The DNA target site for the Brn-3 POU family transcription factors can confer responsiveness to cyclic AMP and removal of serum in neuronal cells

V.Budhram-Mahadeo, T.Theil, P.J.Morris, K.A.Lillycrop, T.Moroy and D.SLatchman*
Medical Molecular Biology Unit, Department of Molecular Pathology, University College London Medical School, The Windyfer Building, Cleveland Street, London W1P 6DB, UK and 1Institut fur Molekularbiologie und Tumorforschung, Philippus Universitat Marburg, Emil-Mankopf-Strasse 2, D-35037 Marburg, Germany

Received April 28, 1994; Revised and Accepted July 8, 1994

ABSTRACT
The POU factors Brn-3a and Brn-3b are closely related transcription factors which are expressed in neuronal cells. The levels of the transcripts encoding these factors are regulated in opposite directions in neuronal cells by specific cellular signalling pathways with dibutyryl cyclic AMP treatment and serum removal enhancing the level of Brn-3a and reducing the level of Brn-3b expression. This opposite expression pattern is paralleled by the ability of Brn-3a to specifically trans-activate a target promoter bearing its DNA binding site whereas this promoter is repressed by Brn-3b. As predicted from these observations this target promoter is strongly activated by serum removal or addition of dibutyryl cyclic AMP. Therefore changes in Brn-3a and b expression can have a functional effect on promoter activity indicating that Brn-3a and Brn-3b can regulate gene expression via a specific binding site in response to the activation of specific cellular signalling pathways. The reasons for the differences in activity between these two related factors and their role in regulating gene activity in the nervous system are discussed.

INTRODUCTION
The POU (Pit-Oct-Unc) family of transcription factors was originally defined on the basis of a common 150–160 amino acid DNA binding domain which was present in the mammalian transcription factors Pit-1, Oct-1 and Oct-2 and in the nematode regulatory protein encoded by the unc-86 locus (for reviews see 1–3). It was subsequently shown that these POU factors played a critical role in regulating growth and differentiation in a number of different cell types, particularly those of neuronal origin. Thus in the nematode the unc-86 mutation results in the failure to develop specific neuronal cell types particularly sensory neurons (4, 5). Similarly, the Pit-1 factor is essential for the correct development of the mammalian pituitary gland and inactivation of Pit-1 by mutation results in congenital dwarfism in both mice (6) and humans (7).

The important role of the founder members of the POU family in neuronal cells led to a search for other members of this family expressed in these cells. Polymerase chain reaction (PCR) experiments using degenerate oligonucleotides derived from the conserved region of the POU domain led to the isolation of several mRNAs encoding novel members of the POU family expressed in the mammalian brain (8).

A similar approach to isolate cDNA clones for the POU factors expressed in the immortalized ND7 cell line derived from sensory neurons (9) led to the isolation of several cDNA clones derived from Brn-3, one of the factors originally isolated by He et al., (8). In addition however, we also isolated several clones which were closely related to Brn-3 but differed at seven amino acids within the POU domain (10). The factor encoded by these clones therefore represents a novel member of the POU family which was designated Brn-3b to distinguish it from the original Brn-3 factor (8) which we now refer to as Brn-3a.

The distinction between these two factors has been confirmed by the isolation of full length cDNA clones of murine Brn-3a and b (11) and of human Brn-3a (also called RDC-1: 12) and Brn-3b (13) which indicate that they are derived from distinct genes showing only limited homology outside the POU domain. The close homology between Brn-3a and b in the POU domain has led to their classification as a separate sub-family (Group IV) within the POU domain together with the product of the unc-86 gene and the Drosophila factors I-POU and twin of I-POU (3).

Despite the strong homology within their POU domains, Brn-3a and b show distinct expression patterns in the developing rodent (10) and human (12, 14) brain. Moreover, in our initial experiments (10) the two factors showed opposite changes in expression pattern during the in vitro differentiation of the ND7 neuronal cell line. Thus when ND7 cells were differentiated to a non-dividing process bearing phenotype by transfer to serum
free medium (9, 15), the expression level of Brn-3a rose dramatically whilst the level of Brn-3b declined (10). Similarly, Brn-3a has been shown to co-operate with Ha-ras in transforming rat primary cells whereas Brn-3b does not have this property and inhibits the transforming action of Brn-3a (11).

This finding and the opposite regulation of Brn-3a and Brn-3b expression during the in vitro differentiation of ND7 cells suggested that these two POU factors might have antagonistic effects on target gene expression similar to the closely related Drosophila POU factors I-POU and twin of I-POU (16, 17). We have therefore further investigated the regulation of Brn-3a and b expression in neuronal cells and their effect on the activity of a target promoter.

MATERIALS AND METHODS

Cell culture
ND7 cells (9) were grown in L15 medium (Gibco) with 10% foetal calf serum, 0.3% D-glucose, 0.37% sodium bicarbonate, 0.2 mM L-glutamine and 1.0% penicillin/streptomycin (10,000 units per ml). To differentiate the cells to a non-dividing phenotype, they were transferred to DMEM/HAMS (1:1, Gibco) medium with 5 μg/ml transferrin, 250 ng/ml bovine insulin, 30 mM sodium selenite in the absence of serum. Treatment with 1 mM dibutyryl cyclic AMP was carried out for the time indicated.

RNA isolation
RNA was isolated as previously described (18) and used as a template for the preparation of cDNA using random hexanucleotide primers (Pharmacia LKB Biotechnology Ltd). The cDNA equivalent to 0.01 μg of total RNA was amplified by polymerase chain reaction (PCR) according to the method of Kawasaki (19). Amplification of Brn-3a and b cDNAs was carried out with the common primers 5'GTGGGCTCGGCGCTGGCC3' and one or other of two specific primers, 5'CGGGGTTGGTGACGGCAAAA3' (Brn-3a) or 5'CTTGGCTGGATGGCGAAGAGCTTCCTCCAGCA-CGC3'.

In initial experiments a series of dilutions of the competitors was used to determine the concentration which would produce an approximately equimolar level of the normal PCR product and the competitor product. This was then used in all subsequent experiments.

Transient infections
Transfections were carried out according to the calcium phosphate method of Gorman (23). Cells were exposed to the calcium phosphate precipitate for three hours, washed and transferred to fresh medium with or without 1 mM dibutyryl cyclic AMP. In all cases cells were harvested seventy two hours later. The amount of DNA taken up by the cells in each case was measured by slot blotting the extract and hybridization with a probe derived from the ampilicillin resistance gene in the plasmid vector (24). This value was then used to normalize the values obtained in the CAT assay as a control for differences in uptake of plasmid DNA in each sample.

Cat assay
Assays of chloramphenicol acetyl transferase activity were carried out according to the method of Gorman (23) using samples which had been equalized for protein content as determined by the method of Bradford (25).

Plasmid DNA
The test plasmid contains the Brn-3 binding site ATGCTAAT-GAGAT cloned into the Bam HI site in the vector pBL CAT 2 which contains the herpes simplex virus thymidine kinase promoter from −105 to +51 driving expression of the chloramphenicol acetyl transferase (CAT) gene (26). The Brn-3a and b expression vectors contain full length cDNA or genomic clones for each of these proteins (11) cloned under the control of the Moloney murine leukaemia virus promoter in the vector pLTR poly which has been modified by deletion of a cryptic splice site in the SV40 3' untranslated region (27). In the case of Brn-3a, two different expression vectors were used containing cDNAs or genomic clones derived from the longer spliced form of the Brn-3a mRNA and from a shorter intronless mRNA which encodes a shorter protein (11). The Brn-3b vector contains a full length cDNA derived from the intronless mRNA which encodes this protein (11).

RESULTS

In our previous experiments (10) a larger increase in the level of the Brn-3a mRNA was observed in ND7 cells differentiated by transfer to low serum-containing medium (0.5% foetal calf serum) together with 1 mM dibutyryl cyclic AMP than in cells that were differentiated by transfer to serum free medium in the absence of dibutyryl cyclic AMP. However, we had not previously investigated whether the level of the Brn-3a mRNA could be modulated by treatment with dibutyryl cyclic AMP alone. We therefore added 1 mM dibutyryl cyclic AMP to ND7 cells growing in serum-containing medium (10% foetal calf serum) and measured the effect on the expression levels of Brn-3a and Brn-3b (Figure 1a and b). The levels of the Brn-3a and b mRNAs were measured as before by reverse transcriptase-
polymerase chain reaction amplification using an upstream primer whose sequence is common to the POU domain of each factor and a pair of downstream oligonucleotides corresponding to the region of the POU domain which is most diverged between the two factors (10). Both these primer pairs showed similar amplification efficiencies when tested with a given amount of template and a similar number of amplification cycles was used in all experiments. Hence the levels of the Brn-3a and Brn-3b mRNAs observed in our experiments can be directly compared with one another. Variations between different samples in the total amount of RNA or in the efficiency of reverse transcription were equalized by carrying out control amplifications using primers specific for the constitutively expressed transcripts for glucose-6-phosphate dehydrogenase or the ribosomal L6 protein.

In these experiments (Figure 1), cells grown in full serum-containing medium with added dibutyryl cyclic AMP showed a clear increase of Brn-3a expression over the low level normally present in cells growing in full serum-containing medium in the absence of cyclic AMP. In contrast the level of Brn-3b expression declined in cells treated with dibutyryl cyclic AMP whereas the level of the control mRNA encoding the ribosomal L6 protein remained unchanged. Similarly the removal of serum resulted in an increase in Brn-3a mRNA levels and a fall in that of Brn-3b (Figure 1) in accordance with our previous results (10). The combined treatment of removing serum and adding cyclic AMP also resulted in an increase in the level of the Brn-3a mRNA and a fall in that of Brn-3b (Figure 1b). The two treatments did not have a synergistic effect however, with the elevation observed in Brn-3a with both treatments being only marginally above that obtained with each treatment independently whilst the fall in the Brn-3b mRNA was similar to that observed upon serum removal in the absence of cyclic AMP.

Hence ND7 cells show an increase in the level of Brn-3a expression and a decline in the level of Brn-3b expression in response to dibutyryl cyclic AMP treatment or serum removal. This results in a significant change in the ratio of Brn-3a to Brn-3b transcripts as detected by PCR with a common oligonucleotide and a pair of discriminatory oligonucleotides.

The comparative PCR method described above, controls for any variations in the amount of total RNA in each sample or in the efficiency of the reverse transcriptase reaction by comparing the level of product produced from the Brn-3 RNAs with that for the mRNAs encoding the constitutively expressed G6PDH or ribosomal L6 proteins. This method does not control however, for tube to tube variations in the amplification efficiency of the PCR itself. In order to confirm the data obtained in this way we
therefore used the method of Celi et al., (21) to synthesize Brn-3a or Brn-3b templates which when amplified would produce a product approximately one hundred bases shorter than the cDNA produced from the natural Brn-3a or Brn-3b mRNAs. Co-amplification of an equal amount of these competitor templates with the experimental samples can therefore provide an internal control for the efficiency of the PCR reaction with the competitor template being distinguishable from the product of the natural mRNA on the basis of its smaller size (22).

These experiments confirmed the results of our earlier experiments using comparative PCR with a clear increase in the Brn-3a mRNA and fall in the Brn-3b mRNA being observed following dibutyryl cyclic AMP treatment or serum removal although the levels of the internal control template remained the same indicating a similar efficiency of PCR amplification in each case (see for example Figure 2). Overall, in four replicate experiments, an increase in the Brn-3a to Brn-3b ratio was observed at twenty four, forty eight and seventy two hours of exposure to cyclic AMP or at similar times following serum removal.

It is clear therefore that both the removal of serum and the addition of cyclic AMP can result in an increase in the level of the Brn-3a mRNA and a decline in the level of the Brn-3b mRNA.

To understand the significance of these changes we wished to investigate the functional activity of Brn-3a and Brn-3b. To do this we inserted the octamer-related motif ATGCTAATGAGAT upstream of the herpes simplex virus thymidine kinase promoter in the vector pHL CAT 2 (26). This motif was chosen since it binds the POU domain of both Brn-3a and Brn-3b (11 and unpublished observations) but not the neuronal octamer binding protein Oct-2 (28).

The resulting CAT reporter construct was co-transfected into BHK-21 fibroblast cells (29) which lack significant endogenous levels of Brn-3a or Brn-3b, together with either the empty expression vector alone (11) or expression plasmids containing clones capable of encoding similar levels of functional Brn-3a or Brn-3b protein (11 and Theil and Moroy, submitted). In these experiments, (Figure 3a and b) a strong stimulation of CAT activity was observed upon co-transfection of the reporter construct with the Brn-3a expression plasmid. A similar stimulation of activity was observed using two distinct Brn-3a expression plasmids encoding either a longer spliced form of the Brn-3a mRNA and a shorter intronless form which encodes a smaller protein that lacks the 84 amino terminal residues but is otherwise identical to the longer protein (11) (Figure 3b). In contrast the Brn-3b expression plasmid consistently repressed activity approximately three fold below that observed with plasmid vector alone (Figure 3a and b) and was also capable of blocking the stimulation of the target promoter by Brn-3a when
the two forms were transfected together confirming that it expressed functional Brn-3b protein (Figure 3b).

Thus the long and short Brn-3a proteins (11) are capable of stimulating the activity of a promoter carrying the appropriate binding site whereas Brn-3b represses such a promoter. This observation suggested that factors such as serum removal and dibutyryl cyclic AMP treatment which produce a rise in the Brn-3a to Brn-3b ratio, should lead to activation of this target promoter. To test this prediction we transfected ND7 cells with the same CAT reporter construct in the presence or absence of ten per cent foetal calf serum and in the presence or absence of 1 mM dibutyryl cyclic AMP.

In these experiments a clear stimulation of promoter activity was observed in the cells deprived of serum (Figure 4) and in the cells treated with dibutyryl cyclic AMP (Figure 5). This effect was dependent upon the presence of the ATGCTAATGAGAT Brn-3 binding site since it was not observed with the parental pBL CAT 2 plasmid alone. Apart from Brn-3, the only other POU factors expressed by ND7 cells are Oct-1 and Oct-2 (10). We have previously shown that the neuronal form of Oct-2 cannot bind to the target site used here (28) whilst although Oct-1 can...
bind, it has no significant effect on promoter activity (our unpublished data) consistent with its ability to act as only a very weak transactivator (30).

Hence Brn-3a and Brn-3b are the only proteins present in ND7 cells which can influence the activity of the target site in the promoter we use. This indicates that effectors which produce a change in the ratio of endogenous Brn-3a and b can indeed modulate the activity of a target promoter which is activated by Brn-3a and repressed by Brn-3b in co-transfection experiments. Moreover, the binding site for Brn-3a and Brn-3b can act as a response element modulating gene expression in response to serum or dibutyryl cyclic AMP.

**DISCUSSION**

Both the Brn-3a and Brn-3b transcription factors have previously been reported to be expressed in neuronal cells within the developing rat (10), mouse (11, 12) and human (12, 14) nervous systems. Here we show that the levels of the mRNAs encoding these two proteins are regulated in opposite directions by treatment of ND7 cells with dibutyryl cyclic AMP and serum. Moreover, a binding site for these two factors can modulate promoter activity in opposite directions in response to addition of dibutyryl cyclic AMP or serum to neuronal ND7 cells.

Interestingly these effects may be cell type specific since Turner et al., (31) observed only a slight decrease in the mRNA encoding Brn-3b (which they refer to as Brm-3.2) in response to cyclic AMP treatment of F9 embryonal carcinoma cells with a slight increase being observed following similar treatment of N18 neuroblastoma cells. Similarly they observed only a slight rise in the mRNA encoding Brn-3a (which they refer to as Brn-3.0) following cyclic AMP treatment of N18 cells (31). Since N18 cells represent one of the two parents of the ND7 hybrid cell line (9), this suggests that the strong response of the Brn-3a and Brn-3b mRNAs to cyclic AMP which we observe is a property derived from the primary dorsal root ganglion neuron fusion partner, although further experiments will be required to confirm this.

Regulation by cyclic AMP treatment has previously been reported to play a critical role in the functioning of the POU factors Pit-1 and SCIP (32, 33) neither of which are expressed in ND7 cells (10). Synthesis of SCIP (also known as Oct-6 or Vmw65 which is normally an exclusive property of Oct-1 only (unpublished observations). This is in agreement with the finding that Brn-3a and b can interact to form heterodimers.

In both these cases, the precise ratio of Brn-3a to Brn-3b in the cell would play a critical role in the level of target gene activity. This point is emphasized by our finding that Brn-3b can prevent the stimulatory effect of Brn-3a on target gene activity in co-transfections (Figure 3b) and can also inhibit the ability of Brn-3a to activate transcription following DNA binding.

This antagonistic activity of two factors which can both bind to DNA could lead to competition between the factors for binding sites in any common target genes. Similarly in view of the ability of POU factors to heterodimerize with one another (38) it is possible that Brn-3a and b can interact to form heterodimers. In both these cases, the precise ratio of Brn-3a to Brn-3b in the cell would play a critical role in the level of target gene activity. This point is emphasized by our finding that Brn-3b can prevent the stimulatory effect of Brn-3a on target gene activity in co-transfections (Figure 3b) and can also inhibit the ability of Brn-3a to activate transcription following DNA binding.

In contrast to I-POU and twin of I-POU which are alternatively spliced products of the same gene and differ by only two amino acids (16, 17), Brn-3a and Brn-3b differ by seven amino acids in the POU domain (10), show extensive differences outside the POU domain (11, 13) and are encoded by two distinct genes (11). These differences may result in only Brn-3a containing a functional activation domain allowing it to directly stimulate gene transcription following DNA binding. Interestingly however, the only amino acid difference between Brn-3a and b in the highly conserved POU homeobox is at position 22 in the first helix which has been shown to be critical in controlling the protein—protein interactions of the POU factors. Thus substitution of the alanine found at this position in the Oct-2 POU factor with the glutamic acid found in the Oct-1 factor confers on Oct-2 the ability to heterodimerize with the herpes simplex virus trans-activator Vmw65 which is normally an exclusive property of Oct-1 only (39). Hence Brn-3a and b may differ in their ability to interact with other proteins allowing Brn-3a but not Brn-3b to function indirectly by recruiting an activating factor to the gene promoter.

In agreement with this idea we have shown that the exchange of the POU domains between Brn-3a and b confers upon Brn-3b the ability to act as an activator of the promoter used here (unpublished observations). This is in agreement with the finding that both the long and short forms of Brn-3a can trans-activate the target promoter used here indicating that this effect is not dependent upon the N-terminus of the protein. In contrast the transforming activity of Brn-3a does appear to be dependent on the N-terminal region since it is a property only of the longer form of the protein (11).
Interestingly a form of Brn-3b with additional N-terminal sequences has recently been detected in the retina (14) and in the CNS (31) although it was absent in the spinal cord (11) raising the possibility that two tissue-specific forms of Brn-3b may exist. Moreover, both Brn-3a (40) and this longer form of Brn-3b (31) have been shown to activate a promoter containing a non octamer motif from the synaptophysin releasing hormone promoter which binds Brn-3. Hence different regions of the Brn-3 proteins may be important for transactivation via different DNA binding sites in different genes with activation via some sites being dependent on the POU domain present in all forms of a specific factor whilst activation via other sites is dependent upon the N-terminal region which is present only in the longer forms of each factor.

Although it remains to be determined whether the Brn-3a and Brn-3b factors modulate gene expression directly or indirectly, it is already clear that these factors are regulated in opposite directions by specific cellular signalling pathways and can have antagonistic effects on the activity of a target promoter. The relative levels of these factors, perhaps in conjunction with the level of a third recently identified member of the Brn-3 family Brn-3c (41) are therefore likely to play a critical role in regulating the activity of specific genes in neuronal cells.

ACKNOWLEDGEMENTS

We thank John Wood for the ND7 cells and much helpful discussion. This work was supported by Action Research; Birthright, the Royal College of Obstetricians and Gynaecologists; The Cancer Research Campaign and the Medical Research Council. VB-M is supported by an SERC Case Studentship. T.M. was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 215, D10) and the Mildred Scheel Stiftung für Krebsforschung.

REFERENCES