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Mouse *BRN-3* family of POU transcription factors: a new aminoterminal domain is crucial for the oncogenic activity of *BRN-3A*

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ABSTRACT

The class IV POU domain genes *Brn-3a, -b* and *-c* are differentially expressed during neural development and at least *Brn-3a* also in neuroectodermal tumors. In contrast to *Brn-3b* and *Brn-3c* , *Brn-3a* encodes two protein variants: *Brn-3a(l)* and *Brn-3a(s)*. *Brn-3a(s)* lacks 84 aminoterminal residues but is otherwise identical to *Brn-3a(l)*. Outside the well conserved carboxyterminal POU domains all three *Brn-3* proteins (*-a, -b* and *-c*) diverge until the aminoterminal end where a new domain of about 100 amino acids is identified. This domain is conserved only between *Brn-3* proteins and other class IV POU factors. *Brn-3a(l)* that contains the complete domain but not *Brn-3a(s)* that lacks 84 amino acids of it is able to tumorigenically transform primary fibroblasts. *Brn-3b* that lacks 40 amino acids of the new domain does itself not transform, but abolishes the oncogenic potential of *Brn-3a(l)* when transfected together. This demonstrates not only that *Brn3-a(l)* is a proto-oncogene and may well be causally involved in the generation of neuroectodermal tumors but also suggests that the intactness of the new aminoterminal domain described here is crucial for oncogenic activity. In addition, our data indicate that *Brn-3b* acts as an inhibitor of *Brn-3a(l)* activiy.

INTRODUCTION

The group of POU domain proteins was originally defined by the sequence homology of four related transcription factors: *Pit-1*, *Oct-1*, *Oct-2* and *Unc 86* (1–5). The POU domain is 150–160 amino acids long and contains a bipartite DNA binding domain, a highly conserved amino terminal region of about 80 amino acids and a carboxy terminal stretch of about 60 conserved amino acids. These stretches represent the POU specific domain and the POU homeodomain, respectively (for a review see 6). Both subdomains are separated by a less conserved linker region of 15–25 amino acids. The regions adjacent to the POU domain are highly divergent between the family members. Via PCR, a number of additional POU domain containing genes related to the four original genes have been isolated. Subsequently, the growing number of POU domain transcription factors were grouped by sequence homology into six classes: POU I–VI (for a review see 6).

The POU domain mediates specific DNA binding in a cooperative manner. While the classic homeodomain binds to short AT rich sequences, the POU domain recognizes longer stretches as for example the classic octamer motif ATGCAAAT and depends in addition on flanking sequences. The POU homeodomain alone binds only weakly to DNA and needs the POU specifc domain for efficient binding. The interaction of both subdomains leads to cooperative binding to an asymmetrical DNA motif with high affinity (for review see 6). The POU domain does not only bind DNA but is also important for interaction with other proteins. The POU factor *Oct-1* for example (see below) binds to the promotor of Herpes simplex virus (HSV) immediate early genes as part of a complex of proteins in which *Oct-1* contacts the HSV transactivator VP16 directly (7). This interaction occurs between the first two helices of the POU homeodomain and the specificity seems to depend on a single amino acid residue in the first helix of the *Oct-1* POU homeodomain (8,9). This example shows that the interaction of POU domain proteins with co-transactivators that are like the POU proteins themselves only present in certain cells and at different developmental stages represents one mechanism to regulate the activity of POU factors.

The human *Brn-3a* gene was originally isolated from a human genomic library based on a fortuitous DNA sequence homology to *N-myc* (10,11) and was then dubbed *RDC-1*. The human *RDC-1* cDNA was found to encode a typical bipartite POU domain that shows strong homology to the POU domain of the rat *Brn-3* gene and the C.elegans *Unc 86* gene (5,8,12). The POU domains of rat *Brn-3*a and human *RDC-1* are almost identical (11–14). Most recently, partial cDNA clones for the human and rat *Brn-3b* (13,15) and for the rat *Brn-3c* (14) have been described. The rat *Brn-3a* gene is differentially expressed during neural development (13); in humans, expression of *Brn-3* genes

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in general is readily detected in brain, eye and spinal cord during the second and third trimester of gestation, but ceases at later stages (11). Similarly, in the mouse, expression was measured with a probe detecting transcripts of all three *Brn-3* familiy members and was found to occur in brain and spinal cord with a peak at day 13 of embryonal development (11). However, this probe could not distinguish the expression of individual family members.

The human *Brn-3a* homologue *RDC-1* is expressed in neuroepitheliomas and Ewing's sarcomas (11). Both are malignant neoplasias derived from neuroectodermal tissue and resemble a distinct type of round cell tumors that were grouped with other neoplasias into one class termed 'PNET' (peripheral neuroectodermal tumors). It has been speculated that this specific expression could reflect a role of *Brn-3a* in the generation of these tumors (10,11).

As a prerequisite to determine their possible involvement in tumorigenesis we studied the gene structure and developmental expression of all members of the *Brn-3* family. We report here the genomic structure and the full sequences of the mouse *Brn-3a, -b* and *3c* genes. We demonstrate that they are differentially expressed during neural development and that the conserved POU domains of *Brn-3a, -3b* and *-3c* bind cooperatively to the heptamer/octamer DNA recognition site. We finally show that a newly identified aminoterminal domain is crucial for *Brn-3a* to aquire oncogenic potential and that *Brn-b* can interfere with the activity of *Brn-3a*.

RESULTS

Structure of murine *Brn-3* family genes

A mouse genomic DNA library was screened with a probe that covered part of the human *Brn-3a/RDC-1* POU domain and 3' untranslated region (11) to isolate genomic clones representing all members of the *Brn-3* gene family . Twenty independent l phage clones were characterised by restriction mapping and hybridisation analysis. Sequence analysis of subgenomic fragments containing the POU domain and comparison to already known sequence portions from rat and human showed that the mouse *Brn-3a, -3b* and *-3c* genes had been isolated (Fig. 1a−c). Fragments derived from genomic clones that cover either the POU domain or are located outside of this region and are representative and specific for the respective *Brn-3* genes were isolated. These fragments were used as probes in Southern analyses (probes I, V, VII, Fig. 1a−c) with mouse genomic DNA to proove the integrity of the isolated phage clones and the completeness of the screening procedure (data not shown).

Characterization of cDNAs and identification of a new aminoterminal domain

cDNA molecules of the corresponding genes were isolated with RACE−PCR techniques using poly A^+ RNA from mouse embryonal day 13 (p.c.) spinal cord where all three *Brn-3* genes are expressed (see below). Genomic fragments as well as cDNA clones were sequenced to gain complete information about the genomic structure of the three *Brn-3* loci (Fig.1a−c): Transcription of the murine *Brn-3a* locus yields two transcripts of about the same length (see below) that could be translated into a long and a short *Brn-3a* protein variant. These proteins differ in 84 amino terminal residues but are otherwise identical. The long and short open reading frames predict proteins with theoretical molecular weights of 33.5 and 43 kDa, respectively. Our analysis of the human *Brn-3a* locus revealed an analogous structure (data not shown).

Transcription of the *Brn-3b* locus gives rise to one transcript (Fig. 1b) with the capacity to encode a protein of 322 amino acids. Comparison of a partial human *Brn-3b* protein sequence (15) with the murine homologue described here show 98% homology on amino acid level and an extension of the open reading frame of 58 amino acids for the murine clone.

For murine *Brn-3c* , a 700 bp cDNA was isolated via RACE−PCR (Fig. 1c). Sequence data derived from genomic clones and cDNA clones predict a protein of 338 amino acids with an M_r of about 35 kDa. Similar to *Brn-3a* the *Brn-3c* coding region is interrupted by an intron (Fig.1a,c). Interestingly, this intron occupies the same position in the *Brn-3a* and *Brn-3c* gene so that the overall structure of both genes is remarkably similar (Fig. 1a, c). Exon one of *Brn-3a* and *Brn-3c* have the capacity to encode 41 and 40 amino acids, respectively.

Within the POU domain the three *Brn-3* proteins show almost complete amino acid identity, only 12 residues out of 160 being exchanged (Fig. 2a). The third helical regions of the homeodomain and of the POU specific domain, which by analogy with the *Antennapedia* homeodomain and the *Oct-1* POU specific domain are involved in DNA binding, are entirely conserved (16,17). Interestingly, one of the amino acid differences refers to the same position (Fig. 2a, arrowhead) that distinguishes *Oct-1* and *Oct-2* in their ability to interact with the HSV transactivator VP16 (8,9,14).

The N-terminal region represents the second conserved part of the *Brn-3* proteins (Fig. 2b). Homology is not only confined to the members of the *Brn-3* family but also extends to the *Drosophila melanogaster I-POU* and *C.elegans Unc 86* proteins. This region of about 100 amino acids can distinguish class IV POU domain proteins from members of other classes and therefore represents a new domain. For *Brn-3a* and *Brn-3c* the homolgy in the N-terminus covers the whole 100 amino acid stretch further emphasizing their similarity in structure. *Brn-3b* is shorter and shows homology over 60 amino acids in this region (Fig. 2b). 84 amino acids of this domain are almost entirely conserved between *Brn-3a*, *Brn-3c* but are not found in the short *Brn-3a* protein (*Brn-3a(s)*) (Fig.2b).

Murine *Brn-3* genes are differentially expressed during neural development

Expression of *Brn-3* genes was examined first by using a general murine *Brn-3* probe coding for the conserved POU domain (probe II, Fig. 1a). Northern transfer hybridisation experiments with total RNA from day 13 (p.c.) mouse embryonal spinal cord revealed transcripts of 3.5 and 2.5 kb (Fig. 3a). In a second step, specific probes originating from the 3' untranslated region of *Brn-3a* and *Brn-3b* (probes III and IV, respectively, see Fig. 1a, b) or from the coding region upstream of the POU domain of *Brn-3c* (probe VI, see Fig. 1c) were used for sequential hybridisation to the same filter and revealed 3.5 kb transcripts for *Brn-3a* and *Brn-3b* and a 2.5 kb transcript for *Brn-3c* (Fig. 3a). Rehybridisation of the filter with a probe specific for transcript 1 of *Brn-3a* (probe VIII, Fig. 1) showed the presence of a 3.5 kb transcript indicating that this transcript is indeed present in spinal cord (Fig. 3a). Thus, the *Brn-3a* transcripts 1 and 2 (Fig. 1a) and the *Brn-3b* transcript (Fig.1b) are very similar in length.

To determine expression of the individual *Brn-3* genes during neural development, total RNA from head parts of various

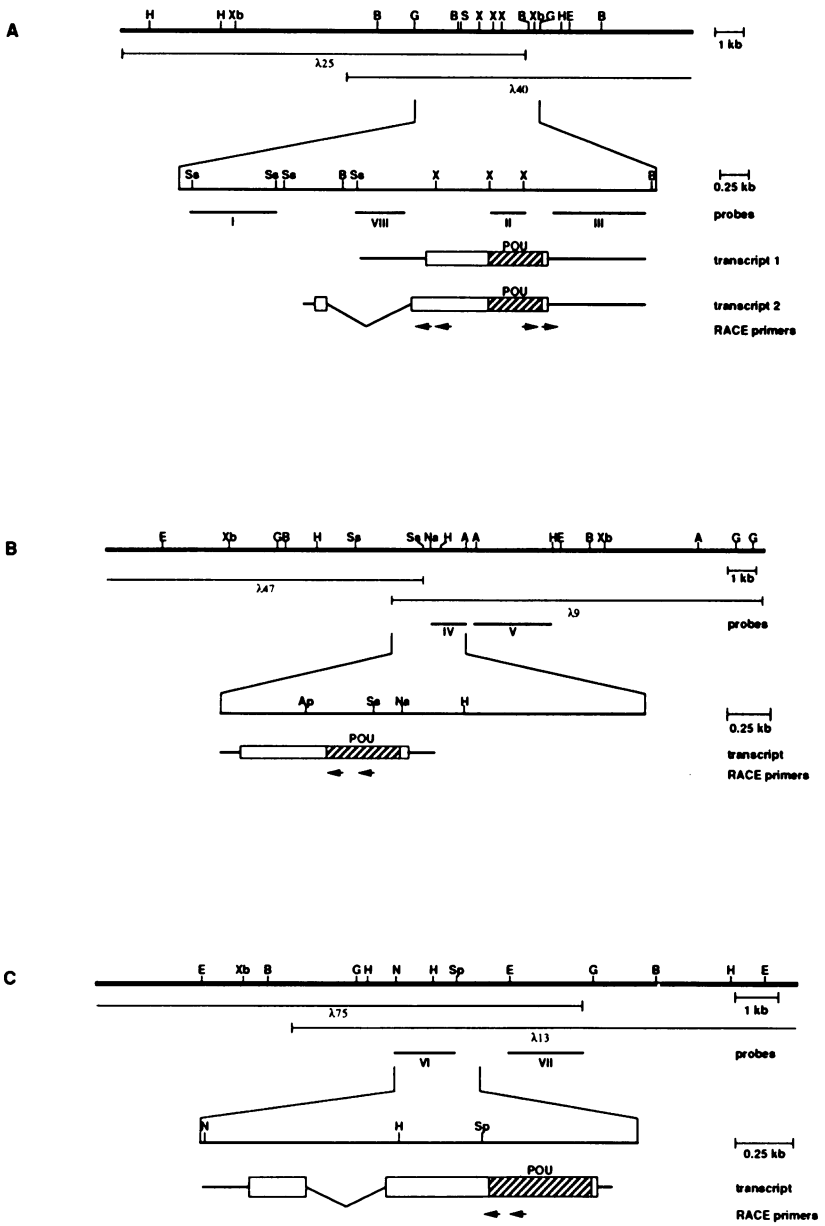

Figure 1. Genomic loci of the murine *Brn-3* genes, partial restriction maps and location of coding regions. (**a**) *Brn-3a.*, the short and the long *Brn-3a* protein variants (*Brn-3a(s)* and *Brn-3a(l)*) are encoded by transcripts 1 and 2, respectively. (**b**) *Brn-3b*. (**c**) *Brn-3c*. Open boxes show the coding regions; the shaded part within represents the POU domains. The different probes and the cDNA stretches that were isolated via RACE−PCR are indicated. The locations of the primers for RACE−PCR are indicated by the tips of the arrows. Restriction enzymes are: A, *Asp*718, Ap, *Apa*I, B, *Bam*HI, G, *Bgl*II, E, *Eco*RI, H, *Hin*dIII, N, *Not*I, Na, *Nae*I, Sp, *Sph*I, Ss, *Sst*I, X, *Xho*I, Xb, *Xba*I. (Note that the scale varies between the three loci.)

embryonic stages and from neonatal and adult brain was isolated and reverse transcribed using the same gene specific oligonucleotides as for 5'-RACE−PCR. PCR amplification of first strand cDNA then revealed the expression of the *Brn-3* isoforms (Fig. 3b). Expression of *Brn-3a* is found during all stages of embryonic brain development from embryonic day 11 (p.c.) until the neonatal stage (P1) and adulthood. In contrast, expression of *Brn-3b* and *Brn-3c* appears to begin at later embryonic stages (day 13 p.c.) compared to *Brn-3a* (Fig. 3b). No expression could be detected in adult mouse brain for both *Brn-3b* and *-3c* (Fig. 3b). These results show a temporally regulated expression for all three *Brn-3* genes.

Similar DNA binding characteristics of individually expressed POU domains

POU domain proteins function as transcription factors through binding to octamer consensus sequences (for a review see 6). DNA binding is mediated by the POU domain which on its own is sufficient to fulfill this function (18,19). To determine whether amino acid changes in the POU domains alter the DNA binding characteristics, the POU domains of all *Brn-3* proteins were individually expressed as GST fusion proteins and used in electrophoretic mobility shift assays. The site preference was determined in these assays using equivalent concentrations of

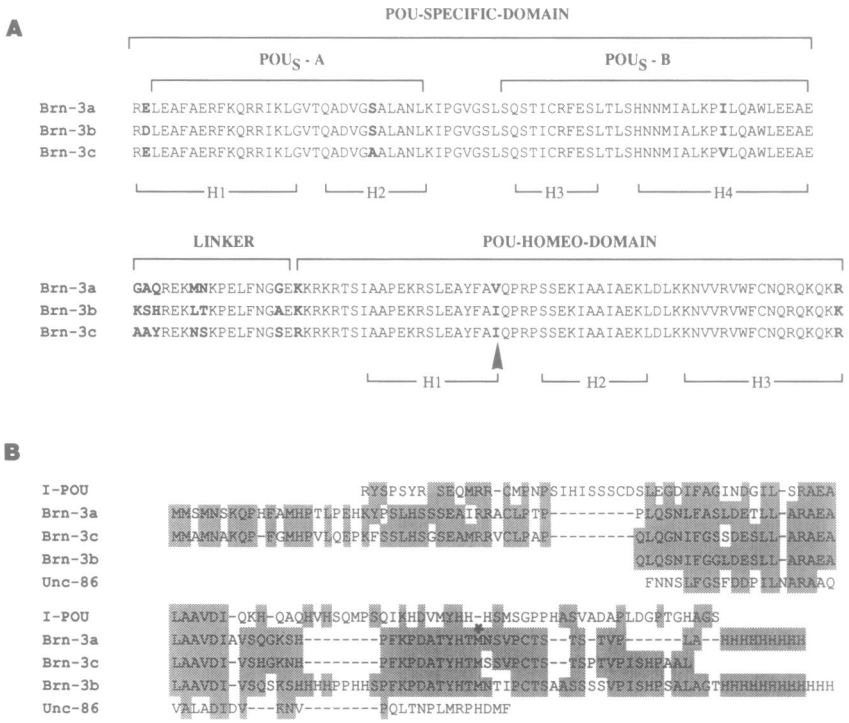

Figure 2. Comparison of amino acid sequences between *Brn-3a, -3b* and *3c*. and other POU factors. (**a**) Comparison of the POU domains of *Brn-3a, -3b* and *-3c*. (**b**) New aminoterminal domain of class IV POU transcription factors. Amino acid sequence comparison of *Brn-3* proteins, *Unc 86* and *I-POU*. The position of the aminoterminal methionine of the short *Brn-3a* variant (*Brn-3a(s)*) is marked by an asterisk.

various radiolabeled oligonucleotide binding sites corresponding to naturally occuring octamer motifs. All *Brn-3* POU domains showed similar DNA binding characteristics: The IgHep oligonucleotide (see Materials and Methods) that contains adjacent octamer and heptamer motifs was bound with highest affinity compared to the single octamer motifs HSV-Oct and wt-Oct (Fig. 4a). No binding could be observed to recognition elements of the *Drosophila* homeodomain gene product *Ubx* (data not shown). Binding experiments with the IgHep oligomer showed two bands indicating the existence of two DNA−protein complexes (Figs. 4a, b) Both complexes disappeared in competition experiments with unlabelled IgHep but not with a random competitor oligonucleotide (RCO) (Fig. 4b). The lower mobility complex (upper band) disappeared with less amounts of unlabelled IgHep competitor oligonucleotide indicating a lower binding affinity of this complex. In this complex two *Brn-3* POU domain molecules may have bound to IgHep similar to the interaction of *Brn-4* with its recognition site (20). The slight residual binding seen with GST-*Brn-3* at a 1:1000 excess of unlabelled IgHep (Fig. 4b) is probably due to an excess of *Brn-3a* fusion protein.

The IgHep oligonucleotide in the normal (Oct^+Hep^+) and in altered versions with either a mutated octamer (Oct^-Hep^+) or a mutated heptamer motif (Oct^+Hep^-) was used to test cooperative binding of the *Brn-3a* POU domain in analogy to the POU domain of *Oct-1* (21,22). Mutation of the heptamer (Oct^+Hep^-) dramatically diminishes binding of the POU domain (Fig. 4c). In addition, the binding appears to be weaker compared to wt-octamer or HSV octamer sequences (Fig. 4a). Mutations in the octamer (Oct^-Hep^+) leads to almost complete loss of both DNA−protein complexes (Fig. 4c). Binding experiments with *Brn-3b* and *-3c* POU domains yielded similar

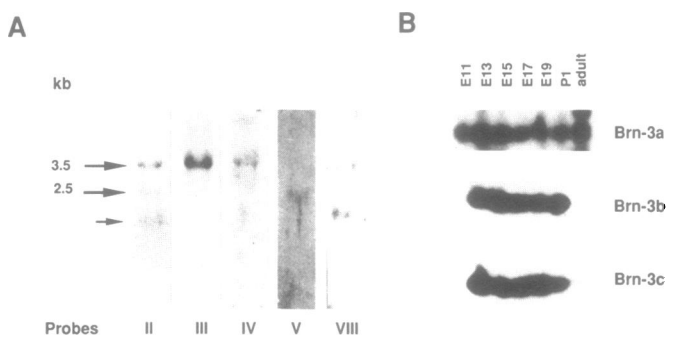

Figure 3. Expression of *Brn-3* genes. (**a**) 30 μg of total RNA from mouse embryo day 13 (p.c.) spinal cord was analysed by Northern blotting with the probes indicated. For the exact location of the gene specific probes see Fig. 1a−c. The probes used were entirely sequenced to ensure their specificity and exclude cross hybridisation between *Brn-3* family members. The specific transcripts for *Brn-3a, -b* and *-c* were detected (arrows). A 2 kb transcript (small arrow) was only detected with the general POU probe (probe II) and probe VIII that is specific for *Brn-3a* transcript 1 and may represent yet another *Brn-3a* transcript. (**b**) Differential expression of individual *Brn-3* genes during neural development was assayed with RT-PCR using RNA from the indicated sources (E, embryonal stages; P, newborn).

results (data not shown). This indicates that the binding of isolated *Brn-3* POU domains to octamer sequences is enhanced by an adjacent heptamer motif probably due to cooperative effects.

Brn-3a shows oncogenic potential

The human *Brn-3a* is expressed in cell lines representative of a subset of neuronal tumors such as neuroepitheliomas and Ewing's sarcomas (10,11), suggesting a role of *Brn-3a* in the

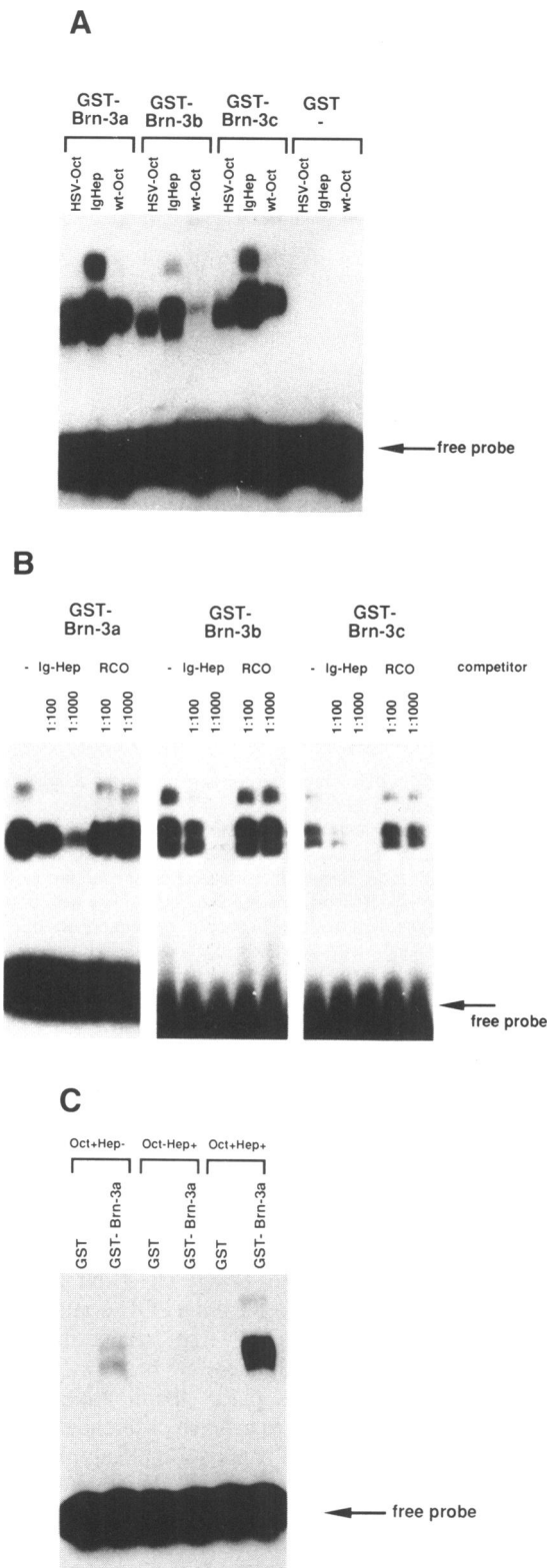

Figure 4. DNA binding activity of the POU domains of *Brn-3a*, *-3b* and *-3c* expressed as GST fusion proteins. (**a**) Specificity of binding to the wild type (wt) octamer, the Herpes simplex virus (HSV) octamer or to the IgHep oligonucleotide containing adjacent octamer and heptamer sequences (GST alone did not show any binding). (**b**) Competition of specific DNA binding to IgHep with an excess of unlabeled IgHep oligonucleotide or with a nonspecific random competitor oligonucleotide (RCO). (**c**) Cooperative binding of GST–*Brn3a* to IgHep (Oct^+Hep^+) and mutated versions (Oct^+Hep^-, Oct^-Hep^+).

development of these malignancies. As both human and murine *Brn-3a* genes code for different forms of *Brn-3a* protein, we aimed to clarify whether these forms have different biological properties and different abilities to induce malignant transformation. A human genomic clone capable to produce both the long and the short form of *Brn-3a* was cloned into the modified eukaryotic expression vector pLTRpoly (23). The resulting construct (p3aLS, see Material and Methods) was then used alone and in combination with a plasmid carrying the activated *Ha-ras* gene (construct pEJ6.6ras, (24)) to transfect primary fibroblasts (REFs) derived from day 14 rat embryos. Foci of transformed cells were observed in the control *c-myc/Ha-ras* cotransfection (constructs pKo-myc and pEJ6.6ras, (24)) and in cells that received both *Ha-ras* and p3aLS (Fig. 5a, b, Table 1). A construct capable of encoding the long and short form of *Brn-3a* from the mouse showed a similar transforming activity as the human p3aLS (data not shown). No induction of foci was seen when p3aLS and *c-myc* were transfected separately (Table 1) or in combination (not shown). In contrast, a construct with a mouse genomic clone encoding only the short *Brn-3a* variant (construct p3aS) or *Brn-3b* (construct p3b) in the same expression vector showed little or no oncogenic activity when cotransfected with activated *Ha-ras* (Table 1). This suggests that the intactness of the newly identified aminoterminal domain is crucial for full transforming activity but also points to functional differences between *Brn-3a(l)* on one hand and *Brn-3a(s)* and *Brn-3b* on the other hand. Interestingly, when p3aLS and p3b are transfected together with pEJ6.6ras, no or little foci formation is seen (Table 1), suggesting that *Brn-3b* inhibits the oncogenic activity of *Brn-3a(l)*.

Independent foci from *c-myc/Ha-ras* and p3aLS/*Ha-ras* cotransfections were selected, grown in medium (DMEM) containing 10% serum and established as stable lines. To demonstrate anchorage independence and tumorigenicity, p3aLS/*Ha-ras* transformed cells were tested for growth in semisolid medium and for the formation of tumors in nude mice. All cell lines derived from p3aLS/*Ha-ras* cotransfections were able to form colonies in soft agar after an average of 13 days (Fig. 5c, d, Table 2). Cell lines derived from foci after *c-myc/Ha-ras* cotransfection were used as a control and showed more colonies that were also larger in size than colonies derived from p3aLS/*Ha-ras* lines (Fig. 5c, d, Table 2). In addition, all *c-myc/Ha-ras* and all p3aLS/*Ha-ras* transformed cell lines gave rise to tumors in nude mice after an average latency period of 13 days (Table 2). In contrast, untransfected REFs were incapable of anchorage independant growth (not shown) and tumor formation in nude mice (Table 2).

Northern blot analysis of RNA from cell lines established from foci after transfection with *c-myc/Ha-ras* (lines 19 and 21), with p3aLS/*Ha-ras* (lines 9 and 24) and with p3aS/*Ha-ras* (line 40) showed the presence of the expected transcripts for *c-myc* and *Ha-ras* (Fig. 5e). Hybridisation with a POU specific probe revealed a 3.6 kb transcript in the lines 9 and 24 and a 3.5 kb transcript in the line 40 (Fig. 5e). Gel shift experiments with whole cell extracts from line 40 and an octamer containing ologonucleotide indicated that a functional short *Brn-3a* protein variant was produced from construct p3aS (not shown). This also demonstrates that the low or absent transforming activity of the short *Brn-3a* variant is an intrinsic effect and not due to the lack of expression in REFs. The same can be assumed for *Brn-3b* as the same vector system was used for all constructs. PCR analysis with primers located in exon one and exon two of *Brn-3a*

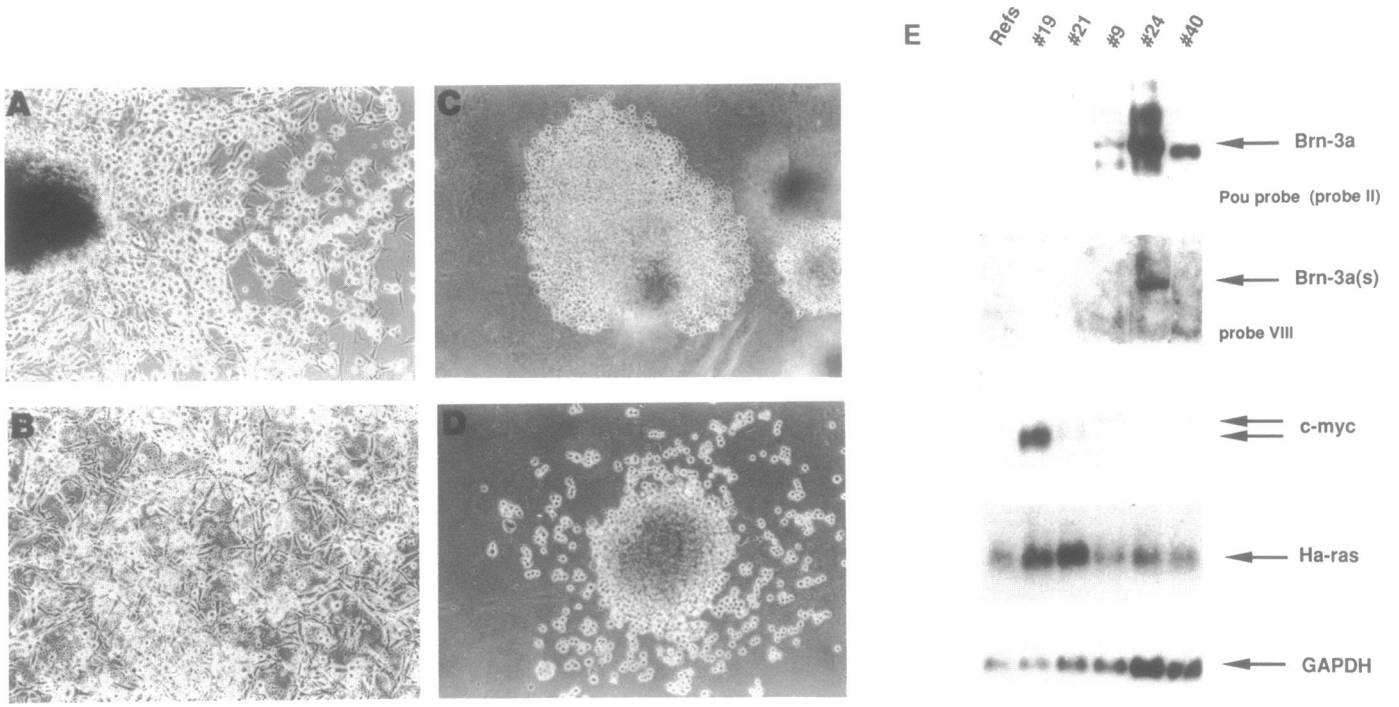

Figure 5. (a) Transformed cells after cotransfection of primary REFs with the plasmids pKo-myc and pEJ6.6 ras that contain the *c-myc* gene and the activated *Ha-ras* gene, respectively (see also Materials and Methods). (b) Transformed cells after cotransfection with p3aLS and pEJ6.6ras. (c) Colony grown in soft agar from cells cotransfected with pKo-myc and pEJ6.6ras. (d) Colony grown in soft agar from cells cotransfected with p3aLS and pEJ6.6ras. (e) Expression of *Brn-3a*, *c-myc*, *Ha-ras* and *GAPDH* in REFs and cell lines established from transformed foci. Cell lines 19 and 21 had been established after transformation of REFs with the constructs pKo-myc and pEJ6.6ras. The lines 9 and 24 were obtained from foci after transformation with p3aLS and pEJ6.6ras, line 40 after transfection of p3aS and pEJ6.6ras. Expression of *Brn-3a* resulting from the constructs p3aLS and p3aS is seen in lines 9, 24 and 40, respectively. Rehybridisation of the same filter with probe VIII that was derived from the untranslated 5′ end of *Brn-3a* transcript 1 and is specific for this transcript (Fig. 1) showed that line 24 but not line 9 expresses this *Brn-3a* mRNA molecule. The construct used to establish line 40 did not contain this region (see Fig. 1 and Material and Methods). The exogenous *c-myc* transcript present in lines 19 and 21 stems from transfected pKo-myc and is shorter (lower arrow) than the endogenous transcript in lines 9 and 24 (upper arrow) because the pKo-myc plasmid contains only exon 2 and 3 but not exon 1 of the murine *c-myc* gene. The endogenous *c-myc* transcript is absent (40) in pKo-myc/pEJ6.6ras transfectants (lines 9 and 21) and present in very low amounts in REFs and the lines 9, 24 and 40 (upper arrow).

confirmed that transcript 2 (Fig. 1a) capable of encoding the long form of the *Brn-3a* protein is present in the transformed cell lines 9 and 24 (not shown). Hybridisation with probe VIII (Fig. 1a) indicated that transcript 1 is present in line 24 but not in line 9 suggesting that expression of the smaller *Brn-3a* protein is not crucial for transformation (Fig. 5e). The nature of the smaller transcript seen in cell line 9 remains to be determined. The cell lines established from foci represent stable transfectants. Therefore, the varying relative levels of expression of the transfected constructs (Fig. 5e) is probably due to different copy numbers.

DISCUSSION

The *Brn-3* family of developmental control genes consists of three members in rat and probably two members in humans (11–15). We describe here the characterisation of all three members of the *Brn-3* family in the mouse *(Brn-3a, -3b and 3c)* and present the full aminoterminal ends of all family members. All three are encoded by single copy genes that are located on different genomic loci. Their expression appears to be regulated differentially during neural development very much like the rat and human homologues (11–14). Consistent with findings in the rat, *Brn-3a* is expressed at earlier and later stages in neural development compared to *Brn-3b* and *-3c*. This suggests that expression of *Brn-3a* is more generalised during neuronal development, whereas *Brn-3b* and *-3c* expression may be more restricted.

Brn-3a and *-3c* contain at least two exons but *Brn-3b* appears to represent another example of a POU domain gene with an intronless coding region. The *Brn-3b* form decribed here resembles in that respect the genes *Brn-1, -2, -4* and *scip* (20,25). It is possible that these genes arose from a POU domain ancestor gene by duplication via a reverse transcribed RNA. Retrotransposition of genes might therefore be a way to duplicate genes or single exons and place them under different transcriptional control elements to achieve differential expression of similar genes. However, we cannot completely rule out that other *Brn-3b* transcripts exist in tissues other than spinal cord or in other developmental stages and might contain aminoterminal exons similar to *Brn-3a* and *-3c*. The genomic loci encoding *Brn-3a* and *Brn-3c* appear very similar in structure as both contain an amino terminal intron. Interestingly however, the *Brn-3c* locus produces only one specific mRNA species whereas the *Brn-3a* gene gives rise to two transcripts of similar length capable of encoding a long and a short form of the *Brn-3a* protein. The transcription start points for the two *Brn-3a* transcripts as far as they can be inferred from the 5′ ends of PCR–RACE clones are located about 500 bp apart on the genomic sequence. This suggest that two different promotors are used for the transcription

Table 1. REF transfection experiments

DNA transfected (constructs)	No. of foci/10^6 cells (5 indep. experiments)	Average number of foci
pKo-myc	0 / 0 / 0 / 0 / 0	0
pEJ6.6(ras)	0 / 1 / 0 / 0 / 1	0.4
pKo-myc+pEJ6.6(ras)	100 / 54 / 51 / 40 / 117	72.4
p3aLS	0 / 0 / 0 / 0 / n.t.	0
p3aLS+pEJ6.6(ras)	6 / 9 / 17 / 13 / 45	18
p3aS	0 / 0 / 0 / 0 / 0	0
p3aS+pEJ6.6(ras)	0 / 2 / 2 / 5 / 1	2
p3b	n.t./n.t./ 0 / 0 / 0	0
p3b+pEJ6.6(ras)	n.t./ n.t./ 3 / 1 / 3	2.3
p3aLS+p3b+pEJ6.6(ras)	n.t./ n.t./ n.t./ 1 / 4	2.5

The number of foci per individual experiment obtained after cotransfection with the indicated DNA constructs is given per 10^6 cells. 5 independent experiments were carried out. The last column shows the average number of foci. For the description of the constructs see Materials and Methods.

Table 2. Cell lines were established from foci obtained after cotransfecting REFs with the indicated constructs

constructs	cell line	soft agar colonies col./10^4cells	soft agar colonies col./10^5cells	tumors in nude mice (days)
pKo-myc+ pEJ6.6(ras)	#19	400	2000	2/2 (11)
pKo-myc+ pEJ6.6(ras)	#21	300	1000	2/2 (13)
p3aLS+pEJ6.6(ras)	#9	200	500	2/2 (16)
p3aLS+pEJ6.6(ras)	#24	150	500	2/2 (13)
-	REFs	0	0	0/2 (25)

Single cells from these lines were seeded out in soft agar to test their ability of anchorage independent growth. The colonies were scored after about 10 days, but at the same time for a given line. Tumorigenicity was tested by injection of about 5×10^6 cells from the established lines intraperitoneally into nude mice. Two animals per cell line were used. Tumors appeared after the latency time given in parentheses.

of the *Brn-3a* locus implicating a mechanism of differential regulation of *Brn-3a* expression and activity.

Specific activity of the individual *Brn-3* isoforms in a distinct cell type or at a certain developmental stage might also depend on the interactions with other proteins through the POU domain itself. Indeed, the high degree of homology between the three *Brn-3* POU domains and their similar DNA binding characteristics do not preclude the occurrence of single amino acid changes particularly at the end of helix 1 of the POU homeodomain. Here, *Brn-3a* contains a valine whereas *Brn-3b* and *-3c* both show isoleucine residues as in the corresponding rat genes (14). The analogous amino acid position has been shown to be critical for the interaction between the POU domain of *Oct-1* and the Herpes simplex virus transactivator VP16 (8,9). The change at this position suggests that *Brn-3a, -3b* and *-3c* might be differentially modulated in their activity through interaction with other cellular transactivators very much like *Oct-1*.

An alternative mechanism of maintaining specificity is the expression of inhibitors: the example of the two class IV POU factors *I-POU* and *tI-POU* shows that a change in two amino acids in otherwise identical products from the same gene distinguishes a transcriptional activator from a repressor (26,27). A similar mechanism of specifiying the activity of POU factors might be the presence of a new aminoterminal domain described here. This domain shows a remarkable degree of homology exclusively confined to three *Brn-3* proteins and to *Unc 86* and *I-POU* that all belong to the same class of POU factors. The region between this conserved aminoterminal domain and the conserved carboxyterminal POU domains diverges between all three *Brn-3* proteins, *Unc-86* and *I-POU*, suggesting strongly that this new aminoterminal domain is of critical functional importance. It is conceivable that the length of this domain regulates specifc biological properties of class IV POU factors, may be through interaction with other co-transactivators. Indeed, only the intact aminoterminal homology domain provides the long *Brn-3a* variant (*Brn-3a(l)*), with transforming and tumorigenic activity. These findings not only establish the POU factor *Brn-3a(l)* as an onco-protein and suggest that *Brn-3a(l)* may indeed be implicated in the generation of neuroepitheliomas and Ewing's sarcomas as has been speculated (11), but may also reflect a general mechanism to regulate *Brn-3a* activity. Interestingly, *Brn-3b* or *Brn-3a(s)* that lack 39 and 84 amino acids in their aminoterminal domains show no or little oncogenic activity. Moreover, *Brn-3b* can inhibit the oncogenic activity of *Brn-3a(l)* suggesting either direct interaction of both proteins or competition of necessary cofactors or DNA recognition sequences as possible mechanism of regulating the activity of *Brn-3* proteins. However, further experiments have to elucidate the exact nature of the inhibition of *Brn3a(l)* transforming activity.

Other POU factors as *Oct-1* or *Oct-3/4* are not able to transform established fibroblasts as for example 3T3 cells or RAT1 cells. In contrast, homeobox and paired box containing genes have been shown to transform mouse 3T3 or RAT-1 fibroblasts (28–30). In addition, the homeobox containing genes *PBX-1* and *Hox 11 (tcl-3)* are activated through chromosomal translocations in different forms of leukemia and are prime candidates to be classified as oncogenes (31–33). Although the oncogenic potential of all these genes has not been evaluated yet in primary cells or in transgenic mice, a common picture emerges that links development and tumorigenesis in that both require the regulation of cell growth, proliferation and invasion. It is therefore conceivable that deregulated expression of some but not all transcriptional transactivators involved in developmental control can lead to the tumorigenic conversion of a cell. It appears now most interesting what target genes are regulated by these new onco-proteins. It is possible that these target genes represent a new class of downstream regulators that are functionally different from targets of non oncogenic developmental control genes.

MATERIALS AND METHODS

Isolation of genomic clones

A genomic library prepared from mouse liver DNA (mouse strain 129, Stratagene, Lambda Fix™ II) was screened using a *Sma*I fragment from the human *RDC-1* cDNA that covered the POU domain and part of the 3′ untranslated sequence. 20 independent

clones were isolated and divided into 3 groups according to restriction mapping. Fragments of each group containing the POU homeodomain were subcloned into the bluescript SK vector and partially sequenced by the Sanger dideoxy chain termination method. These fragments included a 4.4 kb *Bgl*II fragment, a 7.5 kb *Xba*I fragment and a 4.5 kb *Not*I fragment for *Brn-3a*, *Brn-3b* and *Brn-3c*, respectively.

Isolation of cDNA clones by RACE–PCR

To isolate 5′ end cDNA clones the 5′ RACE system from GIBCO BRL was used and the manufacturers instructions were followed. Poly A^+ RNA from spinal cord derived from mice at embryonal day 13 (p.c.) was prepared as described (35) and reverse transcribed using gene specific oligonucleotides residing within the POU domain except for *Brn-3a* (see Fig. 1). After tailing of the first strand cDNA, primary and secondary amplification was performed with primers residing upstream of the forementioned oligonucleotides used for cDNA synthesis. Finally, cDNA molecules for each of the three genes were isolated, subcloned into bluescript vector and entirely sequenced. Control reactions without reverse transcriptase in the first strand synthesis were used in amplification reactions under the same conditions and did not yield any products.

Oligonucleotides for first strand cDNA synthesis included for *Brn-3a*, *Brn-3b* and *Brn-3c*, respectively:
5′-GCACGGTGGACGTGGAC-3′
5′-GCGATCATGTTGTTGTGTGACAG-3′
5′-GGGATCTTAAGATTGGCTAAAG-3′.
Primary amplification was performed with:
5′-CTATTCATCGTGTGGTACGTG-3′
5′-GCGTGAGAGACTCAAACCTG-3′
5′-TTGATGCGCCTCTGCTTGAAG-3′.
The following oligonucleotides were used in a second amplification step:
5′-AATGAATTCGCTTGAAAGGGTGGCTCTTG-3′
5′-AATGAATTCATCCACGTCGCTCATGCAG-3′
5′-AATGAATTCGGGTGACTCATGCCCATG-3′.

A cDNA clone from the 3′ region of the murine *Brn-3a* gene was isolated using a described protocol (34). Oligonucleotides for primary and secondary amplification of cDNA are:
5′-TTCTCTGCCACTTACTGAG-3′
5′-GAGGATCCGAATTCCCAGCCTAGAGAACAGG-3′.

After amplification the obtained PCR products were subcloned into bluescript SK vector and entirely sequenced by the Sanger dideoxy chain termination method.

DNA and RNA analyses

Mouse genomic DNA was isolated according to standard procedures (35) and digested with restriction enzymes to completion, separated on a 0.8% agarose gel, denatured and transferred to nitrocellulose or nylon filter membranes using standard protocols. Hybridisations were carried out in buffer with 50% formamide at 42°C. All probes were double stranded DNA fragments and were radiolabeled with [^{32}P] dCTP and a random hexamer primer mix using the large fragment of DNA polymerase I. Filters were washed under stringent conditions with 0.2×SSC, 0.1% SDS at 65°C and exposed at −70°C to Kodak XAR 5 films with enhancing screens. RNA was isolated using the AGPC method (36), fractionated on 0.8% agarose formaldehyde gels and directly transferred onto nylon filters. Hybridisation and washing conditions were as described above. RT–PCR experiments were as described (35). RNA from head parts, neonatal and adult stages was reverse trancribed using the same gene specific oigonucleotides that were used in RACE PCR. 5′ oligonucleotides for the amplification of first strand molecules were as follows: for *Brn-3a*: CACTTTGCCGCGACTTTG; for *Brn3-b*: CTGAGCGTAATGTGTGCCTTC; for *Brn-3c*: GCAAGAACCCAAATTCTC. 3′ oligonucleotides were the same as in the RACE–PCR reactions. The PCR products were separated on a gel, transferred to a filter and hybridised to oligonucleotide probes specific for the individual *Brn-3* genes. Control reactions without reverse transcriptase in the first strand synthesis were used in amplification reactions under the same conditions. Neither specific bands nor hybridisation signals could be detected in these reactions (not shown).

Expression and purification of glutathione-S-transferase (GST) fusion proteins

DNA fragments containing the complete POU domain coding sequence were generated by PCR amplification from plasmid DNA using the following oligonucleotide pairs for *Brn-3a*, *Brn-3b* and *Brn-3c*, respectively.
5′-AATGGATCCTGCGACTCGGACACGGACCCG-3′
5′-GAGGATCCGAATTCCCTCAGTAAGTGGCAGAG-3′
5′-AATGGATCCCACATGGGCTGCATGAGCGAC-3′
5′-AATGGATCCCTTCACTGCGAAACCGGTTCAA-3′
5′-AATGGATCCGCGTGTCTCAGCGATGTGGAG-3′
5′-AATGGATCCCTGGAGTGTCCCGTAAGCTC-3′

After digestion with *Bam*HI these fragments were subcloned into the bacterial expression vector pGEX-2T and expressed as a fusion protein with gluthathione-S-transferase. The fusion protein was isolated via gluthathione-S-affinity chromatography according to the suppliers instructions (SIGMA). The eluates from the affinity collumns showed one single band on SDS–PAGE analysis after Coomassie staining indicating successful purification of the fusion proteins.

DNA binding studies

The probes used for bandshift assays were oligonucleotides, 5′ endlabelled with T4 polynucleotide kinase and purified by gelfiltration. The oligonucleotides represent the following DNA binding sites (sense sequences shown): the octamer binding site from the Herpes simplex virus immediate-early gene promoter (HSV-Oct, 37), 5′-GCATGCTAATGATATTCTTT-3′; The octamer binding site from the mouse Ig heavy chain enhancer containing adjacent octamer and heptamer motifs (IgHep, 38), 5′-CTGATTTGCATATTCATGAGAC-3′; the wild type octamer which is identical to the octamer motif containing region of the mouse heavy chain enhancer (25,39) 5′-GGTAATTTGCATTTCTAA-3′.

To demonstrate specificity and cooperativity of binding to the IgHep DNA binding site either the octamer (Oct^-Hep^+) or the heptamer motif (Oct^+Hep^-) were mutated. The oligonucleotides were as described (20). Binding reactions were performed on ice in 20 μl binding buffer (10 mM Hepes–KOH, pH 7,9; 60 mM KCl; 1 mM EDTA; 1 mM DTT; 4% Ficoll) in the presence of 1 μg poly(dIdC). About 50 ng of purified fusion protein was used in each binding experiment. Free DNA and protein–DNA complexes were resolved on a native 6% polyacryamide gel run in 0.5×TBE. After electrophoresis, the gel was dried and exposed. Competition assays were done under the same conditions but with unlabelled oligonucleotides added.

REF assay and constructs

The construct p3aLS contains a 5 kb *Bam*HI/*Eco*RI human genomic DNA fragment (11) that is capable to encode the long and the short *Brn-3a* protein and as backbone the modified eukaryotic expression vector pLTRpoly (23) with an LTR from Moloney murine leukemia virus at the 5′ end and splice and polyadenylation sequences from SV40 at the 3′ end. A 2.2 kb *Pvu*II/*Bam*HI mouse genomic fragment only capable of encoding the short form of *Brn-3a* (*Brn-3a(s)*) and a 1.6 kb *Sal*I/*Pvu*II mouse genomic fragment capable of encoding *Brn-3b* were subcloned into the same vector and gave rise to the constructs p3aS and p3b, respectively.

Pregnant Fisher rats were killed at day 14, the embryos were removed and placed in sterile PBS. Cells from the torso were dispersed using normal trypsin/EDTA+1% chicken serum. They were separated from debris by low speed centrifugation. The final cell pellet was resuspended in DMEM/10% serum (FCS) and plated at 1 million cells per 10 cm petri dish. After the cells had reached confluency, they were replated at 1 million again and used for transfections the following day (1 million cells per transfection). Transfections were done by the conventional calcium phosphate precipitation method overnight with 20 μg of DNA. This included 10 μg of pEJ6.6ras (containing the activated *Ha-ras* gene from a human bladder carcinoma, 24) and 10 μg pKo-myc (containing exon 2 and 3 of the mouse *c-myc* gene under the control of the SV40 early promoter/enhancer) or an equal amount of DNA of the different *Brn-3a* or *Brn-3b* constructs together with either *c-myc* or *Ha-ras* plasmids. DNA from pUC vector was used to reach a total of 20 μg of DNA in transfections with a single oncogene. 24 h after transfection the cells were shocked with 25% glycerol in Medium for 1−2 min and another 24 h later were split into four plates and assayed for focus formation. The test for anchorage independance was done in 6 cm petri dishes containing 0.5% agar and 10% FCS in DMEM. This basal layer was then overlaid with 1.5 ml of 0.33% agar and 10% FCS in DMEM containing 10^4 and 10^5 REFs or the same number of cells from *c-myc*/*Ha-ras* or p3aLS/*Ha-ras* cotransfection and incubated. Colonies were scored after about 10 days.

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Note added

While this paper was in press Xiang *et al.* (*Neuron* **11**, 689−701, 1993) isolated an aminoterminally spliced *Brn-3b* mRNA from rat retina and Gerrereo *et al.* (*PNAS*, **90**, 10841−10845, 1993) reported a conserved domain (termed POU IV box) in the amino terminus of the *Brn-3a* protein very similar to the domain described here.

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