The differentiation of the ND7 neuronal cell line to a nondividing phenotype bearing numerous neurite processes is accompanied by a dramatic increase in the levels of the activating POU family transcription factor Brn-3a and a corresponding fall in the levels of the closely related inhibitory factor Brn-3b. We have previously shown that the artificial overexpression of Brn-3a in these cells can induce neurite outgrowth and the activation of genes encoding synaptic vesicle proteins in the absence of a differentiation-inducing stimulus. Here we show that overexpression of Brn-3b can reduce process outgrowth and synaptic vesicle gene expression following exposure to a stimulus which would normally induce differentiation. These inhibitory effects are abolished by altering a single amino acid in the POU homeodomain of Brn-3b to its equivalent in Brn-3a. The converse mutation in Brn-3a allows it to inhibit process outgrowth in response to a differentiation-inducing stimulus. Hence a single amino acid difference results in these closely related factors having opposite effects and allows the balance between them to regulate differentiation.

The Brn-3a, Brn-3b, and Brn-3c transcription factors (also known as Brn-3.0, 3.2, and 3.1, respectively) (1, 2) are closely related members of the POU family of transcription factors which are encoded by three distinct genes (3–6) (for review of POU factors see Refs. 7 and 8). Among the mammalian POU factors, the Brn-3 proteins are the most closely related to the nematode POU factor Unc-86, which plays a critical role in neuronal development in the nematode (9, 10) suggesting that the Brn-3 factors may play a similar role in mammals. In agreement with this idea the three members of the Brn-3 family are expressed in distinct but overlapping populations of neuronal cells during development and in the adult organism (1–5, 11) with Brn-3a for example being expressed in the first differentiated neurons to appear in the midbrain, hindbrain, and spinal cord during development (12). Interestingly Brn-3a has been shown in co-transfection experiments to activate several promoters including those of the neurally expressed genes encoding pro-opiomelanocortin (1), α-internexin (13), and the presynaptic vesicle protein SNAP-25 (14) as well as a thymidine kinase promoter containing an added synthetic binding site for the various forms of Brn-3 (tk-Oct) (15, 18). In contrast Brn-3b represses these promoters (13, 15, 18) and also inhibits their activation by Brn-3a. Similarly Brn-3c has only a weak activating effect on these promoters (13, 15, 18).

When the proliferating ND7 cell line (obtained by fusing primary dorsal root ganglion neurons with C1300 neuroblastoma cells) (17) is induced to cease dividing and differentiate to a mature neuronal-like phenotype by treatment with cyclic AMP or removal of serum from the medium (18), the levels of Brn-3a rise dramatically, and those of Brn-3b fall (3, 15), while Brn-3c levels remain unchanged. This differentiation process is likely to require the induction of specific genes involved in the outgrowth of neurite processes and the acquisition of a more neuronal-like phenotype. If such genes were targets for activation by Brn-3a and repression by Brn-3b, the changes in Brn-3 expression could potentially play a key role in the differentiation process by activating the expression of such genes. Hence the ND7 cell system offers a convenient model system for studying the role of the Brn-3 factors in neuronal differentiation.

Indeed we have previously used this system to show that the inhibition of Brn-3a expression using an antisense approach prevents the neurite outgrowth and elevated expression of SNAP-25, which normally occur following exposure of ND7 cells to a differentiation inducing stimulus (14). Moreover, the stable overexpression of Brn-3a in ND7 cell clones resulted in neurite outgrowth and the induction of several synaptic proteins including SNAP-25 even in the absence of a differentiation inducing stimulus (19).

Given the ability of Brn-3a to induce the SNAP-25 promoter in co-transfection experiments (14), it is therefore likely that the rise in Brn-3a, which occurs during ND7 cell differentiation, directly activates the SNAP-25 promoter and is thus responsible for the rise in SNAP-25 expression, which occurs during the differentiation event. Moreover, the finding that SNAP-25 expression is essential for neurite outgrowth in several different types of neuronal cells in vitro and in vivo (20) indicates that the exocytosis of synaptic vesicles, which is required for synaptic transmission, and the constitutive exocytosis, which is required for axon outgrowth, may have several components such as SNAP-25 in common. Hence, the induction by Brn-3a of SNAP-25 gene expression and that of other synaptic proteins is likely to be responsible, at least in part, for its ability to induce neurite outgrowth.

Although these findings indicate a critical role for the rise in Brn-3a expression in inducing neuronal differentiation in ND7 cells, they also focus attention on the role of Brn-3b. Thus the levels of Brn-3b fall dramatically during ND7 cell differentiation (3, 15), while in co-transfection experiments it both represses the basal activity of a number of different promoters, which are activated by Brn-3a, and prevents their activation by...
Brn-3a (13, 15, 16). We have therefore investigated the response of ND7 cell clones overexpressing Brn-3b to a stimulus which would normally induce differentiation and have compared this to the response of clones overexpressing either Brn-3a or Brn-3c.

MATERIALS AND METHODS

Preparation of Cell Lines and Analysis of Brn-3 Expression—cDNA clones of wild type and mutant forms of Brn-3a, Brn-3b, and Brn-3c, as well as the POU domains of Brn-3a and Brn-3b, were inserted in the sense orientation under the control of the dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter of the mammalian expression vector PJ5 (21). Following co-transfection into ND7 cells (17) together with a plasmid conferring neomycin resistance (pMSG-NEO), stable transfecants were selected by the supplementation of culture medium with G418 to a final concentration of 800 µg/ml and individual clones were isolated after 7–14 days of selection. Putative clones were treated with dexamethasone at a final concentration of 1 µM for 24 h to induce expression of the MMTV promoter to allow screening for clones capable of expressing the exogenous construct. RNA was isolated from cells by the guanidinium thiocyanate method (22), treated with DNase to remove any contaminating DNA, and subsequently used as the template for cDNA synthesis. Resultant cDNA was amplified by PCR essentially as described by Kawasaki (23). In initial screening experiments to confirm that exogenous constructs were producing sense mRNAs in the cell lines, PCR was performed using a forward primer internal to the POU domain (Brn-3a, 5'-GACCTGGGACACCGACCCCGG-3'; Brn-3b, 5'-GACCTGGATGCACCCCGG-3'); Brn-3c, 5'-GATGTTGGATGCACCCCG-3') and a reverse primer internal to the vector sequence (5'-AGATGTCGTTACCATCATG-3') so as not to amplify endogenous Brn-3mRNA, with product detected by Southern hybridization using appropriate probes. cDNA from clones capable of expressing the exogenous construct were subsequently subjected to PCR to determine the total level of expression of that member of the Brn-3 family by using forward and reverse primers internal to the POU domain as described by Kawasaki (23). In initial screening experiments to confirm that exogenous constructs were producing sense mRNAs in the cell lines, PCR was performed using a forward primer internal to the POU domain (Brn-3a, 5'-GACCTGGGACACCGACCCCGG-3'; Brn-3b, 5'-GACCTGGATGCACCCCGG-3'); Brn-3c, 5'-GATGTTGGATGCACCCCG-3') and a reverse primer internal to the vector sequence (5'-AGATGTCGTTACCATCATG-3') so as not to amplify endogenous Brn-3mRNA, with product detected by Southern hybridization with appropriate probes. cDNA from clones capable of expressing the exogenous construct were subsequently subjected to PCR to determine the total level of expression of that member of the Brn-3 family by using forward and reverse primers internal to the POU domain as described by Kawasaki (23). In initial screening experiments to confirm that exogenous constructs were producing sense mRNAs in the cell lines, PCR was performed using a forward primer internal to the POU domain (Brn-3a, 5'-GACCTGGGACACCGACCCCGG-3'; Brn-3b, 5'-GACCTGGATGCACCCCGG-3'); Brn-3c, 5'-GATGTTGGATGCACCCCG-3') and a reverse primer internal to the vector sequence (5'-AGATGTCGTTACCATCATG-3') so as not to amplify endogenous Brn-3mRNA, with product detected by Southern hybridization with appropriate probes. cDNA from clones capable of expressing the exogenous construct were subsequently subjected to PCR to determine the total level of expression of that member of the Brn-3 family by using forward and reverse primers internal to the POU domain as described by Kawasaki (23).

RESULTS

When ND7 cells are induced to differentiate by removal of serum, the level of Brn-3a rises and that of Brn-3b falls (Fig. 1a). To test the effect of Brn-3b on the differentiation of ND7 cells we used cell lines prepared by stably transfecting ND7 cell lines with cDNA clones for Brn-3a, Brn-3b or Brn-3c under the control of the dexamethasone inducible MMTV promoter, allowing us to regulate the level of Brn-3 in each cell line by
steroid treatment. Three cell lines of each type selected on the basis of their neomycin resistance (encoded on a co-transfected plasmid) were used to control for clonal variation. Each showed basal and steroid inducible expression of the appropriate transfected form of Brn-3 in cells proliferating in full-serum containing medium as assayed by PCR using one primer specific for the RNA transcript derived from a transfect plasmid and one primer specific for the appropriate form of Brn-3 (Fig. 1b). Similarly increased basal levels and steroid inducibility of each form of Brn-3 in the proliferating cells could be demonstrated by PCR using primers which would specifically amplify both the endogenous and exogenous mRNAs encoding each form of Brn-3 (Fig. 1b). Most importantly the levels of the exogenous Brn-3 in each cell line were unaffected by the induction of cell differentiation by removal of serum. Thus the fall in endogenous Brn-3b that occurs in these circumstances (see Fig. 1a) in all the cells resulted in the Brn-3b-transfected cells having a much higher level of Brn-3b than control cells following exposure to a differentiation-inducing stimulus (Fig. 1b). Moreover, the differentiated Brn-3a-transfected cells still maintained a higher total level of Brn-3a than control cells (Fig. 1b) despite the rise in endogenous Brn-3a levels that occurs upon differentiation (see Fig. 1a) (3, 15). Similarly, enhanced levels of Brn-3c were also observed in the Brn-3c transfected cells (Fig. 1b).

We therefore investigated the effect of Brn-3a, Brn-3b, or Brn-3c over expression on the outgrowth of neurites which normally occurs following removal of serum from the ND7 cells (18). Cells were induced to differentiate by removal of serum and the effect of each form of Brn-3 assessed. As shown in Fig. 2 and Table 1 the Brn-3a- and Brn-3c-expressing cell lines exhibited enhanced neurite length compared to control cells following the induction of differentiation and exposure to steroid treatment to fully induce expression of Brn-3a or Brn-3c (p < 0.05 for Brn-3a or Brn-3c cell lines compared to vector, in a paired t test). This extends our previous conclusion (19) that Brn-3a and to a lesser extent Brn-3c can induce neurite outgrowth even in full serum-containing medium and indicates that exogenous Brn-3a can enhance outgrowth even in the presence of the enhanced levels of endogenous Brn-3a produced by the differentiation-inducing stimulus.

In contrast however, all three Brn-3b-expressing cell lines showed decreased levels of neurite outgrowth compared to control cells following induction of differentiation even without steroid treatment (Fig. 2 and Table 1, p < 0.001 in a paired t test). Following steroid treatment, these cells showed a further reduction in neurite length which was significant both in comparison to the neurite length observed in these cells in the absence of steroid treatment (p < 0.01 in a Mann Whitney test) and by comparison to the neurite length in similarly treated control cells (p < 0.01 in a paired t test) (Fig. 2b and Table 1). This resulted in these processes being considerably shorter.

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In all cases values are the mean ± the standard error of the mean obtained in triplicate determinations of neurite length in each of three different clonal cell lines of each type.

| Brn-3 isoform | Average neurite length when differentiated in the absence of dexamethasone μm | Average neurite length when differentiated in the presence of dexamethasone μm |
|---------------|------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Control vector | 55 ± 9                                                                      | 58.6 ± 13                                                                     |
| Brn-3a        | 62 ± 10                                                                     | 72.7 ± 15                                                                     |
| Brn-3b        | 46 ± 2                                                                     | 36 ± 11                                                                      |
| Brn-3c        | 49 ± 13                                                                     | 71 ± 9                                                                      |

The first promoter that is inducible by all three forms of Brn-3 (26). As expected no change in GAP43 expression was noted in the Brn-3b-expressing cells (Fig. 4b), paralleling our previous findings that neither Brn-3a or Brn-3b alter the level of this protein in undifferentiated ND7 cells or regulate its promoter in co-transfections (19).

Hence as well as reducing the length of neurite processes, which form upon differentiation, Brn-3b can also inhibit some but not all of the changes in gene expression that occur during this process. In previous experiments, using chimeric proteins containing different regions of Brn-3a or Brn-3b, we have shown that the POU domain plays a key role in the different effects of these factors on target promoters in co-transfection assays (15, 16). Thus, although the POU domain of both factors can bind to DNA (5, 27), only the isolated POU domain of
Brn-3a can activate the tk-Oct promoter (13), whereas the POU domain of Brn-3b cannot do so. Hence as well as acting as the DNA binding domain, the POU domain of Brn-3a can also act as a distinct activation domain. We therefore prepared ND7 cell lines overexpressing the isolated POU domain of Brn-3a or Brn-3b and tested their effect on neurite outgrowth. As shown in Fig. 5 the differentiated cells expressing the Brn-3a POU domain showed a similar enhancement of neurite length compared to control cells as did those expressing full-length Brn-3a (p < 0.05 in a paired t test comparing Brn-3a POU domain cells with controls). Moreover, the differentiated cells expressing the POU domain of Brn-3b showed a similar steroid inducible reduction in neurite length as occurred in cells expressing full-length Brn-3b (p < 0.01 in a paired t test comparing Brn-3b POU domain cells with controls). As expected no effects on the number of processes formed were observed in the cells overexpressing the POU domain paralleling the lack of effect of the full-length factors in differentiated cells (Fig. 6).

Interestingly, as well as affecting neurite length in a similar fashion to the full-length protein, the POU domains also either enhanced (Brn-3a) or reduced (Brn-3b) the expression of SNAP-25 in a manner similar to that observed in differentiated cells expressing the full-length factors (Fig. 7). Hence the Brn-3b POU domain appears to be able to inhibit process outgrowth and SNAP-25 expression, whereas the Brn-3a POU domain has the opposite effect.

This indicates that one or more of the seven amino acid differences between the POU domains of Brn-3a and Brn-3b (3, 16) plays a critical role in this effect. Six of these differences are in the relatively nonconserved linker region joining the POU-specific and POU homeodomains, which together make up the POU domain (7, 8). We have therefore focused our attention on the single difference in the POU homeodomain at position 22 and have shown that this plays a critical role in the different effects of Brn-3a and Brn-3b on a target promoter in co-transfection assays. Thus alteration of the isoleucine at this position in Brn-3b to the valine found in Brn-3a allows the mutant Brn-3b to activate the tk-Oct promoter (28).

We therefore tested the effect of overexpressing mutant forms of full-length Brn-3a or Brn-3b in which the amino acid
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Moreover, cells expressing Brn-3a with isoleucine instead of valine at position 22 showed a much smaller enhancement of SNAP-25 expression than did cells expressing intact Brn-3a (Fig. 7). More dramatically, these cells not only failed to show the enhancement of process formation characteristic of Brn-3a but actually showed a steroid-inducible reduction in process formation compared to control differentiated cells which was almost as large as that observed in cells expressing Brn-3b (Fig. 5). Thus the mutant Brn-3a isoleucine shows a dramatically different effect on neurite outgrowth compared to wild type Brn-3a ($p < 0.01$ for Brn-3a isoleucine compared to Brn-3a in a paired $t$ test). Hence alterations at a single amino acid can dramatically affect the ability of Brn-3a or Brn-3b to regulate ND7 cell differentiation.

**DISCUSSION**

Our previous studies (14, 19) have shown that the overexpression of Brn-3a can enhance both the number and length of neurite processes, which form in ND7 cells under conditions that do not normally promote differentiation, and can also activate the expression of several genes encoding proteins involved in the synaptic vesicle cycle. The differentiation process in ND7 cells, however, normally involves both a rise in the level of Brn-3a and a fall in the level of Brn-3b (3, 15). In the experiments reported here we have investigated the role of Brn-3b in this process and have shown that, under conditions which normally promote differentiation, overexpression of Brn-3b can reduce the length of the neurites that form and reduce the expression of some but not all of the genes that are activated during Brn-3a-induced differentiation.

These findings indicate therefore that regulation of ND7 cell differentiation involves a balance between the differentiation-inducing factor Brn-3a and the inhibitory factor Brn-3b. As Brn-3a and Brn-3b are expressed in distinct but overlapping patterns in the developing and adult nervous system (1–3, 5, 11, 12), it is possible that they play a similar role in regulating neurite outgrowth during development and in response to nerve injury in the adult.

Although further studies will be required to confirm this possibility, our findings clearly establish Brn-3b as the first transcription factor to have an inhibitory effect on neurite outgrowth. Our recent identification of the gene encoding the neuronal nicotinic acetylcholine receptor α2 subunit as the first example of a gene whose promoter is activated by Brn-3b and not Brn-3a (29, 30) raises the possibility that Brn-3b might inhibit neurite outgrowth by activating the expression of other genes whose products play a directly inhibitory role in outgrowth.

However, it is more likely that the inhibitory effect of Brn-3b is brought about by its ability to repress the activity of specific genes. Thus in co-transfection experiments we have previously shown that Brn-3b can repress the basal activity of several different promoters, which are inducible by Brn-3a, and can also interfere with their activation by Brn-3a (13, 15, 16). Moreover, in the experiments reported here the inhibition of neurite outgrowth was accompanied by a reduction in SNAP-25, synaptophysin, and synaptotagmin expression in the Brn-3b-overexpressing cells.

The ability of Brn-3b to prevent the rise in SNAP-25 expression which normally occurs during ND7 cell differentiation is of particular interest since this presynaptic vesicle protein (31) has itself been shown to be necessary for process formation by different neuronal cell types both in *vivo* and *in vitro* (20). It is likely therefore that the ability to inhibit the expression of SNAP-25 and potentially of other genes whose protein products are involved in the process of neurite outgrowth underlies the inhibitory effect of Brn-3b on process formation.
This association of the inhibitory effect of Brn-3b on specific gene expression and on neurite outgrowth is further supported by the critical role of the amino acid at position 22 in the homeodomain in this process. Thus alteration of the isoleucine at this position to the valine in Brn-3b prevents the inhibitory effect of Brn-3b not only on a co-transfected promoter (28) but also, as shown here, on endogenous SNAP-25 expression and on neurite outgrowth. Moreover, the reciprocal substitution in Brn-3a not only abolishes its ability to enhance neurite outgrowth but actually allows it to inhibit neurite outgrowth during differentiation.

We previously suggested (28) that Brn-3b may inhibit Brn-3a-induced genes simply by binding to the DNA and being unable to activate transcription. It would therefore passively inhibit gene expression by preventing Brn-3a binding to the DNA. In this model position 22 in the homeodomain which is known to have no effect on DNA binding (28) would be involved in allowing Brn-3a to act as an activator perhaps by allowing Brn-3a to recruit a second activating factor (for further discussion, see Dawson et al. (28)), while Brn-3b could not do so. However, the finding that mutation of this position in Brn-3a allows it to actually inhibit neurite outgrowth suggests that Brn-3b (and the mutant Brn-3a) may actually act as an active repressor by interacting with wild type Brn-3a or the basal transcriptional complex (for discussion of the mechanisms of transcriptional repression see Refs. 32 and 33). In this case isoleucine at this position would allow this inhibitory interaction to occur, whereas valine would not do so.

Whatever the precise mechanism of this effect, however, it is already clear that Brn-3b is the first example of a transcription factor which can inhibit neurite outgrowth. The further analysis of this factor and its interaction with Brn-3a should greatly enhance our understanding of the mechanisms that regulate neurite outgrowth in development and may potentially have therapeutic implications for the treatment of human spinal injury when nerve regeneration is very poor.

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