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All *Brn3* genes can promote retinal ganglion cell differentiation in the chick

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SUMMARY

Targeted gene disruption studies in the mouse have demonstrated crucial roles for the *Brn3* POU domain transcription factor genes, *Brn3a, Brn3b, Brn3c* (now called *Pou4f1*, *Pou4f2*, *Pou4f3*, respectively) in sensorineural development and survival. During mouse retinogenesis, the *Brn3b* gene is expressed in a large set of postmitotic ganglion cell precursors and is required for their early and terminal differentiation. In contrast, the *Brn3a* and *Brn3c* genes, which are expressed later in ganglion cells, appear to be dispensable for ganglion cell development. To understand the mechanism that causes the functional differences of *Brn3* genes in retinal development, we employed a gain-of-function approach in the chick embryo. We find that Brn3b(l) and Brn3b(s), the two isoforms encoded by the *Brn3b* gene, as well as Brn3a and Brn3c all have similar DNA-binding and transactivating activities. We further find that the POU domain is minimally required for these activities. Consequently, we show that all these Brn3 proteins have a similar ability to promote development of ganglion cells when ectopically expressed in retinal progenitors. During chick retinogenesis, cBrn3c instead of cBrn3b exhibits a spatial and temporal expression pattern characteristic of ganglion cell genesis and its misexpression can also increase ganglion cell production. Based on these data, we propose that all Brn3 factors are capable of promoting retinal ganglion cell development, and that this potential may be limited by the order of expression in vivo.

Key words: Brn3, POU domain, Transcription factor, Retinal ganglion cell, Chick

INTRODUCTION

The vertebrate retina is a highly organized sensorineural epithelium consisting of six classes of neurons and one class of glial cell. Owing to its accessibility, well-characterized cell classes and neural tube origin, the retina has provided an excellent system in which to study determination and differentiation mechanisms of the central nervous system. During development, different retinal cell classes are generated from multipotential progenitors (Turner and Cepko, 1987; Turner et al., 1990) in a loose temporal order, with the ganglion cells being the first cell type to be produced (Sidman, 1961; Young, 1985). Several other studies on the role of environmental factors have led to the suggestion that the environment, as well as intrinsic differences among progenitor cells, contribute to the determination of at least several types of retinal cells (reviewed in Cepko, 1999).

Recent advances have begun to unravel the molecular bases that control the specification and differentiation of retinal ganglion cells. The neurogenic gene, *Notch,* is expressed in retinal progenitors and was found to suppress ganglion cell differentiation (Austin et al., 1995). In conditions minimizing cell-cell contacts, the majority of progenitors in the early retina differentiated into ganglion cells (Austin et al., 1995). In contrast, forced expression of the proneural basic helix-loop-helix (bHLH) transcription factors, Xath5 and Xath3, has been shown to promote ganglion cell formation in *Xenopus* (Kanekar et al., 1997; Perron et al., 1999).

Brn3b, also known as Brn3.2 (Xiang et al., 1993; Turner et al., 1994) and now called Pou4f2, is a POU domain transcription factor required for retinal ganglion cell development. The *Brn3b* gene is a vertebrate homolog of the *C. elegans Unc-86*, a gene essential for proper development of multiple neural lineages, including mechanosensory neurons (Finney et al., 1988; Finney and Ruvkun, 1990; Baumeister et al., 1996). In the mouse, *Brn3b* encodes two isoforms of polypeptides – a long form Brn3b(l) and a short one Brn3b(s), due to the usage of alternative translation initiation sites (Theil et al., 1993, 1995; Fig. 1A). During mouse retinal development, Brn3b proteins are found in postmitotic retinal ganglion cell precursors as well as differentiated ganglion cells (Xiang et al., 1993, 1995; Gan et al., 1996; Xiang, 1998). In *Brn3b* targeted null mice that lack expression of both Brn3b(l) and Brn3b(s), a large number of retinal ganglion cells fail to undergo proper early and terminal differentiation. They eventually degenerate by apoptosis (Gan et al., 1996, 1999; Erkman et al., 1996; Xiang, 1998). Despite this demonstration of the requirement for Brn3b in vivo, it is unknown whether Brn3b is sufficient to

promote ganglion cell differentiation. It is also unknown whether there are any functional differences between the two isoforms. Previous work has led to the suggestion that Brn3b(l) acts as a transcriptional activator in vitro (Turner et al., 1994; Trieu et al., 1999), and that Brn3b(s) lacks DNA-binding activity and may function as a repressor (Morris et al., 1994; Theil et al., 1995).

Brn3a and *Brn3c*, also known as *Brn3.0* and *Brn3.1*, respectively, are two other *Brn3* genes (Gerrero et al., 1993; Theil et al., 1993; Xiang et al., 1995). Similar to Brn3b, both Brn3a and Brn3c are expressed in differentiated ganglion cells during mouse retinogenesis (Xiang et al., 1995; Xiang, 1998). However, their expression is initiated 2 days after the onset expression of Brn3b and they are absent from migrating ganglion cell precursors (Xiang, 1998). Targeted disruption of *Brn3a* and *Brn3c* in mice does not cause retinal defects, although it does result in severe sensory deficiencies elsewhere (Erkman et al., 1996; McEvilly et al., 1996; Xiang et al., 1996, 1997). It is speculated that Brn3b may be able to assume the functions of Brn3a and Brn3c in retinal ganglion cell genesis, but it may play a role early in ganglion cell development that cannot be performed by Brn3a and Brn3c because of their belated expression (Xiang, 1998). One prediction of this hypothesis is that the three Brn3 factors would be functionally equivalent if they had the same spatiotemporal expression pattern during retinal development.

To test this hypothesis, we took a gain-of-function approach to investigate the roles of Brn3 proteins in retinal ganglion cell development. In contrast to earlier reports (Morris et al., 1994; Theil et al., 1995), we show that Brn3b(s) can not only bind DNA but can transactivate reporter gene expression. In the chick retina, overexpression of Brn3b(l) and Brn3b(s) in progenitors promotes development of ganglion cells. Moreover, misexpression of Brn3a and Brn3c exert similar effects. During chick retinogenesis, cBrn3c instead of cBrn3b is the first Brn3 factor to be expressed in the ganglion cells, suggesting that cBrn3c plays a similar role in the chick as the mouse Brn3b in mouse retinal development. Together, these data reveal a potential for all Brn3 factors to promote retinal ganglion cell development provided that they are expressed in the right place at the right time during embryogenesis.

MATERIALS AND METHODS

Plasmid constructs

For examining transcriptional properties, the human Brn3b(l) expression plasmid was described previously (Xiang et al., 1993). To construct the Brn3a and Brn3c expression plasmids, artificial cDNA for the human Brn3a and mouse Brn3c was generated by joining their two exons via PCR amplification, followed by inserting the cDNA into the pRK5 vector modified from pCIS (Gorman et al., 1990). The expression plasmids of Brn3b(s) and all other Brn3 truncations were constructed in pRK5 using PCR-derived DNA fragments. The luciferase reporter construct was described in Qi (1997), containing the IE110 promoter from +33 to −164 bp of the herpes simplex virus (HSV). The pCMV-β-gal plasmid, used as an internal control for transfection efficiency, was described in Spaete and Mocarski (1985). For generating retroviral plasmid constructs, cDNA fragments of Brn3b(l), Brn3b(s), Brn3b POU domain, Brn3a, Brn3c, Brn3cΔ8, and cBrn3c were first subcloned into the shuttle plasmid Slax12NCO and subsequently transferred into the RCASBP(A) vector at the *Cla*I site (Morgan and Fekete, 1996). The *cBrn3c* cDNA was derived by joining its two exons via PCR amplification of the chicken genomic DNA. RCAS-AP, carrying the human placental alkaline phosphatase gene (Fekete and Cepko, 1993), was used as a control plasmid. All PCR-derived constructs were verified by DNA sequencing.

Transfection and luciferase assay

The 293S human embryonic kidney cells and ND7 neuronal cells were utilized for transient transfection. Expression and reporter plasmids were cotransfected into these cells by lipofection using the Lipofectamine reagent following manufacturer’s instructions (Life Technologies). Luciferase activities were measured 48 hours after transfection using a luciferase assay system according to the manufacturer’s protocol (Promega). All luciferase activities were normalized with the β-galactosidase activities derived from the control plasmid pCMV-β-gal. Experiments were performed in triplicate and repeated a minimum of three times.

Gel mobility shift assay

Brn3b(l) and Brn3b(s) proteins were produced by the coupled TNT transcription/translation system (Promega) using the aforementioned expression plasmids. DNA oligonucleotides containing Brn3-binding sites were end-radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions were carried out at room temperature for 20-30 minutes in a final volume of 20 μl containing 10 mM Hepes (pH 7.5), 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 5% glycerol, 0.1 mM DTT, 0.1 mM PMSF, 1 μg poly(dI-dC), 5×10^5 cts/minute of labeled probe, and 3 μl of desired protein lysates. Competition was performed by adding to the reactions desired amount of cold oligonucleotides. Free and bound probes were resolved in a 5% nondenaturing polyacrylamide gel. The following two DNA oligonucleotides were used for probe and competition, containing the consensus Brn3-binding site (Gruber et al., 1997) and the HSV IE110 Brn3-binding site (Fig. 1B), respectively: 5′CACGCATAATT-AATCGC and 5′CAATGCTAATGATATGC. The nonspecific oligonucleotide M4 was described in Xiang et al. (1995).

Generation and purification of the anti-cBrn3c antibody

The DNA fragment corresponding to amino acids 124-183 of cBrn3c (Lindeberg et al., 1997) was amplified by PCR, and inserted in frame into the pGEMEX (Promega) and pMAL-cR1 (New England Biolabs) vectors to express proteins fused with the bacteriophage T7 gene 10 protein and bacterial maltose-binding protein, respectively. Production of rabbit antiserum and affinity-purification of the antibody were performed as previously described in Xiang et al. (1995).

Preparation and injection of retroviruses

Replication-competent avian retroviruses were prepared in chick embryonic fibroblasts (CEFs). Retroviral plasmid constructs were transfected into CEFs using the Lipofectamine reagent. After 7-10 days of amplification, the viruses were harvested, concentrated and titered as described (Morgan and Fekete, 1996). Titers of all viruses used were in the range of 1×10^8-1×10^9 CFU/ml. Fertilized chicken eggs were purchased from Spafas, Inc. and staged according to Hamburger and Hamilton (1951). Injection of retroviruses into chick optic vesicles was performed at stages 9-11 as described (Morgan and Fekete, 1996). Briefly, the chick embryos were exposed by cutting a window in the shell. Concentrated RCAS retroviruses containing target genes were mixed with 1/10 volume of 0.05% Fast Green, which functioned as a tracking dye. Approximately 0.1 μl of viruses were injected into each optic vesicle of the embryo and both vesicles were injected. Following injection, the embryos were sealed and incubated for 4 to 5 more days before harvest.

Dissociation of chick retinal cells

The procedure for dissociation of retinal cells was modified from

Altshuler and Cepko (1992). At E5.5-E6.5, control and injected embryos were collected and their eyeballs were isolated. For comparison, only eyeballs with similar size were used for analysis. Retinas were dissected from eyeballs and digested with 1 mg/ml trypsin in Mg^{2+}- and Ca^{2+}-free Hank's balanced salt solution (HBSS) for 20 minutes at 37°C, followed by treatment with 2 mg/ml soybean trypsin inhibitor to stop the reaction. Each digested retina was then centrifuged and triturated in HBSS with 0.1 mg/ml DNaseI. The dissociated retinal cells were centrifuged and resuspended in 500 µl HBSS, from which 30 µl (retinas at E5.5) or 10 µl (retinas at E6.5) was plated in each well of Vectabond (Vector Labs)-coated, 8-well slides (Cel-line Associates). Slides were kept at room temperature for 1 hour in a wet chamber to allow cells to settle down before further analysis.

Immunohistochemistry and immunocytochemistry

For immunostaining chick retinal sections, embryos were collected at various developmental stages and eye cups were made as described previously (Xiang et al., 1993, 1995). Following fixation in 4% paraformaldehyde, eye cups were cryoprotected in sucrose, equilibrated and embedded in OCT, and sectioned at 10-14 µm in a cryostat. Immunostaining was carried out using the ABC system (Vector Laboratories) and the NovaRED or VIP substrate kits (Vector Laboratories) were used for color development. For quantitation of Islet1-positive and Brn3a-positive cells on sections of retinas infected with RCAS viruses, the number of immunoreactive cells were scored in a high-power optic field using a reticule mounted on the microscope. A minimum of two fields of each type was counted for each retina and at least three retinas were analyzed each time. The experiment was repeated three times.

For immunostaining dissociated retinal cells on slides, cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature, blocked in 5% normal goat serum overnight at 4°C, and then incubated with primary antibodies for 3 hours at room temperature. For 5-bromodeoxyuridine (BrdU) immunocytochemistry, the cells were incubated in 1 N HCl for 1 hour at 37°C after fixation. Following incubation with primary antibodies, the cells were immunostained using the ABC system and the NovaRED substrate kit while their nuclei were simultaneously labeled with 4′, 6-diamidino-2-phenylindole (DAPI, 5 µg/ml). For quantitation, the number of immunoreactive cells and the total number of DAPI-labeled cells were scored in a high-power optic field. Ten randomly selected fields were counted for each retina and a minimum of three retinas were analyzed for each type. For retinas injected with RCAS viruses, counts were done only for those highly infected (≥70% of cells were p27-positive), as judged by immunoreactivity with the anti-p27 gag antibody. Each experiment was repeated at least three times and all data were tested for significance using two sample Student's *t*-test with unequal variances.

Antibodies were obtained from the following sources: anti-cBrn3c (this work), anti-Brn3b (Xiang et al., 1993), anti-Brn3c (Xiang et al., 1995), anti-Brn3a (Xiang et al., 1995; Chemicon), anti-Islet1 (Yamada et al., 1993), anti-NF 200 (Boehringer Mannheim), anti-BrdU (Sigma) and anti-p27 (Spafas).

BrdU and TUNEL labeling

For BrdU labeling, 50 µl BrdU (1 µg/µl in HBSS) was dropped onto embryos at E5.5, 4 hours before embryo collection. Labeled retinas were then dissociated and processed for BrdU immunocytochemistry as described above.

The terminal dUTP nick-end labeling (TUNEL) was performed as described (Xiang, 1998; Xiang et al., 1998) with modification. In brief, dissociated retinal cells were fixed in 4% paraformaldehyde, permeated in 0.1% Triton X-100, and incubated for 1 hour at 37°C in terminal transferase buffer containing 4 µM biotin-16-dUTP and 0.25 units/ml terminal transferase. Following washes in PBS, the cells were subsequently processed and stained using the ABC system and the NovaRED substrate kit, and counterstained with DAPI. Quantitation was performed as described above.

RESULTS

DNA-binding and transactivation activities of Brn3b(l) and Brn3b(s)

To understand whether the two isoforms of Brn3b proteins have similar or different functions, we first examined their transcriptional and DNA-binding properties. In transient transfection assays, we used a luciferase reporter gene under the control of an HSV IE110 promoter containing a single Brn3-binding site (Fig. 1B). In human embryonic kidney cells (293S), transfection of Brn3b(l) (1-410) resulted in a 4-fold increase in luciferase activity (Fig. 1A,D), consistent with previous studies showing that Brn3b(l) is a transcriptional activator (Turner et al., 1994; Trieu et al., 1999). Interestingly, the construct containing only the DNA-binding POU domain plus the five C-terminal amino acid residues (249-410) displayed comparable transactivation activity as the full-length Brn3b(l) (Fig. 1A,D). In addition, truncation up to 123 N-terminal amino acid residues (98-410 and 124-410) enhanced transactivation activity by more than 2-fold compared with that of the full-length Brn3b(l) (Fig. 1A,D). Similar results were observed in ND7 cells, a neuronal cell line derived from rat dorsal root ganglia (Wood et al., 1990), although all constructs exhibited overall weaker transactivation activities in these cells (Fig. 1E).

Brn3b(s) lacks 97 N-terminal amino acid residues of Brn3b(l) but acquires nine unique N-terminal residues (Fig. 1A). It is thought that Brn3b(s) does not bind DNA and acts as a transcriptional repressor (Morris et al., 1994; Theil et al., 1995). However, transfection of Brn3b(s) into both 293S and ND7 cells activated the luciferase reporter as effectively as Brn3b(l) (Fig. 1D,E), suggesting Brn3b(s) may be capable of transactivating the promoter via DNA binding. To rule out the possibility that Brn3b(s) per se could not bind DNA but activated luciferase expression through interacting with an unknown DNA-binding protein in the cell, we examined the ability of Brn3b(s) to bind a Brn3 consensus site (ATAATTAAT; Gruber et al., 1997) by gel mobility shift assay. As shown in Fig. 1C, in vitro translated Brn3b(l) and Brn3b(s) bound specifically to the consensus site and the binding could be completely abrogated by 100-fold excess of unlabeled consensus sites, but was not inhibited by up to 500-fold excess of a nonspecific site. In a similar experiment, we were able to show that Brn3b(l) and Brn3b(s) could bind specifically to the Brn3 recognition site found in the HSV IE110 promoter (Fig. 1B; data not shown). Together, these data indicate that Brn3b(s), like Brn3b(l), possesses specific DNA-binding and transcriptional activation activities.

The POU domain can confer both DNA-binding and transactivation activities of Brn3 factors

We next investigated transcriptional properties of the other two Brn3 proteins – Brn3a and Brn3c. In the three Brn3 proteins, the POU domain is located at the very C-terminal end followed by only 5 amino acid residues (Xiang et al., 1995; Figs 1A, 2A). Transfection of Brn3c into both ND7 and 293S cells increased luciferase activity by 4- to 5-fold (Fig. 2A,B). Similar to the truncated Brn3b polypeptides, N-terminal truncations did not diminish the transactivation activity and the

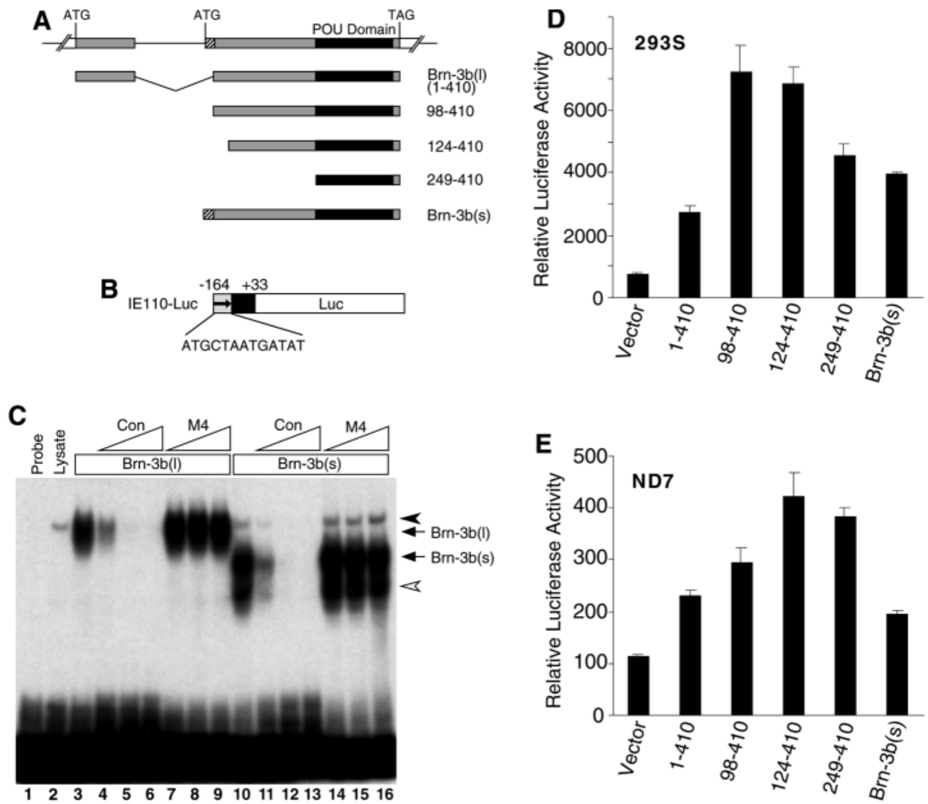

Fig. 1. DNA-binding and transcriptional activities of Brn3b(l) and Brn3b(s). (A) Schematic diagram of expression constructs. The human *Brn3b* locus contains two exons and one intron. The coding region is indicated by three gray boxes, one black box (POU domain) and one hatched box [the nine unique N-terminal amino acid residues of Brn3b(s)]. The open boxes represent the 5′ and 3′ untranslated regions. Indicated also are translation initiation (ATG) and termination (TAG) codons. Brn3b(l) has 410 amino acid residues and the truncated constructs are named by the positions of deletions. (B) Schematic of the reporter construct IE110-Luc. The luciferase reporter gene (Luc) is placed under the control of an HSV IE110 promoter from +33 to −164 bp containing the indicated Brn3-binding site. (C) Gel mobility shift assay using a consensus Brn3-binding site (Con) as probe. Reactions contained unprogrammed lysate (lane 2), or in vitro translated Brn3b(l) (lanes 3-9) or Brn3b(s) (lanes 10-16) in the absence (lanes 3 and 10) or presence of specific (Con; lanes 4-6 and 11-13) or nonspecific (M4; lanes 7-9 and 14-16) competitors. Each cold competitor oligonucleotide was used in 10-, 100-, or 500-fold molar excess of the probe (triangles). The Brn3b(l) and Brn3b(s) protein complexes, which can be abrogated by excessive consensus sites, are indicated by arrows while the filled arrowhead points to a specific complex derived from the lysate. The complex indicated by the open arrowhead presumably resulted from protein degradation. (D,E) Relative luciferase activities following cotransfection of different expression constructs with IE110-Luc. Cotransfection was conducted in 293S (D) or ND7 (E) cells and luciferase activity was measured 48 hours after transfection. Transfection efficiency was controlled by measuring β-galactosidase activity following cotransfection with the pCMV-β-gal plasmid, which constitutively expresses β-galactosidase. Results represent means±s.d. of triplicate assays in a single experiment.

truncation containing only Brn3c POU domain plus its five C-terminal amino acid residues (179-338) exhibited approximately the same transactivation activity as the full-length Brn3c (Fig. 2A,B). Similar assays showed that a truncated polypeptide containing only the Brn3a POU domain plus its five C-terminal amino acid residues was transcriptionally as active as the full-length protein (data not shown). Thus, the DNA-binding and transactivation activities of Brn3 proteins largely reside in the POU domain plus the five C-terminal amino acid residues.

To test whether the POU domain of Brn3 proteins has transactivation activity in addition to its DNA-binding property, we measured transcriptional activities of POU domains of the three Brn3 factors. As shown in Fig. 2C, the POU domains derived from Brn3a, Brn3b and Brn3c clearly activated the IE110 promoter, although they displayed significantly less transactivation activities than those of the corresponding POU domains plus the five C-terminal amino acid residues. Therefore, these data together demonstrate that the Brn3 POU domain is not only a DNA-binding domain, but also an activation domain.

Promotion of retinal ganglion cell differentiation by ectopic expression of Brn3b(l) and Brn3b(s)

Targeted deletion studies of *Brn3* genes in mice have implied that there is an early role for *Brn3b* in retinal ganglion cell development that cannot be performed by the later-expressed *Brn3a* and *Brn3c* (Gan et al., 1996; Erkman et al., 1996; McEvilly et al., 1996; Xiang et al., 1996, 1997; Xiang, 1998). To understand this role, we first examined the consequences of forced expression of Brn3b(l) in chick retinal progenitors. The replication competent RCAS retroviral vector was employed to overexpress Brn3b(l). As most of the ganglion cells are produced between E2 and E7 during chick retinogenesis (Prada et al., 1991), we infected optic vesicles with RCAS-Brn3b(l) viruses at stage 9-11 (approx. E1.5), prior to the initiation of ganglion cell birth. At E7.5, most of the retinas were found to be effectively infected by the virus as indicated by the intense p27 gag immunoreactivity (Fig. 3A). As a result, exogenous Brn3b(l) was highly expressed throughout the retina (Fig. 3B).

To determine the effect of Brn3b(l) misexpression in retinal progenitors, we checked the number of cells positive for the LIM homeodomain transcription factor Islet1, a ganglion-cell-specific marker at early times in development (Austin et al., 1995). At E7.5, a minor fraction of retinas infected with RCAS-Brn3b(l) viruses contained patches of uninfected regions as judged by immunolabeling of retinal sections with the anti-p27 gag antibody (Fig. 3C). When adjacent sections were immunostained with the anti-Islet1 antibody, we found a significant increase in the number of Islet1-positive cells within

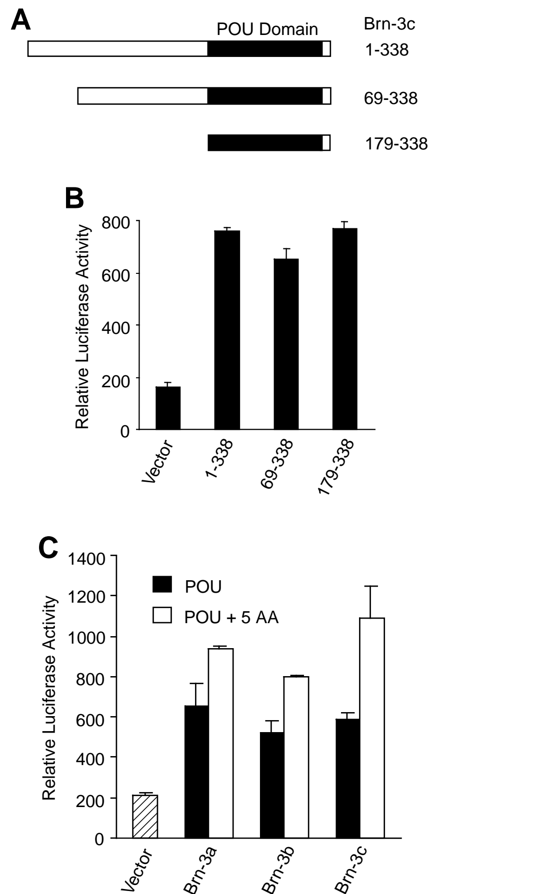

Fig. 2. Transcriptional activities of Brn3c and Brn3 POU domains. (A) Schematic of Brn3c full-length and truncated protein constructs. The filled box represents the POU domain. Brn3c contains 338 amino acid residues and the truncated expression constructs are named by the positions of deletions. (B) Relative luciferase activities following cotransfection of different Brn3c expression constructs with IE110-Luc in ND7 cells. (C) Relative luciferase activities following cotransfection of expression constructs for POU domains (POU) and the corresponding POU domains plus the five C-terminal amino acid residues (POU + 5 AA) of Brn3 proteins with IE110-Luc in ND7 cells. Luciferase activities in B and C were measured 48 hours after transfection. Transfection efficiency was controlled by measuring β-galactosidase activity following cotransfection with the pCMV-β-gal plasmid, which constitutively expresses β-galactosidase. Results represent means±s.d. of triplicate assays in a single experiment.

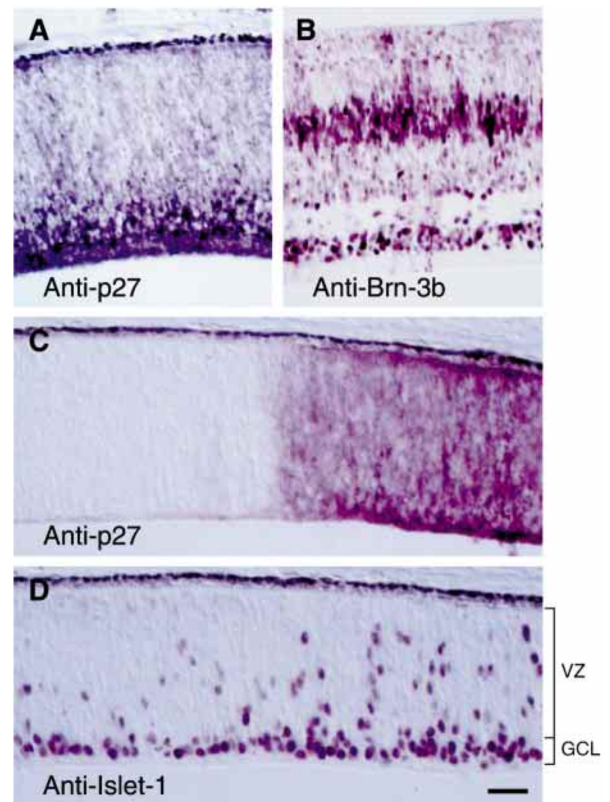

Fig. 3. Ectopic expression of Brn3b(l) in retinal progenitors increases Islet1-positive cells. (A,B) Sections from E7.5 retinas infected with RCAS-Brn3b(l) viruses were immunostained with anti-p27 gag (A) or anti-Brn3b (B) antibodies. Both antibodies labeled cells throughout most of the infected retinas. (C,D) Adjacent sections from an E7.5 retina infected with RCAS-Brn3b(l) viruses were immunostained with anti-p27 gag (C) or anti-Islet1 (D) antibodies. In this retina, more Islet1-immunoreactive cells were found in the patch stained with the anti-p27 gag antibody than in the patch negative for p27 gag. GCL, ganglion cell layer; VZ, ventricular zone. Scale bar, 18.8 μm in A,B and 25 μm in C,D.

the ganglion cell layer as well as the ventricular zone in the infected region compared to the uninfected area (Fig. 3C,D). The Islet1-positive cells in the ventricular zone were presumably newly generated ganglion cells migrating towards the ganglion cell layer. To ask if this effect was specific to Brn3b(l), we found that ectopic expression of the human alkaline phosphatase (AP) by infection with RCAS-AP viruses did not cause any increase in the number of Islet1-positive cells (data not shown). By quantitation of cells immunoreactive for Islet1 and Brn3a on retinal sections in both the peripheral and intermediate regions, we observed an approximately 30-50% increase in the number of Islet1-positive and Brn3a-positive cells in retinas infected with RCAS-Brn3b(l) viruses, compared to those infected with RCAS-AP viruses (Fig. 4). Therefore, ectopic expression of Brn3b(l) can increase retinal ganglion cell production.

Given the ability of Brn3b(s) and the Brn3b POU domain to bind DNA and activate gene expression (Figs 1, 2D), we investigated whether their misexpression could also promote retinal ganglion cell formation. In these experiments, the number of ganglion cells was determined by dissociating retinal cells followed by immunocytochemistry for Islet1 and neurofilament 200 (NF 200), another marker specifically expressed in all ganglion cells shortly after their exit from the cell cycle (Austin et al., 1995). At E5.5-6.5, approximately 10-13% of all retinal cells were NF 200-positive in the control uninfected retina (Fig. 5B-E), consistent with the number reported previously (Austin et al., 1995). However, only 3-4% were found to be Islet1-positive (Fig. 5B-E), suggesting the Islet1 antibody labeled only a subpopulation of dissociated ganglion cells. Consistent with the analysis on retinal sections, retinal infection with RCAS-Brn3b(l) viruses increased Islet1-positive and NF 200-positive cells by 20-50% (Figs 4, 5A,B). Similarly, infection with RCAS-Brn3b(s) and RCAS-

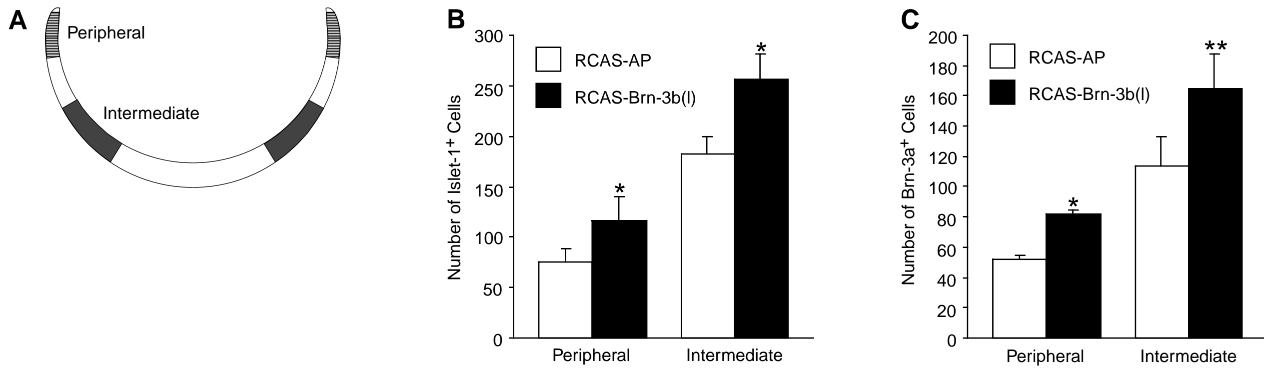

Fig. 4. Quantitation of Islet1-positive and Brn3a-positive cells in E7.5 retinas infected with RCAS-Brn3b(l) viruses. (A) Schematic retinal diagram indicating the peripheral and intermediate regions analyzed. (B,C) Increase in the number of Islet1-positive (B) and Brn3a-positive (C) cells in retinas infected with RCAS-Brn3b(l) viruses compared to those infected with RCAS-AP viruses. Each histogram represents the mean±s.d. for at least three retinas. **P*<0.01; ***P*<0.05.

Brn3bPOU viruses resulted in more than 20% increase in Islet1-positive and NF 200-positive cells (Fig. 5A,C,D). By contrast, infection with control RCAS-AP viruses did not alter the number of Islet1-positive and NF 200-positive cells (Fig. 5E). In addition, retinal infection with RCAS-Brn3b(l) viruses had no effect on the number of cells positive for visinin (data not shown), a photoreceptor-specific marker, consistent with the specific expression of Brn3 proteins in ganglion cells. Thus, both Brn3b(l) and Brn3b(s) are capable of specifically promoting retinal ganglion cell formation and the POU domain is minimally required for this activity.

The effect of Brn3b protein misexpression on retinal ganglion cell generation could be a result of: (1) promoting cell proliferation, (2) promoting cell differentiation, or (3) inhibiting cell death. To distinguish these possibilities, we examined the effect of Brn3b(l) misexpression on retinal cell division and apoptosis by BrdU and TUNEL labeling, respectively. At E5.5, approximately 50% of all retinal cells

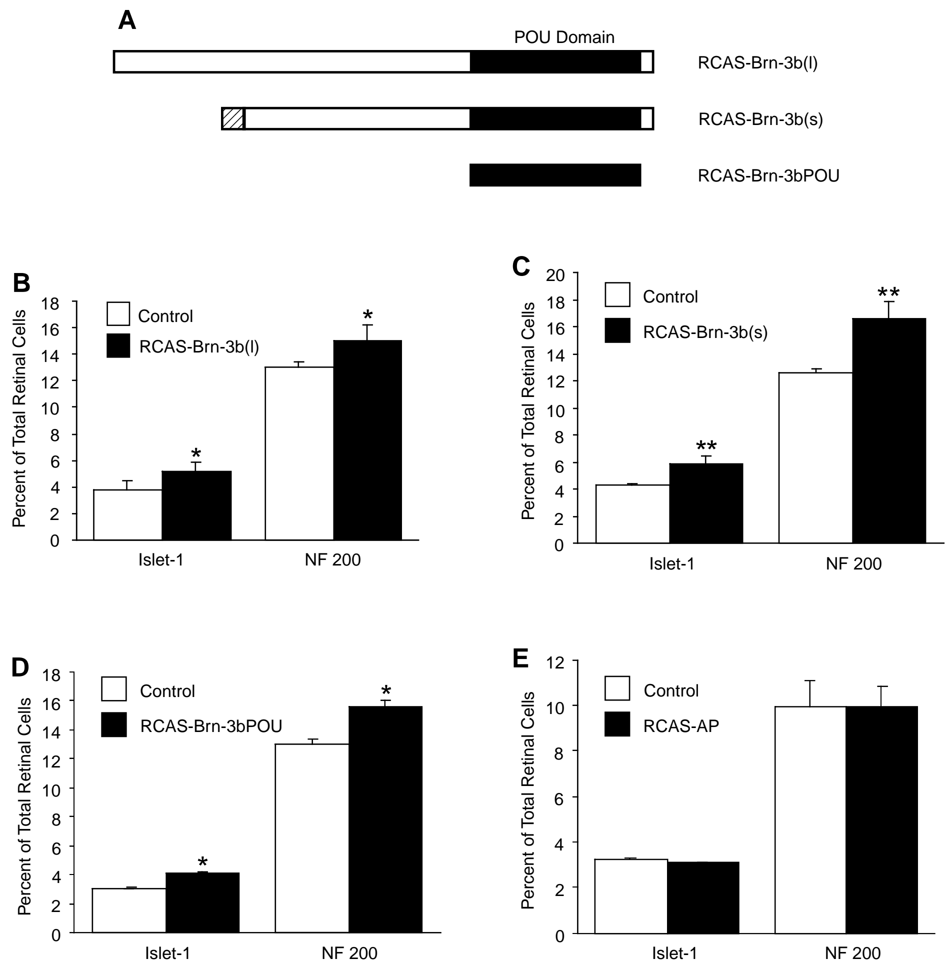

Fig. 5. Effect of misexpression of Brn3b(l), Brn3b(s) and the Brn3b POU domain on ganglion cell production. (A) Schematic diagram of Brn3b(l), Brn3b(s) and the Brn3b POU domain. The filled box indicates the POU domain and the hatched box represents the nine unique N-terminal amino acid residues of Brn3b(s). (B-E) Quantitation of Islet1-positive and NF 200-positive cells in retinas infected with RCAS-Brn3b(l) (B), RCAS-Brn3b(s) (C), RCAS-Brn3bPOU (D), and RCAS-AP (E) viruses. Control and infected retinas were collected at E5.5-E6.5, dissociated and immunostained with anti-Islet1 and anti-NF 200 antibodies. Immunoreactive cells were scored and plotted as percentage of total ganglion cells. Misexpression of Brn3b(l), Brn3b(s) and their POU domain significantly increased the number of Islet1-positive and NF 200-positive cells in the retina, whereas, misexpression of AP had no effect. Each histogram represents the mean±s.d. for at least three retinas. **P*<0.005; ***P*<0.05.

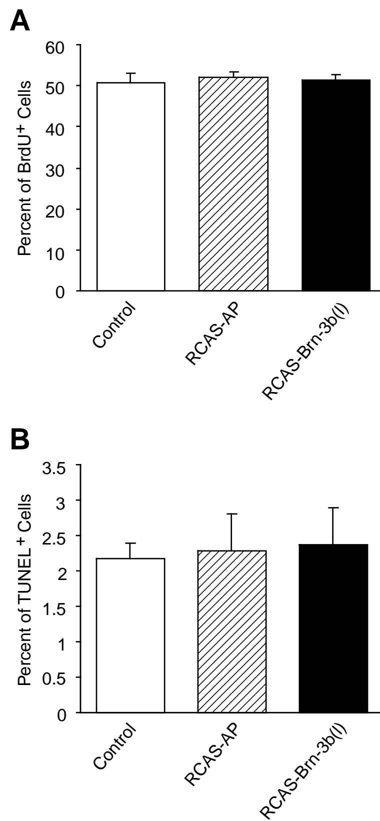

Fig. 6. Misexpression of Brn3b(l) in the retina has no effect on cell proliferation or apoptotic cell death. (A) Quantitation of BrdU-positive, dividing cells in E5.5 retinas infected with RCAS-Brn3b(l) or RCAS-AP viruses, or in control uninfected retinas. (B) Quantitation of TUNEL-positive cells in E5.5 retinas infected with RCAS-Brn3b(l) or RCAS-AP viruses, or in control uninfected retinas. Each histogram represents the mean±s.d. for at least three retinas.

were BrdU-positive, mitotic progenitors in the control uninfected retina (Fig. 6A). Infection of retinas with RCAS-Brn3b(l) or RCAS-AP viruses did not cause any change in the number of BrdU-positive cells (Fig. 6A), indicating Brn3b(l) may have no effect on proliferation of retinoblasts. TUNEL labeling revealed a very low level of apoptotic cell death (approx. 2%) in the E5.5 control retina, which was not affected by infection with RCAS-Brn3b(l) or RCAS-AP viruses (Fig. 6B). Therefore, misexpression of Brn3b(l) in retinal progenitors appears to affect neither cell proliferation nor cell death, suggesting that Brn3b(l) and Brn3b(s) may exert their retinal effects by promoting differentiation of ganglion cells.

Misexpression of Brn3a and Brn3c in retinal progenitors promotes ganglion cell differentiation

Given Brn3 factors share the same DNA-binding sites and all activate gene expression (Gruber et al., 1997; Figs 1, 2), we tested whether Brn3a and Brn3c could also promote retinal ganglion cell development as the Brn3b proteins. As shown in Fig. 7B, infection of retinas with RCAS-Brn3a viruses led to a 30-50% increase in the number of NF 200-positive cells, and a 30-60% increase in the number of Islet1-positive cells by E5.5-6.5. A similar increase in the number of NF 200-positive

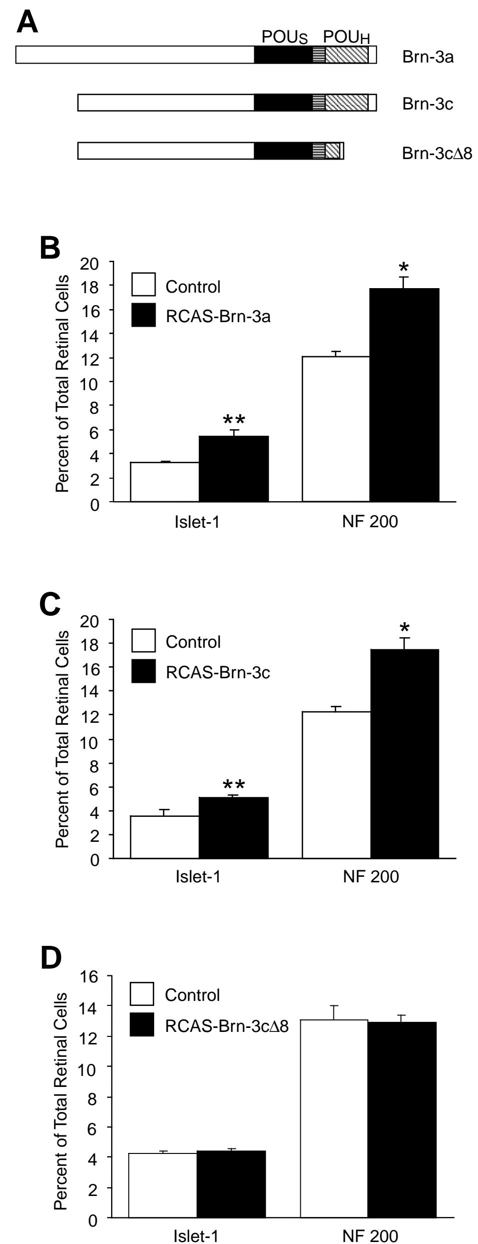

Fig. 7. Effect of misexpression of Brn3a and Brn3c proteins on ganglion cell production. (A) Schematic diagram of Brn3a, Brn3c and Brn3cΔ8. The POU-specific (POU_S) and POU-homeo (POU_H) domains of the POU domain are indicated by the filled and hatched boxes, respectively. (B-D) Quantitation of Islet1-positive and NF 200-positive cells in retinas infected with RCAS-Brn3a (B), RCAS-Brn3c (C) or RCAS-Brn3cΔ8 (D) viruses. At E5.5-E6.5, misexpression of Brn3a and Brn3c resulted in a significant increase in the number of Islet1-positive and NF 200-positive cells in the retina, whereas, misexpression of Brn3cΔ8 had no effect. Each histogram represents the mean±s.d. for at least three retinas. *P<0.005; **P<0.05.

and Islet1-positive cells was observed when retinas were infected with RCAS-Brn3c viruses (Fig. 7C), indicating that both Brn3a and Brn3c are able to promote development of retinal ganglion cells.

To test if the POU domain of Brn3c is also required for this

activity, we generated RCAS viruses that produce a mutant Brn3c protein (Brn3cΔ8) containing an 8 bp deletion in the POU-homeodomain (Fig. 7A). This deletion results in a truncated Brn3c protein that lacks the second and third helices of the POU-homeodomain (Fig. 7A), and has been shown to cause inherited progressive hearing loss in the human (Vahava et al., 1998). When misexpressed in the retina, Brn3cΔ8, in contrast to the wild-type protein, exerted no effect on the number of NF 200-positive and Islet1-positive cells (Fig. 7D), demonstrating an intact POU domain is essential for Brn3c to promote ganglion cell development. Together, these data reveal a potential for Brn3a and Brn3c to perform the early developmental function that normally requires Brn3b during differentiation of mouse retinal ganglion cells and that only their belated expression in vivo may limit this potential.

cBrn3c is expressed in migratory ganglion cell precursors and promotes ganglion cell differentiation

To investigate whether the endogenous chicken Brn3 factors play a conserved role in promoting retinal ganglion cell development, we first examined their spatial and temporal expression patterns during chick retinogenesis. As in the mouse and human, three *cBrn3* genes have been isolated from the chicken genome (Lindeberg et al., 1997; Artinger et al., 1998). In our previous studies, we showed that our specific antibodies against human Brn3a and Brn3b could cross-react with cBrn3a and cBrn3b in the adult chicken retina, respectively (Xiang et al., 1993, 1995). However, our antibody against human Brn3c displayed no cross-immunoreactivity for cBrn3c, presumably because cBrn3c is more diverged in sequence from human Brn3c than cBrn3a and cBrn3b from their human orthologs. Thus, we generated a polyclonal antibody against a cBrn3c fusion polypeptide containing amino acid residues 124-183. In this antigen region, cBrn3c shares only 32-40% amino acid sequence identity with Brn3a and Brn3b. By Western blot assay, the anti-cBrn3c antibody did not show any cross-reactivity with Brn3a and Brn3b fusion proteins containing the antigen region (data not shown), demonstrating the specificity of this antibody.

The expression patterns of cBrn3 proteins during chick retinogenesis were examined by immunostaining retinal sections from various embryonic stages using the anti-Brn3a, anti-Brn3b, and anti-cBrn3c antibodies. At E3, a small number of cBrn3c immunoreactive cells are seen in the central region of the retina, the area of initial ganglion cell birth. By E4.5, cBrn3c expression spreads over the entire retina, with cBrn3c immunoreactivity observed in both the ganglion cell layer and the migrating ganglion cell precursors within the ventricular zone (Fig. 8G). Although cBrn3c is still found in many migrating cells in the ventricular zone by E7.5, its expression is completely localized to the ganglion cell layer by E11 (Fig. 8H,I). On the other hand, neither cBrn3b nor cBrn3a is expressed in the retina by E3. They begin to express by E4.5 but their expression is found in only a small number of cells within the ganglion cell layer of the central retina (Fig. 8A,D). From E7.5 to adult, the expression of cBrn3a and cBrn3b persists in the ganglion cells including occasional displaced ganglion cells in the inner nuclear layer (Fig. 8B,C,E,F). Since all ganglion cells are born between E2 and E9 (Prada et al., 1991) from the ventricular zone cells, beginning in the central retina, cBrn3c expression appears to be spatiotemporally coincident with the first stages of the genesis of ganglion cells. In contrast, cBrn3a and cBrn3b are expressed only in ganglion cells after they have arrived in the ganglion cell layer. Therefore, unlike in the mouse, cBrn3c rather than cBrn3b is the first Brn3 factor expressed in the developing chick retina and is hence likely to play an early role in ganglion cell differentiation.

To determine if cBrn3c indeed has the ability to promote retinal ganglion cell differentiation, we ectopically expressed

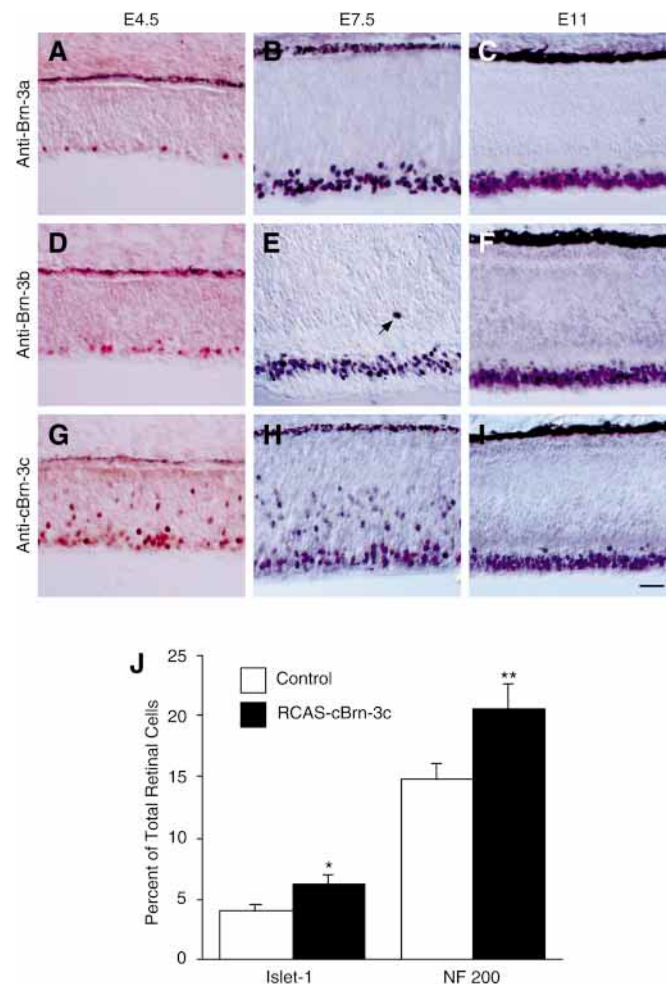

Fig. 8. Expression and activity of cBrn3 factors in the developing chick retina. (A-I) Spatial and temporal expression patterns of cBrn3 proteins during chick retinogenesis. Retinal sections from the indicated developmental stages were immunostained with anti-Brn3a (A-C), anti-Brn3b (D-F) and anti-cBrn3c (G-I) antibodies. At E4.5-E7.5, cBrn3c is found in the ganglion cell layer as well as in many scattered cells in the ventricular zone, while it is localized to only the ganglion cell layer at E11. Starting at E4.5, the expression of cBrn3a and cBrn3b is restricted to cells in the ganglion cell layer including occasional displaced ganglion cells in the inner nuclear layer (indicated by an arrow in E). Scale bar, 25 μm. (J) Effect of misexpression of cBrn3c on ganglion cell differentiation. At E5.5-E6.5, a significant increase in the number of Islet1-positive and NF 200-positive cells was seen in retinas infected with RCAS-cBrn3c viruses compared with control uninfected retinas. Each histogram represents the mean±s.d. for at least three retinas. $^{*}P<0.01$; $^{**}P<0.05$.

cBrn3c in retinoblasts by infection with RCAS-cBrn3c viruses. At E5.5-6.5, viral infection resulted in approx. 30-50% increase in the number of NF 200-positive and Islet1-positive cells (Fig. 8J), indicating that cBrn3c and Brn3b have not only a similar retinal expression pattern but a similar potency in the promotion of ganglion cell development.

DISCUSSION

In the experiments reported here, we have systematically analyzed the transcriptional properties of Brn3 factors and their ability to promote retinal ganglion cell differentiation. We show that the two isoforms of Brn3b, Brn3b(l) and Brn3b(s), can similarly bind DNA and activate gene expression and that the POU domain is minimally required for these activities. Correspondingly, ectopic expression of Brn3b(l), Brn3b(s), as well as the POU domain in chick retinoblasts via RCAS viruses, can promote differentiation of ganglion cells. Moreover, Brn3a and Brn3c share similar transactivation and differentiation activities. During chick retinogenesis, cBrn3c is first expressed in the migrating ganglion cell precursors prior to their arrival in the ganglion cell layer, suggesting that cBrn3c instead of cBrn3b may play an early role in ganglion cell differentiation. Consistent with this notion, cBrn3c and Brn3b show a similar potency in the promotion of ganglion cell differentiation. Therefore, our data reveal a potential for all Brn3 factors to promote differentiation of retinal ganglion cells but this potential may be limited by their spatiotemporal order of expression in vivo.

Brn3 proteins exhibit similar DNA-binding and transactivation activities

In the present work, we have shown that Brn3b(l), Brn3a and Brn3c can function as transcriptional activators, consistent with previous studies (Turner et al., 1994; Morris et al., 1994; Trieu et al., 1999). Analyses of truncated Brn3 proteins have allowed us to further define the activation domains of these proteins. Interestingly, both the POU domain and the five C-terminal amino acid residues make contributions to the transactivation activity of a Brn3 protein, and together they appear to confer all of the transactivation activity. Thus, the POU domain of all Brn3 proteins can act not only as a DNA-binding domain, but also an activation domain. Consistent with this observation, the Brn3a POU domain by itself (Morris et al., 1994), and the Brn3b POU domain in association with the estrogen receptor (Budhram-Mahadeo et al., 1998) have been shown to be capable of conferring transactivation activity. In addition, our analyses reveal a motif containing the five C-terminal amino acid residues in a Brn3 protein that can confer additional transactivation activity. In this motif, a serine and an alanine residue are conserved in all Brn3 proteins isolated thus far from the human (Xiang et al., 1993, 1995), mouse (Gerrero et al., 1993; Turner et al., 1994) and chicken (Lindeberg et al., 1997), suggesting that they may be important for transactivation activity.

Brn3b(s) was previously reported to have no intrinsic DNA-binding capacity, but instead to form a heterodimer with Brn3a, rendering it inactive for DNA binding and transactivation (Theil et al., 1995). This notion is however not supported by the present study. We can clearly show that Brn3b(s) specifically binds DNA sites recognized by Brn3b(l) and other Brn3 proteins, suggesting that Brn3b(s) and Brn3b(l) may regulate gene expression by a similar mechanism. In fact, both proteins are shown to have a similar ability to activate reporter gene expression (Fig. 1). In *Drosophila*, the Brn3 homologous gene *I-POU* also encodes two alternatively spliced products, I-POU and tI-POU, which display similar DNA-binding properties (Turner, 1996). In the present work, Brn3b(s) functioned only as a transcriptional activator, and we did not observe any inhibitory effect of Brn3b(s) on the transactivation activity of Brn3a (S. L. K. and M. X., unpublished results). However, early studies have indicated that Brn3b(s), in some cases, is able to repress gene expression depending on the specific promoters used (Morris et al., 1994; Budhram-Mahadeo et al., 1996, 1999).

Misexpression analyses reveal a potential for all Brn3 factors to promote retinal ganglion cell differentiation

During mouse embryogenesis, Brn3a, Brn3b and Brn3c are all expressed in retinal ganglion cells (Gerrero et al., 1993; Turner et al., 1994; Xiang et al., 1993, 1995; Xiang, 1998). Targeted disruption of *Brn3b* in mice causes downregulation of retinal ganglion cell markers, production of a large set of ganglion cells without axons and their eventual apoptotic death (Erkman et al., 1996; Gan et al., 1996, 1999; Xiang, 1998). However, mice lacking *Brn3a* or *Brn3c* do not appear to have any retinal defects (Erkman et al., 1996; McEvilly et al., 1996; Xiang et al., 1996, 1997). The lack of a retinal phenotype in *Brn3a* and *Brn3c* mutant mice is thought to be a result of functional redundancy among the three Brn3 factors (Xiang, 1998). Due to this redundancy, analyses of the *Brn3* loss-of-function mutants have so far provided little insight into the role of Brn3a and Brn3c in retinal development. Therefore, the present work employed a gain-of-function approach to study the retinal functions of Brn3 factors.

In our previous analyses of *Brn3b* null mice, we have proposed that *Brn3b* may play an early role in retinal ganglion cell differentiation (Gan et al., 1996; Xiang, 1998). Consistent with this speculation, ectopic expression of Brn3b(l) and Brn3b(s) in retinal progenitors increases ganglion cell generation. This effect is likely to result from promotion of ganglion cell genesis because overexpression of Brn3b(l) does not appear to stimulate proliferation of retinoblasts or inhibit their apoptotic death. Given that Brn3b(l) and Brn3b(s) have nearly the same DNA-binding and transcriptional properties, and are indistinguishable in their capacity to promote differentiation of ganglion cells, they may control retinal ganglion cell development by regulating expression of similar target genes. Although targeted deletion of *Brn3a* or *Brn3c* in mice resulted in no obvious retinal defect, Brn3a and Brn3c proteins appear to promote ganglion cell differentiation as effectively as the Brn3b proteins, as assayed by ganglion cell induction in chick retinal progenitors. Thus, the gain-of-function approach in the chick embryo nicely complements the loss-of-function approach in the mouse to reveal important functions for Brn3 proteins in retinogenesis.

The in vivo role of a Brn3 factor in retinal development depends on the timing of its expression

Since all three Brn3 proteins can promote ganglion cell genesis

by forced expression, they may be functionally interchangeable during retinal development. However, their order of expression during retinogenesis appears to limit their functions. Thus, Brn3a and Brn3c are unable to substitute for the early function of Brn3b in retinal ganglion cell differentiation in *Brn3b* null mice, most likely due to their late onset of expression. Conversely, Brn3b may be able to compensate for the absence of Brn3a and Brn3c since it is turned on early in migratory ganglion cell precursors and persists in differentiated ganglion cells (Xiang et al., 1998). As a consequence, gene targeting experiments in mice have indeed shown that Brn3a and Brn3c are dispensable for proper development of retinal ganglion cells (Erkman et al., 1996; McEvilly et al., 1996; Xiang et al., 1996, 1997). These data closely resemble those accumulated for the myogenic bHLH transcription factors Myf5 and myogenin. In tissue culture, forced expression of both factors can convert non-muscle cells into myoblasts and eventually into myotubes (Braun et al., 1989; Edmondson and Olson, 1989). During mouse embryogenesis, Myf5 is expressed in the somites prior to myogenin (Sassoon et al., 1989; Ott et al., 1991). In *Myf5* null mice, formation of myogenic precursors in the myotome is delayed for 2 days (Braun et al., 1992), whereas, in myogenin null mice, myoblasts are generated normally but they are blocked from differentiating into muscle fibers (Hasty et al., 1993; Nabeshima et al., 1993). Interestingly, the early defect of *Myf5* null mice can be rescued by targeting *myogenin* into the *Myf5* locus so that myogenin can be expressed in the same spatial and temporal pattern as Myf5 (Wang et al., 1996; Wang and Jaenisch, 1997). These results demonstrate that the potential for myogenin to determine myogenic precursors is limited by its belated expression during mouse myogenesis. Similarly, the potential for Brn3a and Brn3c to promote ganglion cell differentiation is limited by their relatively late expression during retinal ganglion cell development.

If all three Brn3 factors have the same functional potential, Brn3a or Brn3c would be able to substitute for Brn3b in its early role in retinal ganglion cell development provided that they had the same early expression pattern as Brn3b. Although this speculation remains to be tested by gene replacement experiments in the mouse, evolution has apparently performed a similar experiment in the nature, lending support to this speculation. In the chick retina, only differentiated ganglion cells express cBrn3b, indicating that cBrn3b, unlike its mouse counterpart, is not required for the initial differentiation of ganglion cells during chick retinogenesis. Instead, cBrn3c is found in the migrating ganglion cell precursors before the onset of cBrn3b expression, suggesting that cBrn3c may play an early role in the differentiation of ganglion cells, which would require Brn3b in the mouse. By ectopic expression in retinal progenitors, we indeed show that cBrn3c and Brn3b have a similar ability to induce ganglion cell development. Thus, all Brn3 proteins are likely to have similar potential in the promotion of retinal ganglion cell development, although this potential may normally be limited by their order of expression during embryogenesis.

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