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The N-terminal domain unique to the long form of the Brn-3a transcription factor is essential to protect neuronal cells from apoptosis and for the activation of Bcl-2 gene expression

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ABSTRACT

The ability of the POU family transcription factor Brn-3a to stimulate neurite outgrowth and the expression of the genes encoding neuronal proteins such as the neurofilaments and SNAP-25 has previously been shown to be dependent upon the C-terminal POU domain which can mediate both DNA binding and transcriptional activation. We show here, however, that the ability of Brn-3a to activate Bcl-2 expression and protect neuronal cells from apoptosis (programmed cell death) requires a distinct N-terminal activation domain. Bcl-2 gene activation and protection from apoptosis are thus produced only by the long form of Brn-3a which contains this domain and not by a naturally occurring short form lacking this domain or by the isolated POU domain, although all these forms of Brn-3a can stimulate neurite outgrowth. Hence Brn-3a is a multifunctional transcription factor with different regions of the factor mediating its different effects and two distinct forms with different properties being generated by alternative splicing.

INTRODUCTION

The three mammalian Brn-3 transcription factors are members of the POU (Pit-Oct-Unc) transcription factor family. This family was originally defined on the basis of a common 150–160 amino acid domain in the mammalian transcription factors Pit-1, Oct-1 and Oct-2 and the nematode regulatory protein Unc-86 which plays a key role in the development of the nervous system in this organism (for review see 1,2). The POU domain constitutes the DNA binding domain of these factors and consists of a POU-specific domain and a POU-homeodomain related to that found in the homeobox factors.

Three different Brn-3 factors have been identified which are encoded by distinct genes and are the most closely related mammalian factors to the nematode Unc-86 protein (3,4). The three factors Brn-3a (also known as Brn-3 or Brn-3.0; 5–7), Brn-3b (also known as Brn-3.2; 6,8) and Brn-3c (also known as Brn-3.1; 5,9) are expressed in distinct but overlapping groups of neurons in the developing and adult nervous system (5–9) with Brn-3a for example, defining the earliest post-mitotic neurons to appear in the CNS (10).

The possibility that these factors play a critical role in the development of specific neuronal cell types which is suggested by their expression patterns and homology to Unc-86 is supported by the results of knock out mouse experiments in which the genes encoding individual Brn-3 factors have been inactivated. Interestingly, whilst mice lacking Brn-3b or Brn-3c show specific defects which are apparently confined to the visual and auditory systems, respectively (11), mice lacking Brn-3a show much more widespread losses of both sensory and motor neurons in both the peripheral and central nervous systems (12,13).

This critical role for Brn-3a *in vivo* is paralleled by its ability to activate the promoters of a number of different genes expressed in neuronal cells in co-transfection experiments as well as to activate expression of the corresponding endogenous gene when Brn-3a is over-expressed in neuronal cells. Genes which are activated in this manner include those encoding pro-opiomelanocortin (5), the neurofilaments (14) and the related intermediate filament protein α-internexin (15), the anti-apoptosis protein Bcl-2 (16) and the synaptic vesicle proteins SNAP-25 and synaptophysin (17–19). In contrast, none of these genes is activated by the closely related Brn-3b factor and, indeed, activation of several of these genes by Brn-3a is inhibited by Brn-3b (17–19). Several of the factors activated by Brn-3a such as SNAP-25 are involved in the process of neurite outgrowth (20) and we have recently shown that over-expression of Brn-3a can stimulate neurite outgrowth under conditions when it would not otherwise occur, whereas Brn-3b cannot do so (19).

The ability of Brn-3a to stimulate neurite outgrowth and activate genes encoding, for example, SNAP-25 and the neurofilaments is dependent upon the C-terminal POU domain which is able to act as both a DNA binding domain and a transcriptional activation domain (14,18–19). Thus the isolated POU domain of Brn-3a is

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able to both stimulate neurite outgrowth and activate these genes. Interestingly, however, these effects are not observed with the isolated POU domain of the closely related Brn-3b factor. We have demonstrated that this is due to a valine versus isoleucine difference at position 22 of the POU-homeodomain in these factors which determines the transcriptional activating ability of the POU domain although it has no effect on the DNA binding ability (21,22). Thus a mutant Brn-3a containing isoleucine instead of valine at this position fails to activate transcription whereas the converse mutant in Brn-3b containing valine activates transcription.

Despite this critical role of the POU domain, it does not entirely account for the properties of Brn-3a. Thus, activation of the α-internexin promoter by Brn-3a is dependent upon an N-terminal region which acts as a discrete activation domain (15,23). Interestingly, this N-terminal domain is found only in the long form of Brn-3a and not in the short form of the factor which is generated by alternative splicing and lacks the N-terminal 84 amino acids found in the long form (3,24). The proportion of these two forms of Brn-3a varies in different parts of the nervous system and in response to specific stimuli (24). Hence it appears that Brn-3a exists in two different forms with some properties mediated by the C-terminal POU domain being common to both forms of the factor and others mediated by the N-terminal region being unique to the long form.

We have therefore investigated the role of the different domains of Brn-3a in activating the expression of the Bcl-2 gene. In addition, in view of the anti-apoptotic effect of Bcl-2 we have investigated whether over-expression of Brn-3a can protect a neuronal cell line from apoptosis and the role of the N-terminal domain in this effect.

MATERIALS AND METHODS

Construction of stable cell lines

The ND7 derived cell lines overexpressing different forms of Brn-3a and other Brn-3 family members were generated by stable transfection of ND7 cells with cDNA clones under the control of the glucocorticoid-inducible mouse mammary tumour virus (MMTV) promoter in the vector PJ5 (25) and have been described previously (19,26). Characterisation of these stable cell lines revealed that exogenous mRNA and protein levels of the various Brn-3 factors are approximately equal (19,26; M.D.Smith and D.S.Latchman, unpublished data). The cells were grown in L15 medium containing 10% foetal 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 1 μM was used to induce expression of the MMTV 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 NaC1, 25% glycerol, 0.2 mM EDTA containing 1 μg/ml pepstatin and 1 mM DTT) and submitted to SDS–polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose filter by western blotting and probed with the primary antibody to Bcl-2, and detected using ECL Western Blotting reagent (Amersham). Replica gels were stained with Coomassie, and filters were stripped and re-probed with a control antibody (pGp9.5) to ensure equal loading.

Plasmid constructs

A full length construct of the human Bcl-2 gene 5′ (P1) promoter region cloned upstream of the luciferase reporter gene has previously been described (27). In order to generate a full length construct of the 3′ (P2) promoter, a *Hin*dIII restriction site was introduced at position –8 (relative to ATG) by polymerase chain reaction, prior to subcloning upstream of the luciferase reporter gene.

Transient transfection

Sensory neuron derived ND7 cells (28) and baby hamster kidney derived fibroblasts (BHK-21 cells) (29) were routinely cultured in L15 medium containing 10% foetal calf serum and DMEM containing 10% foetal calf serum, respectively. Transient transfection was carried out according to the method of Gorman (30). Routinely, 1×10^6 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 CMV promoter, and cells 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 CAT assays.

Analysis of cell death

Trypan blue exclusion and the preparation of genomic DNA was carried out as previously described (31). For colorimetric assay of DNA fragmentation, 1×10^6 cells were harvested by centrifugation, the pellet lysed in lysis buffer (10 mM Tris, 1 mM EDTA, pH 7.5 containing 0.2% Triton X-100), following which lysates were centrifuged at 13 000 *g* to separate intact from fragmented chromatin. Supernatant was then transferred to a fresh tube and both pellet and supernatant precipitated at 4°C overnight in 12.5% trichloroacetic acid. Precipitates were sedimented by centrifugation at 13 000 *g* for 10 min, hydrolyzed by heating to 90°C for 10 min in 80 μl of 5% TCA, following 0.16 ml of DPA reagent (0.15 g diphenylamine, 0.15 ml sulphuric acid, 0.05 ml acetaldehyde in 10 ml glacial acetic acid) was added to each tube. Optical density was determined at 600 nm after overnight color development (32).

Staining of apoptotic cells was performed by the end-labelling of DNA 3′ ends with dUTP-FITC using a modification of the TUNEL method (33).

Cell cycle analysis

Single cell suspensions were fixed in 70% ethanol at –20°C and resuspended in PBS containing 1 mg/ml RNase, 20 μg/ml propidium iodide (PI) and 5 μg/ml fluorescein isothiocyanate (FITC). An EPICS flow cytometer (Coulter Electronics) was used for all studies.

Statistics

Microstat software (Biosoft) was used for statistical analysis. Pairwise comparisons were performed using *t*-test or Mann–Whitney analysis.

Values are the means of those obtained from two independent preparations, each analyzed in duplicate. Data from three

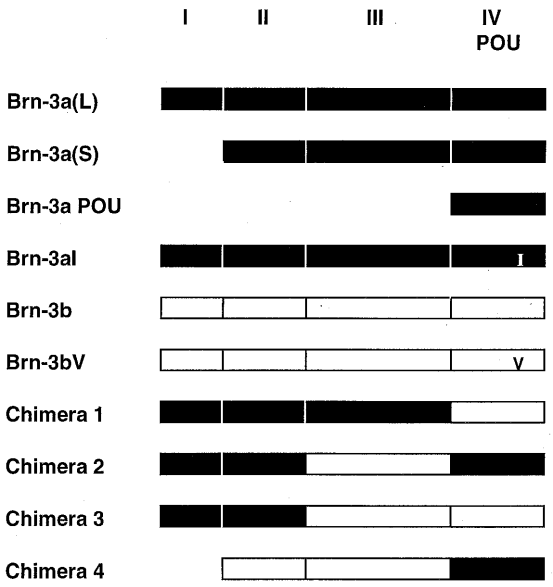


Figure 1. Schematic diagram of the Brn-3a constructs used in this study. Brn-3a is shown as a solid box, Brn-3b as an open box. I and V indicate the amino acid substitutions used to generate mutant forms of Brn-3a and Brn-3b.

individual clones expressing each construct are combined. Bars indicate the standard deviation of the mean.

RESULTS

We have previously demonstrated that the Bcl-2 P2 promoter (which is the predominant promoter used in neuronal cells) is strongly activated by Brn-3a in co-transfections carried out in the ND7 neuronal cell line whilst the P1 promoter is also activated, although more weakly (16). To test whether this activation was dependent upon the N-terminal domain of Brn-3a, we co-transfected luciferase reporter constructs containing either the P1 or P2 Bcl-2 promoters with expression vectors encoding either the long or short form of Brn-3a or the isolated POU domain (Fig. 1). Similar experiments were also carried out with chimeric constructs containing different regions of Brn-3a linked to regions of Brn-3b (which has no effect on the Bcl-2 promoter; 16) (Fig. 1).

In these experiments, activation of both the P1 (Fig. 2a) and P2 (Fig. 2b) Bcl-2 promoters was dependent upon the N-terminus of Brn-3a being present (see Fig. 2c for a schematic diagram of the Bcl-2 promoters). Thus activation was observed with the long form of Brn-3a which contains this domain but not with the short form or the isolated POU domain. Similarly, activation was observed with Brn-3a/Brn-3b chimeras 1–3 which contain this domain of Brn-3a but not with chimera 4 which lacks it. Hence in cells of neuronal origin, activation of the two Bcl-2 promoters by Brn-3a is dependent upon the presence of the N-terminal domain with the C-terminal POU domain being insufficient for activation.

In parallel with these experiments, we also carried out similar co-transfections into BHK fibroblast cells in which we had not previously tested the effect of Brn-3a on the Bcl-2 promoter. As illustrated in Figure 2, in these cells, Brn-3a had no effect on the P1 promoter and only a small effect on the P2 promoter which was much less than that observed in ND7 cells. This apparent neuronal cell specificity of Bcl-2 promoter activation is in

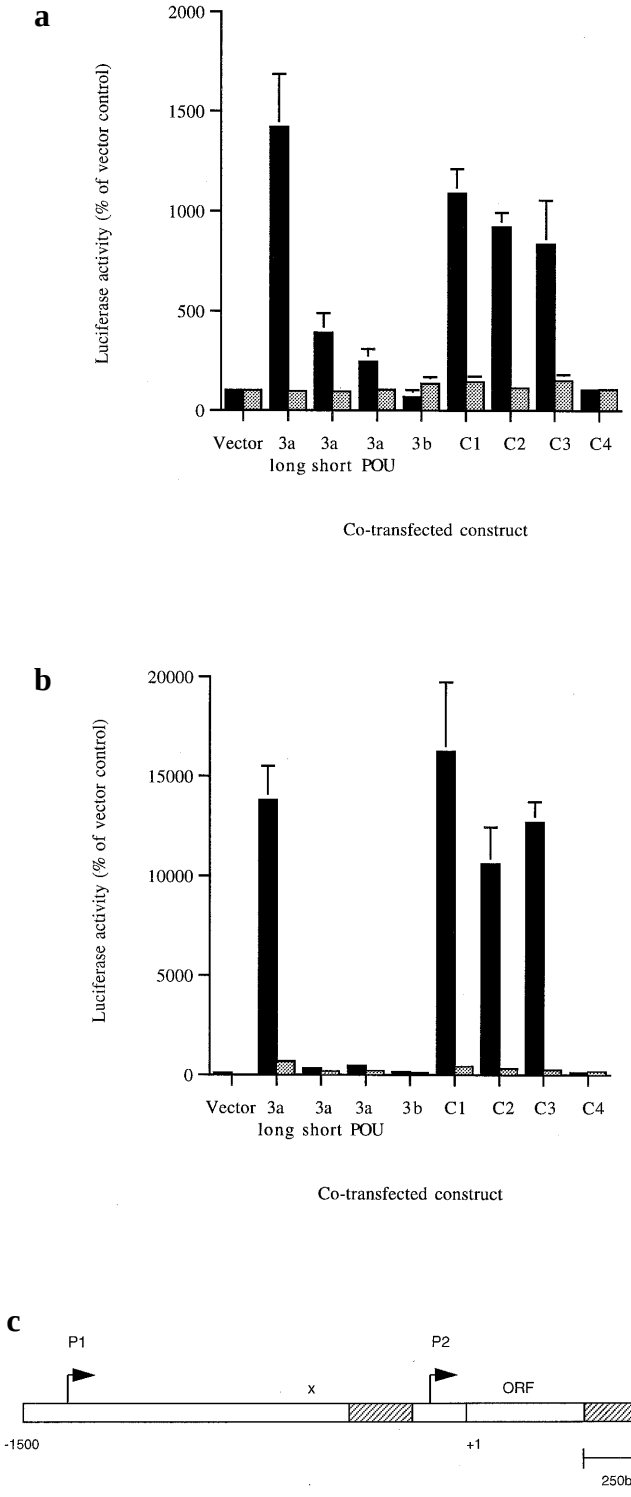


Figure 2. The human Bcl-2 promoter is regulated by the N-terminal activation domain of the Brn-3a transcription factor. Results of the reporter gene assay following the co-transfection into ND7 cells (filled boxes) or BHK cells (stippled boxes) of the indicated plasmids with (**a**) P1, (**b**) P2 Bcl-2 promoter constructs. C1, C2, C3 and C4 indicate the chimeric constructs of Brn-3a and Brn-3b drawn schematically in Figure 1. (**c**) Schematic representation of the 5′ end of the human Bcl-2 gene indicating the two promoter regions (P1 and P2). ORF indicates the start of the open reading frame; hatched regions indicate introns; x indicates the Brn-3a binding site.

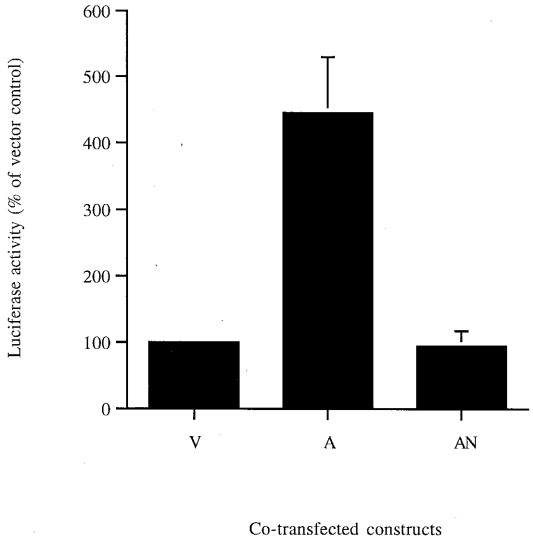


Figure 3. Effect of the isolated N-terminal domain on the activation of the P2 Bcl-2 promoter by Brn-3a. Assay of luciferase activity of the P2 promoter following transfection of ND7 cells with expression vector lacking any insert (V) expression vector encoding the intact long form of Brn-3a (A) or separate expression vectors containing the intact long form of Brn-3a and the isolated N-terminus (AN). All transfections were equalised for DNA content using expression vector lacking any insert.

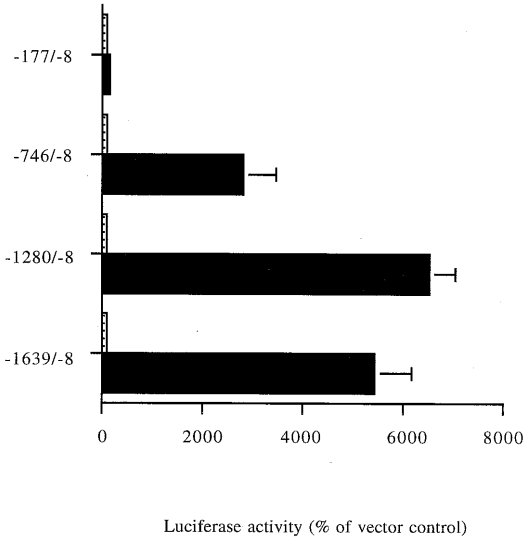


Figure 4. Deletion constructs of the Bcl-2 promoter identify a region necessary for activation of reporter constructs by Brn-3a. Results of luciferase assays following the co-transfection of the long form of Brn-3a with the indicated deletion constructs of the human P2 Bcl-2 promoter regions identify a 569 bp region (–746 to –178) required for activation. Stippled boxes are the appropriate vector control transfections for each deletion construct.

contrast to the effect of Brn-3a on other promoters which appear to be activated equally well in co-transfections into BHK or ND7 cells regardless of whether their activation is dependent upon the POU domain as in the case of the neurofilament genes (14) or by the N-terminal domain as in the case of α-internexin (15).

It is possible therefore that Brn-3a may activate the Bcl-2 promoter by binding to the promoter via the POU domain with the N-terminal domain then interacting with a neuronal specific factor bound at another site in the promoter or which is recruited to the promoter via the N-terminal domain of Brn-3a. To test this possibility we investigated the effect of a construct expressing the isolated N-terminal domain of Brn-3a in the absence of any DNA binding domain. This construct was included in co-transfections of the long form of Brn-3a and the Bcl-2 promoter into ND7 cells to determine if it could interfere with promoter activation by competing with intact Brn-3a for the putative neuronal specific factor. This was indeed the case with activation of both the full Bcl-2 promoter (data not shown) and the isolated P2 promoter (Fig. 3) by Brn-3a being clearly inhibited by inclusion of the construct expressing the isolated N-terminal domain.

Hence the N-terminal domain of Brn-3a is required together with the DNA binding domain for activation of the Bcl-2 promoter and appears to act by interacting with a neuronal-specific co-factor. We previously identified a site located at –584 to –594 in the Bcl-2 promoter with the sequence CATCAATCTTC which is able to bind Brn-3a in a DNA mobility shift assay with similar affinity to other previously characterized sites and whose inactivation by mutation greatly reduces the responsiveness of the Bcl-2 promoter to Brn-3a (16).

To investigate the role of this sequence in the induction of the Bcl-2 promoter by Brn-3a, we used a series of deletion constructs containing different regions of the P2 promoter linked to a luciferase reporter. As illustrated in Figure 4, deletion of the region from –746 to –177 of the promoter which contains this element, produced a significant fall in the activation of the promoter by Brn-3a. Hence the region containing this element plays an important role in the inducibility of the Bcl-2 promoter by Brn-3a, although other sequences further upstream are required for maximal inducibility. To test whether this Brn-3a binding sequence could mediate the response to Brn-3a in isolation from the rest of the promoter, a 173 bp *Bgl*II–*Nsi*I fragment of the Bcl-2 promoter containing this region or an oligonucleotide containing the isolated binding site itself were separately cloned upstream of the heterologous thymidine kinase promoter in the vector pBL Cat2 (34). In these experiments, Brn-3a was able to activate both the promoter containing the fragment of the Bcl-2 promoter (Fig. 5a) and that containing the isolated binding site (Fig. 5b), although it had no effect on the isolated thymidine kinase promoter in the parental vector (35,36 and data not shown).

This demonstrates for the first time that this binding site for Brn-3a from the Bcl-2 promoter can confer a response to Brn-3a on a heterologous promoter when present either alone or in a short region of the Bcl-2 promoter. This activation was not affected by altering the valine at position 22 in the POU homeodomain of Brn-3a to isoleucine which prevents activation of promoters which are dependent on the POU domain of Brn-3a although it does not prevent DNA binding of the mutant factor. Similarly, Brn-3b carrying the converse isoleucine to valine change was not able to activate the promoter, although this change allows Brn-3b to activate POU domain-dependent promoters (22) (Fig. 5).

The results of our co-transfection experiments indicate that the activation of the transfected Bcl-2 promoter is dependent upon the N-terminal domain. To determine if the N-terminal domain was similarly required for the activation of endogenous Bcl-2 gene expression, we used derivatives of the ND7 neuronal cell line which we have stably transfected with expression vectors encoding different forms of Brn-3a under the control of the dexamethasone-inducible MMTV promoter (19,26). In these

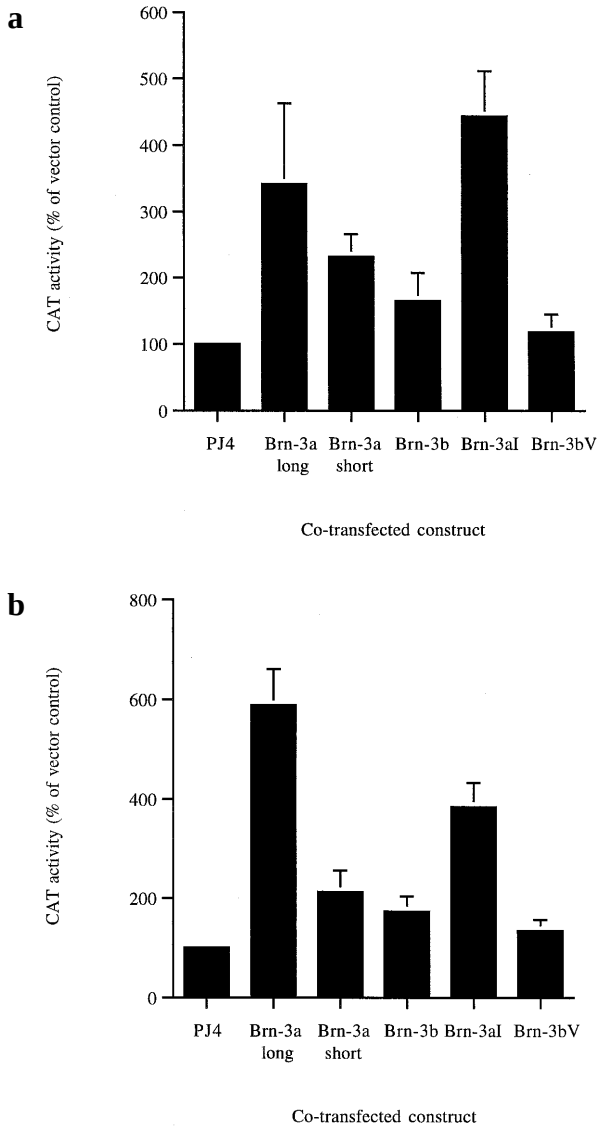


Figure 5. Identification of a motif within the human Bcl-2 promoter region that is sufficient for activation of a heterologous promoter by Brn-3a. (**a**) Chloramphenicol acetyltransferase (CAT) assay of the ability of the indicated Brn-3 factors to transactivate a heterologous thymidine kinase promoter containing a 173 bp *Bgl*II–*Nsi*I fragment from the human P2 Bcl-2 promoter region (–744 to –571). (**b**) Results of a CAT assay to determine the ability of the indicated Brn-3 factors to transactive a heterologous thymidine kinase promoter containing the minimal CATCAATCTTC motif contained within the *Bgl*II–*Nsi*I fragment (–594 to –584).

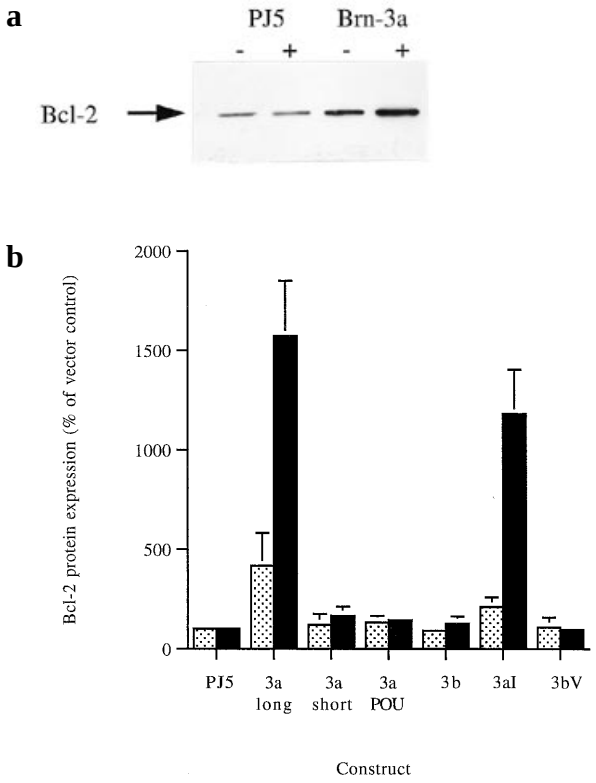
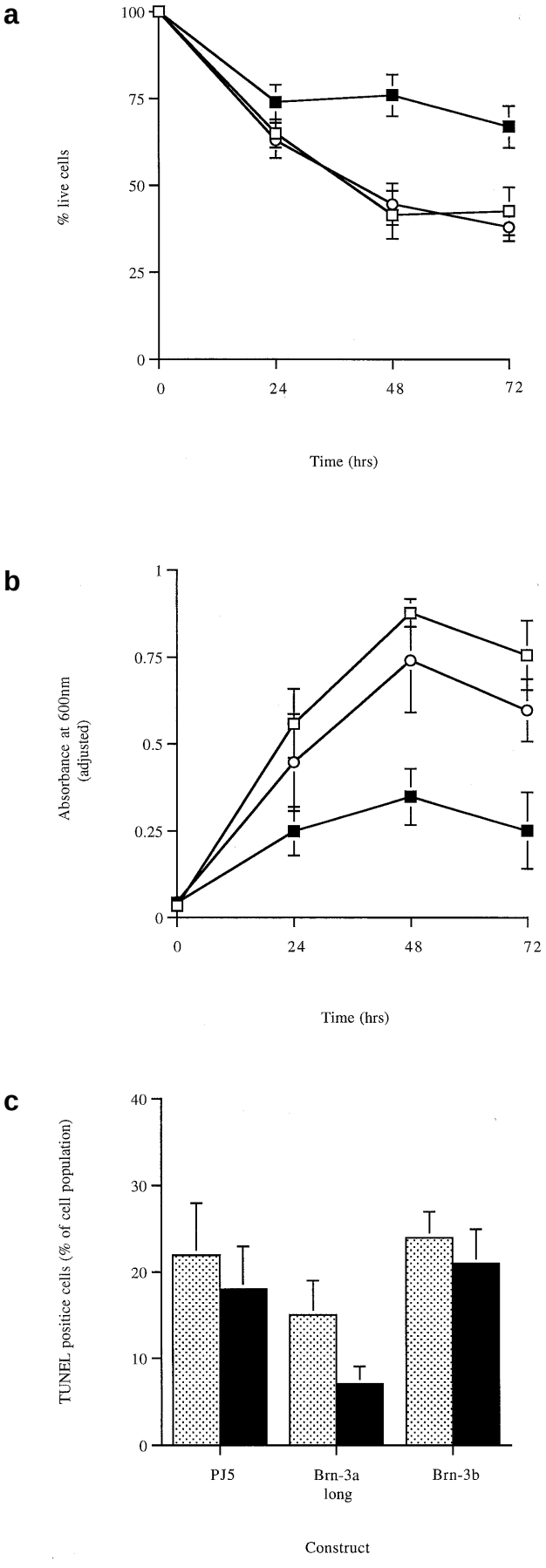


Figure 6. Bcl-2 protein expression is regulated by Brn-3a in ND7 cells. (**a**) Representative western blot of Bcl-2 protein expression in ND7 cell lines stably overexpressing either Brn-3a or the empty PJ5 expression vector as indicated, in the absence (–) or presence (+) of dexamethasone. (**b**) Bcl-2 protein expression in cell lines expressing the indicated constructs compared to that in ND7 cells transfected with vector alone maintained in the absence (stippled boxes) or presence (filled boxes) of dexamethasone. Data are pooled from three independently isolated cell lines expressing each construct.

experiments (Fig. 6), clear overexpression of endogenous Bcl-2 was observed in three independently isolated cell lines over-expressing the long form of Brn-3a with a greater elevation being observed following dexamethasone induction of maximal Brn-3a expression. However, no activation of Bcl-2 expression was observed in the cells overexpressing the short form of Brn-3a or the isolated POU domain indicating that, as in the co-transfection experiments, the activation of endogenous Bcl-2 gene expression requires the N-terminal domain. This conclusion is reinforced by the fact that Brn-3a containing isoleucine at position 22 was not impaired in its ability to activate the Bcl-2 gene compared to wild type Brn-3a whilst Brn-3b containing valine was inactive, like wild type Brn-3b (Fig. 6). Hence mutations affecting the ability of the POU domain to modulate transcription of its target promoters do not affect the ability of Brn-3a to activate Bcl-2 expression.

Having established the importance of the N-terminal domain for activating the Bcl-2 promoter, we wished to determine whether the ability of Brn-3a to induce the anti-apoptotic Bcl-2 protein allowed it to protect a neuronal cell line from apoptosis. To take advantage of our stable ND7-derived cell lines expressing different forms of Brn-3a, we wished to use this system. We have previously shown that upon serum removal, parental ND7 cells undergo either apoptotic programmed cell death or differentiation to a non-dividing phenotype bearing neuritic processes, whilst retinoic acid (RA) enhances the degree of apoptosis during the differentiation event (31). We therefore wished to test the protective effect of Brn-3a in this system.

Following the transfer of the stable cell lines containing the parental PJ5 vector with no insert to serum-free media containing dexamethasone to induce the MMTV promoter, a proportion of cells ceased to proliferate and began to extend processes, in agreement with previous studies (31). The degree of cell death in these control cells was assayed by trypan blue exclusion and found to be similar to that in parental ND7 cells at all timepoints (data not shown). The addition of 1 µM all-*trans* RA to the differentiated cultures resulted in a further decrease in the number of viable cells at all timepoints. These data demonstrate that the



addition of dexamethasone has no significant effect on the proportion of viable cells when compared to the effects observed in parental ND7 cells under the same experimental conditions. These similarities were observed in all vector alone control cell lines studied (data not shown).

However, significant increases (24 h $P < 0.05$; 48 h $P < 0.005$; 72 h $P < 0.05$) in the number of viable cells were observed in the three independent cell lines stably overexpressing high levels of full length Brn-3a (Brn-3aL), following the induction of the MMTV promoter upon the addition of 1 μM dexamethasone to the RA-containing serum-free media (Fig. 7a; Table 1, Brn-3aL). Similar protection was afforded by overexpression of Brn-3a in serum-free media which was not supplemented with RA (Table 1), although, as expected, less cell death was observed in the absence of RA in accordance with our previous results (31).

Thus, as transfer of ND7 cells to serum-free media with or without RA is known to induce apoptotic death, these data argue that high levels of Brn-3a are capable of rescuing ND7 cells from apoptotic death. Such a reduction in the number of apoptotic cells was confirmed by flow cytometric analysis of PI/FITC stained cells which identified an apoptotic population with less than 2N DNA content in differentiated control ND7 cells (empty vector alone) which was reduced following the induction of Brn-3a expression in the three Brn-3a overexpressing lines (data not shown). No significant alteration in the number of viable cells or in the degree of apoptosis was observed in all cell lines overexpressing Brn-3b (Fig. 7a and Table 1).

To extend these observations, we utilized a number of techniques to assay the degree of nucleosomal fragmentation of genomic DNA characteristic of apoptotic death. The overexpression of Brn-3a resulted in a decrease in the proportion of fragmented DNA within the cell populations as determined by both the colorimetric DNA fragmentation assay of Sellins and Cohen (32) (Fig. 7b) as well as the degree of nucleosomal laddering following agarose gel electrophoresis of genomic DNA (data not shown). No significant alteration in the degree of chromatin fragmentation was observed in cell lines over expressing Brn-3b (Fig. 7b and data not shown).

These observations were extended by the labelling of the cultured cells by the TUNEL technique which utilizes the labelling of free 3′ DNA ends by terminal deoxynucleotide transferase to visualize the DNA fragments generated by the characteristic endonucleolytic cleavage which occurs during apoptosis. As shown in Figure 7c, the overexpression of Brn-3a resulted in a 3-fold decrease in the number of TUNEL positive cells as compared to the cell line overexpressing the vector alone, at 24 h (45 ± 7%, $P < 0.005$), 48 h (35 ± 6%, $P < 0.001$) and 72 h following transfer to serum-free media containing RA (37 ± 8%, $P < 0.001$).

Figure 7. Brn-3a expression rescues ND7 cells from apoptotic programmed cell death following serum removal. (**a**) Cell survival in cell lines overexpressing Brn-3a (filled squares) or Brn-3b (open circles) maintained in serum free media containing 1 μM RA for 72 h, compared to the values in ND7 cells transfected with vector alone (open squares). (**b**) Relative amounts of DNA fragmentation within cells expressing either vector alone (open squares), Brn-3a (filled squares) or Brn-3b (open circles) maintained in serum free media containing 1 μM RA for 24, 48 and 72 h. (**c**) TUNEL positive nuclei as a proportion of the total number ND7 cells expressing either empty vector alone Brn-3a or Brn-3b in the absence (stippled bars) or presence (filled bars) of dexamethasone, when maintained in serum-free media containing 1 μM RA for 48 h.

Table 1. Overexpression of Brn-3a but not Brn-3b, rescues ND7 cell following serum removal

Transfected construct	Cell survival (viable cells as % or original cell population)[a]			
	–RA		+RA	
	Dex –	Dex +	Dex –	Dex +
PJ5	62 ± 6	59 ± 3	49 ± 5	41 ± 8
Brn-3aL	64 ± 3	87 ± 5*	61 ± 8	80 ± 9**
Brn-3b	58 ± 7	63 ± 5	40 ± 9	43 ± 5
Brn-3aS	57 ± 6	63 ± 4	51 ± 9	45 ± 12
Brn-3aP	61 ± 4	63 ± 5	45 ± 6	40 ± 6
Brn-3aI	68 ± 3	78 ± 7	57 ± 9	82 ± 5**
Brn-3bV	54 ± 6	58 ± 3	41 ± 6	44 ± 7

[a]Cell survival at 48 h following transfer to serum-free media with or without the addition of 1 μM retinoic acid determined by trypan blue exclusion. Values are means ± standard deviation of the mean determined in duplicate for three independent cell lines expressing each construct cultured in the absence (Dex –) or presence (Dex +) of dexamethasone. L, long form; S, short form; P, isolated POU domain; I, isoleucine mutant; V, valine mutant.
*Statistically significant increase from appropriate vector control ($P < 0.05$).
**Statistically significant increase from appropriate vector control ($P < 0.005$).

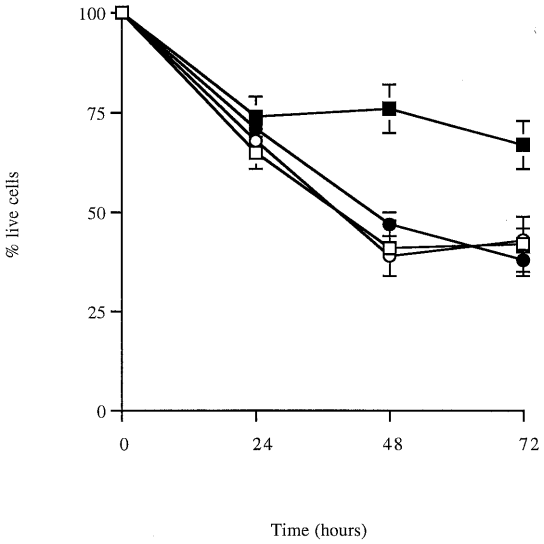


Figure 8. Protection of ND7 cells requires the presence of an N-terminal activation domain within Brn-3a. Cell survival in ND7 cell lines overexpressing the long form of Brn-3a (filled squares), the short form of Brn-3a (open circles) or the isolated POU domain of Brn-3a (filled circles) when maintained in serum-free media containing 1 μM RA for 24, 48, 72 h and following induction of the MMTV promoter with dexamethasone compared to the value in ND7 cells transfected with vector alone (open squares).

To test the effects of the N-terminal domain on the ability of Brn-3a to rescue ND7 cells, we tested the stable cell lines overexpressing the isolated POU domain of Brn-3a or the naturally occurring short form of Brn-3a which lacks the N-terminal activation domain. When these cell lines were analyzed, neither the Brn-3a POU domain alone or the short form of Brn-3a were able to confer the protection afforded by Brn-3a (long form) (Fig. 8, Table 1). Hence the ability of Brn-3a to protect ND7 cells from apoptosis is dependent upon the presence of the N-terminal activation domain that is present in the long form of Brn-3a (Brn-3a long).

To further extend the observation that the N-terminal domain of Brn-3a is apparently critical for Brn-3a to the rescue ND7 cells from apoptosis, we tested the stable cell lines overexpressing the mutant forms of Brn-3a (Brn-3aI) and Brn-3b (Brn-3bV) containing mutations at position 22 in the POU homeodomain. The Brn-3aI cell line, in which the expressed protein contains the N-terminus of Brn-3a but a POU domain equivalent to that of Brn-3b, maintained the ability to rescue ND7 cells when assayed by all criteria described above (Table 1). This contrasts with the inability of this construct to induce neurite outgrowth due to the lack of a functional Brn-3a POU domain (19). Conversely, the overexpression of the Brn-3bV construct which contains a POU domain capable of stimulating transcription in conjunction with the N-terminus of Brn-3b (Brn-3bV), was unable to rescue ND7 cells from apoptosis (Table 1). Hence, as in the case of Bcl-2 gene activation, protection from apoptosis is dependent on the presence of the N-terminal domain.

DISCUSSION

In this study, we have shown that both the activation of Bcl-2 gene expression and protection from apoptosis require the N-terminal activation domain of Brn-3a. This is in contrast to events such as the stimulation of neurite outgrowth (19) and the activation of the SNAP-25 (18) and neurofilament (14) promoters which require only the C-terminal POU domain. In the case of the Bcl-2 promoter, the POU domain is required for binding to the promoter since activation is not observed with the isolated N-terminal domain lacking any linked DNA binding domain (Fig. 3). However, unlike the neurofilament or SNAP-25 promoters, the POU domain alone cannot mediate activation of the promoter which requires the additional N-terminal domain.

Interestingly, the isolated N-terminal domain not only fails to activate Bcl-2 transcription but interferes with activation by full length Brn-3a. This is likely to involve the removal of an essential co-factor from the DNA by its interaction with the N-terminus, suggesting that transcriptional activation of the Bcl-2 promoter by Brn-3a involves the interaction of the N-terminus with another factor. Moreover, our results (Fig. 2) indicating that the Bcl-2 promoter is preferentially activated by Brn-3a in ND7 cells compared to BHK cells suggest that this factor may be expressed specifically in neuronal cells.

These considerations suggest a so far unique mechanism for the activation of the Bcl-2 promoter by Brn-3a since the other promoter characterized so far which is dependent upon the N-terminal domain, that of the gene encoding α-internexin, is activated equally well in co-transfections into BHK and ND7 cells (15). Further studies on the architecture of the Bcl-2 promoter and on the nature of the factor(s) with which the N-terminal domain of Brn-3a interacts will be necessary to fully characterize these effects.

It is already clear, however, that both the activation of Bcl-2 gene expression and the protective effect against neuronal apoptosis require the N-terminal domain of Brn-3a in contrast to the majority of the previously defined effects of Brn-3a. This suggests that these two events are linked and that Brn-3a protects against apoptosis at least in part, via the activation of Bcl-2 gene expression. Moreover, these findings indicate that Brn-3a is a multi-functional molecule with two distinct regions of the factor

being involved in its different functional effects. Thus, the N-terminal activation domain is involved in the stimulation of Bcl-2 gene expression and protection from apoptosis whereas the C-terminal domain mediates DNA binding and transcriptional activation of other genes and the stimulation of neurite outgrowth.

Most importantly, the dependence of Bcl-2 gene activation and protection from apoptosis on the N-terminal domain means that they will only be produced by the long form of Brn-3a and not by the short form which lacks this domain (3). In contrast, the stimulation of neurite outgrowth and of genes associated with this process will be produced by both forms since they both contain the common POU domain. The different ratio of the long and short form in different neuronal tissues and the alteration of this ratio in response to different stimuli (24) indicates that the alternative splicing event producing these two isoforms can be regulated. When taken together with the different properties of the two forms of the protein which we have characterized here, this indicates that the distinct functional domains of Brn-3a will allow the production, in different circumstances, of different forms of Brn-3a which either stimulate survival of neurons and promote neurite outgrowth or promote neurite outgrowth without increasing survival.

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