Coordinate Induction of the Three Neurofilament Genes by the Brn-3a Transcription Factor*

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The POU domain transcription factor Brn-3a is able to stimulate neurite outgrowth when overexpressed in the neuronal ND7 cell line, whereas the closely related Brn-3b factor does not have this effect. We show that Brn-3a overexpression also enhances the expression of the three neurofilament genes at both the mRNA and protein levels, whereas Brn-3b overexpression has no effect. In addition Brn-3a activates the three neurofilament gene promoters in co-transfection assays in both neuronal and non-neuronal cells. As observed for enhanced neurite outgrowth, the stimulation of neurofilament gene expression and activation of the neurofilament gene promoters is observed with the isolated POU domain of Brn-3a. A single amino acid change in the POU homeodomain of Brn-3a to the equivalent amino acid in Brn-3b abolishes its ability to activate the neurofilament promoters, whereas the reciprocal change converts Brn-3b to an activator of these promoters.

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we have recently obtained evidence that the rise in Brn-3a and the fall in Brn-3b occurring during ND7 cell differentiation play a critical role in the differentiation process itself. Thus preventing the rise in Brn-3a using an antisense approach prevents ND7 cell differentiation and the rise in SNAP-25 expression which normally occurs in response to differentiation-inducing stimuli such as serum removal (18). Moreover, artificial overexpression of Brn-3a in ND7 cells results in clonal cell lines which extend neurite processes when grown in full serum-containing medium which does not normally induce the differentiation of these cells (25). Conversely ND7-derived cell lines overexpressing Brn-3b fail to show neurite outgrowth when removed from serum which would normally induce such outgrowth in parental ND7 cells (26).

These findings indicate therefore that the balance between Brn-3a and Brn-3b is likely to play a critical role in the differentiation of ND7 cells, and they allow the use of these cells as a model system to understand the role of these factors in neuronal differentiation and to elucidate the mechanism by which they act. In view of the finding that SNAP-25 is essential for neurite outgrowth by a variety of different neuronal cells in vitro and in vivo (27), it is likely that the ability of Brn-3a to induce SNAP-25 expression plays a role in its ability to induce neurite outgrowth. However, it is likely that this dramatic effect of Brn-3a on the phenotype of ND7 cells involves more than its ability to induce SNAP-25 and that other target genes encoding proteins involved in neurite process formation are also likely to be activated by Brn-3a. We previously found, however, that the growth-associated protein GAP-43 shows no change in its expression in the Brn-3a or Brn-3b overexpressing ND7 cells, in agreement with our finding that its promoter is unaffected by co-transfection with Brn-3a (25). In view of the importance of the neurofilament proteins in neuronal process formation and particularly in specifying axonal calibre (for review see Ref. 28) and the simultaneous appearance of neurofilament and Brn-3a expression during early post-mitotic neuronal differentiation (15), we have investigated the expression of the neurofilament heavy, medium, and light chain genes in our ND7 cells overexpressing different Brn-3 transcription factors and have also studied the effect of the Brn-3 factors on the promoters of these genes in co-transfection experiments carried out in both neuronal and non-neuronal cells.

MATERIALS AND METHODS

Cell Culture—BHK-21 cells (29) and ND7 cells (21) were routinely cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and L15 medium containing 10% fetal calf serum, respectively. The ND7-derived cell lines overexpressing Brn-3a, Brn-3b, or Brn-3c were constructed as described previously (25, 26) by stable transfection of ND7 cells with expression vectors containing each form of Brn-3 which had previously been shown to overexpress Brn-3a, Brn-3b, or Brn-3c under the control of the glucocorticoid-inducible mouse mammary tumor virus promoter in the vector pJ5 (30). These 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 1 μM was used to induce expression of the murine mammary tumor virus promoter.

Plasmid DNA—The neurofilament reporter gene constructs containing various regions of the NF-H, NF-M, and NF-L promoter linked to the chloramphenicol acetyltransferase gene have been described previously (31, 32). In the transient transfections, the Brn-3a and Brn-3b expression vectors contain full-length cDNA or cDNAs encoding various regions of the molecule cloned under the control of the Moloney murine leukemia virus promoter in the vector pJ4 (30). The mutant forms of Brn-3a and Brn-3b containing an altered amino acid at position 22 in the homeodomain were constructed as described previously using the pALTER-1 vector (Promega) and were then subcloned into the pJ4 vector for use in transient transfection experiments (33). Transient Transfection—Transient transfection of DNA was carried out according to the method of Gorman (34). Routinely 1 × 10⁶ BHK-21 cells (29) or ND7 cells (21) were transfected with 10 μg of the reporter plasmid and 10 μg of the Brn-3 expression vectors. In all cases cells were harvested 72 h later. The amount of DNA taken up by these cells in each case was measured by slot blotting the extract and hybridization with a probe derived from the ampicillin resistance gene in the plasmid vector (35). This value was then used to equalize the values obtained in the CAT assay as a control for differences in uptake of plasmid DNA in each sample. In parallel experiments, we also included an internal control plasmid in which the cytomegalovirus promoter drives β-galactosidase expression allowing equalization of the samples on the basis of β-galactosidase activity prior to assay of CAT activity.

CAT Assay—Assays of chloramphenicol acetyltransferase activity were carried out according to the method of Gorman (34) using samples that had been equalized for protein content as determined by the method of Bradford (36).

RNA Isolation and Quantitation—RNA was isolated from stably transfected cells by the guanidinium thiocyanate method (37). The expression of the neurofilament RNAs was then quantitated using a reverse transcriptase/polymerase chain reaction assay. Initially all samples were amplified using primers for the constitutively expressed RNA encoding the L6 ribosomal protein. Subsequent mRNA were amplified using specific primers for each mRNA. Primers were as follows: NFL 5'-CCCTACTTCCTTTGACCTGCTTA-3' and 5'-TCTCTTGTTGGTCCTGATGAC-3'; NFM 5'-ACACCATCCGCGCATGTCGCGTTACCTGTTCCCTGTTG-3' and 5'-TACCTCGTTACCTGTTCCCTGTTG-3'; NFH 5'-CCCAAGGGAGATAACCTGAG-3' and 5'-TCAATGTCCAGGGCCACTTCT-3'; N M 5'-ATGCTCTTCTCAAACTTGACC-3' and 5'-AATCTACACACCTCATGCC-3'. All amplifications were carried out using a number of cycles and amount of mRNA that had been determined in preliminary experiments to be in the range of the assay where the amount of product formed was linearly related to the amount of input RNA.

Protein Isolation and Western Blotting—Protein was isolated from cells by freeze-thaw extraction in protein buffer (20 mM HEPES, pH 7.9, 0.45 mM NaCl, 25% glycerol, 0.2 mM EDTA contains 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml dithiothreitol) and submitted to analysis by SDS-polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose filter by Western blotting, and the neurofilament proteins were detected by probing with the mouse monoclonal antibodies NM-08-304, NM-08-307, and NM-08-306 (Genosys). To ensure equal loading of protein samples, gels were stripped and reprobed using a control antibody, in this case pGp9.5.

RESULTS

The ND7-derived cell lines overexpressing individual forms of Brn-3 were constructed (25, 26) by stably transfecting ND7 cells with expression vectors containing each form of Brn-3 under the control of the dexamethasone-inducible murine mammary tumor virus promoter in the vector pJ5 (30). Both by RNA analysis (25, 26) and DNA mobility shift assays using an oligonucleotide containing a binding site for the Brn-3 factors (data not shown), all the cell lines showed some overexpression of the appropriate form of Brn-3 in the absence of dexamethasone with enhanced expression being inducible upon addition of 1 μM dexamethasone. Although expression is therefore somewhat leaky and induced severalfold by hormone, it is possible to use these cells to examine the effects of overexpressing different forms of Brn-3 by studying these effects both in the absence or presence of dexamethasone and comparing the results to similarly treated control cells. We therefore prepared mRNA and protein from the various ND7-derived cell lines proliferating in full serum-containing medium either in the presence or absence of dexamethasone. In each case mRNA and protein was prepared from three independent cell lines of each type which had previously been shown to overexpress Brn-3a, Brn-3b, or Brn-3c (25, 26). Initially, the expression of each of the neurofilament proteins was quantitated using a Western blotting assay.

As shown in Fig. 1, each of the neurofilament proteins showed a somewhat enhanced level in the Brn-3 overexpressing cells in the absence of steroid compared with control cells

¹ The abbreviation used is: CAT, chloramphenicol acetyltransferase.
stably transfected with the pJ5 vector alone. Moreover, upon steroid induction of exogenous Brn-3a gene expression, the levels of each of the neurofilament proteins increased in the Brn-3a cell lines but not in the control cells. This resulted in overexpression in the Brn-3a cell lines of 3.7-fold for NF-H ($p < 0.01$), 2.2-fold for NF-M ($p < 0.05$), and 3-fold for NF-L ($p < 0.01$) relative to control cells. Hence, the expression of all three neurofilament proteins is enhanced in the Brn-3a expressing cells. In contrast no increase in expression of any of the neurofilament proteins was observed in the Brn-3b expressing cells, whereas only small (less than 2-fold) increases were observed in the Brn-3c expressing cells.

Having established that Brn-3a can enhance neurofilament protein levels, we wished to determine whether this was paralleled by increased neurofilament mRNA levels as would be expected if Brn-3a directly activates the neurofilament gene promoters. The relative expression of each of the neurofilament mRNAs was therefore quantified in the cells using a reverse transcriptase/polymerase chain reaction assay.

In these experiments (Fig. 2) the neurofilament heavy chain mRNA was overexpressed approximately 2-fold in the Brn-3a overexpressing cells compared with control cells stably transfected with the pJ5 vector alone ($p < 0.01$). Upon steroid induction this overexpression of the neurofilament heavy chain gene rose to approximately 6-fold over that observed in similarly treated control cells ($p < 0.01$). Similar overexpression of the neurofilament light chain of approximately 2-fold before steroid treatment ($p < 0.01$) and of approximately 5-fold after steroid induction ($p < 0.01$) was also observed in the Brn-3a overexpressing cells, whereas the neurofilament medium chain mRNA showed only marginal overexpression in the Brn-3a cells before steroid treatment but was overexpressed approximately 3-fold ($p < 0.01$) after steroid treatment (Fig. 2). In contrast no significant overexpression of any of the neurofilament genes was observed in the Brn-3b or Brn-3c overexpressing cells either before or after steroid treatment.

These findings indicate therefore that the overexpression of Brn-3a in the ND7 cells can induce the overexpression of all three of the neurofilament genes at both the mRNA and protein levels. Thus prior to dexamethasone treatment when some overexpression of Brn-3a is observed in the ND7 cells (25), there is also some overexpression of the different neurofilament genes. Moreover, when enhanced expression of Brn-3a is induced by steroid treatment, this results in significant overexpression of all three neurofilament genes above the level observed in comparably treated control cells with the neurofilament heavy chain showing the greatest effect at both the mRNA and protein levels, followed by the light chain and then the medium chain.

In previous experiments we have shown that the ability of Brn-3a to induce target genes in co-transfection experiments is dependent upon two activation domains, one of which is located at the N terminus of the molecule (17), and the other is located at the C terminus coincident with the POU domain. The relative importance of these different activation domains varies with the target promoter tested (17, 22, 24). In our previous experiments (25) we showed that transfection of ND7 cells with a naturally occurring form of Brn-3a (Brn-3a short) that lacks the N-terminal activation domain (9, 38) still results in the promotion of neurite outgrowth, although to a somewhat lesser following treatment with 1 μM dexamethasone to induce expression of each form of Brn-3 (solid bars). All values are expressed relative to the level of the appropriate neurofilament protein in cells stably transfected with the pJ5 expression vector alone and are the average of three determinations in each of three different cell lines of each type with the standard deviation of the mean being shown by the bars.
extent than that observed with the full-length Brn-3a, suggesting therefore that the N-terminal activation domain plays only a small role in stimulating the expression of the genes required for neurite outgrowth. In contrast, overexpression of the POU domain of Brn-3a alone is able to promote neurite outgrowth, although to a somewhat lesser extent than that observed with full-length Brn-3a (25). This suggests that the POU domain alone can stimulate the expression of the genes that are required for neurite outgrowth.

In view of this we therefore investigated the expression of the various neurofilament mRNA in cells overexpressing either the POU domains of Brn-3a or Brn-3b or the short form of Brn-3 lacking the N-terminal domain. In these experiments (Fig. 2) the short form of Brn-3a was able to promote significant overexpression of the different neurofilament genes particularly following steroid treatment to induce the expression of Brn-3a ($p < 0.01$ for NF-H and NF-L and $p = 0.01$ for NF-M when cells transfected with the short form of Brn-3a are compared with control cells following steroid treatment). This suggests that the N-terminal domain is dispensable for induction of the neurofilament genes. Interestingly, cells transfected with the POU domain of Brn-3a alone showed overexpression of the different neurofilament genes of between 2- and 3-fold following steroid treatment ($p < 0.01$ for NF-L, NF-M, and NF-H when Brn-3a POU overexpressing cells are compared with control cells following steroid treatment). In contrast no overexpression of any of the neurofilament genes was observed in the cells transfected with the Brn-3b POU domain. This indicates therefore that overexpression of the POU domain alone is sufficient to induce overexpression of all three neurofilament genes, whereas Brn-3b POU does not have this effect.

These findings indicate therefore that the ability of full-length Brn-3a to induce the overexpression of the neurofilament genes is very little impaired by the deletion of its N-terminal activation domain and that a significant effect on these genes can be obtained with the POU domain alone. These findings closely parallel our previous findings (25) which suggested that neurite outgrowth could be induced by full-length Brn-3a, the short form of Brn-3a lacking the N-terminal domain, and by the isolated POU domain alone suggesting that the POU domain had the prime role in this effect.

Although our data thus clearly indicate the overexpression of the different neurofilament genes in the Brn-3a expression cells, they do not indicate whether this is a direct effect of Brn-3a on the promoters of the various genes or simply represents a downstream effect of the ability of Brn-3a to induce neurite outgrowth which in turn somehow induces overexpression of the neurofilament genes. To distinguish these possibilities, we therefore investigated whether Brn-3a could have a direct effect on the activity of the neurofilament gene promoters in transiently transfected cells. Thus we co-transfected neurofilament reporter gene constructs containing the promoters of each of the neurofilament genes (31, 32) with expression

Fig. 2. Quantitation of the mRNA encoding the neurofilament heavy chain (a), medium chain (b), and light chain (c) mRNAs in ND7 cell lines overexpressing Brn-3a, Brn-3b, or Brn-3c and in similar cell lines overexpressing either a naturally occurring short form of Brn-3a lacking the N-terminal exon (Brn-3as) or overexpressing either the POU domain of Brn-3a or that of Brn-3b. Values are shown for cells either in the absence of dexamethasone induction (dotted bars) or following treatment with 1 μM dexamethasone to induce expression of each form of Brn-3 (solid bars). All values are expressed relative to the level of the appropriate mRNA in cells stably transformed with the pJ5 expression vector alone and are the average of three determinations in each of three different cell lines of each type with the standard deviation of the mean being shown by the bars.
Brn-3b had no significant effect on the promoter. Although the samples in these experiments were equalized on the basis of plasmid uptake in each sample, similar results were obtained when the samples were equalized on the basis of the activity of a co-transfected cytomegalovirus-β-galactosidase plasmid (data not shown). These experiments indicate therefore that Brn-3a but not Brn-3b can directly stimulate the NF-H promoter in both neuronal cells and in fibroblast cells where any indirect effect exerted via the stimulation of neuronal differentiation can be excluded. These results indicate therefore that Brn-3a is likely to have a direct stimulatory effect on the NF-H promoter.

To investigate the regions of the NF-H promoter that were responsible for this effect, we utilized reporter constructs that contain progressively smaller regions of the NF-H promoter linked to the CAT gene. In these experiments a construct containing the region of the NF-H promoter from −385 to +46 relative to the transcriptional start site was stimulated by Brn-3a in a similar manner to the longer construct (data not shown). Similarly, a construct that contains only 63 bases upstream of the start site of transcription was also stimulated by Brn-3a but not by Brn-3b in both BHK cells (data not shown) and ND7 cells (Fig. 4a). Hence Brn-3a can stimulate a minimal NF-H promoter containing the basic sequences required for transcription of the neurofilament heavy chain gene.

In view of this we tested whether similar stimulation of the NF-M and NF-L promoters could also be observed with Brn-3a. In these experiments a minimal NF-M (Fig. 4b) promoter containing the region from −98 to +27 relative to the transcriptional start site was stimulated by Brn-3a and not by Brn-3b. Similarly a minimal NF-L (Fig. 4c) promoter containing the region from −60 to +82 relative to the transcriptional start site was also stimulated by Brn-3a and not by Brn-3b. Hence constructs containing relatively short regions of the three neurofilament gene promoters around the transcriptional start site can be directly stimulated by Brn-3a but not by Brn-3b in co-transfection assays.

In our previous experiments (Fig. 2) using stably transfected ND7 clonal cell lines, the isolated Brn-3a POU domain was also able to stimulate the expression of the endogenous neurofilament genes, although not as strongly as full-length Brn-3a. We therefore tested whether the isolated Brn-3a POU domain could similarly stimulate the minimal neurofilament gene promoters in co-transfection assays. As illustrated in Fig. 4, clear stimulation of the NF-H, NF-M, and NF-L promoters was observed in co-transfections carried out in ND7 cells, although the extent of stimulation of each of the promoters was less than observed with the full-length Brn-3a construct paralleling the results in stably transfected cell lines. In contrast no significant stimulatory effect was observed with Brn-3b POU domain confirming that this effect is specific for Brn-3a (Fig. 4). Similar results were also observed when co-transfections were carried out in the BHK-21 fibroblast cells (data not shown).

These experiments indicate therefore that the isolated POU domain of Brn-3a can directly activate the minimal neurofilament promoters in co-transfection experiments and that such a direct effect on the promoters is likely to be responsible for the observed activation of the endogenous neurofilament genes in ND7 cells stably transfected with the Brn-3a POU domain construct. The fact that the Brn-3b POU domain construct cannot reproduce these effects in either stably transfected cell lines or in transient transfection experiments indicates that one of the seven amino acid differences between the POU domains of Brn-3 and Brn-3b (9, 12) must play a critical role in this effect. The POU domain consists of two distinct subdomains, the POU-specific domain and the POU homeodomain
FIG. 4. CAT assays showing the results of co-transfecting ND7 cells with promoter constructs containing the minimal NF-H (a), NF-M (b), or NF-L (c) reporter constructs together with either pJ4 expression vector alone or the same vector containing full-length inserts for Brn-3a or Brn-3b or inserts encoding only the POU domain of Brn-3a or Brn-3b. Values indicate the CAT activity obtained in each case and are expressed relative to the CAT activity obtained in the co-transfection of each reporter construct with pJ4 vector alone (set at 100%).
22 of the homeodomain critically regulates the ability of Brn-3a vector alone. Hence this single amino acid difference at position below the basal level observed upon co-transfection of plasmid and indeed appeared to inhibit the activity of these promoters was now unable to activate any of the neurofilament promoters. Conversely the Brn-3a(I) mutant wise intact Brn-3b suffices to convert Brn-3b to an activator of the test promoter this single amino acid difference within other (1, 2), we have concentrated our attention on the single mutation in which the valine at this position in Brn-3a is mutated to the isoleucine residue in Brn-3b. Although the POU-specific domain of Brn-3a and Brn-3b is identical, the POU homeodomain contains a single amino acid difference at position 22 in the first α-helix, whereas the linker domain contains six amino acid differences. Since the linker region is very variable between different POU factors and is believed to allow the two major domains to move relative to one another (1, 2), we have concentrated our attention on the single amino acid difference at position 22 in the first α-helix.

We have previously shown that altering the isoleucine residue at this position in Brn-3b to the valine residue located at the equivalent position in Brn-3 converts Brn-3b from a repressor to an activator when it is co-transfected with an artificial test promoter containing a binding site for both Brn-3a and Brn-3b (33). The effect of this mutation on a naturally occurring promoter which is normally regulated by Brn-3a/Brn-3b has not, however, been tested. Similarly, we have not previously investigated on any promoter the effects of the converse mutation in which the valine at this position in Brn-3a is mutated to the isoleucine residue in Brn-3b.

The effect of these mutant constructs Brn-3a(I) and Brn-3b(V) on the three minimal neurofilament promoters was therefore tested in co-transfection assays. As illustrated in Fig. 5, the Brn-3b(V) mutant strongly transactivated all three minimal neurofilament promoters to a degree greater or equal to that observed with intact Brn-3a. Hence as with the artificial test promoter this single amino acid difference within otherwise intact Brn-3b suffices to convert Brn-3b to an activator of the neurofilament promoters. Conversely the Brn-3a(I) mutant was now unable to activate any of the neurofilament promoters and indeed appeared to inhibit the activity of these promoters below the basal level observed upon co-transfection of plasmid vector alone. Hence this single amino acid difference at position 22 of the homeodomain critically regulates the ability of Brn-3a to stimulate the neurofilament promoters, whereas Brn-3b does not normally do so.

**DISCUSSION**

In our previous work (25) we showed that overexpression of the Brn-3a transcription factor can induce ND7 cells to extend numerous neurite processes even in full serum-containing medium, thus mimicking the differentiation of these cells which normally occurs when they are placed in serum-free medium. This ability of a single transcription factor to induce the complex phenotypic changes involved in neurite outgrowth is likely to depend on its ability to activate target genes whose protein products are required for this purpose. Indeed we previously demonstrated that overexpression of Brn-3a can activate the endogenous genes encoding the synaptic vesicle proteins SNAP-25, synapsin, synaptotagmin, and synaptophysin (25) as well as directly stimulating the SNAP-25 promoter (18) and that of the neuronal intermediate filament protein α-internexin (17) in co-transfection experiments. As the expression of SNAP-25 is essential for neurite outgrowth in different neuronal cell types (27), it is likely that its stimulation by Brn-3a is involved in the process of neurite outgrowth. Target genes for Brn-3a are essential for diverse aspects of early neuronal differentiation, including neurite outgrowth (27), synaptogenesis, as well as establishing the early axonal cytoskeleton, suggesting that Brn-3a may act as a master control complex promoting early neuronal differentiation.

In agreement with this view, we show here that expression of the three neurofilament genes is greatly enhanced in cell lines overexpressing Brn-3a. Activation of neurofilament (and α-internexin) genes provides the cytoskeletal framework for neurite elongation and for the radial growth of axons (28, 39). During development, neurofilaments became the major cytoskeletal component in large axons, and their levels of expression are major determinants of axonal size (for review see Ref. 40). Moreover, neurofilaments are obligate heteropolymers in vivo (41, 42), so it is necessary for subunit expression to be coordinated to ensure a continuous supply and delivery of neurofilaments along the axon. Increases (or decreases) of individual subunit expression are disruptive to assembly and/or transport, leading to accumulations of neurofilaments and reduced axonal size (43–48).

It is therefore likely to be of critical importance that Brn-3a can stimulate the endogenous genes encoding all three neurofilament proteins in stably transfected cell lines. As illustrated in our co-transfection experiments, this effect is likely to involve a direct stimulation of the neurofilament promoters by Brn-3a rather than being a consequence of the neuronal differentiation induced by Brn-3a. Brn-3a can stimulate the minimal promoters of all three neurofilament genes in co-transfection experiments not only in the neuronal ND7 cell line but also in BHK-21 fibroblast cells. Since such BHK cells evidently do not show neuronal differentiation in response to Brn-3a, this indicates that the changes in neurofilament gene expression which occur in response to Brn-3a are likely to be due to a direct effect on the neurofilament promoter.

Interestingly, we have previously shown that Brn-3a can also activate the promoter of the α-internexin gene (17, 49) which encodes a closely related type IV intermediate filament protein (26, 50). In contrast we have previously observed no transactivation by Brn-3a of the promoter encoding the vimentin protein which belongs to a different class of intermediate filament proteins.² Interestingly, vimentin is expressed in proliferating neuronal precursor cells that line the ventricular

² M. D. Smith, P. J. Morris, S. J. Dawson, and D. S. Latchman, unpublished observations.
surface of the neural tube but is rapidly down-regulated upon terminal neuronal differentiation.

Moreover, we previously reported that induction of the α-internexin promoter by Brn-3a could be observed using a reporter construct containing only 77 bases of upstream promoter sequence (17), and subsequently we identified a response element for Brn-3a between positions −77 and −60 of the α-internexin promoter (49). Similarly, in the experiments reported here we have shown that neurofilament promoter constructs containing relatively small amounts of upstream sequence are responsive to Brn-3a, suggesting that the promoters of the genes encoding the type IV intermediate filament proteins contain Brn-3a-responsive elements located close to the transcriptional start site.

As well as showing a similar location of the Brn-3a-responsive region, the neurofilament promoters also resemble that of α-internexin in being stimulated by Brn-3a but not by the closely related transcription factor Brn-3b (this paper and Ref. 17). However, despite these similarities, the region of Brn-3a which is required for stimulation of the promoter differs in the case of the neurofilament genes and that of α-internexin. Thus, we previously showed (17) that stimulation of the α-internexin promoter requires an activation domain located at the N terminus of the protein and is not observed when the promoter is co-transfected with the POU domain alone. In the experiments reported here, we have shown, however, that the neurofilament promoters resemble that encoding SNAP-25 (19) and a artificial promoter containing a synthetic binding site for Brn-3a and Brn-3b (24) in that they can be stimulated by co-transfection with the Brn-3a POU domain but not by the closely related Brn-3b POU domain. In previous experiments (49) we showed that transfer of the Brn-3a response element from the α-internexin promoter to the same site in the test promoter as normally occupied by the synthetic Brn-3a/Brn-3b binding site resulted in the α-internexin response element becoming responsive to the POU domain of Brn-3a. Thus the difference in the responses of the neurofilament promoters and that of α-internexin is likely to involve a different context of the Brn-3a response element in these various promoters.

The stimulation of the neurofilament promoters by the POU domain of Brn-3a but not by that of Brn-3b prompted us to examine the effect on these promoters of mutant forms of Brn-3a and Brn-3b containing a single amino acid substitution at position 22 in the first α-helix of the POU homeodomain. We have thus demonstrated that this amino acid plays a critical role in the ability of Brn-3a and not Brn-3b to stimulate the neurofilament promoters. The ability of Brn-3b containing a valine rather than an isoleucine residue at this position to activate the neurofilament promoter parallels our previous finding (33) that this mutant can also activate an artificial promoter containing a synthetic binding site for the Brn-3 factors and, moreover, extends this finding to a naturally occurring neuronal promoter. Similarly, the data reported here show for the first time that the converse mutation in which isoleucine is substituted for valine in Brn-3a completely abolishes the ability of Brn-3a to activate a target promoter.

Although the POU domain of the Brn-3 factors directs their binding to DNA, the critical role of this amino acid within the POU domain in the different effects of Brn-3a and Brn-3b does not appear to be due to differences in the DNA binding abilities of the various wild type and mutant forms of Brn-3. Thus, the POU domains of both Brn-3a and Brn-3b are able to bind to DNA (9, 13, 49), and the mutations do not appear to have any effect on this DNA binding ability (33). Similarly, position 22 of the homeodomain is located at the C terminus of the first α-helix and is thus not in contact with the DNA according to crystallographic analysis of the POU domain of the related Oct-1 protein (51). Indeed such crystallographic analysis indicates that this amino acid is located on the surface of the POU domain that could be involved in protein-protein interactions.

Such a role for the amino acid at this position is supported by the finding that in the related POU proteinsOct-1 and Oct-2, the amino acid at this position plays a critical role in determining their ability to interact with the herpes simplex virus virion protein VMW65 (52). Thus substitution of the alanine found at position 22 of the homeodomain in Oct-2 with the glutamic acid found at the equivalent position in Oct-1 allows Oct-2 to interact with VMW65 which is normally a property of Oct-1 only. By analogy therefore, it is likely that the presence of a valine at this position in Brn-3a or the mutant Brn-3b allows the protein to interact with and thus recruit an activating molecule to the promoter, whereas Brn-3b or Brn-3a containing isoleucine cannot do so. Alternatively Brn-3b and Brn-3a containing isoleucine at this position can recruit an inhibitory molecule which does not interact with molecules containing valine at this position.

In either case, it is clear that the nature of the amino acid at this position in Brn-3a and Brn-3b plays a critical role in the ability of Brn-3a alone to activate the three neurofilament gene promoters, whereas it is not involved in the activation of the α-internexin promoter which is dependent upon the N terminus of Brn-3a. The different alternatively spliced forms of Brn-3a which either possess or lack the N-terminal domain (9, 38) may thus be involved in the disparate expression of the neurofilament genes and theα-internexin gene in different subsets of neurons and their dissimilar expression pattern over time. Conversely the similar activation mechanism of the three neurofilament subunit genes by Brn-3a may help to explain their co-expression in the same subset of neurons.

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Coordinate Induction of the Three Neurofilament Genes

Coordinate Induction of the Three Neurofilament Genes by the Brn-3a Transcription Factor
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