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 multipotent 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 related 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 precursors to differentiate into ganglion cells.

**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 into the pRK5 vector modified from pCIS (Gorman et al., 1990). The procedure for dissociation of retinal cells was modified from W. Liu and others.
dissociated retinal cells were centrifuged and resuspended in 500 μl trypsin inhibitor to stop the reaction. Each digested retina was then incubated 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 μl 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. 1A,D), 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, we examined the ability of Brn3b(s) to bind a Brn3 consensus site (ATAATTAT; 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
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 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, 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).
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 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.

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 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-
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...
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, or in control uninfected retinas. Each histogram represents the mean±s.d. for at least three retinas.

**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 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,1). 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
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 as 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, 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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