Bcl-2 Transcription from the Proximal P2 Promoter Is Activated in Neuronal Cells by the Brn-3a POU Family Transcription Factor*

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The BCL-2 protein is able to protect neuronal and other cell types from apoptotic programmed cell death and plays a key role in regulating the rate of apoptosis during development of the nervous system. We have previously demonstrated that the Brn-3a POU domain transcription factor protects sensory neurons from apoptotic programmed cell death induced by nerve growth factor withdrawal. We report here that Bcl-2 transcription is predominantly initiated from the Bcl-2 P2 promoter in both the ND7 neuronal cell line and primary dorsal root ganglion neurons, in contrast to the predominant use of the Bcl-2 P1 promoter in other cell types. Moreover, Bcl-2 transcription initiated from the P2 region increases in ND7 cells stably overexpressing Brn-3a, resulting in enhanced BCL-2 protein levels. Similarly, the Bcl-2 P2 promoter is directly activated by Brn-3a in co-transfection assays in both ND7 cells and dorsal root ganglion neurons. Analysis of the Bcl-2 regulatory sequence revealed a binding site for Brn-3a that is required for maximal activation by Brn-3a both in transfected cells and during differentiation of ND7 cells. Together these data identify Brn-3a as the first transcription factor regulating Bcl-2 activity specifically in neuronal cells and indicate that the anti-apoptotic effect of Brn-3a is likely to be mediated, at least in part, via the up-regulation of Bcl-2 expression.

The survival of specific sets of neurons during embryonic and postnatal remodeling of the nervous system is thought to be regulated by the presence of neurotrophic factors. In the absence of these trophic signals, neurons die by apoptotic programmed cell death (1, 2). An increasing number of genes have been shown to coordinate apoptosis in mammalian cells, with several lines of evidence implicating the members of the Bcl-2 family as regulators of neuronal survival (3, 4).

The bcl-2 gene was originally isolated at the chromosomal breakpoint in t(14;18) bearing follicular B cell lymphoma (5), the translocation of bcl-2 to the immunoglobulin heavy chain locus leading to the deregulation of Bcl-2 expression. Bcl-2 is a membrane-bound 26-kDa protein that is located in several subcellular locations including the outer mitochondrial, outer nuclear, and endoplasmic reticulum membranes (6, 7). Although the function of the Bcl-2 family of proteins remains to be elucidated, recent evidence suggests that Bcl-2 may act to target the protein kinase Raf-1 to the mitochondrial membrane (8), whereas Bcl-xL is able to form ion channels in lipid membranes (9). Bcl-2-related proteins have been demonstrated to regulate cell survival in response to a variety of apoptotic stimuli, including growth factor withdrawal and genotoxic damage, in a number of cell types (10). The homodimeric form of Bcl-2 has been shown to enhance the survival of both central and peripheral neurons in culture and has been shown to counter the effects of a variety of apoptotic stimuli including neurotrophic factor removal (3). Evidence suggests that the susceptibility of the cell to apoptotic programmed cell death in neuronal cells can be affected by the ratio of the levels of Bcl-2 to Bax (11) and is, in the case of neuronal cells, regulated by the levels of various neurotrophic factors (11, 12). In vivo, Bcl-2 mRNA transcripts and protein are detectable within the murine nervous system as early as embryonic day 10. Although levels peak during the periods in which remodeling of the nervous system occurs, the continued expression into adulthood suggests a role for Bcl-2 in the maintenance of the nervous system (Ref. 13 and references therein).

Whereas Bcl-2 is regulated in both a tissue- and temporal-specific manner, little is known about the regulatory mechanisms governing its transcription. Two promoter regions have been identified in the 5′-regulatory region of the bcl-2 gene. P1, the predominant promoter in B cells, is a TATA-less, GC-rich region containing multiple initiation sites and several SP1 and π1 recognition elements (14–16). In contrast, the second promoter, P2, located approximately 1.3 kilobase pairs downstream of P1, contains a CCAAT box, an octamer motif (ATG-CAAAG), and a TATA element. This promoter is responsible for the production of only a small percentage of the Bcl-2 transcripts in the cell types, such as B cells (15), so far studied in detail. The relative activity of the P1 and P2 promoters in neuronal cells has not been characterized. Although transcriptional regulators of Bcl-2 identified to date include the p53 tumor suppressor gene product (17, 18), the products of the cellular and viral myb genes (19, 20), and the product of the Wilms tumor gene wt1 (21, 22), none of these have been shown to play a role in the regulation of Bcl-2 expression in neuronal cells. Hence Bcl-2 promoter usage in neuronal cells, together with the identity of the transcription factors which regulate its expression in these cells, remains uncharacterized.

The three members of the Brn-3 family of type IV POU domain transcription factors, Brn-3a (23–25), Brn-3b (25), and Brn-3c (24, 26), are expressed in distinct but overlapping sets of neurons in the developing and adult nervous systems (26, 27) suggesting that, like the highly homologous nematode Unc-86 POU factor, they may play a key role in the regulation of gene expression in neuronal cells. Such a function has been con-
firmed by knock-out studies that demonstrate that the various members of the Brn-3 family are critical for a number of aspects of central nervous system development (28–30). Brn-3a and Brn-3c are generally activating factors, whereas Brn-3b generally represses promoters that are activated by Brn-3a (31). Interestingly, our recent studies have implicated a role for Brn-3a in the development of the mature process bearing phenotype of neurons that occurs during differentiation of these cells. First, the levels of Brn-3a expression increase upon the differentiation of neuronal cells whereas levels of Brn-3b decrease (32–34). More importantly, however, is the observation that the overexpression of Brn-3a promotes neurite outgrowth in these cells (34), whereas antisense treatment inhibits process formation (35). Furthermore, we have recently demonstrated that Brn-3a is capable of protecting cells of neuronal origin, as well as primary cultures of sensory neurons, against apoptosis induced by either serum withdrawal (in the neurally derived ND7 cell line) or by withdrawal of nerve growth factor (in primary cultures of trigeminal and dorsal root ganglion neurons). In view of the known role of Bcl-2 in promoting neuronal cell survival, we have therefore investigated promoter usage in neuronal cells and have tested the effect of Brn-3a on the two Bcl-2 promoters.

MATERIALS AND METHODS

Construction of Stable Cell Lines—The ND7-derived cell lines overexpressing Brn-3a were generated by stable transfection of ND7 cells with cDNA clones under the control of the glucocorticoid-inducible mouse mammary tumor virus promoter in the vector PJ5 (36) and have been described previously (34, 37). The cells were grown in L15 medium containing 10% fetal calf serum supplemented with G418 to a final concentration of 800 μg/ml to maintain expression of the transgene. Treatment with dexamethasone at a final concentration of 800 μM was used to induce expression of the mouse mammary tumor virus promoter.

Protein Isolation and Western Blot Analysis—Protein was isolated from cells by freeze-thaw extraction in protein buffer (20 mM Hepes, 0.45 M NaCl, 25% glycerol, 0.2 mM EDTA containing 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM diithiotreitol) and submitted to SDS-polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose membranes by Western blotting, and antibodies were probed with the primary antibodies to Bcl-2, Bcl-xL, Bax, Bad, and Mcl-1 and detected using ECL Western blotting reagent (Amersham Pharmacia Biotech). Replica gels were stained with Coomassie, and filters were stripped and reprobed with a control antibody (pGp9.5) to ensure equal loading.

Nuclear Run-on Assays—Assays using human BCL-2 promoter target sequences were performed essentially as described by Young and Korosezer (34). Briefly, isolated nuclei were incubated in a reaction mixture containing 250 μCi of [3H]dCTP (3000 Ci/mmol), 2.5 mg/ml phosphocreatine, and 50 μg/ml creatine phosphokinase at 37 °C for 30 min. Following its isolation, radiolabeled run-on RNA from these reactions was purified over Sephadex G-50 and recovered by ethanol precipitation.

For run-on targets, 1 μg of the following were added: (i) an 888-bp SmaI/XhoI fragment including the P1 promoter region; (ii) a 489-bp XhoI/AccI fragment from the 5′-untranslated region; (iii) a 489-bp AccI/PstI fragment including the P2 promoter region; and (iv) a 350-bp fragment including exon 2 were applied to nylon membrane (Amersham Pharmacia Biotech) using slot-blotting apparatus. Positive controls in parallel included actin, and negative control was 3 μg of Bluescript KS+ vector.

Equivalent amounts of radiolabeled RNA from each sample were hybridized to target sequences for 24 h at 42 °C in 5 ml of hybridization buffer (50% formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 μM NaH2PO4, 0.1% sodium dodecyl sulfate, and 2× Denhardt’s solution), following prehybridization for 2 h. Membranes were subsequently washed for 20 min in 2× SSC, 0.1% SDS at room temperature, twice for 20 min in 0.1× SSC, 0.1% SDS at 65 °C, and once in 2× SSC containing 2 μg/ml RNase A at 37 °C, prior to exposure to Hyperfilm (Amersham Pharmacia Biotech) with intensifying screens for 7 days.

Phosphorimaging Constructs—A full-length construct of the human BCL-2 gene 5′ (P1) promoter region cloned upstream of the luciferase reporter gene has been described previously (16). In order to generate a full-length construct of the 3′ (P2) promoter a HindIII restriction site was introduced at position −8 (relative to ATG) by polymerase chain reaction, prior to subcloning upstream of the luciferase reporter gene. Deletions of both the 5′ and 3′ constructs were generated using the appropriate restriction sites within the promoter sequences (see Fig. 2, A and B).

Transient Transfection—Sensory neuron-derived ND7 cells (38) and baby hamster kidney-derived fibroblasts (BHK-21 cells) (39) were routinely cultured in L15 medium containing 10% fetal calf serum and Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, respectively. Transient transfection was carried out according to the method of Gorman (40). Routinely, 1 × 106 cells were transfected with 10 μg of the reporter plasmid and 10 μg of expression vectors together with 2 μg of pCMVβ plasmid containing the Escherichia coli lacZ gene under the control of the constitutive cytomegalovirus promoter, and cells were harvested 48 h later. The efficiency of transfection of each sample was determined using a chemiluminescent assay for β-galactosidase activity using a commercial kit (Galactolight Plus, Tropics), and these values were used to subsequently equalize the values obtained from the luciferase and chloramphenicol acetyltransferase assays.

Culture and Transfection of Primary Neurones—Dorsal root and trigeminal ganglia were dissected from newborn Sprague-Dawley rat pups at postnatal day 1 (P1) and, following the removal of connective tissue sheaths, were incubated in trypsin (0.1% (Wortington) in calcium and magnesium-free Earle’s balanced salt solution). Ganglia were washed twice in phosphate-buffered saline, dissociated, and plated onto sterile 35-mm dishes precoated with polyornithine (0.5 mg/ml) and laminin (20 μg/ml). Cells were cultured in defined medium (41) supplemented with recombinant nerve growth factor (NGF; Life Technologies, Inc.) at a final concentration of 20 ng/ml. Expression and reporter constructs (1 μg each), together with 1 μg of CMVβ plasmid, were introduced to cultured cells by liposome-mediated transfection. Control cultures were similarly transfected with 1 μg of empty expression vector. The efficiency of transfection of each sample was determined using a chemiluminescent assay for β-galactosidase activity using a commercial kit (Galactolight Plus, Tropics), and these values were used to subsequently equalize the values obtained from the luciferase and chloramphenicol acetyltransferase assays.

Virus Construction and Growth—The full-length Brn-3a cDNA was cloned downstream of the Rous sarcoma virus promoter, and in reverse orientation to the cDNA encoding green fluorescent protein (GFP) cloned under control of the cytomegalovirus promoter. This expression cassette (pR20.5-Brn-3a) was subcloned into a shuttle vector so that it was flanked by herpes simplex virus 1 (HSV-1) UL43 gene (32–34) and the 9.4-kb shuttle vector was co-introduced into BHK cells together with HSV-1 strain 16716 DNA which contains an inactivating insertion in the VMW65 gene as well as deletion of both copies of the ICP34.5 gene (42). Recombinant virus was subsequently plaque-purified on the basis of the visualization of GFP under ultraviolet conditions. Western blot analysis was performed to confirm that high levels of Brn-3a protein were produced in infected cultures before a high titer stock was grown.

Control virus containing the bacterial lacZ gene under the Rous sarcoma virus promoter (that is, containing pR20.5-lacZ-UL43) was similarly generated.

Dorsal root ganglion neurons from stage embryonic day 17 mouse and rat embryos were isolated and maintained in culture as described above on glass coverslips at a density of approximately 200 neurons per coverslip. Cultures were infected in duplicate with approximately 1 × 106 plaque-forming units/coverslip of Brn-3a or control virus for 60 min, washed, and then maintained in medium supplemented with NGF for 24 h. The efficiency of viral infection was determined by visualization of GFP (typically 100% of neurons were GFP-positive) before media were replaced with fresh media with or without the addition of NGF. Neuronal cell counts were then performed 24 h later.

Luciferase and Chloramphenicol Acetyltransferase Assays—Assays of luciferase activity were carried out using a commercially available kit (Promega) and a Turner luminometer, whereas assays of chloramphenicol acetyltransferase activity were carried out according to the method of Gorman (40). All samples were equalized for DNA uptake as above. Electron Mobility Shift Assay—Two pairs of complementary oligonu-

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2 The abbreviations used are: bp, base pairs; PCR, polymerase chain reaction; BHK, baby hamster kidney; GFP, green fluorescent protein; HSV-1, herpes simplex virus 1; NGF, nerve growth factor.
Regulation of Bcl-2 by Brn-3a

RESULTS

Brn-3a Enhances Expression of Bcl-2 but Not Bad, Bax, or Bax in ND7 Cells—We have recently described the ability of transfected Brn-3a to confer protection against apoptosis in ND7 cells following serum removal, as well as in primary cultures of NGF-dependent sensory neurons following the removal of NGF from culture media.1 To confirm and extend these observations, we have generated a safe and efficient recombinant herpes simplex virus (HSV-1) vector that is capable of long term, high level co-expression of both Brn-3a and GFP in neuronal cells both in vitro and in vivo.2 Table I shows that virally delivered Brn-3a is capable of protecting embryonic mouse dorsal root ganglion neurons from apoptosis induced by the withdrawal of NGF from culture medium (Table 1). Similar protection is afforded to cultures of trigeminal ganglion neurons (data not shown).

To test whether these protective effects involve members of the Brn-2 family, we examined the expression of these proteins in ND7 cells stably transfected with an expression vector in which high levels of Brn-3a are expressed under the control of the glucocorticoid-inducible mouse mammary tumor virus promoter (see Ref. 34 for detailed description). The analysis of protein extracts from these ND7 cells stably overexpressing Brn-3a revealed a large and significant increase in BCL-2 protein levels in these cells following induction of the mouse mammary tumor virus promoter upon the addition of dexamethasone to the culture medium (Fig. 1; p < 0.005). BCL-2 protein levels were not altered significantly in ND7 cell lines expressing either empty expression vector or vectors containing other members of the Brn-3 family, and no effect of dexamethasone on Bcl-2 expression was observed in any of these cells (Fig. 1 and data not shown).

In order to extend these observations, we wished to investigate whether other Bcl-2-related proteins were regulated by Brn-3 in a similar manner to that seen in the case of Bcl-2. Protein extracts from the cell lines were subject to Western blotting with antibodies raised to Bad, Bax, and Mcl-1. No significant alterations in the level of Bad or Bax (Fig. 1) protein expression was observed, and neither Mcl-1 nor both Bad and Mcl-1 were regulated by the glucocorticoid promoter region cloned upstream of the chloramphenicol acetyltransferase gene (clone TM-666 (18)) was not significantly altered following co-transfection with Brn-3a into ND7 cells (data not shown).

Bcl-2 Promoter Activity in Sensory Neurons—The 5′-flanking region of the human BCL-2 gene contains two distinct promoters (P1 and P2) that are required for initiation of transcription (Fig. 2A). The P1 region is situated 1.3–1.5 kilobase pairs upstream of the open reading frame and is a GC-rich, TATA-less promoter containing several SP1, STAT, and B elements with multiple start sites (14, 15). The second promoter (P2) is located 1.3 kilobase pairs downstream of P1 and includes an octamer motif, as well as CCAAT and TATA elements. Although it is not known which of these two promoter regions is utilized in cells of neuronal origin, studies have demonstrated that in other cell types in which Bcl-2 promoter activity has

**TABLE I**

| Virus | Cell survival, number of neurons as % of original population* | + NGF | - NGF |
|-------|---------------------------------------------------------------|-------|-------|
| Control |                                                              | 98.25 ± 0.4 | 32 ± 6.6 |
| Brn-3a |                                                              | 86.25 ± 12.6 | 80 ± 9.0 |

* Survival of virally infected dorsal root ganglion neurons from mouse embryonic stage day 17 embryos at 24 h following transfer to media with or without the addition of 20 ng/ml NGF. Values are means ± S.D. of the mean determined in duplicate in two independent experiments.
**Regulation of Bcl-2 by Brn-3a**

**Fig. 2.** The P2 region of the Bcl-2 promoter is the predominant origin of transcription in neuronal cells. **A,** schematic representation of the fragments of the Bcl-2 promoter used as probes for nuclear run-on analysis of Bcl-2 promoter activity in neuronal cells. ORF indicates the start of the open reading frame, and hatched regions indicate intronic sequences. Roman numerals indicate the fragments used as targets for run-on assays. **B,** nuclear run-on analysis of Bcl-2 promoter activity in proliferating ND7 cells (lane 1), differentiated ND7 cells (lane 2), ND7 cells overexpressing Brn-3a (long form) (lane 3), the non-neuronal BHK cell line (lane 4), and primary rat dorsal root ganglion cells (lane 5). Equivalent counts per min of radiolabeled run-on RNA were hybridized to the indicated targets derived from the Bcl-2 promoter (I–IV) as shown in A. Actin (A) and tubulin (T) targets are used as positive controls, and Bluescript KS+ DNA serves as a negative control (−).

been characterized (predominantly cells of hemopoietic lineage), transcription is predominantly initiated from the P1 region (14–16). We therefore wished to determine the extent of P1 and P2 promoter usage in cells of neuronal origin prior to analyzing the effect of Brn-3a on the Bcl-2 promoters.

Nuclear run-on analysis in the ND7 neuronal cell line indicates that Bcl-2 transcription is predominantly initiated at low but detectable levels from within the P2 promoter region in the undifferentiated, proliferating region (Fig. 2B, lane 1), and that the level of transcription increases upon the induction of differentiation into a non-dividing, process-bearing phenotype following the removal of serum from the culture medium (lane 2). Longer exposure of autoradiograms suggests a low level of P1 usage in ND7 cells. Of particular interest is our previous observation that the level of expression of Brn-3a increases dramatically upon the induction of differentiation in ND7 cells (32), suggesting a link between the levels of the Brn-3a factor and Bcl-2 promoter activity in this cell line. Such a direct role for Brn-3a in the regulation of Bcl-2 transcription was confirmed by the observation that the level of transcription from this P2 region significantly increased in cell lines stably overexpressing Brn-3a (lane 3). Both promoter regions were silent in the non-neuronal BHK cell line (lane 4). P2 promoter dominance in primary sensory neurons was confirmed using run-on RNA isolated from primary cultures of both trigeminal and dorsal root ganglion neurons from rat neonates (lane 5) indicating that this effect is not confined to the immortalized ND7 cell lines. The ability of the assay to detect P1 promoter activity was confirmed using extracts from the B cell line DHL-4 (data not shown).

**The Bcl-2 Promoter Is Regulated by Brn-3a in the ND7 Cell Line—**Luciferase reporter constructs containing either the full-length Bcl-2 promoter region (that containing both P1 and P2 regions and spanning between −3934 to −8 bp relative to the ATG) or the isolated P1 regions (spanning from −3934 to −1280 bp) have previously been described (Ref. 16 and references therein). In order to generate a full-length construct of the P2 promoter (spanning −746 to −8 bp) a HindIII restriction site was introduced at position −8 (relative to ATG) by polymerase chain reaction, prior to subcloning upstream of the luciferase reporter gene. Co-transfection experiments into the parental ND7 cell line revealed that Brn-3a is capable of strongly activating both the full-length P1/P2 construct (Fig. 3A) as well as the construct containing the P2 region (Fig. 3C). Brn-3a only weakly activated the construct containing the isolated P1 region (Fig. 3B), whereas the related members of the Brn-3 family, Brn-3b and Brn-3c, had no significant effect on these constructs (Fig. 3, A–C, and data not shown). Hence these results confirm that the enhanced transcription of the bcl-2 gene, as well as the increase in BCL-2 protein levels, in cells overexpressing Brn-3a is dependent upon the direct effect of Brn-3a on the Bcl-2 P2 promoter.

**The Bcl-2 Promoter Is Regulated by Brn-3 in Primary Neuronal Culture—**The ND7 cell line was originally established via the fusion of N18TG2 azaguanine-resistant neuroblastoma cells with neonatal dorsal root ganglion neurons (38). Therefore, in view of the ability of Brn-3a to activate the Bcl-2 promoter reporter gene constructs in ND7 cells, we wished to determine whether these observations were paralleled in primary neurons. We therefore performed co-transfection experiments in primary cultures of neurons isolated from dorsal root ganglia of adult rats (see “Materials and Methods”). In these experiments Brn-3a significantly activated reporter constructs containing both the full-length Bcl-2 promoter (that is P1 and P2) (Fig. 4A) as well as the construct containing the P2 region (Fig. 4C). Brn-3a, however, did not activate the isolated P1 region (Fig. 4B). The related factor Brn-3b had no significant effect on the activity of all three constructs (Fig. 4, A–C).

**Mutation of a Putative Brn-3a Binding Element Reduces but Does Not Abolish Brn-3a Responsiveness—**The 600 bp of regulatory sequence immediately upstream of the Bcl-2 P2 promoter region contained in the P2 construct spanning −746 to −8 bp (this construct will now be referred to as clone −746WT) contains two repeat elements, CTTTCGCTTC and CAT-CAATCTTC, similar in sequence to the internexin CTTCTTCT motif identified in the a-internexin gene promoter.
as a response element mediating induction by Brn-3a (44). To test whether these elements are capable of binding Brn-3a, we performed electromobility shift assay on these sequences. Although the first of these two sequences did not bind any Brn-3 family members, a synthetic oligonucleotide containing the second of these two motifs (the sequence 5′-CATCAATCTTC-3′ located at −584 bp to −594 bp of the Bcl-2 promoter) was able to bind Brn-3a (Fig. 5).

To determine the requirement for this motif in the Bcl-2 promoter for the regulation by Brn-3a, the sequence CATCAATCTTC was altered to CAGAAATAGTC by PCR-mediated mutagenesis of the construct containing 2584 bp to 2594 bp of the Bcl-2 promoter (clone 2746WT) to create clone 2746MUT. This double mutation of both halves of the bipartite motif resulted in a 43% decrease in the ability of Brn-3a to activate the construct containing this region of the promoter following transfection into ND7 cells (Fig. 6A).

We next tested whether this mutation also affected the increase in activity of the Bcl-2 promoter upon the differentiation of ND7 cells that occurs when endogenous Brn-3a levels rise. Under these conditions the activity of clone −746WT increases in differentiated cells to 600% of its activity in proliferating ND7s (Fig. 6B). It is likely that this observation is due to the increase in the level of expression of Brn-3a and the concomitant decrease in Brn-3b levels that occurs during differentiation of ND7 cells (32). However, the increase in reporter gene activity upon differentiation of cells transfected with this same region of promoter containing clone −746MUT was only 50% of the wild type clone (Fig. 6B).

A similar reduction but not inability of Brn-3a to activate −746MUT was observed following transfection into primary cultures of neonatal rat dorsal root ganglion neurons (Fig. 7). Hence this mutation in a Brn-3a-binding site reduces the response of the Bcl-2 promoter to transfected Brn-3a in dorsal root ganglion neurons and ND7 cells and also similarly its response to ND7 cell differentiation when endogenous Brn-3a levels rise.

Oct-1 and Oct-2 Do Not Activate the Bcl-2 Promoter—The
specificity of these effects to the neuronally expressed Brn-3 family of POU factors was confirmed by co-transfection experiments using the Bcl-2 promoter construct in combination with constructs expressing the more distantly related POU domain transcription factors Oct-1, Oct-2.1, Oct-2.4, and Oct-2.5. No activation or repression of Bcl-2 promoter activity was observed upon co-transfection of these type II POU factors into either ND7 cells (Fig. 8), the BHK fibroblastic cell line, or primary cultures of neonatal rat dorsal root ganglion neurons (data not shown).

Interestingly, although they did not activate the constructs independently, Oct-1 and to a lesser extent Oct-2.5 did, however, act synergistically with Brn-3a on all constructs tested, enhancing the degree by which Brn-3a activated these constructs when transfected into neuronal cells (Fig. 8).

DISCUSSION

By having made an initial observation that BCL-2 protein levels are increased approximately 10–20-fold in ND7 cells stably overexpressing Brn-3a (Fig. 1), we wished to determine whether this increase in protein levels was due to alterations in the transcriptional activity of the bcl-2 gene in these cells. However, we first wished to determine the precise nature of Bcl-2 transcription in neuronal cells as this has yet to be documented in the literature. The data presented here comprise the first report of Bcl-2 promoter usage in cells of neuronal lineage and indicate that the two Bcl-2 promoter regions are differentially regulated in a cell type-specific manner.

Importantly, the number of transcripts initiated from the P2 region (and to a lesser extent from the P1 region) increased significantly in ND7 cells induced to overexpress Brn-3a (Fig. 2, lane 3). This observation not only provides evidence that the increase in BCL-2 protein levels in the Brn-3a overexpressing cell lines is due to transcriptional activation but also suggests that a different set of transcription factors and enhancers are utilized in the regulation of Bcl-2 expression in neurons. Co-transfection experiments confirmed that Brn-3a (a transcription factor that is predominantly expressed in the developing nervous system (27)) is capable of activating Bcl-2 expression via the enhancement of transcriptional initiation within the P2 promoter (Figs. 3 and 4). In particular, the reporter construct containing the P2 region and approximately 600 bp of upstream regulatory sequence (~8 to ~746 bp in clone 746WT) or mutant sequence CAGAAATAGTC spanning ~8 to ~746 bp, in ND7 cells. B, comparison of the activity of the reporter gene constructs containing wild type clone 746WT (stippled bars) and mutant clone 736MUT (filled bars) spanning ~8 to ~746 bp of the Bcl-2 promoter in proliferating and differentiated ND7 cells. Values are the means of those obtained from two independent preparations, each analyzed in duplicate. Bars indicate the standard deviation of the mean.

Fig. 6. Disruption of the sequence CATCAATCTTC within the P2 region reduces the response of the Bcl-2 P2 promoter to Brn-3a in ND7 cells. A, comparison of the ability of Brn-3a to activate the fragment containing either wild type sequence CATCAATCTTC in clone 746WT (stippled bars) or mutant sequence CAGAAATAGTC in clone 746MUT (filled bars) within the Bcl-2 promoter spanning ~8 to ~746 bp, in ND7 cells. B, comparison of the activity of the reporter construct spanning wild type clone 746WT (stippled bars) and mutant clone 736MUT (filled bars) spanning ~8 to ~746 bp of the Bcl-2 promoter in proliferating and differentiated ND7 cells. Values are the means of those obtained from two independent preparations, each analyzed in duplicate. Bars indicate the standard deviation of the mean.

Fig. 5. Identification of a motif within the human Bcl-2 promoter region that is bound by Brn-3a. Electromobility shift assay using in vitro transcribed and translated Brn-3a protein and 32P-labeled probe derived from double-stranded oligo P2 (lanes 2–5) is shown. Samples were incubated alone (lane 3), in the presence of 100-fold excess of unlabeled homologous oligonucleotide (lane 4), or in the presence of 100-fold excess of unlabeled, unrelated oligonucleotide (lane 5). Lane 2 is the corresponding labeled probe incubated with control in vitro transcribed and translated product. Lane 1 is double-stranded oligo P2 probe alone (no translate added).
Whereas the Bcl-2 P2 promoter does contain the octamer motif ATGCAAAG (which is bound by octamer binding POU domain factors) approximately 120 bp upstream of the ATG, members of the Brn-3 family, including Brn-3a, show only a very weak affinity for this sequence. However, the P2 region contained two motifs similar to that previously identified in the α-internexin promoter as mediating a response to Brn-3a (44). The most 3' of these elements specifically bound Brn-3a, and mutagenesis of this site in the context of the −746- to −8-bp fragment (in clones −746WT and −746MUT) results in a reduction of the responsiveness to transfected Brn-3a and ND7 differentiation by approximately 50–60% but not a complete inhibition of the effect. Whereas this reduction is reproducible and is statistically significant (p < 0.005 in a Mann Whitney test), it is thus likely that the action of Brn-3a is not solely dependent on its binding through this particular site. Interestingly, we have shown that the oligonucleotide containing this site conferred a low responsiveness to Brn-3a onto a heterologous promoter in co-transfection experiments but to a lesser extent than was observed with larger fragments containing the Bcl-2 P2 promoter region (data not shown) suggesting that this single site is involved in the action of Brn-3a but that other adjacent sequences are also necessary.

We have thus identified a site in the Bcl-2 promoter that is involved in its response to transfected Brn-3a. We are currently investigating the roles of adjacent sites within this region upstream of the P2 promoter in the regulation of the promoter by Brn-3a. Most importantly, mutation of this site that affects responsiveness to Brn-3a in transfection assays also reduces to a similar extent the induction of the Bcl-2 promoter during ND7 cell differentiation, suggesting that the rise in endogenous Brn-3a levels during the differentiation process plays a key role in this enhanced activity.

We have previously demonstrated that the coordinate expression of Brn-3a and Brn-3b plays a role in the regulation of process outgrowth during neuronal differentiation (34, 37). In the present study we have demonstrated that the Brn-3a POU domain transcription factor is capable of activating the bcl-2 gene promoter. Together with the observation that BCL-2 protein levels are significantly increased in neuronally derived ND7 stable cell lines overexpressing Brn-3a, these data suggest that this regulation of a factor known to promote cell survival

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\(^4\) M. D. Smith and D. S. Latchman, unpublished observations.
is likely to be one pathway by which Brn-3a is able to confer neuronal cells with resistance to apoptosis. In particular, this phenomenon may explain our observations that Brn-3a over-expression is able to protect (i) the ND7 cell line from serum withdrawal-induced apoptotic programmed cell death and (ii) primary cultures of sensory neurons from the trigeminal and dorsal root ganglia of neonatal rat pups from apoptosis induced by withdrawal of nerve growth factor. Indeed, it is possible that this ability of Brn-3a to protect neurons from apoptosis via the regulation of bcl-2 gene expression may be one explanation as to why significant numbers of sensory neurons fail to survive the period of target field innervation in Brn-3a null mice (29, 30).

Thus, in conclusion, these data suggest that Brn-3a is an important regulator of the bcl-2 gene in neuronal cells and is the first factor shown to specifically regulate the proximal P2 promoter region which we have shown to be the predominant promoter in neuronal cells. Both Brn-3a and Bcl-2 are capable of promoting the differentiation of neurally derived cell lines (34, 46), suggesting that the regulation of bcl-2 gene expression provides a possible pathway by which Brn-3a is able to both promote neurite outgrowth (34) and neuronal survival.1

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