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Septamer Element-Binding Proteins in Neuronal and Glial Differentiation

Albert Dobi,[1,2] Miklos Palkovits,[3] Christina G. Palkovits,[1] Mary A. Ring,[1,2] and Denes v. Agoston[1,2]

[1]*Laboratory of Developmental Neurobiology, National Institute of Child Health and Development, National Institutes of Health, Bethesda, Maryland 20892,* [2]*Department of Anatomy and Cell Biology and Program in Neuroscience, School of Medicine, Uniformed Services University, Bethesda, Maryland 20814, and* [3]*Laboratory of Genetics, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892*

Differentiation of progenitors into neurons and glia is regulated by interactions between regulatory DNA elements of neuron- and glia-specific genes and transcription factors that are differentially expressed by progenitors at progressive stages of neural development. We have identified a novel DNA regulatory element (TTTGCAT = septamer) present on the enkephalin (ENK), neuronal cell adhesion molecule, neurofilament of 68 kDa (NF68), growth-associated protein of 43 kDa, glial high-affinity glutamine transporter, tyrosine hydroxylase, etc., genes. When septamer function was blocked by introducing septamer competitor DNA into primary differentiating neural cultures, mRNA levels of ENK, NF68, and glial fibrillary acidic protein decreased by 50–80%, whereas no effect was seen using a control DNA. Septamer elements serve as binding sites for lineage-specific multimeric complexes assembled from three distinct nuclear proteins. Progenitors express a 16 kDa protein (*p-sept*) which binds to DNA as a homodimer (detected as the 32 kDa P-band). Cells that entered the neuronal lineage express an additional 29 kDa protein (*n-sept*) that binds to the homodimerized *p-sept*, and together they form a 62 kDa multimer (detected as N-band). Cells that entered the glial lineage express a distinct 23 kDa protein (*g-sept*), which along with the homodimerized *p-sept* form a 56 kDa multimer (observed as G-band). The binding of the distinct protein complexes (P, G, and N) to the septamer site causes a lineage-specific DNA bending (P = 53°; G = 72°; and N = 90°), which may contribute to the regulatory effect of the septamer interaction. In summary, septamer and its binding proteins represent novel protein–DNA interactions that may contribute to the regulation of neuroglial differentiation in the developing mammalian CNS.

Key words: progenitors; neuron; glia; development; DNA molecular decoy; DNA bending

Neurons and glia of the adult mammalian CNS are derived from multipotent progenitors that develop into neurons and glia (Brustle and McKay, 1996; Lillien, 1998; Pincus et al., 1998). At the level of transcriptional control, neurodifferentiation is envisaged as a cascade of spatiotemporally coordinated and cooperative interactions between regulatory DNA elements of neuron- or glia-specific genes and transcription factors that are differentially expressed in developing cells at progressive stages of neural development (Lemke, 1993). However, the identity of these transcription factors, as well as their coordinated interactions that must occur during development, is poorly understood because of the enormous complexity of the mammalian CNS. One approach to overcome this complexity is to search for mammalian homologs of genes identified in *Drosophila* or *Caenorhabditis elegans* (He and Rosenfeld, 1991). Despite some setbacks, e.g., *gcm* (Kim et al., 1998), this strategy has been successful (Rubenstein and Beachy, 1998). Another approach is to directly probe regulatory DNA fragments from genes that specify a neuronal or glial phenotype with nuclear proteins. Nuclear proteins isolated from distinct brain regions obtained at different developmental stages will include transcription factors that act at various stages of neural differentiation to specify a neuronal phenotype (Dobi et al., 1997). These transcription factors can be identified by the complexes that they form with their cognate DNA targets in electrophoretic mobility shift assays (EMSAs) and by DNA footprinting and can be purified and/or cloned. This approach can therefore be used to identify the DNA motifs and their transcription factors whose interactions guide neural precursors into a given phenotype.

We have focused on the enkephalin (ENK) gene as our model system for defining the transcriptional events underlying neural differentiation. Enkephalinergic neurons mediate social behavior, reward and aggression (Martin et al., 1991; Konig et al., 1996). Because of their spatially contrasting distribution they represent a convenient model system to explore transcriptional regulation of phenotypic differentiation in neural progenitors. We anticipated that interactions between nuclear proteins derived from early embryonic brain [embryonic days 12–14 (E12–E14)] and DNA elements from the ENK gene would identify DNA motifs that are involved in controlling general differentiation steps such as the entry of progenitors into the neuronal or glial lineage. DNA fragments used in our initial studies (Dobi et al., 1997) encompassed the entire regulatory region of the rodent ENK gene (Rosen et al., 1984; Dobi et al., 1995a; Agoston et al., 1998).

Our screening identified a novel DNA element (septamer), which, in addition to the ENK gene, is also present on the regulatory regions of neuron- and glia-specific genes. The sep-

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Correspondence should be addressed to Dr. Denes v. Agoston, Department of Anatomy and Cell Biology and Program in Neuroscience, School of Medicine, Uniformed Services University of the Health Sciences, B2036, Bethesda, MD 20814. E-mail: vagoston@helix.nih.gov.

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tamer element serves as a binding site for developmentally expressed nuclear proteins. Here we describe the developmental distribution of septamer element recognition factors, termed sept proteins, and their association with neuronal and glial differentiation in the developing rat brain. Importantly, we provide evidence that blocking septamer function by introducing specific competitor DNA molecules into differentiating neural cultures results in the downregulation of the expression of neuron- and glia-specific genes. These results suggest a general regulatory role for septamer interactions during neuroglial differentiation.

MATERIALS AND METHODS

Microdissection of rat brain and preparation of nuclear extracts

Dissection of the rat brain into various ontogenetically and phenotypically distinct brain regions between embryonic stages E10–E21 and postnatal ages P2–P28 as well as the preparation and characterization of nuclear extracts from the dissected brain regions, peripheral tissues, and cultured cells were performed as described (Dobi et al., 1997). The broad-range protease inhibitor [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride] (AEBSF) was used throughout the preparation of nuclear extracts and the subsequent experiments. HeLa cell nuclear extracts were purchased from Stratagene (La Jolla, CA) and CellTech (Minneapolis, MN).

Generation of restriction DNA fragments and synthetic oligonucleotides

A DNA fragment corresponding to nucleotides -542 and -384 of the rat ENK (rENK) gene was generated (Dobi et al., 1997) from the plasmid pRESS1 (Joshi and Sabol, 1991) by PCR using sequence-specific primers and was cloned into a pCRII plasmid vector (Invitrogen, Carlsbad, CA). For binding studies, the 160 bp fragment rENK-542;-379 was released from the plasmid by *Eco*RI digest, labeled by [α-^{32}P]dATP incorporation with Klenow polymerase, and purified on 4% polyacryamide gel (acrylamide/bisacrylamide, 40:1) in 1× Tris acetate-EDTA buffer. The DNA fragment was excised from the gel and eluted. Ten femtomoles of DNA probe were used per EMSA. Partly overlapping subfragments were designed based on the consensus sequence information available for the rENK gene (Durkin et al., 1992; S. Sabol and J. Joshi personal communication; A. Dobi and D. v. Agoston, unpublished results). A subfragment (rENK-496;-467) 5′-AAATATTGGTTTGCATAATCATTGACTGCC-3′ retained all the binding activity and therefore was used in subsequent studies. Synthetic oligonucleotides were radioactively labeled by [γ-^{32}P]dATP and polynucleotide kinase, annealed to the complementary strand, and gel-purified as described (Dobi et al., 1995a).

EMSA

Binding reactions were performed as described (Dobi et al., 1995a). Briefly, 10 fmol of rENK-542;-379 or the synthetic probe rENK-496;-467 (Fig. 1) was added to 3 μg of nuclear extracts in the presence of 0.2 mg/ml poly(dI·dC) (Boehringer Mannheim GmbH, Heidelberg, Germany) in binding buffer (12 mM HEPES, pH 7.9, 60 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1 mM AEBSF, and 5% glycerol) in a total volume of 10 μl. The reaction mixture was incubated at 25°C for 20 min, and the complex was separated from the free probe by electrophoresis on a 4% polyacrylamide gel (acrylamide/bisacrylamide, 40:1) in 1× Tris borate-EDTA (TBE) buffer at 12 W of constant power for 2 hr at room temperature. Gels were fixed in 10% acetic acid, transferred to Whatman (Maidstone, UK) 3MM paper, dried, subjected to autoradiography, and analyzed in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In the competition assay, 1 pmol of unlabeled competitor DNA was added to the reaction mixture before addition of the nuclear extract, resulting in a 100-fold excess of the competitor DNA.

Methylation interference assay

Radioactively labeled rENK-542;-379 (see above) was further cut by *BsmAI* restriction endonuclease and gel-purified. The G residues were methylated by dimethyl sulfate using the Maxam–Gilbert sequencing kit according to the manufacturer's instructions (DuPont, Boston, MA). Eighty femtomoles of probe (6×10^6 cpm/pmol) were combined with 40 μg of nuclear extracts derived from E16 striatum in the presence of 15 μg of poly(dI·dC) in binding buffer (see above) in a total volume of 100 μl. The optimal amount of protein required was determined by titration. The mixture was incubated at room temperature for 30 min. The bound and free fractions were separated on a 4% polyacrylamide gel as above. The gel was autoradiographed, and subsequently the bound and free fractions were excised and eluted by soaking overnight at 37°C in 400 μl of elution buffer (0.5 M NH_4OAc, 0.1% SDS, 2 mM EDTA, and 10% methanol). The eluted probes were extracted with phenol/chloroform and precipitated with ethanol twice in the presence of 10 μg of glycogen. The pellets were washed with 70% ethanol, dried, and subjected to chemical cleavage (Maxam and Gilbert, 1977). The DNA was lyophilized twice and dissolved in H_2O, and the activity was adjusted to 2000 cpm/μl. Three microliters of samples were mixed with 3 μl of 90% formamide and loading dyes, heated to 95°C for 3 min. Three thousand counts per minute of sample were analyzed in each lane of a 6% polyacrylamide, 7 M urea, TBE sequencing gel. After the separation, the gel was exposed to x-ray film at −70°C for 72 hr. The identity of the core binding site, designated septamer motif (underlined sequence, see above), was independently identified using EMSA in combination with the left-to-right and right-to-left truncated probe strategy (Dobi et al., 1995b; data not shown).

Antibody supershift assay

Antibodies raised against Oct-1 (a kind gift from Dr. L. Staudt, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD) and Oct-2 proteins (Santa Cruz Biotechnology, Santa Cruz, CA) were tested under two conditions: (1) 3 μg of nuclear extracts were incubated with 10 ng of either anti-Oct-1 rabbit polyclonal IgG or anti-Oct-2 rabbit polyclonal IgG in binding buffer (see above) in the presence of 10 μg of BSA and 0.2 mg/ml of poly(dI·dC) at 0°C for 40 min in a total volume of 10 μl; 10 fmol of radioactively labeled rENK-542;-379 probe were added to the reaction mixture and incubated at room temperature for an additional 30 min. ("pre"); and (2) in a second set of experiments, 10 fmol of the radioactively labeled probe were first incubated with 3 μg of nuclear extracts as above, followed by addition of 10 ng of either anti-Oct-1 rabbit polyclonal IgG or anti-Oct-2 rabbit polyclonal IgG; the reaction mixtures were incubated at 4°C for 40 min. In control reactions, 3 μg of HeLa nuclear extract was tested under identical conditions. Complexes were separated and analyzed by EMSA as above.

Cell cultures

Astrocytes. Cortical astrocytes were obtained from 1-d-old neonatal (Sprague Dawley) rats as described (McCarthy and de Vellis, 1980). After replating confluent cells, astrocytes were grown in DMEM with high glucose and 20 mM HEPES, pH 7.4, containing 10% fetal bovine serum. Cells were harvested; nuclear proteins and cytoplasmic total RNA were prepared as previously described (Dobi et al., 1995a).

Expanding cultures of striatal progenitors. Cells derived from E16 striatum were isolated and cultured in the presence of 10 ng/ml basic fibroblast growth factor (bFGF) as mitogen as described (Johe et al., 1996). After 6 d of mitotic expansion in the presence of bFGF, differentiation was initiated by removing bFGF and culturing cells in serum-free medium. Some cultures were harvested at the end of the expansion period (+bFGF); others were harvested 3 d after withdrawal of the mitogen (bFGF withdrawn). Cell culture medium was removed by aspiration; cultures were washed with 1 × PBS, and after removal of PBS culture dishes were frozen on dry ice. Nuclear extracts were prepared using the microprocedure adopted for cultured cells, and total cellular RNA was prepared from cytoplasmic supernatants as described (Dobi et al., 1995a).

Differentiating neural cultures of rat embryonic forebrain. Forebrains were dissected from E15.5 embryos and collected in ice-cold D1 solution as previously described (Agoston et al., 1991). After several steps of washing with equilibrated Earl's balanced salt solution the brains were minced with sterile blades and dissociated by using the papain dissociation system according to the manufacturer's instructions (Worthington, Lakewood, NJ). The resulting cell suspension was diluted with equilibrated MEM containing 10% bovine serum, 10% heat-inactivated horse serum, N3 nutrient mixture (Brenneman et al., 1987), 2.5 mM glutamine, and anibiotic-antimycotic mixture (Life Technologies, Gaithersburg, MD), and the number of viable cells was determined by the trypan blue exclusion technique. Cells were plated at the density of 400,000/ml in poly-L-lysine-precoated tissue culture flasks. Medium was changed 12 hr after plating, corresponding to 0.5 d *in vitro* (DIV), to MEM containing 5% heat-inactivated horse serum, N3, 2.5 mM glutamine, and antibiotic-antimycotic mixture (Life Technologies). Some cultures received a mix-

ture of 5′-fluoro-2′-deoxyuridine and uridine (FUDR) at final concentrations of 15 and 35 μg/ml, respectively, to block cellular proliferation. At this concentration FUDR completely blocks proliferation without causing death of the mitotically arrested cells (Brenneman et al., 1987). Cultures were harvested at 2.5 DIV by the removal of the medium by aspiration and placing the flasks on dry ice. Nuclear extracts and cytoplasmic supernatants were prepared using the microprocedure as above. Total cytoplasmic RNA was extracted and reverse-transcribed for PCR characterization of gene expression pattern of the cultures as above. As RT-PCR analysis has demonstrated, these culture conditions were permissive for nestin-expressing as well as neurofilament of 68 kDa (NF68)- and glial fibrillary acidic protein (GFAP)-expressing cells.

Embryonic striatal cultures for DNA competition assay. Striata were dissected from E17 embryonic rat brains and dissociated by using the papain dissociation system as above. Cells were plated in MEM containing 5% heat-inactivated horse serum, N3, 2.5 mM glutamine, and anibiotic-antimycotic mixture (Life Technologies) at the density of 150,000 cells/ml in poly-L-lysine-precoated 24 well plates. Medium was changed 12 hr after plating.

DNA competition assay. The assay was performed essentially as described for DNA molecular decoy (Yamashita et al., 1998). As competitor DNA molecule the synthetic DNA fragment rENK-496;-467 previously tested in EMSA was used (see above). For control competitor DNA, a transversion mutant of the septamer DNA element was created (the core TTTGCAT was replaced by GGGTACG), leaving the flanks identical (septamer^{mut}). EMSA showed that the mutated fragment failed to bind nuclear proteins (data not shown). The competitor and control DNA molecules were introduced into cells by using the polyethyleneimine delivery system as described (Boussif et al., 1995). Four to 6 hr after transfection, the medium was changed, and cells were cultured as above. Cultures were harvested 2 d after DNA decoy and processed for RNA extraction followed by reverse transcription.

RNA preparation, reverse transcription, and PCR

Total cellular RNAs were prepared by using the RNeasy technique (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Equal amounts of RNA were reverse-transcribed using oligo-dT priming in the Superscript II system according to the manufacturer's instructions (Life Technologies). PCR conditions and primers for ENK, NF68, GFAP, and cyclophilin primers were as described earlier (Dobi et al., 1995a). Cyclin D2 (cycD2) primers were as published (Freeman et al., 1994). Nestin primers (5′-ACTGAGGATAAGGCAGAGTTGC-3′ and 5′-GAGTCTTGTTCACCTGCTTGG-3′) were designed using the GeneWorks program (Oxford Molecular, Campbell, CA) using the rat nestin gene sequence (GenBank accession number M34384). PCR reactions were performed as described (Dobi et al., 1995a). PCR amplicons were separated on 5–20% acrylamide gels (Novex, San Diego, CA) or thin agarose gels (Dobi et al., 1997). Bands were visualized and quantified either in a PhosphorImager (Molecular Dynamics) or in an Eastman Kodak (Rochester, NY) SP700 imaging system after SYBR green-I (Molecular Probes, Eugene, OR) staining.

UV cross-linking in solution

A synthetic septamer oligonucleotide for UV cross-linking was designed to form a partial hairpin structure with a recessed 3′ end (5′-GTCAATGATTATGCAAACCAATATTTGCGTCAGGCTAGCCTGACG-3′; also see Fig. 1). The purified oligonucleotide was annealed to allow formation of the hairpin in Klenow polymerase buffer (80 mM Tris-HCl, pH 8.3, 25 mM $MgCl_2$) at room temperature for 10 min. The recessive end was filled in with deoxynucleotides and [α-^{32}P]dATP using Klenow polymerase. The labeled double-stranded DNA was gel-purified, and ~30 fmol of probe (2×10^7 cpm/pmol) was combined with 3 μg of nuclear extract from either E14 or E16 rat striatum in the presence of 0.2 μg/μl poly(dI·dC) in binding buffer in a total volume of 30 μl and incubated at room temperature for 30 min. The UV cross-linking in solution was performed by exposing the binding reaction mixtures to 0.12 J of UV light for 4 hr at 4°C. Part of the reaction mixture was incubated with 1 U of DNase I (Worthington Biochemical, Freehold, NJ) at room temperature for 5 min. To remove phosphate groups from the proteins, 0.3 U of protein tyrosine phosphatase 1B and 0.3 U of protein phosphatase type-2A (specific for phosphoseryl and phosphothreonyl proteins; Upstate Biotechnology, Lake Placid, NY) were added to the reaction mixtures, which were then incubated for 15 min at 30°C. All solutions included 0.5 mM of the protease inhibitor AEBSF. The complexes were analyzed on a 4–20% gradient Tris-glycine/SDS gel (Novex).

Combined chemical and UV cross-linking

A synthetic oligonucleotide containing the septamer element for UV cross-linking was designed to form a partial doublet hairpin structure with a recessive 3′ end and prepared as above. Approximately 10 fmol of probe (2×10^7 cpm/pmol) was combined with 3 μg of nuclear extract from either E14 or E16 striatum in the presence of 0.2 μg/μl of poly(dI·dC) in binding buffer (see above) in a total volume of 10 μl and incubated at room temperature for 30 min. The DNA–protein complex was separated from the free probe on a 4% polyacrylamide gel in TBE buffer. After electrophoresis, the gel was laid on a plastic wrap and slid onto a previously developed x-ray film and transferred onto the top of a ready pack X-Omat AR film (Kodak) along with position markers. The gel–film sandwich was put in a UV Stratalinker oven (Stratagene) and covered with a thin layer of 1% glutaraldehyde (Sigma, St. Louis, MO) where indicated. Before the actual experiment, the optimal concentration of glutaraldehyde was determined by titration. The UV cross-linking was performed with 0.12 J of energy for 4 hr at 4°C so that exposure to the x-ray film, the DNA-protein cross-linking with UV light, and the protein–protein cross-linking with glutaraldehyde were performed simultaneously. The positions of the DNA–protein complexes and free DNA were identified after developing the film. The DNA–protein complexes and the free probe were excised from the gel and analyzed on 4–20% gradient Tris-glycine/SDS gel (Novex).

Circular permutation assay

A 26 mer synthetic oligonucleotide containing the septamer element along with flanks (-467;-496 bp of the ENK gene; Fig. 1) was inserted into the pBend5 plasmid (a generous gift from Drs. Sankar Adhya and Jin Kim, National Cancer Institute, National Institutes of Health, Bethesda, MD) using *Xba*I and *Sal*I restriction sites. The resulted pBend5/sept plasmid was cut by *Nru*I, *Eco*RV, *Bam*HI, *Cla*I, and *Mlu*I restriction endonucleases. The resulting 146-bp-long fragments were radioactively labeled at the ends by polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) and [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) and gel-purified. Reaction mixtures for DNA–protein binding reactions were prepared as described above, except that a 5 fmol labeled fragment was combined with 1 μg of nuclear extracts derived from either E14 or E15 striatum. The electrophoretic separation was performed in 8% polyacrylamide gel (acrylamide/bis, 40:1, w/w) in 1× TBE buffer under 13 W of constant power. After separation the gel was fixed, dried, and subjected to autoradiography. To estimate the DNA bending angles, the relative migration values were measured, and the approximate "α" values were calculated as described (Kim et al., 1989). Because the mobilities of the free probes showed only insignificant variations, corrections for free probe mobility differences were unnecessary.

RESULTS

The septamer motif is the binding site for nuclear proteins of the developing brain

The initial screening for specific protein–DNA interactions using the PCR fragment rENK-542;-379 corresponding to the 5′ regulatory region of the rENK gene and nuclear extract derived from E18 striatum showed specific and abundant DNA–protein duplexes marked as N- and G- bands (Fig. 1*A*). Toward the identification of the binding site, synthetic subfragments of rENK-542;-379 were tested in EMSA,and rENK-496;-467 formed a doublet that could be specifically competed only by excess original fragment (rENK-542;-379) but not by unrelated DNA.

To determine the site of base-specific interactions, we performed DNA methylation interference footprinting (Fig. 1*B*). Footprinting showed a strong specific interaction between the binding proteins and the internal G-nucleotide at position -484 (star) of an octamer-like motif (TTGG^{488}TTTG^{484}CATAATC, underlined sequence here and *boldface* in Fig. 1*B*). Very importantly however, the 5′ purine base at position 488 (*arrow*) was not involved in DNA–protein interactions, unlike the case for octamer-binding proteins. Because there was no base-specific interaction at the 5′ purine base, we named the DNA element septamer motif.

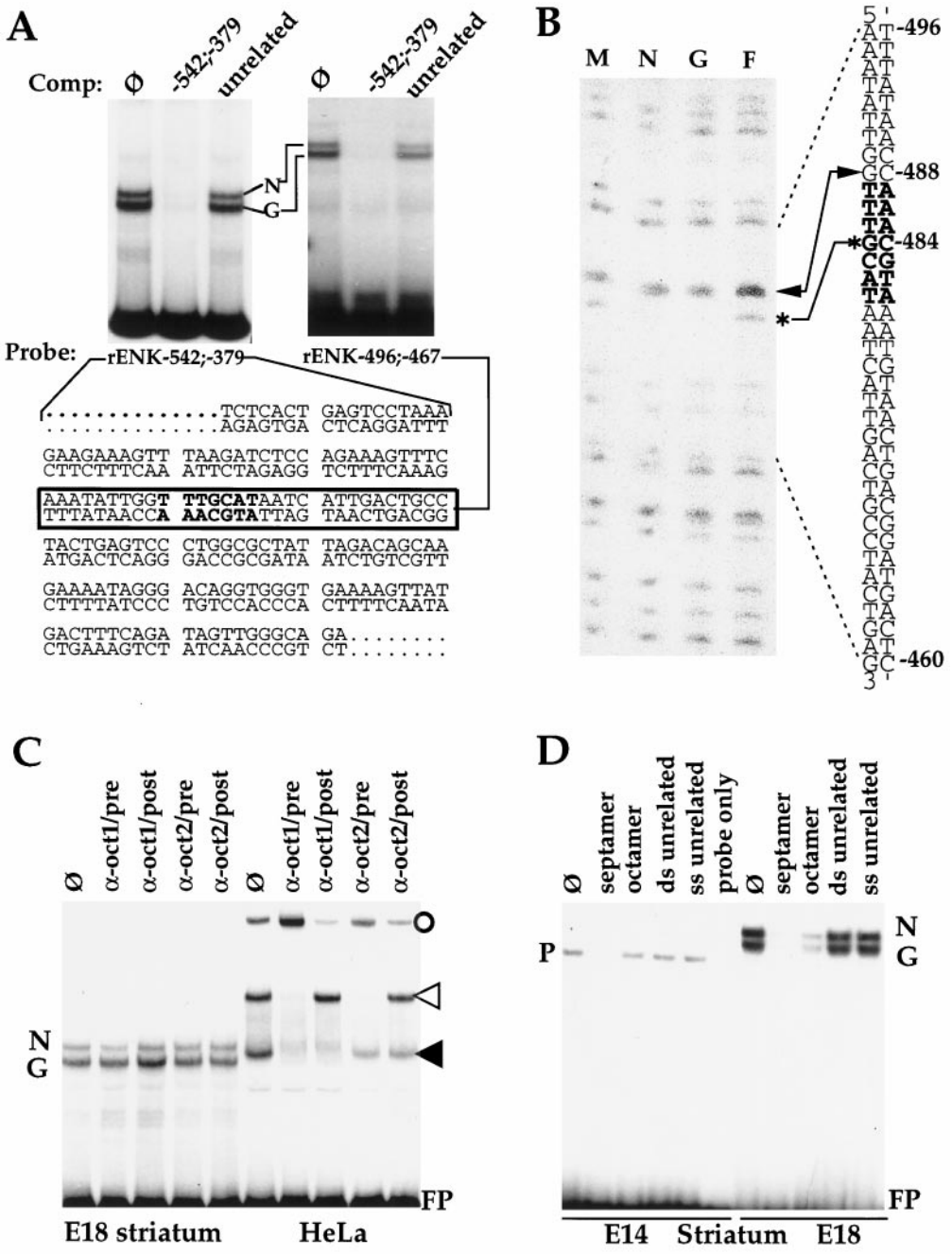


Figure 1. Nuclear proteins from embryonic brain bind to septamer, a novel DNA motif, in gel mobility shift (EMSA) and footprinting assays. *A*, EMSA with nuclear extract from E17 striatum and either the entire 163 bp (rENK-542;-379) fragment or the 30 bp septamer-containing subfragment (reNK-496;-467) as radiolabeled probes. The cold competitors were either the rENK-542;-379 fragment or unrelated dsDNA. The sequence of the DNA probe rENK-542;-379 containing the septamer motif (in *boldface*) is depicted. The *box* marks the position and sequence of the 30 bp synthetic DNA oligonucleotide related to rENK-542;-379. Each probe displays a similar pattern of specific protein–DNA complexes: two closely spaced complexes, with the lower-mobility band expressed in lower amounts. The difference in the relative mobilities of complexes formed from the two probes is the result of the differences in length between the two probes (163 vs 30 bp). *Ø*, No competitor; *FP*, indicates free probe. *B*, DNA methylation interference footprinting of rENK-542;-379 with nuclear extract for E18 striatum identifies the septamer motif (*boldface*) as the binding site. The G nucleotide positioned at 484 showed interference (*asterisk*), whereas no interference was seen at the G residue at 488 (*arrow*). *M*, G reaction marker; *N,* neuron-specific complex; *G,* glia-specific complex; *F,* free probe. *C,* Antibody supershift assay with nuclear extracts from E18 striatum and HeLa cells and rENK-542;-379 as probe. Nuclear proteins were preincubated with the antibodies overnight before addition of the probe (*pre*), or antibodies were added after formation of the DNA–protein complex (*post*). As a positive control, nuclear extracts derived from HeLa cells were used. HeLa cells express both Oct-1 and Oct-2-binding proteins, which form complexes of distinct mobilities (*arrow*, *open arrowhead*). Preincubation of the nuclear extracts with Oct-1 or Oct-2 antibodies (*pre*) resulted in a dramatically altered mobility of the complexes (supershift, marked by *circle*). *D*, The P complex present in E14 striatum can only be competed with septamer but not with octamer sequence. The N and the G complexes present in E18 striatum could, however, be competed by septamer as well as to some extent by the octamer sequence. Unrelated double-stranded (*ds*) and single-stranded (*ss*) DNA did not compete. *FP,* Free probe.

Because of the sequence similarities between octamer DNA elements and the septamer motif, we anticipated that Oct-1 polyclonal antibody would recognize the septamer-binding proteins forming the N- and G-bands (Fig. 1*A*), and this recognition would result in the supershifting of the formed DNA–protein complexes. However, neither the anti-Oct-1 nor anti-Oct-2 antibodies recognized the nuclear proteins in the N and G complexes (Fig. 1*C*). This experiment suggested that proteins that bind to the septamer motif are distinct from the known proteins that bind to the octamer-element. In control experiments, the presence of anti-Oct-1 or anti-Oct-2 antibodies resulted in supershifted complexes when nuclear extracts from HeLa cells were tested (Fig. 1*C*).

We performed competition assays using the rENK-496;-467 synthetic fragment that contains the septamer site or a DNA fragment of equal length that contained the consensus oct-1 site as competitors (Fig. 1*D*). These experiments showed that the early, P-band (see below) was highly specific to the septamer motif. Binding was eliminated when the septamer-containing DNA competitor was used. The presence of excess octamer competitor did not affect binding The N- and G-bands detected in E18 striatum were also successfully competed with septamer

DNA. However, some degree of competition was also observed with octamer-containing DNA.

The different septamer complexes are associated with distinct cellular phenotypes

To obtain the precise spatiotemporal distribution of the septamer-binding proteins, we probed nuclear proteins derived from various brain regions at distinct stages of neurodifferentiation with rENK-496;-467 (for sequence, see Fig. 1*A*). This screening revealed an interesting spatiotemporal distribution of the three previously observed protein–DNA complexes P, N, and G (Fig. 1*D*). The single, medium-mobility band marked as P that was detected in the striatal primordium at E14 was also seen in all tested brain regions except the pons and medulla oblongata at E14 (Fig. 2*A*). Subsequent analysis, however, showed the presence of the P-band in E10–E12 rhombencephalon (data not shown). RT-PCR analysis of the corresponding cytoplasmic supernatants demonstrated the expression of nestin and cycD2 mRNA in these brain regions, suggesting the presence of proliferating neural progenitors (Fig. 2*B*). Neither NF68 nor GFAP mRNA, the markers for differentiated neurons and astroglia, was detected at this stage. Because of the expression of nestin and cycD2 mRNA and because the spatiotemporal pattern of P-band correlated with the generation of neural progenitors in the various brain primordia, we designated this complex "P" for progenitors. In the developing brain, the P-band was not detected after E14 except in the cerebellum, where the P-band reappeared between P2 and P8 (Fig. 2*A*). The P-band was not detected in any brain regions of the adult brain except the olfactory bulb and the hippocampus (Fig. 2*D*).

After E14, the lower-mobility double band (Fig. 1*D*, marked as *N*, *G*) was also detected in all other brain regions tested (Fig. 2*A*). Again, this doublet was observed in the ontogenetically earliest region, the pons and medulla oblongata, at E14 when other brain primordia (diencephalon, striatal primordium, and telencephalon) contained only the P-band (Fig. 2*A,C*). However, the spatiotemporal pattern of appearance and disappearance of the N- and G-bands closely resembled the developmental appearance of developing neurons and glia, which varies among ontogenetically distinct brain regions (Fig. 2*A*). For example, in the striatum, the most abundant N-band was observed at E18, which is the peak activity of neurogenesis (Bayer and Altman, 1995; Fig. 2*A*). In the developing cerebral cortex, the N-band was most abundant between E16 and E18, but it remained detectable at early postnatal ages, consistent with a longer period of neurogenesis in this brain region. There were no detectable complexes in the adult (>P28) cerebral cortex or any other brain regions. RT-PCR analysis of the gene expression pattern showed that the decrease and disappearance of nestin mRNA expression and the appearance of the neuronal marker NF68 mRNA corresponded to the developmental appearance of the N-band in the various brain regions (Fig. 2*A,B*). Similarly, the spatiotemporal appearance of the G-band preceded the developmental appearance of GFAP mRNA, the marker for astroglia (Fig. 2*A,B*). These observations supported the hypothesis that the appearance of N-band marks an early developmental event for neuronal precursors, such as the entry of neural progenitors into the neuronal lineage or an early stage of neuronal differentiation. We also hypothesized that the appearance of the G-band marks the entry of progenitors into the glial lineage or an early stage of glial differentiation. In addition to the N- and G-bands, we frequently observed high-mobility complexes in several brain regions at E16 (Fig. 2*A*). These high-mobility complexes, which were also detected in astrocytic cultures (Fig. 2*E*) and were enhanced after mitogen withdrawal of striatal cultures (Fig. 2*F*), probably consist of dissociated and cleaved components of the septamer multimers (see below).

The association of P-, N-, and G-bands with distinct developmental stages was further investigated by using various cell culture systems. We compared the presence of the various (P-, N-, and G-) bands in microdissected cerebral cortex at P8 to age-related cortical astrocytes in culture. Cerebral cortex contained both N and G complexes; however, only the G-band was detected in age-equivalent cortical astrocyte cultures (Fig. 2*E*). In accord with the EMSA results, RT-PCR analysis demonstrated the expression of GFAP mRNA in astrocytic cultures whereas both GFAP and NF68 mRNA were detected in P8 cerebral cortex (Fig. 2*E*). None of the three complexes (P-, N-, and G-bands) was observed in any peripheral tissues at any ages tested (Fig. 2*D*; data not shown).

We also tested a striatal culture system developed by Johe et al. (1996). The culture originated from E16 striatum and was treated with the mitogen bFGF for 6 d. When these cultures were analyzed at the end of bFGF expansion (corresponding to E22 *in vivo*), the P-band as well as abundant N- and G-bands were detected (Fig. 2*F*). After withdrawal of mitogen, all cells differentiated into astrocytes, as indicated by the abundant G-band and GFAP mRNA. P-band, N-band, and NF68 mRNA were undetectable; however, a low amount of nestin mRNA was detected (Fig. 2*F*).

A positive association of the N-band with differentiating neurons was also established by taking advantage of the distinct onset of neurogenesis and gliogenesis in the developing CNS. Because blocking cellular proliferation after the majority of neurons are postmitotic but gliogenesis is ongoing should reduce G-band and GFAP mRNA levels but should leave N-band and NF68 mRNA relatively unchanged, developing neural cultures derived from E15 rat forebrain were treated with FUDR starting at 1 DIV (E16) for 2 d. Blocking cellular proliferation within this developmental period resulted in a substantial decrease in the intensity of the G-band complex on EMSA and the GFAP mRNA by RT-PCR, whereas the intensities of N-band and NF68 mRNA remained virtually unaffected (Fig. 2*G*). These results further support the hypothesis that N- and G-bands are associated with differentiating neurons and glia, respectively.

The septamer motif is present on various neuronal- and glial-specific genes

The relative abundance and the developmentally regulated appearance of the P-, N-, and G-complexes in the developing rat CNS suggested that the septamer motif may be a DNA regulatory element central to neural differentiation, and as such, it should be present within the regulatory regions of neuronal- and glial-specific genes. Searching the database (GenBank) for genes containing the septamer motif confirmed this assumption. The septamer motif was found on the 5′ regulatory regions of many neuronal- or glial-specific genes. These genes include substance P/neurokinin, NF68, growth-associated protein of 43 kDa (GAP-43), neuron cell adhesion molecule, and glial high-affinity glutamate transporter and vimentin (Table 1).

Blocking the binding of septamer-binding proteins alters gene expression

To gain insight as to the possible function of the septamer motif and its binding proteins, we used a competitor double-stranded

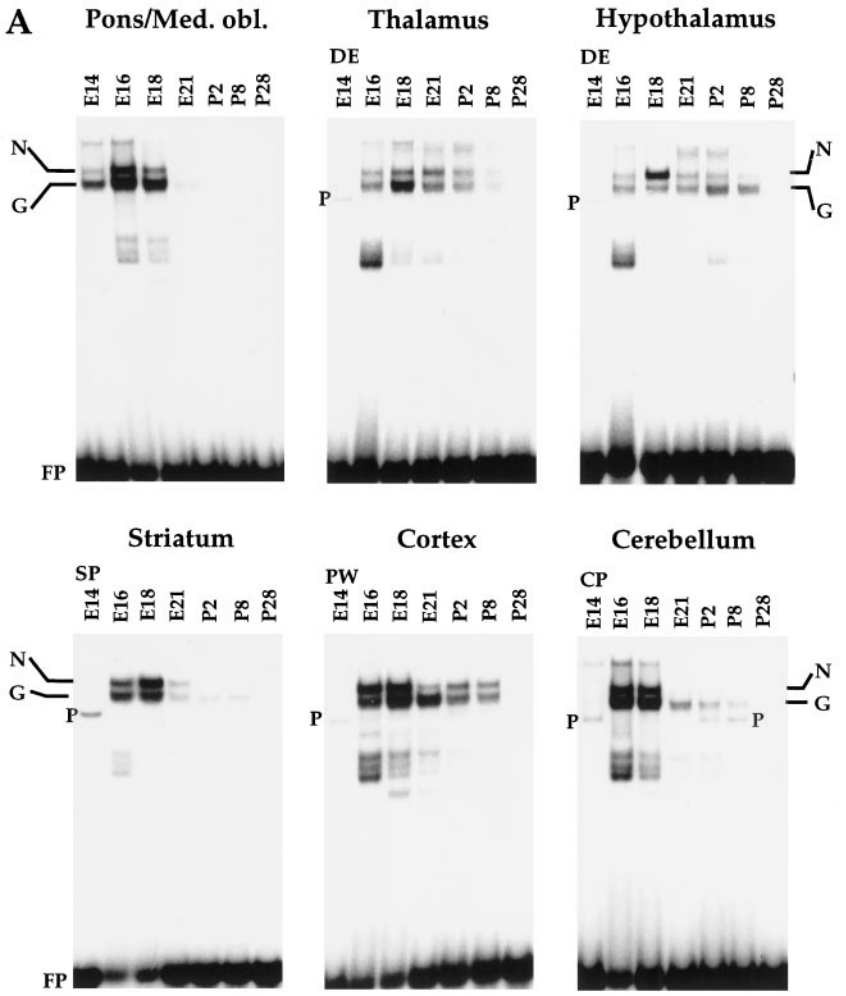


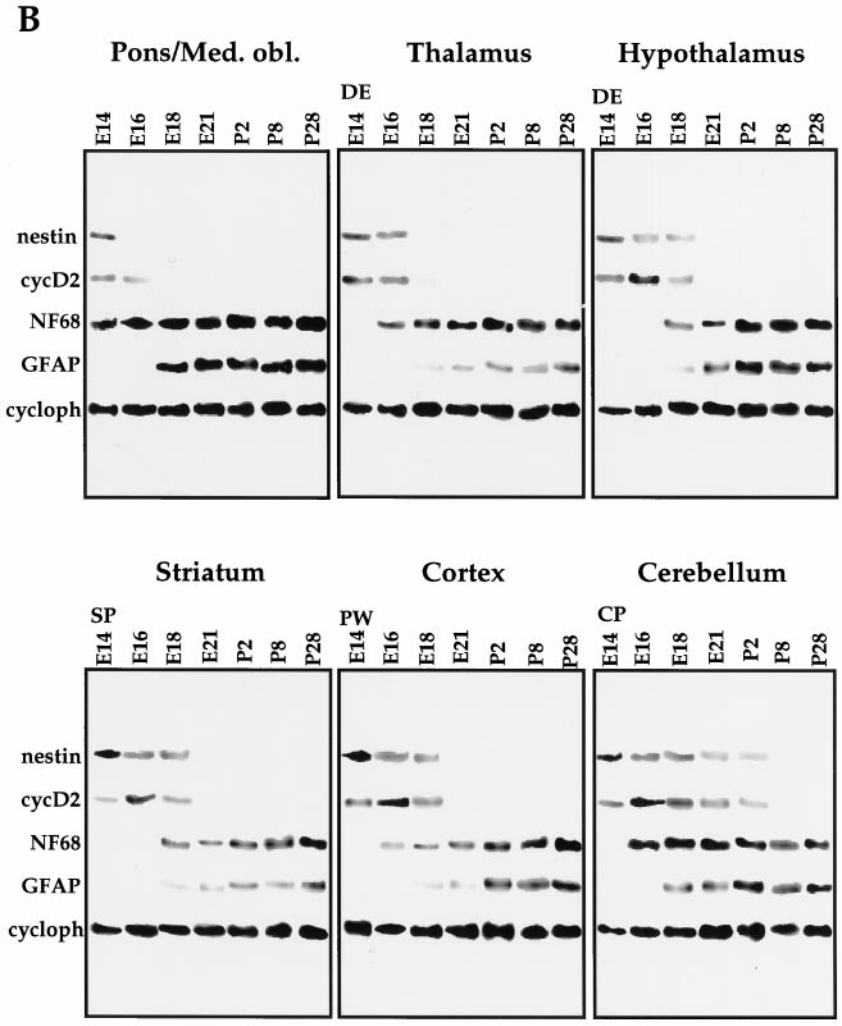
Figure 2. Septamer-binding proteins are associated with different cellular population in the developing rat CNS. *A,* Spatiotemporal distribution of septamer-binding proteins using the rENK-496;-467 fragment as probe in various brain regions between E14 and P28 by EMSA. *P* indicates the formation of a distinct mobility at early stages of neurodevelopment marking the generation of neuroepithelial progenitor cells. *N* indicates a complex whose formation coincides with neurogenesis, and *G* band indicates a complex whose formation coincides with gliogenesis; *DE,* diencephalon; *SP,* striatal primordium; *PW,* prosencephalic wall; *CP,* cerebellar primordium; *Cortex,* frontoparietal cortex. *B,* PCR amplicons indicating the abundance of selected markers as measured by RT-PCR; *Cyclop*, cyclophilin. *C,* P complex is formed in all primordial regions of the forebrain (striatal primordium, prosencephalic wall, and diencephalon), which all express nestin and cyclin D2 mRNA (*bottom panels*). *D,* Hippocampus and olfactory bulb contain the P-band both in early postnatal age (P2) and in adult (P28). Liver does not contain any of the septamer-binding proteins. *E,* Cultured primary cortical astrocytes contain only G-band, whereas the age-equivalent cortex has both N- and G-bands. *F,* EMSA of nuclear proteins derived from a striatal progenitor cell culture 6 d after mitogen treatment (*+bFGF*) shows the presence of all three bands, but 3 d after the mitogen was removed (*bFGF withdrawn*), only the G-band can be detected. The high-mobility bands were primarily detected when the abundant G-band was also seen. *G,* EMSA of nuclear proteins derived from E16 rat forebrain cultures grown for 2 d in the absence or in the presence of the mitotic inhibitor FUDR. Substantially decreased G-band and GFAP mRNA levels were measured in the FUDR-treated cultures, whereas N-band and NF68 mRNA remained unchanged. *C–G, bottom panels,* Abundance of markers as in *D*.

(ds) DNA molecule to decoy septamer proteins from their endogenous DNA binding sites. This approach has been successfully used in numerous model systems and permits functional studies before the cloning of the DNA-binding protein or transcription factor of interest (Morishita et al., 1998). Differentiating striatal cultures at E18 were transfected with the competitor dsDNA. The competitor molecule contained the septamer motif (rENK-496;-467; for sequence information, see Fig. 1*A*). The control DNA contained a transversion mutant (purine to pyrimidine substitution) of the septamer motif (septamer^{mut}) in which the purine to pyrimidine substitution resulted in the loss of protein binding as tested by EMSA (data not shown). The relative abundance of neuronal and glial markers (ENK, NF68, and GFAP) was decreased in cultures after transfection with the wild-type septamer motif containing dsDNA (Fig. 3). ENK mRNA levels decreased by 80%, whereas NF68 and GFAP mRNA levels were ~60% lower than in cultures transfected with septamer^{mut}. The abundance of cyclophilin mRNA did not changed significantly. There were no significant difference between cultures that were transfected with the septamer^{mut} DNA and nontransfected controls. The effects were highly reproducible ($n = 6$).

Lineage-specific protein components of the Septamer-binding complex

To identify the proteins that bind to the septamer motif and form the P-, N-, and G-bands, we performed both solution- and EMSA-mediated (two-dimensional) UV cross-linking experiments with and without simultaneous chemical cross-linking reactions. A DNA probe containing the septamer motif (rENK-496;-467) was UV cross-linked to nuclear proteins derived from E14 or E16 striatum. By EMSA, only the P-band was observed at E14, whereas at E16 no P-band but both N- and G-bands were detected (Fig. 2*A,C*). After UV cross-linking, however, there were no differences seen in the size of the protein–DNA complex derived form the E14 or E16 striatum (Fig. 4*A*; compare *lanes 1, 4*). After deducting the size of the DNA probe, the size of the DNA-binding proteins was found to be 32 kDa in both E14 and E16 striatum. In addition to the identical size, incubation with DNase I and/or dephosphorylation of cross-linked proteins from E14 and E16 striatum resulted in identical changes in gel electrophoretic mobilities (Fig. 4*A*; *lanes 2, 5*, *3, 6*). These observations suggested that the core DNA binding protein may be identical in all three complexes (P, N, and G).

The identical appearance of UV cross-linked DNA–protein complexes in both E14 and E16 striatum (Fig. 4*A*) sharply contrasts with the distinct mobility of P, N, and G complexes detected by EMSA (Fig. 2, compare *A, C*). The identity of proteins forming the various complexes was investigated by a novel combination of EMSA-mediated chemical and UV cross-linking experiments (see Materials and Methods). EMSA-mediated UV cross-linking experiments confirmed that the basic DNA-binding protein in all three complexes (P, N, and G) is 32 kDa (Fig. 4*B, EMSA I*). In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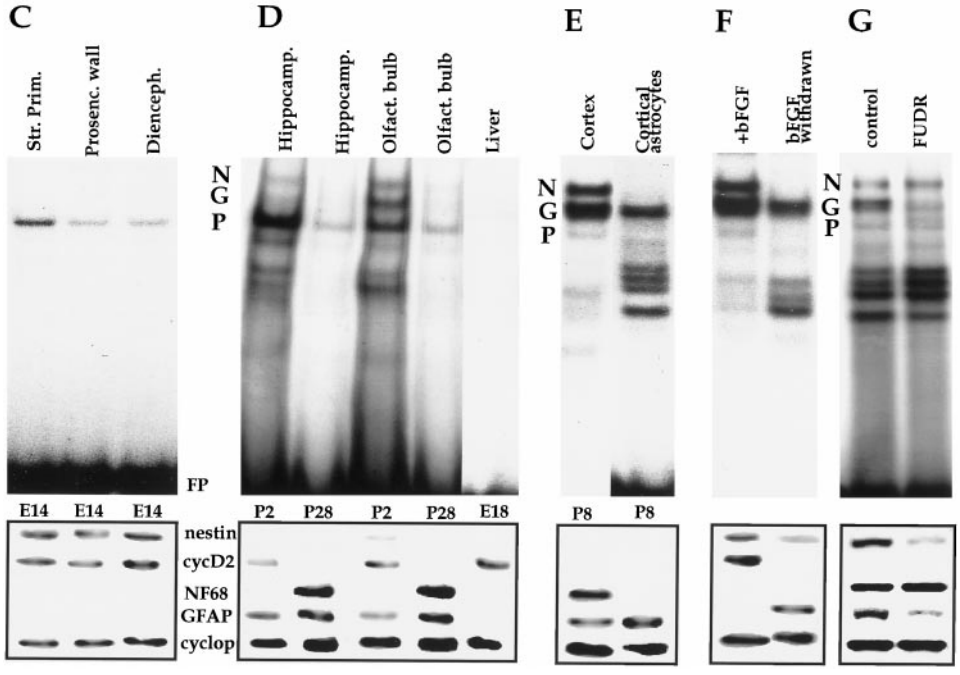
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FUDR
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P

Table 1. Genes with septamer element

Gene	Sequence	Accession no.
Enkephalin[a]	TATTGG**TTTGCAT**AATCAT	X59136
Substance P[a]	AAATGT**TTTGCAT**GTGTTA	L07328
VIP[b]	TATGTC**TTTGCAT**AATGTT	X74297
Tyrosine hydroxylase[c]	CAGGGG**TTTGCAT**GGACCC	M18114
Neurofilament 68kD[b]	AAAAGT**TTTGCAT**GTCTTT	U80021
Necdin[b]	ATCTGC**TTTGCAT**GGATCT	D76440
Synapsin I[c]	TGTACC**TTTGCAT**GTGTTG	M55301
(m1) muscarinic ACh receptor[b]	ACGCTT**TTTGCAT**TCCCGC	AJ02973
GAP-43[a]	CTCCTT**TTTGCAT**TTTCCT	S71492
N-CAM[c]	CAGCAG**TTTGCAT**ATTTTT	X53243
glial high-affinity glutamate transporter[b]	AGACTC**TTTGCAT**CTCAGT	D63816
vimentin[b]	TACAGG**TTTGCAT**CACGTT	D50805

Septamer sequences within the 5′ regulatory region of genes are in boldface; the 5′ base is underlined. N-CAM, Neuronal cell adhesion molecule; VIP, vasoactive intestinal peptide.

Accession numbers are from GenBank.

[a]Rat; [b]mouse; [c]human.

addition, a smaller protein–DNA complex was also detected in all three (P-, N-, and G-) bands (Fig. 4*B*, *EMSA I, diamond*, *lanes 2–4* and markers) after UV cross-linking. The deduced size of this smaller protein is 16 kDa, half the size of the larger protein (32 kDa). The 16 kDa protein most likely represents the monomer unit of the septamer-binding protein, because after chemical cross-linking, the smaller form was undetectable (Fig. 4*B*, *EMSA II*, *lanes 6–8*). Loss of the 16 kDa monomeric form would occur as the equilibrium shifts from monomer to dimer in the presence of chemical cross-linker, and in the P-band only the 32 kDa form was detectable after chemical cross-linking (Fig. 4*B, lane 6, filled circle*). The 16 kDa protein was named *p-sept*. Performing a combination of chemical and UV cross-linking on the P-, N-, and G-bands that were excised after EMSA resulted in the identification of additional proteins (Fig. 4*B*, *EMSA II, lanes 7, 8*). The combination of chemical and UV cross-linking of the excised N-band identified an 84 kDa complex (*filled triangle*). After deducting 32 kDa for the homodimerized *p-sept* and 22 kDa for the free DNA probe from 84 kDa, the size of the N-specific protein was calculated to be 29 kDa (Fig. 4*B, molecular size panel*). This protein was named *n-sept*. Similar calculations indicated that the size of the G-specific protein (*filled rectangle*) is 23 kDa (Fig. 4*B*, *lane 8*). This protein was named *g-sept*. Similar procedures with the excised P-band resulted only in the detection of the 32 kDa *p-sept* homodimer (Fig. 4*B*, *lane 6, filled circle*), but no additional proteins were apparent. The molecular size of *p-sept* was independently confirmed by Southwestern analysis using nuclear proteins derived from E15.5 striatum (data not shown).

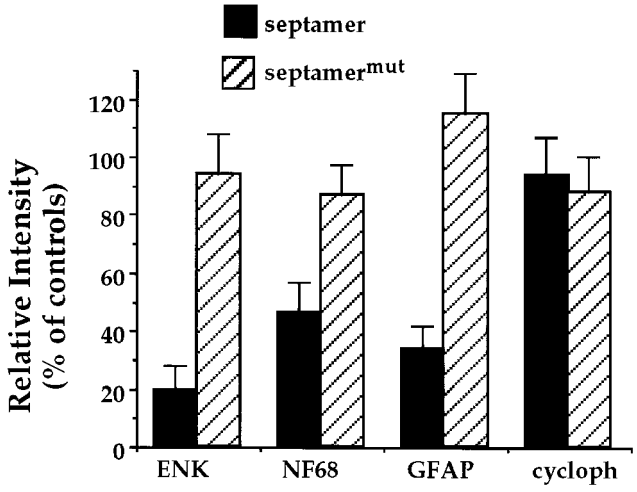

Figure 3. Effect of blocking septamer function in differentiating neural cultures by introducing exogenous competitor DNA molecule. Shown are results of RT-PCR analysis of gene expression patterns after the introduction of rENK-496;-467 as competitor DNA (septamer, *solid bar*) and a mutated control DNA (septamer^{mut}, *hatched bars*). *cycloph,* Cyclophilin. Values are expressed as percentage of the relative intensities obtained from control cultures that did not receive DNA. Error bars indicate SEM ($n = 6$).

The various Sept complexes bend the DNA in a lineage-specific manner

Using a circular permutation assay we addressed the question of whether the distinct Sept-binding protein complexes can specifically affect DNA structure as measured by DNA bending. As the DNA changes its linear shape to a more "V"-shape configuration, so does the migration of the complex. A bending site closer to the center of the probe results in a higher degree of change in the overall shape of the DNA than a bending site closer to either end. The resulting change in the overall shape of the complex can be detected by electrophoretic mobility shift assay; furthermore, the bending angles can be estimated from the electrophoretic migration differences. The circular permutation assay indicated that septamer-binding proteins indeed bend the DNA on DNA–protein complex formation (Fig. 5*A*). Moreover, the bending angle was distinct for each of the lineage-specific complexes (P, G, and N). The P-binding protein(s) present at E14 bent the DNA for 53 ± 4° (Fig. 5*B*). The formation of G complex (made of two P proteins plus the G-specific protein) resulted in an increased bending angle up to 72 ± 3° of the DNA. The N complex (the addition of the N protein to the P homodimer) made the DNA bend even more, with a bending angle of 90 ± 2°.

DISCUSSION

Our approach to elucidate the transcriptional controls underlying neural differentiation using the ENK gene as a model system has yielded the following findings: (1) the identification of septamer, a novel DNA element present within the regulatory regions of

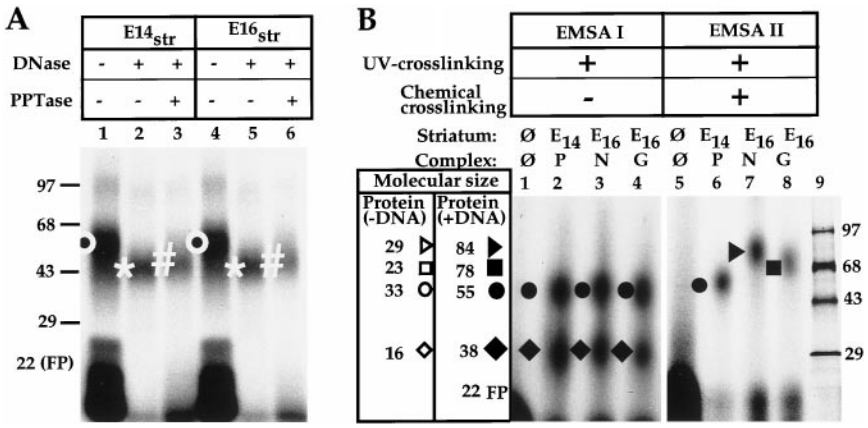

Figure 4. Identification of septamer-binding proteins by UV cross-linking. *A*, Radioactively labeled rENK-496;-467 probe was combined with nuclear extracts from E14 and E16 striatum and was irradiated in solution with UV light. One-third of the cross-linked DNA–protein complex was analyzed without further treatments (*lanes 1, 4*); one-third was additionally incubated with DNase I (see Materials and Methods; *lanes 2, 5*); and one-third of the cross-linked mixture was incubated with both DNase I and protein phosphatases (*PPTase*; *lanes 3, 6*). The complexes were analyzed on a 4–20% SDS-polyacrylamide gel. A *filled circle* indicates the UV cross-linked protein-DNA complex; an *asterisk* marks the cross-linked and subsequently DNase I-treated complex; a *number sign* indicates UV cross-linked complexes after dephosphorylation. *Numbers* at the *left* figure indicate the position of molecular size markers in kilodaltons. *B*, Septamer-binding complexes were separated in the first dimension by EMSA followed by UV cross-linking the probe to the core-binding protein. The *filled circle* indicates a 32 kDa core DNA-binding protein,which is identical in P, N, and G complexes (*lanes 2–4, 6, filled circles*; also see *A*). A *diamond* indicates a protein of 16 kDa, likely the monomeric unit of the DNA-binding protein. Chemical cross-linking enhances the dimerization of this protein (*lane 6*). An *arrowhead* indicates the N complex of 84 kDa (*lane 7*), from which the size of the N-specific protein can be calculated as 29 kDa (see Materials and Methods for details). A *square* marks the G-specific complex (*lane 8*; 78 kDa) from which the size of the G-specific protein has been calculated to be 23 kDa. Molecular weight markers are in *lane 9*.

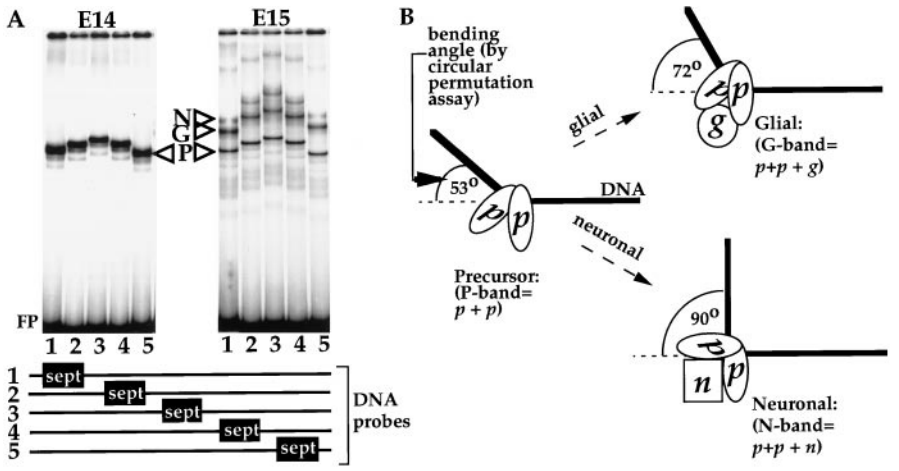

Figure 5. Distinct bending effects of the various septamer binding protein complexes. *A*, In a circular permutation assay five radioactively labeled DNA fragments contain the septamer element at the various positions relative to fr.C derived from pBEN-DrENK (probes *1–5, bottom panel*). All five fragments were tested with proteins present at E14 (*P*) and E15 (*P, G, N*). Different extents of DNA bending are shown as migrational differences of protein–DNA complexes using probes 1–5. *FP,* Migration of the free probe. The *box* indicates the relative position of the septamer element within the fragment. *B*, Diagram summarizing the protein components that bind to the septamer motif and bend the DNA in lineage-dependent manner that can regulate gene expression. The P-band observed by EMSA in primordial brain regions (also see Fig. 2*A,C*) is composed of the homodimerized 16 kDa *p-sept* protein (also see Fig. *4B*). The G-band associated with glial precursors also contains the homodimerized *p-sept* as the DNA binding domain to which the *g-sept* of 23 kDa in molecular weight binds (also see Fig. *4B*). Together they form a 55 kDa multimer detected by EMSA as G-band. The DNA binding unit of the N-band associated with neuronal precursors is also composed of the homodimerized *p-sept* to which a 29 kDa protein, *n-sept,* binds (also see Fig. *4B*). Together they form the 62 kDa multimer detected as N-band by EMSA.

some neuronal- and glial-specific genes; (2) the functional indication from "decoy" experiments that septamer proteins may act as positive regulators during neurodifferentiation; (3) the biochemical characterization of three distinct nuclear proteins (*p-sept, n-sept,* and *g-sept*) that form distinct septamer DNA-containing complexes specific to cells of the progenitor, neuronal and glial lineages; and (4) the demonstration that the distinct septameric protein complexes bend the DNA in a lineage-specific manner that can significantly contribute to the regulatory action in the developing CNS.

The binding site was named septamer because it required seven nucleotides in the core sequence for specific binding (TTT**G***CAT; boldface and asterisk indicating the base-specific interaction). The site is similar to octamer elements, such as the Oct-1 motif (**A**TTTGCAT) present within the regulatory regions of large numbers of genes with diverse functions (Rosenfeld, 1991; Verrijzer and Van der Vliet, 1993). Of particular note is the crucial requirement of the 5′ purine nucleotide (**A**, see above boldface and underlined) for octamer binding (Verrijzer and Van der Vliet, 1993), which is not needed for septamer binding (see Fig. 1*B*). This level of binding specificity is not without precedent (Ryoo and Mann, 1999), because sequences flanking the core-binding site may contribute to binding specificity (Saade et al., 1997; Swanson and Yang, 1999). Additional studies using purified sept proteins are required to identify the role of flanking nucleotides in septamer binding. Proteins of the *Brn* family are predominantly expressed in the developing mammalian CNS, but their recognition site is distinct from the oct-1 motif (Wegner et al., 1993); on the other hand, *Oct-1* and *Oct-2* are not expressed in the brain (Wegner et al., 1993; Eraly et al., 1998). Importantly, *p-sept* that directly binds to the DNA does not bind to the octamer motif (Fig. 1*C*). Moreover, sept proteins were not recognized by any of the different polyclonal anti-Oct-1 and Oct-2 antibodies. These findings, along with the estimated molecular mass for the

various septamer proteins, strongly suggest that these are distinct from any known POU and octamer-binding proteins (Wegner et al., 1993).

The septamer element has been found within the regulatory regions of genes encoding neurotransmitters, transporters, receptors, surface molecule, and neuron-specific cytoskeletal elements (Table 1). The list most likely is incomplete because of the low number of GenBank entries for regulatory DNA regions. Therefore it is conceivable that septamer-binding proteins will be found on many more neuronal- and glial-specific genes. We have performed functional studies using a septamer-containing DNA competitor molecule (also called decoy DNA), which permits experiments before the availability of antisense molecules (Clusel et al., 1995; Morishita et al., 1998). These studies suggested that septamer interactions are required for the developmental expression of the tested neuronal- and glia-specific genes and that septamers are likely positive regulators of neurodifferentiation events. Two of the three markers tested in these studies (ENK and NF68) are known to contain septamer motif (see Table 1). However, because of limited GenBank entrees, we could not confirm the presence of the septamer element on the GFAP gene.

Progenitors populating distinct brain primordia express the 16 kDa *p-sept* that is homodimerized and forms the P-band as observed by EMSA (Figs. 2*A,C,* 4*A,B*). Additional evidence for the association of P-band with progenitors was provided by manipulating the differentiation of progenitors in culture (Johe et al., 1996). When E16 striatal cultures were treated with bFGF for 6 d, progenitors persisted, as indicated by P-band, nestin, and cycD2 mRNA expression. None of these markers could be detected in age-equivalent striatum (E22) or in cultures without bFGF treatment. After withdrawal of bFGF, cells differentiated into the astroglial fate (marked by G-band and GFAP mRNA), in agreement with previous studies showing that the default differentiation pathway is astroglial (Craig et al., 1996; Johe et al., 1996). During early postnatal age the P-band was also detected in the cerebellum (Bayer and Altman, 1995). Because postnatal neurogenesis in the cerebellum gives rise to granule cells, this may explain the presence of the P-band in this period. The N-band was missing at these times; that, however, would not be the first molecular difference between granule cells of the cerebellum and other brain regions (Osborne et al., 1993; Salinas et al., 1994; Dahlstrand et al., 1995).

In the adult brain, the P-band was detected only in the hippocampus and olfactory bulb (Fig. 2*A,D*). This finding agrees with previous descriptions of neural progenitor cell populations in these regions of the adult mammalian CNS (Calof, 1995; McKay, 1997; Gage, 1998; Luskin, 1998). The adult subventricular zone was not examined because of the limited amounts of tissue available.

The appearance and disappearance of the P-, N-, and G-bands were closely correlated with the known developmental gradient of neurogenesis and gliogenesis and differentiation in ontogenetically distinct brain regions (Jacobson, 1993; Bayer and Altman, 1995; Fig. 2*A*). For example, both N- and G-bands appeared first in the medulla oblongata (E13–E14) and were not detectable postnatally, indicating the early cessation of neurogenesis and gliogenesis and differentiation in this part of the CNS. Further evidence documenting the association of N- and G-bands with differentiating neurons and glia was obtained by blocking cellular proliferation in embryonic forebrain cultures. Neurons and glia in the mammalian CNS are generated at different time points of development. Accordingly, in the E16 rat forebrain the majorityof neurons are already postmitotic, but gliogenesis and differentiation are just on the rise (Jacobson, 1993; Bayer and Altman, 1995). A mitotic blocker applied at developmental age E16 consequently did not have a major effect on the abundance of N-band and the corresponding NF68 mRNA expression but substantially blocked gliogenesis, as measured by a drop in the abundance of G-band and GFAP mRNA. The incomplete elimination of the G-band and GFAP mRNA can be explained by the fact that some progenitors had already differentiated into glia before antimitotic treatment. Positive association of the G-band with glia was demonstrated by analyzing primary cultures of cortical astrocytes, in which only the G-band and GFAP mRNA were present (Fig. 2*E*). G-band was also detected in the nuclear extracts of primary cultured oligodendrocytes (data not shown).

By using a novel combination of UV cross-linking (Tang et al., 1997) and chemical cross-linking (Korn et al., 1972), we found that all three septamer complexes (P-, N-, and G-bands) share an identical DNA-binding unit. This unit is formed by the homodimerized *p-sept* (Fig. 4*A,B*). However, the available data are insufficient to determine whether homodimerization of *p-sept* is a prerequisite for DNA binding. As cross-linking studies demonstrated, *p-sept* continued to be expressed in differentiating neuronal and glial cells. These studies also demonstrated that differentiating neurons express a 29 kDa protein (*n-sept*), whereas glia express a 23 kDa protein (*g-sept*). Binding of the 29 kDa *n-sept* to the *p-sept* homodimer resulted in the formation of a 62 kDa neuron-specific multimer observed as N-band. Likewise, binding of the 23 kDa *g-sept* to the homodimerized *p-sept* resulted in the formation of the glia-specific multimer of 56 kDa observed as G-band (Fig. 5).

Sept-binding proteins may exert their regulatory effects through altering DNA structure (Spana and Corces, 1990). For example, the binding of the N complex to its DNA site resulted in DNA bending of 90° (Fig. 5*A,B*). This can be permissive for far-distant protein–DNA interactions (Tjian, 1996). These interactions are critical in cellular differentiation as demonstrated in various cells (Grosschedl et al., 1994; Perez et al., 1994; Armstrong et al., 1995; Dyer et al., 1996). The significantly lesser degree of DNA bending (72°) caused by the G complex creates a different structural environment for far-distant DNA–protein interactions. This could be less permissive for far-distant interactions.

We envisage the role of the various septamer-binding proteins in regulating the expression of septamer motif-bearing genes (Table 1) as similar to the regulation of Ig gene expression in the B-cell lineage. The B-cell-specific coactivator *OCA-B*/*OBF-1*/*BOB-1* is expressed in differentiating B-cells and binds to the *Oct-1* protein that directly binds to the octamer motif (Luo et al., 1992; Wirth et al., 1995; Cepek et al., 1996; Kim et al., 1996; Knoepfel et al., 1996; Matthias 1998). As suggested by the inactivation of the *OCA-B*/*OBF-1*/*BOB-1* gene, a precise, lineage-specific assembly of octamer-binding protein complexes is critical in regulating cell-specific expression of Ig genes (Nielsen et al., 1996; Schubart et al., 1996). Similarly, the *p-sept*-positive multipotent neuroepithelial progenitor cells will differentiate into either the neuronal or the glial fate depending on the expression of either the *n-sept* or the *g-sept* proteins. By analogy to *OCA-B*/*OBF-1*/*BOB-1*, septamer-binding proteins could contribute to the coordinated expression of septamer motif-bearing genes required to regulate the emergence of cellular diversity in the developing nervous system.

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