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A novel POU family transcription factor is closely related to Brn-3 but has a distinct expression pattern in neuronal cells

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ABSTRACT

The use of the polymerase chain reaction in conjunction with degenerate oligonucleotides has allowed the isolation of cDNA clones derived from each of the POU family transcription factors expressed in the proliferating ND7 neuronal cell line. In addition to the previously characterized Oct-1, Oct-2 and Brn-3 factors, ND7 cells have been shown by this means to express a novel POU factor. This factor is closely related to Brn-3 but differs at seven amino acids in the POU domain, one of which is located in a region which is critical for protein-protein interactions between different POU proteins. Like Brn-3, the novel factor is expressed at high levels in embryonic brain and declines in abundance during neuronal development. In contrast to Brn-3 however, it is absent in mature sensory neurons and its expression declines during the *in vitro* differentiation of ND7 cells to a non-dividing phenotype whilst Brn-3 expression increases. The significance of these distinct but overlapping expression patterns is discussed in terms of the possible role of these two factors in regulating neuronal gene expression.

INTRODUCTION

The POU family of transcription factors was originally defined on the basis of a common domain present in the mammalian transcription factors Pit-1 Oct-1 and Oct-2 and the nematode regulatory gene *unc*-86 (for review see 1, 2). This common POU (Pit-Oct-Unc) domain consists of a POU-specific region and a POU-homeodomain with homology to the *Drosophila* homeobox-containing developmental control proteins (for reviews see 3, 4). The POU domain mediates the DNA binding of these factors, allowing them to modulate the activity of specific genes and hence play a critical role in normal development (5, 6). For example, mutations in the Pit-1 gene result in a failure of pituitary gland development leading to the production of dwarf mice (7) whilst the nematode *unc*-86 mutation results in a failure to form specific neuronal cell types especially sensory neurons (8, 9).

The particular importance of POU family factors in neuronal development suggested by these observations was confirmed by the work of He et al (10) who used degenerate oligonucleotides corresponding to conserved regions of the POU domain to isolate several novel POU factors expressed specifically in the rat brain. One of these Brn-3 is highly expressed in sensory neurons in dorsal root, trigeminal and spiral ganglia as well as in sensory nuclei in the brain stem and is particularly closely related to the nematode *unc*-86 gene product indicating the evolutionary conservation of the POU proteins.

The role of POU proteins in regulating gene expression in neuronal cells from a range of different organisms is also indicated by the identification of a *Drosophila* POU protein CFla which is required for the expression of the DOPA decarboxylase gene in specific dopaminergic neurons (11). Interestingly the activity of this protein is regulated by another co-expressed POU factor, I-POU, which lacks two basic residues at the N-terminus of the homeodomain (12). It cannot therefore bind to DNA but can dimerize with CFla and inhibit its activity by preventing it binding to DNA. More recently another factor known as twin of I-POU has also been characterized which retains the two basic residues and can therefore bind to DNA but cannot interact with CFla (13).

These findings indicate that complex interactions between different POU factors can determine which target genes are expressed in specific neuronal cells expressing different POU proteins and reinforce the need to fully characterize all the POU proteins expressed in a particular neuronal cell type. For this reason as well as the apparent importance of POU proteins in

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sensory neurons, we have used the method of He et al (10) to isolate cDNA clones for all the POU factors present in cells of this type. In view of the presence of non-neuronal cells in tissues such as dorsal root ganglia which contain sensory neurons, we chose to use the ND7 neuronal cell line as a source of material for this procedure. These cells are a hybrid cell line prepared by the fusion of a proliferating neuroblastoma cell with non-dividing dorsal root ganglion sensory neurons (14). Thus ND7 cells can be grown in large amounts whilst retaining many characteristics of sensory neurons not found in the parental neuroblastoma (14, 15). Moreover, these cells can be induced by various treatments to differentiate to non-dividing, process-bearing cells with the characteristics of mature sensory neurons (15).

We report here that in addition to Oct-1, Oct-2 and Brn-3, these cells also express a novel POU factor which is closely related to Brn-3 but which shows a distinct expression pattern both during the *in vitro* differentiation of ND7 cells and in primary neuronal cells.

MATERIALS AND METHODS

Cells and tissues

ND7 cells were obtained by fusing the N18 TG2 azaguanine-resistant neuroblastoma (16) with non-dividing cultures of neonatal rat dorsal root ganglion neurons and selecting the resulting hybrid cells in HAT-containing medium (14). Routinely ND7 cells were grown in RPMI medium supplemented with 10% foetal calf serum. Differentiation of ND7 cells to a non-dividing phenotype was carried out as previously described (15) by transfer to medium containing reduced serum (0.5%) together with 1mM dibutyryl cyclic AMP and 200 ng/ml nerve growth factor (NGF) or to serum free medium without these additives. Rat tissues were dissected directly from adult Sprague-Dawley rats.

RNA isolation and PCR

RNA was isolated from cells and tissues by the guanidinium thiocyanate method (18) and used as a template for production of cDNA using random hexanucleotide primers (Pharmacia). The resulting cDNA was amplified by thirty cycles of the polymerase chain reaction (PCR) according to the method of Kawasaki et al (19). In experiments aimed at isolating novel POU factors from ND7 cells, PCR amplification was carried out with degenerate oligonucleotides containing all the possible nucleotide sequences encoding two nine amino acid regions of the POU domain which are highly conserved in all POU proteins. One of these regions, Phe-Lys-Val/Gln-Arg-Arg-Ile-Lys-Leu-Gly, is located near the N terminus of the POU-specific domain whilst the other, Arg-Val-Trp-Phe-Cys-Arg-Gln/Arg-Arg-Gln is located near the C terminus of the POU homeodomain allowing amplification of virtually the whole POU domain (10). The PCR product obtained in this way was cloned into the Bam H1 site of the Bluescript vector (20) using overhanging GATC present in the original oligonucleotides. In subsequent experiments designed to amplify the Brn-3A and Brn-3B mRNAs in the RNAs of various tissues, PCR was carried out using an upstream oligonucleotide with the sequence 5′ GTGGCTCGGCGCTGGC 3′ common to Brn 3A and B and one of two downstream oligonucleotides from the most diverged region of Brn 3A and B (see Figure 1). The specific oligonucleotides used were 5′ CGGGGTTGTACGGCAAAA 3′ (Brn 3A) and 5′ CTTGGCTGGATGGCGAAAG 3′ (Brn 3B). Following PCR amplification with each pair of primers the product was run on a 2% agarose gel, which was blotted onto a Hybond N nitrocellulose filter (Amersham) and hybridized with a mixture of labelled inserts obtained by Pst I digestion of Brn-3 A and B cDNA clones.

The identity of the PCR product obtained in each case was confirmed by digestion with the restriction enzyme FokI which distinguishes the two forms of Brn-3 with Brn-3A giving fragments of 154 and 118 base pairs whilst Brn-3B gives fragments of 218, 43 and 11 base pairs.

DNA sequencing

POU factor clones in the Bluescript vector were sequenced by the chain termination method of Sanger et al (21) using double stranded plasmid DNA and a Sequenase DNA sequencing kit (Cambridge Bioscience). Clones were sequenced on both strands using flanking primers located in the plasmid sequence.

RESULTS AND DISCUSSION

Amplification of cDNA prepared from ND7 cell RNA with degenerate primers containing all the possible sequences encoding two highly conserved nine amino acid regions of the POU domain (10) resulted in a PCR product of approximately 400 bases in size consistent with the distance apart of the primers in the POU domain (data not shown). As expected the band obtained was slightly heterogenous in size consistent with the known differences in the exact size of the POU domain between different factors

Table 1. POU factor clones isolated by polymerase chain reaction.

Class	Number	Comment
1	4	Closely related to human (23) and mouse (22) Oct-1
2	7	Identical to mouse Oct-2 (22)
3	12	Identical to rat (10) and mouse Brn-3 (22)
4	8	Seven amino acid differences from mouse Brn-3 (22) and class 3 clones
5	14	Unrelated to POU factors

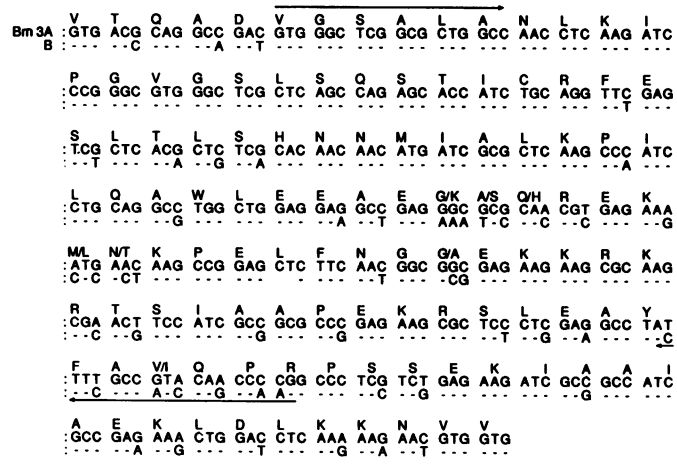

Figure 1. Sequences of the Brn-3A and Brn-3B POU boxes. The predicted amino acid sequence of Brn-3A is shown together with the differences in Brn-3B. The positions of the oligonucleotides used to amplify Brn-3A and Brn-3B mRNAs is indicated.

(10, 22). The PCR product was sub-cloned into the Bluescript vector to facilitate further analysis of the individual products.

The 45 plasmid clones obtained in this way fell into five classes upon DNA sequence analysis and hybridization with clones for known POU factors (Table 1). The largest class (Class 5) did not hybridize to any known POU factor probes and when each of these clones was analyzed by DNA sequence analysis they did not show any conserved regions homologous to the POU domain beyond the oligonucleotides used in the PCR. These clones therefore arise by amplification of sequences unrelated to POU factors due to random homologies to the PCR oligonucleotides and were not studied further. A similar high abundance of spurious clones was noted by He et al (10).

The remaining groups of clones all showed specific sequence and hybridization features characteristic of POU factor cDNAs. Thus one group (Group 1) hybridized strongly to an Oct-1 cDNA probe (23) and showed strong homology to the sequence of the mouse Oct-1 mRNA (22). These clones are therefore likely to be derived from the rat Oct-1 mRNA (24) which like human Oct-1 would be expected to be present in all proliferating cell types including ND7 cells (25, 26). Similarly another group of clones (Group 2) hybridized strongly to Oct-2 cDNA probes (27, 28) and was identical in sequence to the previously published POU domain sequence of mouse Oct-2 confirming the validity of the PCR procedure (22, 29). Although Oct-2 was originally considered to be expressed only in B lymphocytes (26, 30) its presence in ND7 cells is in agreement with its subsequent detection in neuronal cells in the brain (10, 31, 32) whilst we have shown that this protein plays a critical role in regulating both viral and cellular gene expression in ND7 cells (29).

The remaining two groups of clones (Groups 3 and 4) hybridized strongly to a Brn-3 cDNA probe (22). Upon DNA sequence analysis, these clones fell into two groups. One group (Group 3) had a DNA and predicted protein sequence identical to the POU domain of rat (10) and mouse (22) Brn-3 and are therefore derived from the mRNA encoding the previously characterized Brn-3 factor. In contrast the other group of clones (Group 4) showed 50 base differences in their DNA sequence from the group 3 clones resulting in seven amino acid differences in the protein sequence of the POU domain (Figure 1). Both sequences were confirmed from several independently isolated clones. This suggests that these clones are derived from a novel POU factor, closely related to but distinct from Brn-3.

In order to indicate the close relationship of these two factors we refer to the original Brn-3 factor as Brn-3A and to the novel factor we have isolated as Brn-3B. Interestingly one of the amino acid differences between Brn-3A and B is located in the first helix of the homeodomain which has been shown to be critical in controlling the protein-protein interaction between CFla and members of the I-POU family (13) suggesting that Brn-3A and B may differ in their ability to interact with other POU family factors, such interactions being mediated by the POU domain (33).

We next examined the expression pattern of Brn-3A and B using the polymerase chain reaction in conjunction with mRNA prepared from different cell lines and tissues. To do this we synthesized two oligonucleotides corresponding to the Brn-3A and B sequences in the region which is most diverged between these two factors (see Figure 1). In initial experiments each of these oligonucleotides when used in conjunction with a common upstream sequence present in both factors, only produced an amplification product from the appropriate plasmid DNA containing either Brn-3A or B. In contrast no PCR product was detectable when the incorrect primer was used in the amplification either by direct visualization of the PCR product on an ethidium

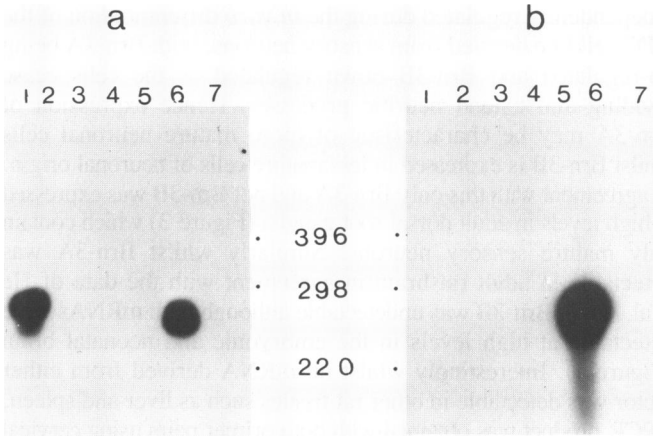

Figure 3. PCR amplification of the Brn-3A (panel a) and Brn-3 B (panel b) mRNAs using RNA prepared from adult rat dorsal root ganglia (track 1), brain (track 2), liver (track 3), and spleen (track 4) mouse 3T3 cells (track 5) human cervix (track 6) or human A20 B lymphocytes (track 7). The positions of DNA size markers are indicated.

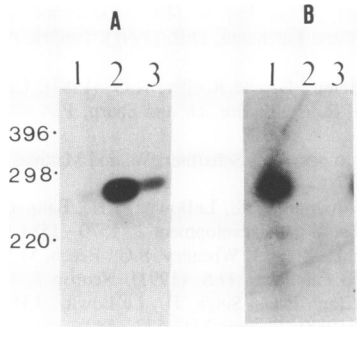

Figure 2. PCR amplification with primers specific for the Brn-3A (panel a) or Brn-3B (panel b) mRNAs using RNA prepared from ND7 cells proliferating in medium containing 10% serum (track 1) or following differentiation in either reduced serum (0.5%) with NGF and cyclic AMP (track 2) or in serum free medium (track 3). The positions of molecular weight markers of the sizes (in base pairs) shown are indicated. The expected products from the Brn-3A or B mRNAs are 272 bases in size.

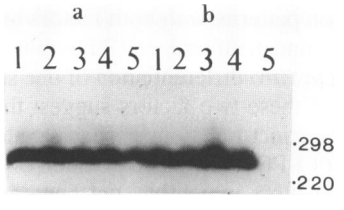

Figure 4. PCR amplification of the Brn-3A (panel a) and Brn-3B (panel b) mRNAs using RNA prepared from rat brain at embryonic days 16 (track 1) and 18 (track 2), at post natal days 2 (track 3) and 7 (track 4) as well as from adult brain (track 5). The positions of DNA size markers are indicated.

bromide stained gel or by subsequent blotting of the gel with mixture of Brn-3 A and B POU domain probes confirming the specificity of the primers (data not shown).

We therefore used each of these pairs of primers to amplify RNA from various sources and visualized the PCR product by electrophoresis on an agarose gel and subsequent hybridization with the Brn-3 POU domain probe. When the two pairs of primers were used to amplify ND7 cell mRNA, the appropriate products of 272 base pairs in size derived from both Brn-3A and B were readily detectable as expected in view of their original isolation from ND7 cells, although Brn-3B was present at a much higher level than Brn-3A. However, the level of Brn-3B decreased significantly when ND7 cells were differentiated to a non-dividing cell type bearing numerous processes (15) by treatment with cyclic AMP and nerve growth factor in reduced serum and also decreased when the cells were similarly differentiated by growth in serum free medium (Figure 2). In contrast the levels of Brn-3A increased during this *in vitro* differentiation process with a greater increase being observed in the cells treated with cyclic AMP and NGF. No change was observed in the level of controls mRNAs such as those encoding actin or the ribosomal L6 protein in these experiments (data not shown). The identify of the Brn-3A and B products obtained in the two PCR reactions was confirmed by restriction enzyme digestion using the restriction enzyme FokI which distinguishes the two sequences (data not shown).

These findings indicate therefore that Brn-3A and B can be independently regulated during the *in vitro* differentiation of the ND7 cell line derived from sensory neurons, with Brn-3A being up-regulated and Brn-3B down regulated as the cells cease dividing and extend neuritic processes. Hence expression of Brn-3A may be characteristic of more mature neuronal cells whilst Brn-3B is expressed in less mature cells of neuronal origin. In agreement with this only Brn-3A and not Brn-3B was expressed at high levels in adult dorsal root ganglia (Figure 3) which contain only mature sensory neurons. Similarly whilst Brn-3A was detectable in adult rat brain in agreement with the data of He et al., (10), Brn-3B was undetectable although both mRNAs were detectable at high levels in the embryonic and neonatal brain (Figure 4). Interestingly whilst no mRNA derived from either factor was detectable in other rat tissues such as liver and spleen, a PCR product was obtained with both primer pairs using cervical mRNA from both human (Figure 3) and rat (data not shown). The PCR product obtained with the Brn-3A primers from human cervical mRNA was slightly different in size to that obtained in neuronal cells however. This appears to represent a difference between the human and rodent forms of Brn-3 since rat cervical mRNA gives a similar size product to that obtained from rat neuronal cells (data not shown).

These data indicate that Brn-3A and B have overlapping but distinct expression patterns with both factors being expressed in primary neurons and neuronal cell lines but being oppositely regulated during *in vitro* differentiation of one such cell line. The close similarity of these two factors suggest that they may also have related but distinct functions perhaps analogous to those of I-POU and twin of I-POU (13). The isolation of full length cDNA clones for these factors which has not been achieved even for the original Brn-3 factor and an analysis of their interactions with other POU proteins should allow an understanding of their functional role in regulating neuronal cell gene expression.

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