The Different Activities of the Two Activation Domains of the Brn-3a Transcription Factor Are Dependent on the Context of the Binding Site*

Vishwanie Budhram-Mahadeo, Peter J. Morris, Nick D. Lakint, Sally J. Dawson, and David S. Latchman§

From the Medical Molecular Biology Unit, Department of Molecular Pathology, University College London Medical School, The Windeyer Building, Cleveland Street, London W1P 6DB, United Kingdom

The POU (Pit-Oct-Unc) family of transcription factors play a critical role in the regulation of gene expression in neuronal cells acting by binding to sequences related to the consensus octamer motif ATGCAAAT in the promoters of their target genes (for review, see Verrijzer and van der Vliet (1993) and octamer motif ATGCAAAT in the promoters of their target cells acting by binding to sequences related to the consensus octamer motif (Budhram-Mahadeo et al., 1995). These factors are of particular interest as the mammalian factors whose inactivation results in the failure to develop specific neuronal cell types particularly sensory neurons (Desai et al., 1993; Ninkina et al., 1993). The Brn-3 family of mammalian POU factors are of particular interest as the mammalian factors most closely related to the nematode POU protein Unc-86 whose inactivation results in the failure to develop specific neuronal cell types particularly sensory neurons (Desai et al., 1988; Finney et al., 1988). Three mammalian Brn-3 factors encoded by distinct genes exist (Theil et al., 1993, 1994) and are known to be expressed in the nervous system. Brn-3a and Brn-3b have been shown to be expressed in the nervous system and to have similar expression patterns (Gerrero et al., 1993; Theil et al., 1993; Turner et al., 1994; Xiang et al., 1993). In addition they show distinct activity as they can act differently in the nervous system of cultured cells (Budhram-Mahadeo et al., 1995, 1995b; Lilleycrop et al., 1992).

These differences in expression pattern are paralleled at the functional level. Thus Brn-3a can activate artificial test promoters bearing octamer-related motifs (Budhram-Mahadeo et al., 1994; Morris et al., 1994) or a binding site derived from the pro-opiomelanocortin promoter (Gerrero et al., 1993) and can also activate the naturally occurring promoters of the pro-opiomelanocortin (Turner et al., 1994) or α-interneurin (Budhram-Mahadeo et al., 1995b) genes. In contrast Brn-3b has been shown to repress both the test promoter bearing the octamer-related motif (Budhram-Mahadeo et al., 1994) and the α-interneurin promoter (Budhram-Mahadeo et al., 1995b) and can interfere with their activation by Brn-3a (Budhram-Mahadeo et al., 1994, 1995b; Morris et al., 1994).

In view of the activation of these two promoters by Brn-3a and not Brn-3b, we have used chimeric constructs encoding different regions of Brn-3a or Brn-3b to map the regions of Brn-3a required for promoter activation. In these experiments, activation of the octamer-containing test promoter is dependent on the DNA-binding POU domain of Brn-3a (Morris et al., 1994). In contrast, activation of the α-interneurin promoter requires a domain at the N terminus of Brn-3a (Budhram-Mahadeo et al., 1995b). In agreement with this difference observed using chimeric constructs, the isolated POU-domain of Brn-3a, when expressed in the absence of other regions can activate the octamer-containing test promoter but not the α-interneurin promoter (Budhram-Mahadeo et al., 1995b).

Hence Brn-3a contains two distinct activation domains which differ in their ability to activate different target promoters. Interestingly, although the α-interneurin promoter contains several copies of the octamer motif (Ching and Lien, 1991), these are not required for activation by Brn-3a. Thus Brn-3a can activate a truncated α-interneurin promoter containing only 77 bases of upstream sequence and lacking any sequences closely related to the octamer motif (Budhram-Mahadeo et al., 1995b). The difference in the effects of different activation domains of Brn-3a on the two test promoters may thus arise from a difference in the sequence between the octamer motif and the target sequence in the α-interneurin promoter. Alternatively it may be dependent on differences in the context of the Brn-3a target site relative to other transcription factor binding sites in the two promoters.
To distinguish these possibilities we have identified the target site for Brn-3a in the α-internexin promoter and have tested the effect of different Brn-3a constructs upon this sequence when it is placed in the same context as the octamer motif within a test promoter.

MATERIALS AND METHODS

Plasmid DNAs—The initial α-internexin promoter construct containing sequences from –77 to +73 relative to the transcriptional start site has been described previously (Budhram-Mahadeo et al., 1995b; Ching and Liem, 1991). Other constructs were derived by linearization at the BglII site at –77 and partial digestion with Bal-31 exonuclease. The end points of the digest within the promoter sequence was determined by DNA sequence analysis. A double-stranded oligonucleotide containing the α-internexin promoter sequence from –77 to –61 with a single-stranded 5′-GATC-3′ sequence at either end was synthesized. This oligonucleotide and the corresponding single-stranded oligonucleotides labeled to equal specific activities with 32P were used in DNA mobility shift assays with in vitro transcribed and translated Brn-3a and β proteins as described previously (Morris et al., 1994).

For the binding assay, 10 fmol of [32P]ATP-labeled oligonucleotide probe was mixed with reticulocyte lysate in the presence of 20 mM Hepes, 5 mM MgCl2, 50 mM KCl, 0.5 mM dithiothreitol, 4% Ficoll, and 2 μg of poly(dI-dC)/20-μl reaction volume. Competitor DNA was added at appropriate molar excess at this stage, as required. The binding reaction was incubated on ice for 40 min prior to electrophoresis on a 4% polyacrylamide gel in 0.25 mM boric acid, 2 mM EDTA, pH 8.3) for 2–3 h at 150 V and 4°C. DNA-protein complexes were visualized by autoradiography of the dried gel.

The double-stranded oligonucleotide was cloned into the BamHI site in the vector pBLCAT2 that contains the herpes simplex virus thymidine kinase promoter driving the cat gene (Lucow and Schutz, 1987). The response of this plasmid reporter was compared with that used in our previous experiments (Budhram-Mahadeo et al., 1994, 1995b), which contains the Brn-3 octamer-related binding site ATGCAATT cloned into the same site in the vector pBLCAT2. The Brn-3a and -3b expression vectors contain full-length cDNA or genomic clones for each of these proteins (Theil et al., 1993) cloned under the control of the Moloney murine leukemia virus promoter in the vector pLTR poly which has been modified by deletion of a cryptic splice site in the SV40 3'-untranslated region.

DNA Transfection—Transfection of DNA was carried out according to the method of Gorman (Gorman, 1985). Routinely 1 × 10⁶ BHK-21 cells (Mashpersion and Stoker, 1962) or NIH3T3 cells (Wood et al., 1990) 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 the 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. This value was then used to normalize the values obtained in the chloramphenicol acetyltransferase assay as a control for differences in uptake of plasmid DNA in each sample.

Chloramphenicol Acetyltransferase Assay—Assays of chloramphenicol acetyltransferase activity were carried out according to the method of Gorman (Gorman, 1985) using samples that had been equalized for protein content as described by the method of Bradford (Bradford, 1976).

RESULTS

In our previous experiments, a construct containing α-internexin promoter sequences from –77 to +73 relative to the transcriptional start site was strongly inducible by Brn-3a (Budhram-Mahadeo et al., 1995b). To further delineate the sequences in the promoter mediating this inducibility, this plasmid was digested with the Bal-31 exonuclease to produce a construct which on sequence analysis contained α-internexin promoter sequences from –46 to +73. As indicated in Fig. 1, inducibility by Brn-3a was completely abolished in the –46 construct, although this construct retained detectable basal promoter activity, indicating that the lack of inducibility was not due to the absence of a functional promoter.

These findings indicate that α-internexin promoter sequences located between –77 to –47 upstream of the transcrip-
whether it was a peculiarity of the sequences had not been reported previously, we wished to test sequences compared with the double-stranded sequence.

The positions of the single- and double-stranded oligonucleotides are indicated by the horizontal lines.

\[
\begin{array}{cccccccc}
A) & A & T & G & C & A & A & T \\
B) & A & T & G & C & A & A & T & T \\
C) & A & T & G & A & A & G & C & T \\
\end{array}
\]

**Fig. 2.** Sequence of the consensus octamer motif (A: Falkner et al., 1996), compared with the octamer-related motif used in our previous studies (B: Ching and Liem, 1991) or the sequence from −68 to −61 in the α-internexin promoter (C: Morris et al., 1994).

As binding of Brn-3a to single- rather than double-stranded sequences had not been reported previously, we wished to test whether it was a peculiarity of the α-internexin sequence or could also be observed with other target sites for Brn-3a. We therefore tested the binding of Brn-3a to the octamer-related sequences used in our previous studies (Morris et al., 1994) (oligonucleotide B in Fig. 2). In accordance with our previous data, Brn-3a bound to this double-stranded oligonucleotide (Fig. 4A). However, as with the α-internexin sequence, much stronger sequence-specific binding was observed to the antisense oligonucleotide (complementary to the sequence illustrated in Fig. 2) with somewhat weaker binding being observed with the other single-stranded oligonucleotide (Fig. 4A), and this was confirmed by competition analysis (Fig. 4B). Most importantly, in these experiments, no competition for binding to the single-stranded sequence was observed with unrelated competitor oligonucleotides confirming that the binding was sequence specific (Fig. 4B). This sequence specificity was observed for both complexes formed by Brn-3a that may represent the binding of monomeric and dimeric Brn-3a.

In these experiments the binding affinity of the octamer-related sequence for Brn-3a appeared to be higher than that of the α-internexin sequence when the amount of either antisense single-stranded or double-stranded probe bound in each case was compared. In agreement with this, unlabeled α-internexin antisense sequence competed relatively poorly for Brn-3a bound to labeled antisense octamer-related sequence compared to the degree of competition observed with unlabeled homologous competitor (Fig. 4B, track 4).

In similar experiments, Brn-3b also exhibited preferential binding to the antisense strand of each sequence compared with its binding to the corresponding sense strand or double-stranded sequence, indicating that this preference is common to Brn-3a and Brn-3b (Fig. 5). Thus these observations indicate that Brn-3a and Brn-3b can bind to at least two specific sequences with higher affinity for one of the two single-stranded sequences than for the corresponding double-stranded sequence.

In our previous experiments (Budhram-Mahadeo et al., 1995b; Morris et al., 1994) both the synthetic octamer-related sequence upstream of the thymidine kinase promoter and the α-internexin promoter were activated by Brn-3a and not by Brn-3b. However these two promoters responded differently to constructs encoding chimeric proteins containing different regions derived from Brn-3a and Brn-3b (Fig. 6), indicating that the α-internexin promoter was activated by an activation domain located at the N terminus of Brn-3a, whereas activation of the synthetic octamer-related sequence was dependent on the POU domain of Brn-3a. Such a difference might be due to the difference in sequence between the two Brn-3a binding sites (Fig. 2) or to the different context of each sequence rela-
tive to other sequences in the two different promoters.

To distinguish between these possibilities, the double-stranded oligonucleotide containing the α-interinexin sequence from −77 to −60 was cloned into the BamHI site in the vector pBLCAT2 (Luckow and Schutz, 1987), thereby placing it at −115 relative to the transcriptional start site of the thymidine kinase promoter the same position as the octamer-related sequence in the pBLCat2-derived construct used in our previous experiments (Morris et al., 1994).

In these experiments (Fig. 7) the thymidine kinase promoter containing the α-interinexin sequence was activated by Brn-3a, confirming that the binding of Brn-3a to the α-interinexin sequence from −77 to −60 was able to produce transcriptional activation of a heterologous promoter. Moreover this promoter construct was not activated by Brn-3b, allowing us to use the chimeric constructs to determine the region of Brn-3a required for activation. Interestingly, the ability of these constructs to activate the thymidine kinase promoter containing the α-interinexin sequence was dependent on the POU domain being derived from Brn-3a and did not depend on the N-terminal region. Thus, for example, the promoter was activated by construct 4, which has the POU domain derived from Brn-3a but lacks the N-terminal domain, whereas construct 3, which has the N-terminal domain of Brn-3a but the POU domain of Brn-3b, did not activate the promoter (Fig. 7).

Hence the response of the α-interinexin sequence to the chimeric constructs is identical to that of the octamer-related sequence when the two are cloned into the same context in the thymidine kinase promoter, although this sequence responds differently when in its natural context in the α-interinexin promoter. We showed previously (Budhram-Mahadeo et al., 1995b) that the octamer-related sequence could also be activated by a construct expressing the Brn-3a POU domain in isolation, indicating that the POU domain of Brn-3a was not only necessary but also sufficient for activation of this construct as well as for DNA binding. In contrast, the natural α-interinexin promoter was, as expected, not activated by this construct (Budhram-Mahadeo et al., 1995b). As shown in Fig. 8, however, when placed upstream of the thymidine kinase promoter, the α-interinexin sequence was able to confer a response to the POU domain of Brn-3a exactly as occurred with the octamer-related sequence in the same promoter context. Moreover, as with the octamer-related sequence, the α-interinexin sequence was not activated by Brn-3b.

**DISCUSSION**

In the work presented here, we have extended out previous studies of the α-interinexin promoter (Budhram-Mahadeo et al., 1995b) to show that its responsiveness to the Brn-3a transcription factor is dependent on a region from −77 to −47 relative to the transcriptional start site. Moreover, a portion of this region from −77 to −60 can bind Brn-3a or Brn-3b and confer responsiveness to activation by Brn-3a upon a heterologous promoter. The best match to the octamer motif (ATGCAAAT), which binds many POU factors in this region, is the sequence AT-GAAGCT, which shows only a five out of eight match. This therefore confirms and extends previous findings that Brn-3a and Brn-3b can regulate promoter activity by binding to sequences either closely (Lillycrop et al., 1995; Morris et al., 1994) or more distantly (Gerrero et al., 1993; Li et al., 1994; Turner et al., 1994) related to the octamer motif.

More interestingly, however, we have shown for the first time that both Brn-3a and Brn-3b bind strongly and in a sequence-specific manner to one single strand of both the α-interinexin promoter sequence and a sequence more closely related to the octamer motif. Moreover, this binding is considerably stronger than that observed to either the corresponding double-stranded sequence or the complementary single strand. Such behavior has never, to our knowledge, previously been described for a POU family transcription factor. Similar preferential binding to one single strand compared with the corresponding double-stranded sequence or the complementary single strand has been reported for proteins binding to specific regulatory sites in a number of other cases. These include proteins binding to the sterol regulatory element in the hydroxymethylglutaryl-CoA reductase and synthase genes (Stark et al., 1991), an inhibitory element in the growth hormone gene promoter (Pan et al., 1990), a regulatory region of the adipin gene promoter (Wilkinson et al., 1990) and an inhibitory region in the androgen receptor gene promoter (Grossman and Tindall, 1995). Similar preferential binding to one single strand has also been reported for the transcription factor Myel-2 (Haas et al., 1995) and for the estrogen receptor either alone or in association with another factor (Mukherjee and Chambon, 1990).

Several of these factors thus appear to bind to sequences that have been shown to have an inhibitory effect on promoter activity (Grossman and Tindall, 1995; Pan et al., 1990; Wilkinson et al., 1990) and binding of the Myel-2 factor to its recognition element in the myelin basic protein gene promoter has also been shown to inhibit promoter activity (Haas et al., 1995). Indeed it has been suggested that binding of such factors could promote or maintain the DNA in a single-stranded form incapable of binding other factors necessary for transcription (Grossmann and Tudall, 1995). Interestingly, Brn-3b can also inhibit promoter activity and interfere with activation by Brn-3a (Budhram-Mahadeo et al., 1994, 1995b; Morris et al., 1994).
Fig. 7. Chloramphenicol acetyltransferase assay following transfection of a construct in which the sequence from −77 to −60 in the α-internexin promoter has been cloned upstream of the TK promoter in the plasmid pBLCAT2. 5 μg of this construct was co-transfected with 10 μg of either expression vector lacking any insert (V) or the same vector containing inserts encoding Brn-3a (A), Brn-3b (B) or the chimeric constructs (1–4) numbered as in Fig. 6. Values are expressed relative to that obtained upon co-transfection with expression vector alone and are the average of three independent experiments whose standard deviation is shown by the bars.

Fig. 8. Chloramphenicol acetyltransferase assay following transfection of 5 μg of the construct containing the −77 to −60 α-internexin sequence in pBLCAT2 together with 10 μg of either expression vector lacking any insert (V) or the same vector containing inserts encoding the isolated POU domains of either Brn-3a (A) or Brn-3b (B).

It is possible, therefore, that binding of Brn-3a and Brn-3b may both produce structural alterations in the DNA, although in the case of Brn-3a, such changes activate transcription, whereas Brn-3b has the opposite effect.

In this regard it is obviously of interest that in some cases the isolated DNA binding domain of Brn-3a can act as an activation domain and stimulate transcription (Morris et al., 1994). In contrast, at least in the case of the α-internexin promoter, other mechanisms must operate which require the activation domain at the N terminus (Budhram-Mahadeo et al., 1995b). In other situations, where the activation domain of a specific factor can activate some promoters but not others, this effect has been shown to depend on either the nature of the binding site in each promoter or its context relative to other binding sites. Thus, for example, the glucocorticoid receptor can activate gene expression by binding to a glucocorticoid response element, but represses rather than activates following binding to a distinct sequence known as the nGRE (Sakai et al., 1988). In contrast the YY1 factor activates the human papilloma virus 18 promoter when an adjacent switching sequence is also present but represses the promoter by binding to the same target sequence when the switching element is absent (Bauknecht et al., 1995). Similarly, the activity of the Drosophila dorsal protein is affected by the presence or absence of adjacent binding sites for the dorsal switch protein (Lehming et al., 1994), while the Ubx protein binds to some DNA binding sites only when a binding site for the extradenticle protein is present (Van Dijk and Murre, 1994).

In the case of Brn-3a, we show here that the different effects of the two activation domains which we described previously (Budhram-Mahadeo et al., 1994, 1995b) are dependent on differences in the context of the binding site rather than its sequence the first time this effect has been demonstrated for two activation domains in the same molecule. Such an effect might depend on the ability of the Brn-3a DNA binding domain to open its binding site to a single-stranded form in the presence of different flanking sequences. Alternatively it could depend upon its ability to produce alterations in the structure of the different sequences adjacent to its binding sites and/or on whether an activating effect was produced by disrupting such sequences. In situations where DNA binding by the POU domain did not produce activation, the N-terminal activation domain would be required and would presumably act in a conventional manner by interacting with other promoter-bound factors.

Alternatively both the POU and N-terminal activation domains may act by interacting with other promoter-bound factors with the two activation domains differing in the factors with which they could interact. Thus promoter-specific activation by the two activation domains would depend upon differences between promoters in the nature of the transcription factors binding to the specific sequences in each promoter or in the composition of the basal transcriptional complex that binds at the TATA box in each promoter. In agreement with this latter possibility the TATA-binding protein-associated factors (TAFs)1 have been shown recently to play a role in promoter selectivity with different complexes assembling at different promoters (Verrijzer et al., 1995). Moreover the two activation domains located within either the estrogen receptor or the glucocorticoid receptor have each been suggested to interact with different TAFs (Tasset et al., 1990) reflecting the general finding that different classes of activation domain can interact with different TAFs (Chen et al., 1994).

Although further studies will evidently be required to clarify the precise mechanism, it is clear that the context of the binding site regulates activation by the two activation domains of Brn-3a. Interestingly the Brn-3a primary transcript can be alternatively spliced to yield mRNAs encoding proteins with and without the N-terminal activation domain, but both containing the POU domain (Gerrero et al., 1993), and the outcome of this process can be regulated by exposure of neuronal cells to specific stimuli.2 Thus the existence of two activation domains in Brn-3a may allow complex patterns of gene regulation in response to specific stimuli with some promoters being activated by both forms of Brn-3a and others only when the full size form is made, with such differential activation being dependent on the context of the Brn-3a binding site in the target promoter.

Acknowledgments—We thank Ron Liem for the −77 α-internexin construct and Tarik Möröy for the Brn-3 constructs.

1 The abbreviation used is: TAF, TATA-binding protein-associated factors.
2 Y.-Z. Liu, S. J. Dawson, and D. S. Latchman, submitted for publication.
REFERENCES

Bauknecht, T., J undt, F., Herr, I., Oehler, T., Delius, T., Shi, Y., Angel, P., and Zur Hausen, H. (1995) J. Virol. 69, 1-12

Bradford, M. M. (1976) Anal. Biochem. 72, 248-254

Budhram-Mahadeo, V., Thel, T., Morris, P. J., Lillycrop, K. A., Möröy, T., and Latchman, D. S. (1994) Nucleic Acids Res. 22, 3092-3098

Budhram-Mahadeo, V., Lillycrop, K. A., and Latchman, D. S. (1995a) Neurosci. Lett. 185, 48-51

Budhram-Mahadeo, V., Morris, P. J., Lakin, N. D., Thel, T., Ching, G. Y., Lillycrop, K. A., Möröy, T., Liem, R. K. H., and Latchman, D. S. (1995b) J. Biol. Chem. 270, 2853-2858

Chen, J. L., Attardi, L. D., Verrijzer, C. P., Yokomori, K., and Tjian, R. (1994) Cell 79, 35-42

Wegner, M., Drolet, D. W., and Rosenfeld, M. G. (1993) Curr. Opin. Dev. Biol. 5, 488-498

Van Dijk, M. A., and Murre, C. (1994) Cell 78, 617-624

Verrijzer, C. P., and van der Vliet, P. C. (1993) Biochim. Biophys. Acta 1173, 1-21

Hekmat, M., Zechner, U., Klett, C., Adolph, S., and Möröy, T. (1993) Cytogeten. Cell Genet. 66, 1177-1187

He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. S., and Rosenfeld, M. G. (1992) Nature 357, 175-179

Mukherjee, R., Chambon, P. (1990) Nature 340, 571-576

Ninkina, N. N., Stevens, G. E. M., Wood, J. N., and Richardson, W. D. (1993) Nucleic Acids Res. 21, 3175-3182

Pan, W. T., Liu, Q., and Bancroft, C. (1990) J. Biol. Chem. 265, 7022-7028

Sakai, D. D., Helms, S., Carstedt-Duke, J., Gustafsson, J. A., Rottman, F. M., and Yamamoto, K. R. (1988) Genes & Dev. 2, 1144-1154

Stark, H. C., Weinberger, O., and Weinberger, J. (1991) Proc. Natl. Acad. Sci. U.S.A. 89, 2180-2184

Tasset, D., Tora, D., Fromental, C., Scheer, E., and Chambon, P. (1990) Cell 62, 1177-1187

Theil, T., McLean-Hunter, S., Zornig, M., and Möröy, T. (1993) Nucleic Acids Res. 21, 5921-5929

Xiang, M., Zhou, L., Peng, Y. W., Caddy, R. L., Shows, T. B., and Nathans, J. (1993) Neuron 11, 689-701