). The orientation of the Brn3a subclone was such that the antisense probe was transcribed with bacteriophage T7 RNA polymerase after cutting the vector with *Bam*HI, and the sense probe was transcribed with SP6 polymerase after cutting with *Not*I; the Brn3c subclone was in the reverse orientation. The radiolabelled probes were reduced to ~150 bp fragments by limited alkaline hydrolysis before hybridization to tissue sections. After in situ hybridization and washing, the sections were coated in Ilford K5 nuclear emulsion, exposed for 1−4 weeks at 4°C and developed in Kodak D19. Sections were photographed under dark-field illumination, counterstained in Hematoxylin (Gills no.3, Sigma), and re-photographed under bright-field illumination. The bright- and dark-field images were separately converted to digital format using a video camera and frame grabber (Neotech) attached to a Macintosh computer, assigned false colors and superimposed using Adobe Photoshop software and photographed directly from the computer screen.

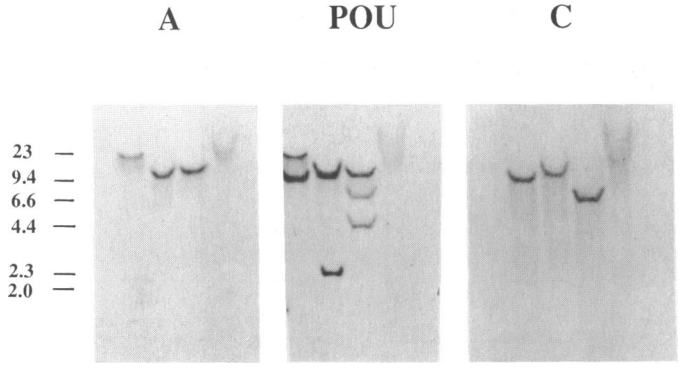

Figure 1. Genomic Southern blots with Brn3a and Brn3c probes. Rat kidney genomic DNA was digested with (from left to right) *Eco*RI, *Bam*HI, *Hin*dIII and *Xho*I, Southern blotted and then probed as described with **a)** a Brn3a-specific probe, **b)** a Brn3 POU domain probe and **c)** a Brn3c-specific probe. Molecular weight markers are shown on the left.

RESULTS

Three Brn3-related POU proteins

Two approaches to identifying novel POU proteins in the rat CNS and PNS were used. First, a PCR approach was used to amplify cDNAs encoding three related members of a Brn3-like family, and obtain sequence across the POU domain. Second, a cDNA library constructed from neonatal rat dorsal root ganglion RNA was screened with a mouse POU-domain probe to isolate additional sequences outside of the POU domains. Both approaches led to the identification of known and novel POU domain sequences. The first mammalian member of the Brn3 family was detected in the rat by He et al. (2), and a second closely related sequence was identified in a rat/mouse hybrid cell line by Lillycrop et al. (8) who named the encoded proteins Brn3a and Brn3b respectively. We detected both of these transcripts in the PCR-generated products derived from neonatal rat brain cDNA, and a third closely related transcript that we have named Brn3c (Table 1). Screening at moderate stringency of 4×10^5 clones from an oligo-dT primed cDNA library derived from rat sensory neurons with a mouse Brn3a POU domain probe resulted in the identification of 15 positive clones. Restriction mapping and partial sequencing demonstrated that the positive clones fell into two groups, corresponding to Brn3a and Brn3c transcripts

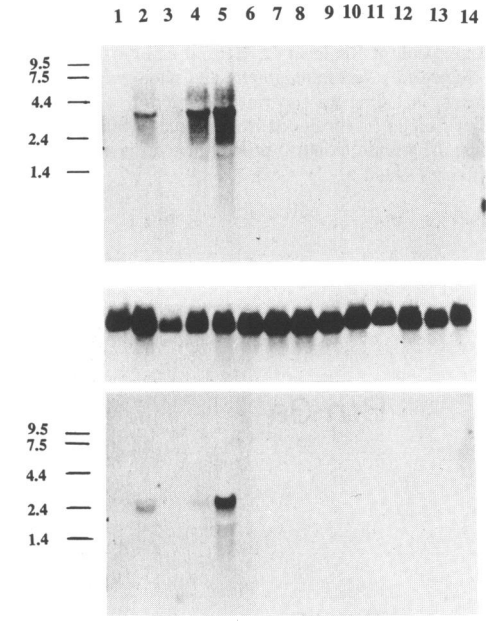

Figure 2 Northern blot of Brn3a and Brn3c transcripts. Total RNA was isolated from neonatal rat cortex (1), cerebellum (2), brain stem (3), spinal cord (4), dorsal root ganglia (5), adrenal glands (6), spleen (7), thymus (8), skin (9), kidney (10), lung (11), heart (12), muscle (13) and liver (14), and northern blotted. The blot was probed consecutively with ^{32}P-labelled probes specific for Brn3a (top panel) or Brn3c (lower panel). To demonstrate that approximately equivalent amounts of RNA were present on the Northern blots, they were re-probed with a ribosomal protein L-27 probe (central strip) The position of RNA size markers is shown on the left.

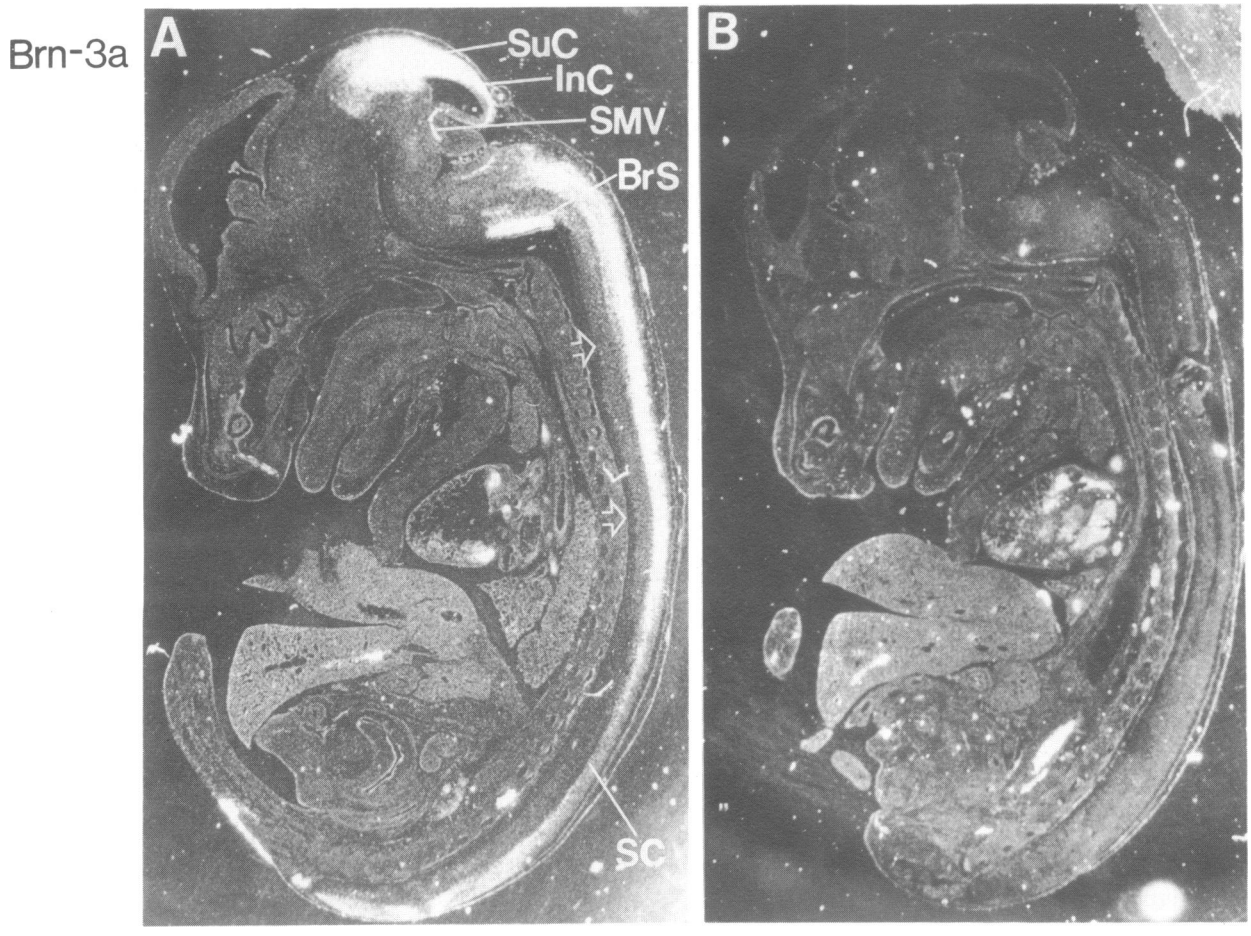

Figure 3. Expression of Brn3a in the E16 rat embryo. Sagittal sections (10 μm nominal thickness) were hybridized *in situ* with either the antisense **(A)** or sense **(B)** probes, subjected to autoradiography and photographed under dark-field illumination. Specific hybridization was observed only in the central and peripheral nervous systems, including the superior and inferior colliculi (SuC and InC), the trigeminal brainstem nucleus (TN), the dorsal half of the spinal cord (SpC) and the dorsal root ganglia (not included in the plane of this section). By comparison, the Brn3c probe gave a specific *in situ* signal only in the DRG and spinal cord (see later Figs). The sense (control) probes gave no *in situ* signal over background. Also indicated in this figure (arrows) are the approximate planes of the sections depicted in figures 4 and 5.

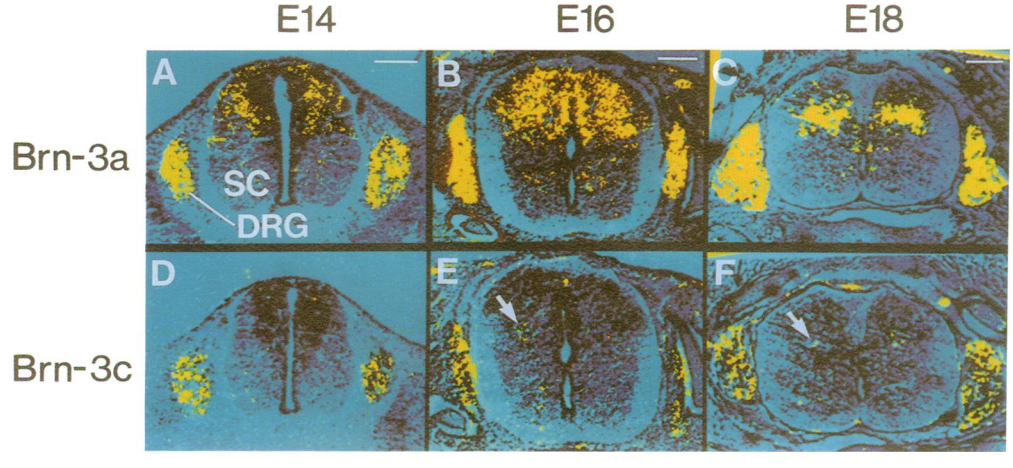

Figure 4. Expression of Brn3a and Brn3c in the developing rat spinal cord and DRG. Each picture is a composite of a dark-field image of an *in situ* autoradiograph (yellow) superimposed on a bright-field image of the same section stained with hematoxylin (blue). Panels **A, B, C** show Brn3a, panels **D, E, F** show Brn3c at E14 (A,D), E16 (B, E) and E18 (C, F). Sections are from the thoracic level of the cord, except those depicted in C, F which are from the cervical level. (See Fig. 3 for the approximate planes of section). Brn3a is expressed in the DRG and the dorsal horn of the spinal cord excluding lamina 1 and the substantia gelatinosa. Brn3c is expressed in a subset of cells in the DRG and in a tiny cluster of cells (arrowed) in the deep dorsal horn (lamina 5) after E14.

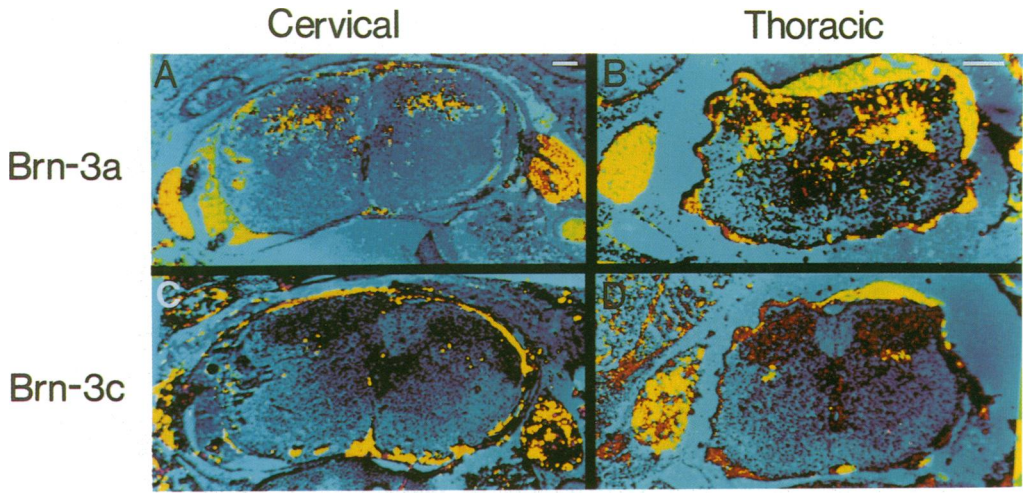

Figure 5. Expression of Brn3a and Brn3c in the newborn rat spinal cord. The *in situ* signal (yellow) is superimposed on a bright-field image of the same section stained with hematoxylin (blue). Panels **A, B** show sections labelled with the Brn3a probe, panels **C** and **D** are labelled with the Brn3c probe. As at earlier ages, Brn3a is expressed mainly in the dorsal horn of the spinal cord and in the DRG; Brn3c is expressed by two small groups of cells in the dorsal horn (about five cells on either side of the midline in these sections which are 15 μm thick), and by a subset of DRG cells.The yellow rim around the perimeter of sections B,C and D is due to light-scattering from red blood cells that accumulate during dissection, rather than silver grains.

encoding the POU domain and C-terminal sequences of these two proteins. The sequences of the POU domains and the C-terminal regions are shown in Table 1, compared to related Class IV POU domain proteins (2) and the octamer-binding proteins Oct1 and Oct2.

Within the POU domain, the three proteins show almost complete amino acid similarity with considerably more variation in the short C-terminal domains. The 3′-untranslated regions of Brn3a and Brn3c are dissimilar (not shown). The third helical regions of the homeodomains, which by analogy with the archetypal Antennapedia homeodomain is the DNA recognition domain, are identical not only in Brn3a and Brn3c but among all known class IV POU domains (Table 1). Interestingly, there is a substitution of valine (Brn3a) for isoleucine (Brn3b and Brn3c) at the same position (C-terminal residue of helix 1) that determines the ability of Oct1, but not Oct2, to interact with the HSV trans-activator VP16 (see below).

Brn3a and Brn3c may be encoded by separate genes

Southern blot analysis of genomic DNA revealed different patterns of hybridization for Brn3a- and Brn3c-specific probes. Single bands were obtained after hybridization with the Brn3a-specific probe to genomic DNA digested with *Eco*RI (23 kb), *Bam*HI (10 kb) *Hin*dIII (11 kb) and *Xho*I (> 23 kb) (Fig. 1). When the same blot was probed with a Brn3c-specific probe, single bands of distinct molecular sizes were obtained; *Eco*RI (9 kb), *Bam*HI (10 kb) *Hin*dIII (7 kb) and *Xho*I (>23 kb) (Fig. 1). Further probing of the same Southern blots with a POU-domain probe that does not distinguish the individual Brn3-related sequences revealed all the fragments that were detected by the specific probes, and additional fragments (a 2.2 kb fragment in the *Bam*HI digest and a 4.3 kb fragment in the *Hin*dIII digest). These data are consistent with the existence of a third Brn3-related gene encoding Brn3b. Alternatively, the data might indicate that the POU domains of Brn3a and/or Brn3c are encoded by more than one exon contained within separate restriction fragments. It is known, for example, that the POU domain of Unc86 is encoded by multiple exons (5).

Distribution of expression of Brn3a and Brn3c

Northern blot analysis

Because the POU domains of Brn3 isoforms show substantial sequence homology, specific probes were constructed using unique 3′ sequence to study the distribution of expression of Brn3a and Brn3c by Northern blotting and *in situ* hybridization. Northern blotting of neonatal rat tissues demonstrated that these transcripts were specific to the nervous system (Fig. 2). A major Brn3a transcript of 3.6 kb was expressed in DRG, spinal cord, cerebellum and, to a lesser extent, brain stem. There was no detectable hybridization to cerebral cortex RNA. Brn3c transcripts were of a smaller size (2.6 kb) and were expressed almost exclusively in DRG, with a low level of transcripts present in the spinal cord. A different 2.4 kb transcript was present in the cerebellum. No Brn3c transcripts were detected in cerebral cortex or non-neural tissues.

In situ hybridization

The expression of Brn3a and Brn3c in embryonic and neonatal rats was investigated by *in situ* hybridization with ^{35}S-labelled RNA probes. In sagittal sections of E14-E18 rats, we could detect a strong signal with the Brn3a antisense probe in the CNS and PNS, but not elsewhere in the embryo. In the CNS, Brn3a is expressed in the midbrain and hindbrain (superior and inferior colliculi and the superior medullary vellum), in the brainstem (medulla) and in the dorsal half of the spinal cord (Fig. 3). In the PNS, Brn3a is expressed in the trigeminal ganglia and in the DRG (not visible in the plane of the section shown in Fig. 3). In contrast, when sagittal sections were hybridized with the Brn3c antisense probe, we could detect a positive signal only in the DRG (not shown). The control (sense) probes gave no significant signals above background (e.g. Fig. 3B).

Coronal (transverse) sections through developing rat embryos provide a clearer picture of Brn3a and Brn3c expression in the spinal cord and DRG. Throughout the period examined, from E12 to the day of birth (P0), Brn3a is expressed strongly in the trigeminal ganglia and DRG (Figs 4 and 5 and not shown). At higher magnification (Fig. 6) it is possible to distinguish DRG

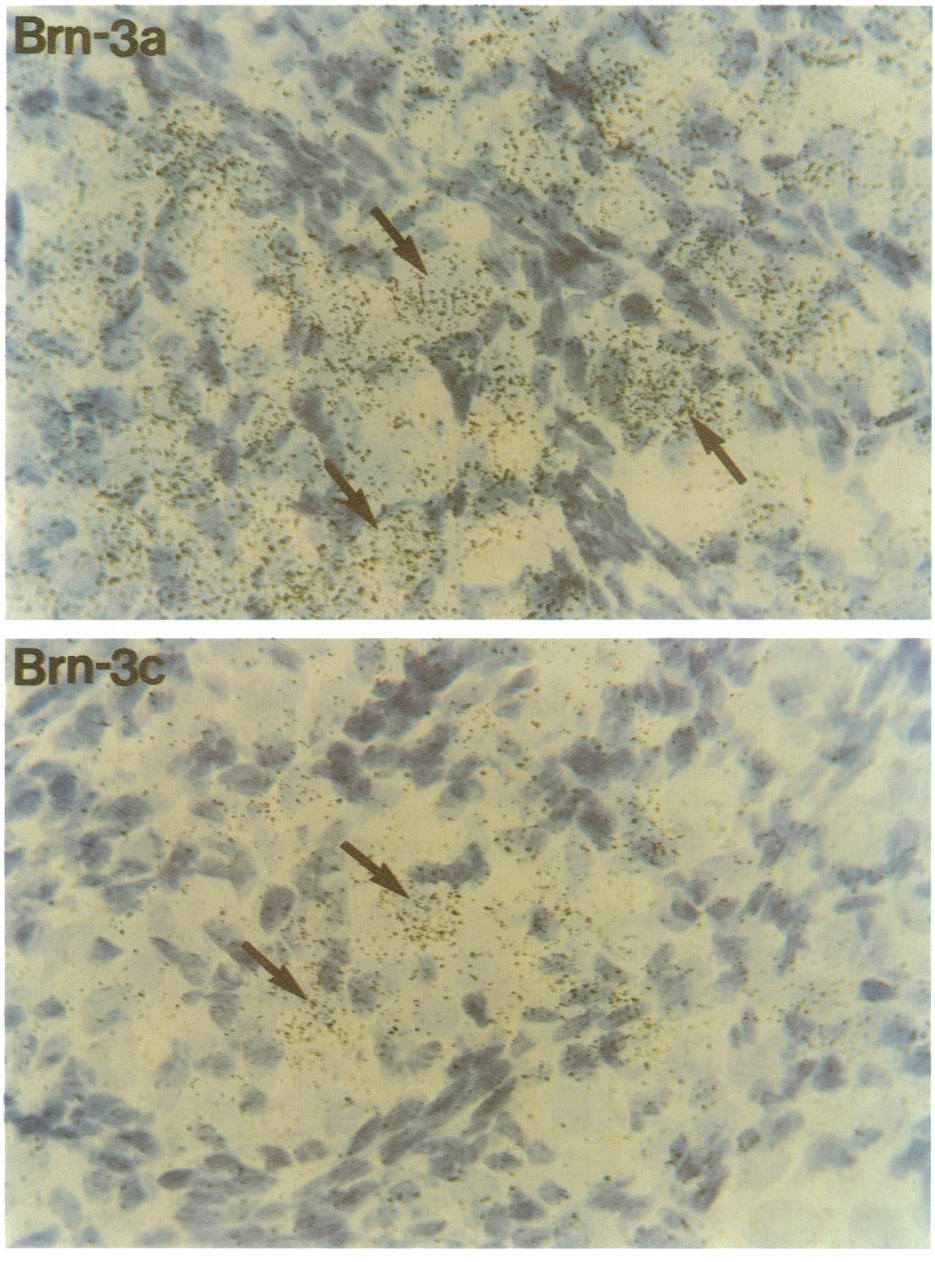

Figure 6. Expression of Brn3a and Brn3c in sections of newborn rat dorsal root ganglia. High magnification bright-field micrographs showing both the *in situ* signal (silver grains) and the associated cell nuclei stained with hematoxylin. For both Brn3a and Brn3c, the silver grains lie predominantly over the lightly stained large light neurons (arrowed) while the small dark cells in this section are unlabelled.

neurons, most of which are positive for Brn3a, from the smaller, more intensely stained cell bodies of Schwann cells and satellite cells, which appear negative.

Brn3a is also expressed in the spinal cord at all ages examined. At E12 (not shown) and E14 (Fig. 4A) the Brn3a hybridization signal is found mainly towards the pial surface in the dorsal half of the cord, outside of the ventricular zone (i.e. in post-mitotic cells). Between E14 and E16, the number of cells expressing Brn3a increases, and the zone of expression comes to occupy most of the dorsal half of the cord (Fig. 4B). By E18, when the vast majority of spinal cord neurons have been formed, the Brn3a-expressing cells are located predominantly in the deep dorsal horn (i.e. in the dorsal half of the cord excluding the most dorsal aspects—lamina 1 and the substantia gelatinosa). Brn3a is expressed along most of the length of the spinal cord. Neither the number of Brn3a-positive cells nor their spatial distribution changes dramatically with developmental time after E16, or with position along the anterior-posterior axis (Fig. 4).

Brn3c is expressed in the DRG at all times examined (E12-P0), but in substantially fewer cells than Brn3a (Figs 4 and 5). At high magnification (Fig. 6) the Brn3c-positive cells can be identified as a subset of neurons. It is not possible to unambiguously distinguish different subclasses of sensory neurons in these sections, but at least some of the Brn3c-expressing cells appear to have large diameter cell bodies, probably corresponding to mechanosensitive neurons (24). Brn3c-expressing cells cannot

be detected in the spinal cord before E16, when a very few positive cells are present in the deep dorsal horn (lamina 4/5) (Fig. 5). At most, five or six cells are present on each side of the midline in these sections (15 μm nominal thickness). This number of Brn3c-positive cells does not change during later development (Fig. 4), or along the longitudinal axis (Fig. 5).

DISCUSSION

We have identified sequences encoding a new Brn3-related POU transcription factor that is expressed in a restricted spatial pattern in the developing rat nervous system. We have named this protein Brn3c to distinguish it from Brn3a and Brn3b, the two previously identified members of this subfamily of POU proteins (2, 8).

The highly conserved nature of Brn3-like proteins and their differential expression in the nervous system supports the view that this family of transcription factors plays an important role in the development and/or function of particular neuronal cell types, for example sensory neurons. The prototypic member of the class IV POU gene family, Unc86, plays a pivotal role in the development of some mechanosensitive neuronal subtypes in *C. elegans*. Another member of this class is I-POU, a Drosophila POU protein that is a dominant-negative repressor of the neuron-specific activating protein Cf1-a (3). The existence of three Brn3-related isoforms suggests that these transcriptional regulators may have distinct functions. Studies with Brn3a [also named Brn3o (11)] have shown that the isolated POU domain is capable of transactivating the corticotrophin releasing factor (CRH) gene, which is also trans-activated by the related POU protein Brn2 (11). Unlike Brn3a, the isolated POU domain of Brn2 does not activate CRH, suggesting that Brn2 sequences outside the POU domain are required for trans-activation. The equivalent activating function of Brn3a may require interaction between the Brn3a homeodomain and a cellular factor, perhaps in a manner analogous to the way that Oct1 interacts with the viral transactivator VP16 (9, 10). Site-directed mutagenesis has identified a single residue in the first helix of the homeodomain of Oct1 that is important for the specific interaction of Oct1 with VP16 (9, 10). This same residue differs between Brn3a (valine) and Brn3c (isoleucine), consistent with the idea that the activities of Brn3a and Brn3c might be differentially modulated by cellular trans-activators. Apart from this change, most of the differences among the Brn3 isoforms are clustered in the region linking the POU box and the homeodomain. However, only a subset of linker residues are substituted (Table 1), suggesting that the conserved linker residues may have functional significance in this subfamily of POU proteins.

DRG neurons are derived from the neural crest, while spinal cord neurons arise from the neural tube (25). Despite these differences in embryonic origin, cell types within lamina 4/5 of the spinal cord as well as DRG neurons express Brn3a and Brn3c transcripts. Therefore, these POU proteins are likely to perform functions other than lineage determination. Brn3a and Brn3c are both expressed in DRG at E13-E14 when some neurons are still dividing (26) as well as in the postmitotic neurons of neonatal animals, suggesting that these factors could be involved in both the establishment and the maintenance of neuronal phenotype. As Brn3a is expressed by most, if not all DRG neurons, it must be present in both mechanosensitive and nociceptive neurons. In contrast, Brn3c is expressed by a minor subset of DRG neurons. Although it has not as yet been possible to unambiguously identify the Brn3c positive cells, some neurons appear to have large cell bodies and probably correspond to mechanosensitive cells.

In the spinal cord, Brn3a and Brn3c transcripts never occur in dividing neuroblasts in the ventricular germinal zone, but are found in a subset of post-mitotic neurons. Brn3a is present within many cells of lamina 4/5 that probably correspond to wide dynamic range cells, which are sites of termination of mechanosensitive sensory neurons (27). Brn3c expression is limited to a much smaller subset of cells within lamina 4/5. Identification of these cell types has not been possible. Spinothalamic neurons are found in laminae 1, 4 and 5, and spinocervical cells and postsynaptic dorsal column cells are predominantly located in lamina 4. Three types of neurons have been defined in laminae 4 and 5 (28), namely cells with transverse dendrites, central cells with longitudinal dendrites, and antennea-type neurons that project dendrites into the substantia gelatinosa. Some preganglionic sympathetic neurons may also occur in this area (28). The neurons that express Brn3a and Brn3c have thus not been identified, but may well correspond to cells that receive input from mechanosensitive sensory neurons which terminate in these laminae. Motor neurons, which are located in the ventral horn, and cells of the dorsal horn (substantia gelatinosa) that receive nociceptive input do not express these transcripts.

We have thus defined a novel isoform of the Brn3 family of POU proteins. We refer to this new isoform as Brn3c to distinguish it from the previously identified members of this family. Brn3c appears to be expressed exclusively in the nervous system in a pattern distinct from that of Brn3a. The patterns of expression of Brn3a and Brn3c, coupled with the known role of the *C. elegans* Unc86 gene product in specifying some neuronal phenotypes, suggests that these mammalian POU factors may be involved in the regulation of development or function of subpopulations of sensory and spinal cord neurons.

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