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The Opposite and Antagonistic Effects of the Closely Related POU Family Transcription Factors Brn-3a and Brn-3b on the Activity of a Target Promoter Are Dependent on Differences in the POU Domain

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The Brn-3a, Brn-3b, and Brn-3c POU family transcription factors are closely related to one another and are members of the group IV subfamily of POU factors. Here we show that despite this close relationship, the factors have different effects on the activity of a target promoter: Brn-3a and Brn-3c stimulate the promoter whereas Brn-3b represses it. Moreover, Brn-3b can antagonize the stimulatory effect of Brn-3a on promoter activity and can also inhibit promoter activation by the Oct-2.1 POU factor. The difference in the transactivation activities of Brn-3a and Brn-3b is dependent upon the C-terminal region containing the POU domain of the two proteins, since exchange of this domain between the two factors converts Brn-3a into a repressor and Brn-3b into an activator.

The POU (named for Pit, Oct, and Unc) family of transcription factors was originally defined on the basis of a conserved region of approximately 150 to 160 amino acids which was identified in the Pit-1, Oct-1, Oct-2, and Unc-86 regulatory proteins (for reviews, see references 12 and 33). This central POU domain constitutes the DNA binding domain of these proteins and allows them to bind to sequences related to the octamer motif ATGCAAAT in their target genes and thereby influence transcription (33, 35).

In several cases, the modulation of gene expression by POU factors has been shown to play a critical role in the development of specific cell types. Thus, of the original POU family members, the Pit-1 factor has been shown to be essential for the correct development of the pituitary gland, and its inactivity results in congenital dwarfism in both mice and humans (14, 24). Similarly the *unc*-86 mutation in the nematode results in the absence of specific neuronal cell types (7).

The critical roles identified for the initial members of the POU family led to efforts aimed at isolating novel members of this family. For example, He et al. (11) used degenerate oligonucleotides derived from conserved regions of the POU domain in a PCR in order to amplify and then clone the mRNAs for any POU factor expressed in the brain. Subsequently, we used a similar approach to isolate cDNA clones for POU factors expressed in the immortalized ND7 cell line derived from sensory neurons (36).

In that experiment (15) we isolated several cDNA clones derived from the Brn-3 POU factor originally isolated by He et al. (11). However, we also isolated several clones which were closely related to Brn-3 but had seven amino acid differences from Brn-3 within the POU domain (15). Hence, the factor encoded by these clones represents a novel POU family member which we refer to as Brn-3b, to distinguish it from the original factor isolated by He et al. (11), which we refer to as Brn-3a (15). A third member of the Brn-3 family, Brn-3c, has recently been isolated by using a similar approach (23).

The close homology of the different forms of Brn-3 within the POU region isolated in the original experiments (11, 15, 23) has led to their being grouped in a separate subfamily (group IV) amongst the POU proteins, together with the product of the *unc*-86 gene and the *Drosophila* factors I-POU and twin of I-POU (35). Interestingly, however, the isolation of full-length murine Brn-3a, Brn-3b, and Brn-3c cDNA clones has shown that the three factors are less closely related outside the POU domain and are encoded by three different genes (29). A similar conclusion has been reached by comparing the cDNA clones for human Brn-3a (also known as RDC-1) (3) and Brn-3b (25).

The existence of three different closely related forms of Brn-3 suggests that they may have different functions. In previous experiments, the POU domains of all these forms of Brn-3 were shown to direct binding specifically to various octamer motifs (29) as well as to other related motifs (8). We have therefore investigated the functional effect of each of the Brn-3 forms on the activity of a target promoter containing an added octamer motif.

MATERIALS AND METHODS

Plasmid DNA. The target plasmids contain the octamer-related motif ATGCAATT, either alone or linked to the adjacent NFI site in the human papillomavirus type 16 (HPV-16) promoter (4, 21), cloned into the *Bam*HI site in the vector pBLCAT2, which contains the herpes simplex virus thymidine kinase promoter from positions −105 to +51 driving the expression of the chloramphenicol acetyltransferase (CAT)

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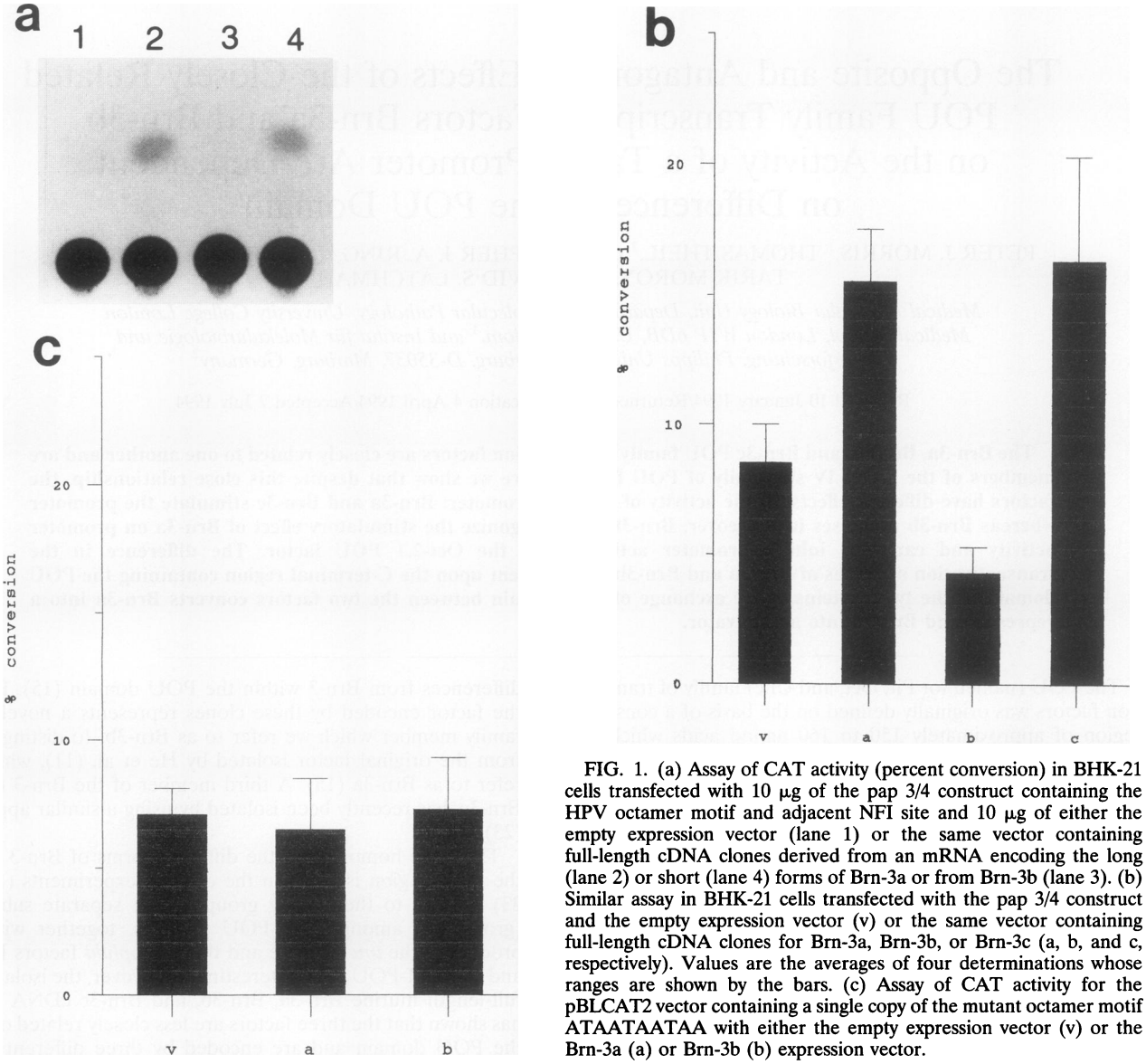

FIG. 1. (a) Assay of CAT activity (percent conversion) in BHK-21 cells transfected with 10 μg of the pap 3/4 construct containing the HPV octamer motif and adjacent NFI site and 10 μg of either the empty expression vector (lane 1) or the same vector containing full-length cDNA clones derived from an mRNA encoding the long (lane 2) or short (lane 4) forms of Brn-3a or from Brn-3b (lane 3). (b) Similar assay in BHK-21 cells transfected with the pap 3/4 construct and the empty expression vector (v) or the same vector containing full-length cDNA clones for Brn-3a, Brn-3b, or Brn-3c (a, b, and c, respectively). Values are the averages of four determinations whose ranges are shown by the bars. (c) Assay of CAT activity for the pBLCAT2 vector containing a single copy of the mutant octamer motif ATAATAATAA with either the empty expression vector (v) or the Brn-3a (a) or Brn-3b (b) expression vector.

gene (18). The mutant octamer motif plasmid was made by similar cloning of the sequence ATAATAATAA, which has been shown not to bind octamer-binding proteins (5, 13a), into the *Bam*HI site of pBLCAT2. The Brn-3a, Brn-3b, and Brn-3c expression vectors contain full-length cDNA clones for each of these proteins (29) cloned under the control of the Moloney murine leukemia virus promoter in the vector pLTRpoly, which has been modified by deletion of a cryptic splice site in the simian virus 40 3′ untranslated region (17). In the case of Brn-3a, two different expression vectors containing cDNAs derived from the longer spliced form of the Brn-3a mRNA and from a shorter intronless form of the mRNA which encodes a shorter protein were used. The Brn-3b vector contains a full-length cDNA derived from the intronless RNA which encodes Brn-3b, while the Brn-3c vector similarly contains a full-length cDNA derived from the mRNA encoding Brn-3c which is produced from the primary transcript by the removal of a single intron (29).

The Oct-1 and Oct-2.1 expression plasmids similarly contain full-length cDNA clones for each of these octamer-binding proteins (4, 22, 36) cloned under the control of the cytomegalovirus immediate-early promoter in the vector pJ7 (20).

DNA transfection. Transfection of DNA was carried out according to the method of Gorman (9). Routinely, 10^6 BHK-21 cells (19) were transfected with 10 μg of the reporter plasmid and 10 μg of the Brn-3 expression vectors. In experiments on the effects of Brn-3a and Brn-3b on the modulation of gene activity by Oct-1 or Oct-2.1, 10 μg of the target plasmid was mixed with 10 μg of either Brn-3a or Brn-3b and 10 μg of either the Oct-1 or Oct-2.1 expression vector or of the pJ7 expression vector without any insert. Forty-eight hours after transfection, cells were harvested for CAT assays. Transfections included a control plasmid in which the myeloproliferative sarcoma virus (MPSV) promoter drives expression of the β-galactosidase gene to control for any effects of the experimental plasmids on transfection efficiency or on gene expression driven by an irrelevant promoter.

CAT assays. Assays of CAT activity were carried out as

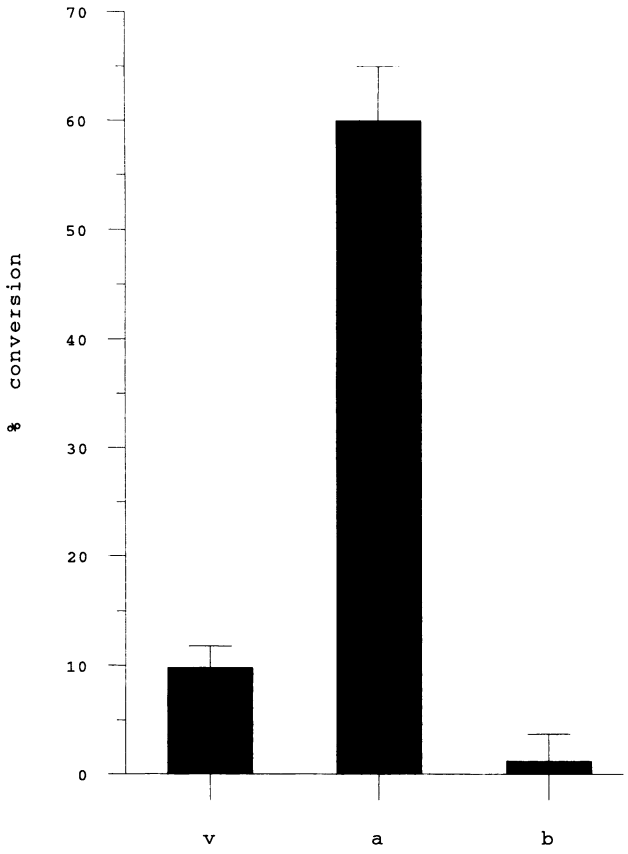

FIG. 2. Assay of CAT activity (percent conversion) upon cotransfection of the pBLCAT2 vector containing five copies of an intact octamer motif with either the empty expression vector (v) or the Brn-3a (a) or Brn-3b (b) expression vector. Values are the averages of two determinations whose ranges are shown by the bars.

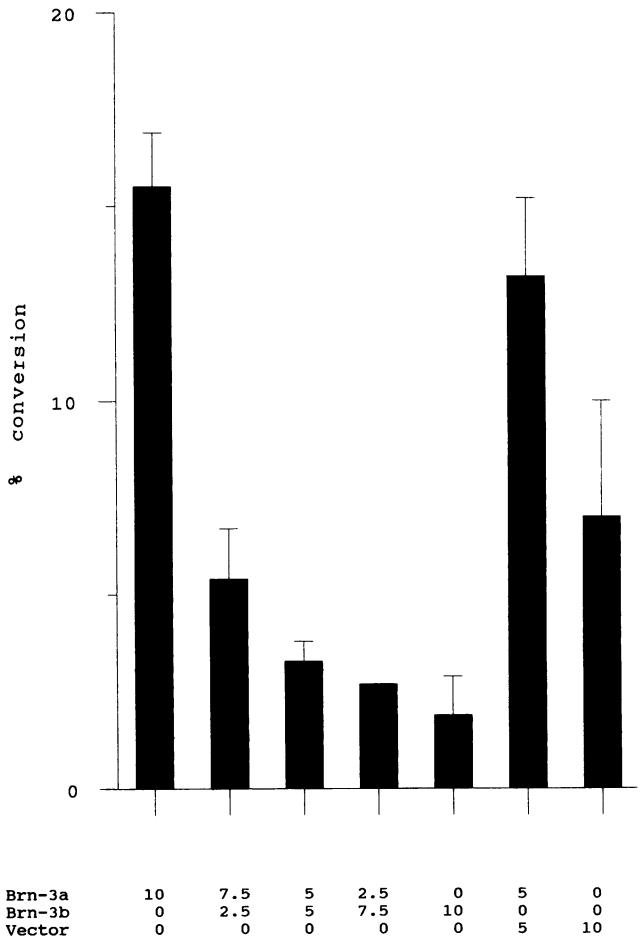

FIG. 3. Assay of CAT activity (percent conversion) for the pap 3/4 construct when cotransfected with the indicated amounts (in micrograms) of either the Brn-3a or Brn-3b expression vector or the empty expression vector. Values are the averages of two determinations whose ranges are shown by the bars.

described by Gorman (9) with extracts which were equalized for protein content, as determined by the method of Bradford (1).

RESULTS

Initially, we cotransfected Brn-3a, Brn-3b, and Brn-3c expression vectors into BHK-21 fibroblast cells together with a reporter plasmid (pap 3/4 [6]) containing an octamer motif (ATGCAATT) and adjacent NFI site derived from the HPV-16 promoter (21, 22) cloned upstream of the thymidine kinase promoter in the vector pBLCAT2. In these experiments (Fig. 1), stimulation (approximately twofold) of CAT activity was observed upon cotransfection of the reporter plasmid with the Brn-3a expression vector, compared with that observed upon cotransfection with the empty expression vector. Enhanced stimulation of promoter activity by Brn-3a was obtained with a plasmid containing five tandem copies of the octamer motif cloned into pBLCAT2, with an approximately sixfold enhancement of promoter activity being observed in this case compared with that obtained with the expression vector alone (Fig. 2). Stimulation of promoter activity was obtained with expression vectors containing cDNAs derived from the longer spliced form of the Brn-3a mRNA and from a shorter intronless form which encodes a truncated protein that lacks the N-terminal 84 amino acids but is otherwise identical to the longer form (29). Hence, both forms of Brn-3a are capable of stimulating promoter activity (Fig. 1a). Similar stimulation was also obtained with Brn-3c, indicating that it is also capable of stimulating promoter activity (Fig. 1b).

In contrast, a reduction in promoter activity was observed when the reporter constructs carrying either single or multiple octamer motifs were cotransfected with the Brn-3b expression vector, compared with the level obtained with the control vector, with an eightfold decline in promoter activity in the presence of Brn-3b being obtained with the construct containing multiple octamer motifs (Fig. 1 and 2). Both stimulation by Brn-3a and Brn-3c and repression by Brn-3b were also obtained with a reporter plasmid (pap 1/2) containing only the HPV octamer-related motif in the absence of the adjacent NFI sequence (data not shown). In contrast, no stimulation of promoter activity by Brn-3a or repression by Brn-3b was observed upon cotransfection with the pBLCAT2 vector alone or with the same vector into which had been inserted a mutant motif containing multiple point mutations in the octamer site which render it incapable of binding Brn-3 or other POU proteins (5, 13a), indicating that this effect was specific to the octamer motif (Fig. 1c).

Hence, Brn-3a and Brn-3c are able to stimulate promoter

activity via the octamer motif, whereas Brn-3b inhibits basal promoter activity. To test whether Brn-3b would inhibit the stimulatory effect of Brn-3a, the pap 3/4 reporter construct was cotransfected with increasing amounts of Brn-3b and correspondingly decreasing amounts of Brn-3a. In these experiments (Fig. 3), Brn-3b was indeed able to inhibit the stimulatory effect of Brn-3a so that when 5 μg of the Brn-3a and 5 μg of the Brn-3b expression vectors were transfected, promoter activity was much lower than that obtained with 5 μg of the Brn-3a vector and 5 μg of the empty expression vector. Indeed, whenever Brn-3b was present at a level equal to or greater than that of Brn-3a, the promoter activity was actually repressed below the level obtained with an equivalent amount of the empty expression vector alone (Fig. 3).

In previous experiments (22), we have shown that the octamer motif in the pap 3/4 construct can also be activated by cotransfection with an expression vector containing a cDNA for the octamer-binding protein Oct-2.1 (16, 36). To investigate the effects of Brn-3a and Brn-3b on this process, 10 μg of the pap 3/4 reporter construct was cotransfected with 10 μg of Oct-2.1 or of the empty plasmid vector in the presence of 10 μg of either the Brn-3a or Brn-3b expression vector.

The presence of Brn-3a or Brn-3b clearly influenced the ability of Oct-2.1 to stimulate the pap 3/4 construct (Fig. 4). The degree of stimulation obtained in the presence of both Brn-3a and Oct-2.1 was considerably greater than that obtained with either factor alone (Fig. 4). Interestingly, Oct-2.1 was able to stimulate the pap 3/4 promoter approximately twofold compared with the levels obtained in the presence of the plasmid vector both in the presence and absence of Brn-3b. However, the degree of promoter activity obtained after Oct-2.1 stimulation was lower in the presence of Brn-3b, indicating that stimulation of the promoter by Oct-2.1 cannot entirely overcome the inhibitory effect of Brn-3b (Fig. 4).

To further investigate the effects of Brn-3a and Brn-3b on the modulation of gene expression by octamer-binding proteins, we used the octamer-binding protein Oct-1. We have previously shown that unlike Oct-2.1, Oct-1 has an inhibitory effect upon the expression of the pap 3/4 construct, since its binding to the octamer motif prevents the binding of the strong transactivator NFI to its adjacent binding site (21). As before, 10 μg of the pap 3/4 construct was therefore cotransfected with either the Brn-3a or Brn-3b expression vector in the presence or absence of the Oct-1 expression vector.

In these experiments (Fig. 5), cotransfection of Oct-1 and Brn-3a was able to reduce the target promoter activity to the basal level obtained in the presence of the empty plasmid vector alone. However, the level of promoter activity obtained in the presence of Oct-1 and Brn-3a was higher than that obtained with Oct-1 alone, indicating that Brn-3a can still enhance promoter activity even in the presence of Oct-1 (Fig. 5). In contrast, it was not possible to assess whether Brn-3b could significantly repress promoter activity in the presence of Oct-1 in these experiments, because of the strong inhibitory effect of Oct-1 alone (Fig. 5). It is likely however, that Brn-3b can do so, since it was able to inhibit the basal promoter activity of the pap 1/2 and pap 3/4 constructs when cotransfected into BHK-21 cells (Fig. 1 and 2), which express significant levels of endogenous Oct-1 (21).

To investigate the basis for the functional difference in activity between Brn-3a and Brn-3b, we utilized various chimeric molecules in which different subdomains of Brn-3a and Brn-3b (designated I to IV) had been exchanged (Fig. 6). Expression vectors which have been shown to direct the

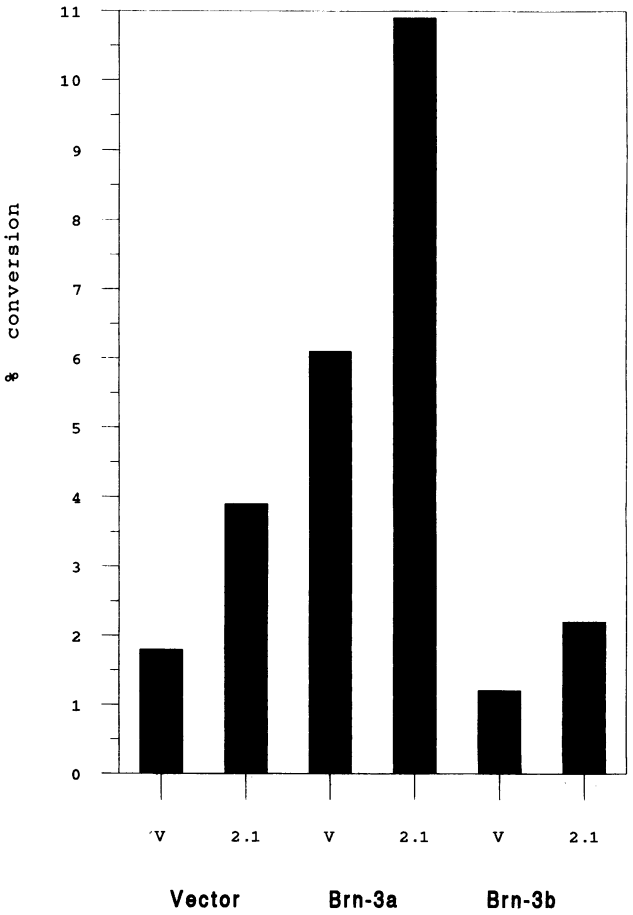

FIG. 4. Assay of CAT activity (percent conversion) for the pap 3/4 construct when cotransfected with 10 μg of either the empty expression vector (v) or the Oct-2.1 expression vector (2.1) together with 10 μg of either the empty expression vector or the Brn-3a or Brn-3b expression vector.

expression of similar amounts of each of these different chimeric proteins in transfection experiments (Fig. 7a) were then cotransfected with the target promoter-bearing plasmid as before. In these experiments (Fig. 7b), the effect on promoter activity of the chimeric Brn-3 molecules was dependent on subdomain IV, which encodes the C-terminal POU domain. Thus, a construct which contains only the POU domain of Brn-3a, with the remainder of the protein derived from Brn-3b (construct 4, which has the pattern -bba), was able to activate gene expression. Similarly, construct 2 (aaba), in which subdomain III was derived from Brn-3b but the other subdomains (I, II, and IV) were derived from Brn-3a, showed a stimulation of target promoter activity similar to that obtained with construct -bba. Conversely, construct 1 (aaab), which has only the POU domain (subdomain IV) derived from Brn-3b, repressed gene expression (Fig. 7). Similar results were obtained with a reporter construct containing multimerized octamer motifs (Fig. 7) and the pap 3/4 construct containing the HPV octamer and the adjacent NFI site (data not shown). Hence, the ability of Brn-3a to stimulate the activity of these target promoters is dependent upon the POU domain, with differences between the POU domains of Brn-3a and Brn-3b resulting in Brn-3b being unable to do so. The constructs which activated gene expression, such as construct 2 (aaba), and those which did not,

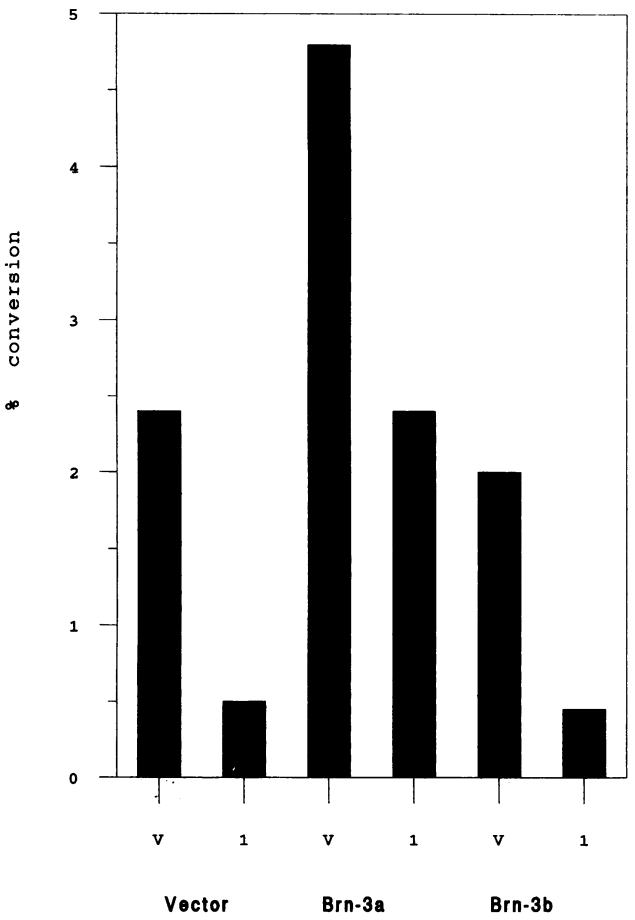

FIG. 5. Assay of CAT activity (percent conversion) for the pap 3/4 construct when cotransfected with 10 μg of either the empty expression vector (v) or the Oct-1 expression vector (1) together with 10 μg of either the empty expression vector or the Brn-3a or Brn-3b expression vector.

such as construct 1 (aaab), were shown to direct similar levels of protein production following transfection, indicating that the differences in their activity did not arise from differences in the levels of protein produced (Fig. 7a) (29a).

DISCUSSION

In this report, we have shown for the first time that the Brn-3a and Brn-3c transcription factors are able to activate a promoter containing an octamer motif, whereas the closely related group IV POU factor Brn-3b inhibits both the basal activity of this promoter and the stimulatory effect of Brn-3a. Opposite activities of closely related POU factors have previously been demonstrated in the case of the *Drosophila* POU factors I-POU and twin of I-POU (31, 32). Twin of I-POU is capable of binding to DNA and stimulating gene expression (32), whereas I-POU binds to the CF1a POU protein and prevents it from binding to DNA, thereby inhibiting the normal stimulatory effect of CF1a on gene expression (31).

In contrast to I-POU and twin of I-POU, which are alternatively spliced products of the same gene and have only two amino acid differences (31), the different forms of Brn-3 show extensive differences outside the POU domain and are encoded by different genes (25, 29) mapping to distinct chromosomal loci (30, 38). Despite this difference, it is possible that Brn-3b, like I-POU, inhibits promoter activity via protein-protein interactions with other activating POU proteins, including Brn-3a and Brn-3c. Interestingly, however, the POU domain of Brn-3b binds to octamer sequences as strongly as that of Brn-3a or Brn-3c (29). Hence, Brn-3b may inhibit activation by Brn-3a by competing for binding to the octamer motif in pap 3/4 but being unable to stimulate promoter activity following binding, thus inhibiting the Brn-3a-mediated stimulation of the promoter.

Interestingly, however, Brn-3b is also able to inhibit the basal activity of the octamer motif in constructs containing single or multiple octamer motifs. This effect may involve the binding of Brn-3b, which then prevents the binding of endogenous stimulatory transcription factors to this motif. However, this is unlikely, since BHK-21 cells with which the assays were carried out do not apparently contain any proteins capable of binding to the octamer motif apart from the constitutively expressed octamer-binding protein Oct-1, which is known to have an inhibitory effect on the pap 3/4 construct (21). It is possible, therefore, that Brn-3b may have a direct inhibitory effect on promoter activity, as has been described for other factors such as the *Drosophila* even-skipped protein (10).

The different abilities of Brn-3a and Brn-3b to activate the target promoter used here are dependent upon differences in subdomain IV of the two factors, which contains the POU domain, since exchange of this domain converts Brn-3a into a

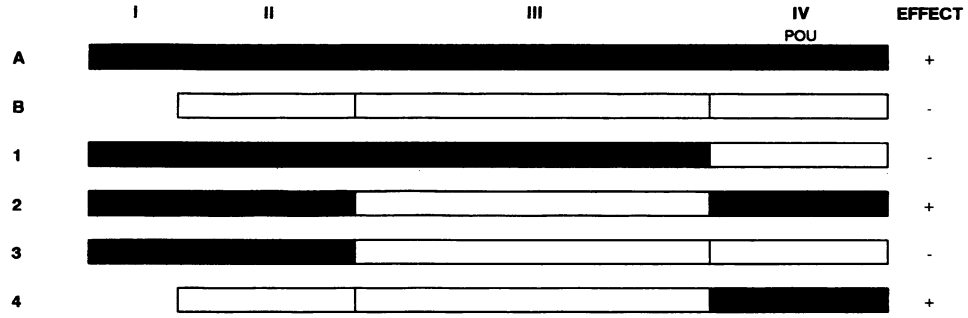

FIG. 6. Summary of gene transactivation data obtained with Brn-3a (construct A) and Brn-3b (construct B) and with constructs 1 through 4 encoding chimeric proteins with different regions derived from Brn-3a or Brn-3b. The division of Brn-3a and Brn-3b subdomains I, II, III, and IV is as follows. Brn-3a: subdomain I, amino acids (aa) 1 to 40; subdomain II, aa 41 to 108; subdomain III, aa 109 to 267; subdomain IV (POU domain), aa 268 to the end. Brn-3b: subdomain II, aa 1 to 92; subdomain III, aa 93 to 169; subdomain IV (POU domain), aa 170 to the end. Subdomains I and II contain the region with similarity to the POU factors I-POU and UNC 86 (8, 29), whereas subdomain IV contains the POU domain.

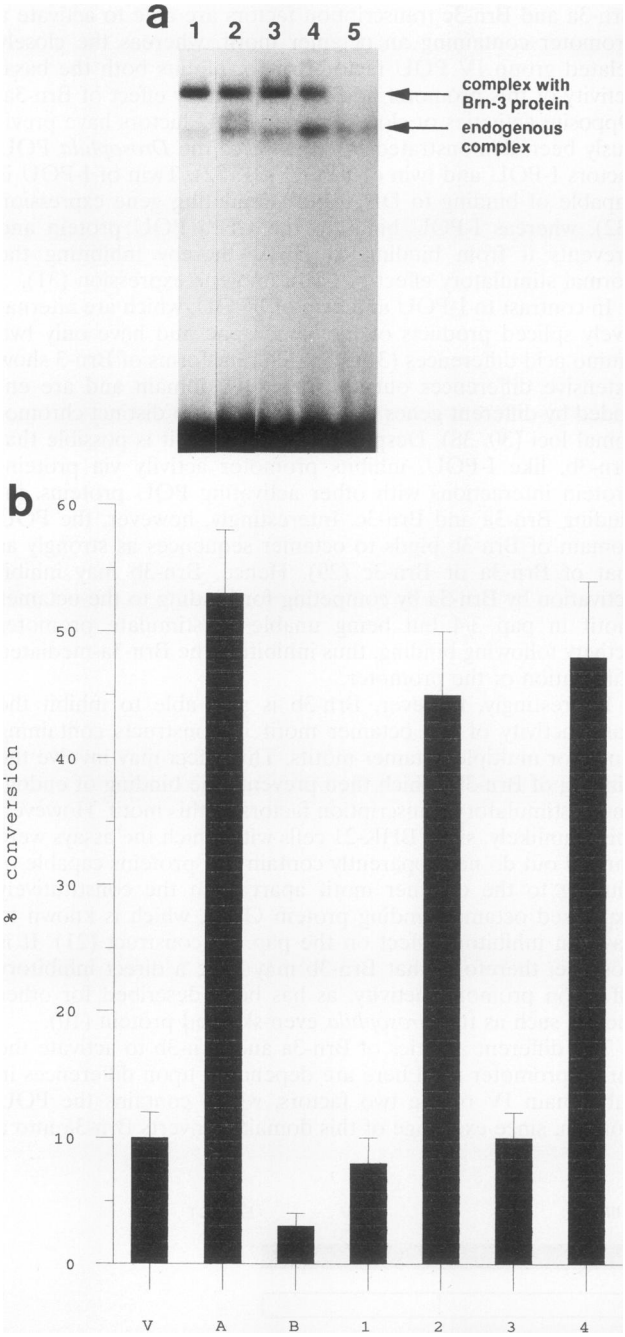

FIG. 7. (a) DNA mobility shift assay with an octamer oligonucleotide and extracts prepared from either untransfected cells (lane 5) or cells transfected with the construct aaab (lane 1), aaba (lane 2), or Brn-3c (lanes 3 and 4). Note the similar expression levels of the aaab and aaba constructs. (b) Assay of CAT activity (percent conversion) upon cotransfection of a construct containing multiple octamer motifs linked to the pBLCAT2 vector with either the empty expression vector or the same vector containing intact Brn-3a (A) or Brn-3b (B) or chimeric constructs 1 through 4 (aaab, aaba, aabb, and -bba, respectively [Fig. 6]). Values are the averages of two determinations whose ranges are shown by the bars.

repressor and Brn-3b into an activator. This subdomain contains five amino acid differences between Brn-3a and Brn-3b in the very short region C-terminal to the POU domain and seven amino acid differences in the POU domain itself. Of the differences in the POU domain, six are in the relatively flexible linker between the POU domain and the homeodomain, with no differences in the POU-specific domain and only one in the POU homeodomain (Fig. 8). Some or all of these differences may result in Brn-3a but not Brn-3b being able to directly activate the target promoter.

Alternatively, since the POU domain is known to control the homodimerization and heterodimerization of different POU family members (34), these differences may control the abilities of different forms of Brn-3 to recruit other activating or inhibiting factors to the promoter, thereby modulating promoter activity indirectly. In this regard, it is of interest that the single amino acid difference between the POU homeodomains of Brn-3a and Brn-3b is at position 22 in the first helix (15), which has been shown to be critical in controlling the interactions of the POU factors with other proteins, such as the herpes simplex virus transactivator Vmw65 (13, 31).

In contrast to the dependence of promoter activation on the POU domain, the ability of Brn-3a and the failure of Brn-3b to cooperate with activated Ha-ras in transforming primary cells has been shown to involve a region at the N-terminus of the Brn-3a protein (29, 29a). Moreover, this N-terminal region is absent in the shorter form of Brn-3a, which lacks the first 84 amino acids and is unable to transform cells but can still stimulate the activity of the simple promoter tested here. Interestingly, a form of Brn-3b with additional N-terminal sequences has recently been detected in the retina (37), although it was absent in the spinal cord (29). It is possible, therefore, that two tissue-specific forms of Brn-3b which differ in their transforming ability but which both contain the same POU domain and therefore cannot stimulate promoter activity exist. The need for the N-terminal region for transformation may reflect a requirement for these N-terminal sequences in the stimulation of promoters different from that tested here. Alternatively, the N-terminus may be required for some other function of the Brn-3 proteins which is necessary for transformation and may possibly involve an interaction with other cofactors.

Although Brn-3a and Brn-3b are both absent in most cell types apart from neuronal cells, they are present in cervical cells of both humans and rats (15). The strong stimulatory activity of Brn-3a on a promoter containing an octamer motif from the HPV-16 promoter may be of significance in cervical cells, particularly since the Oct-2.1 transactivator is also present in these cells (22). We have previously shown that the octamer-related motif in the pap 3/4 construct directs a high level of activity in cervical cells, whereas it is repressed by Oct-1 in other cell types (21). In turn, the cervical-cell-specific activity of this motif may be involved in the cervical-cell-specific activity of the HPV-16 upstream regulatory region (2, 4, 26), since this activity is dependent upon a 100-bp subregion of the upstream regulatory region which contains this octamer-related motif (3, 28). Thus, the stimulatory activity of Brn-3a and Oct-2.1 appears to be capable of overcoming the inhibitory effects of Brn-3b and Oct-1, thereby resulting in the high level of promoter activity obtained with cervical cells compared with that seen in noncervical cells, which express Oct-1 alone.

In addition to having potential significance for cervical cells, members of the Brn-3 family are likely to play an important role in the regulation of cellular gene expression in neuronal cells. Thus, Brn-3a, Brn-3b, and Brn-3c all show specific and distinct expression patterns in the developing rat (11, 15, 23)

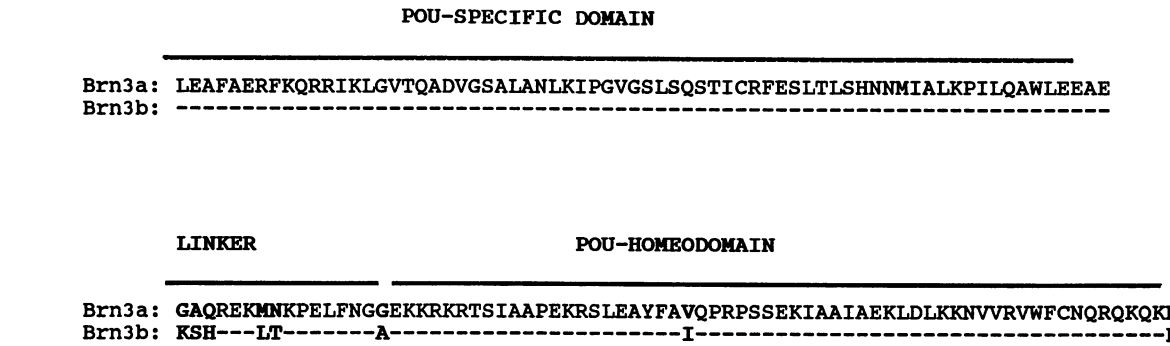

FIG. 8. Comparison of the C-terminal subdomain IV in the Brn-3a and Brn-3b proteins. The positions of the POU-specific domain, linker, and POU-homeodomain components of the POU domain are indicated. The proteins contain a short region of eight amino acids C-terminal to the POU domain which is followed by the translational stop codon.

and human (3, 39) brains. Moreover, during the differentiation of the proliferating ND7 cell line to a nondividing phenotype (27, 37), the levels of Brn-3a rise dramatically, whereas the levels of Brn-3b fall (15). Hence, the opposite functional activities of Brn-3a and Brn-3b are paralleled at least in this case by opposite changes in their expression patterns. It is therefore possible that specific cellular signals act by producing a change in the ratio of Brn-3a to Brn-3b, resulting in a dramatic change in the activities of their target promoters.

Although it will obviously be necessary to identify the cellular target genes for the different members of the Brn-3 family in both cervical and neuronal cells in order to understand their functional role in these cell types, it is already clear that these two related factors have opposite and antagonistic activities and that the balance between them may therefore play a critical role in determining the activities of their target promoters in specific cell types.

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