

# Single-stranded DNA-binding Proteins and Neuron-restrictive Silencer Factor Participate in Cell-specific Transcriptional Control of the *NMDAR1* Gene\*

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Our previous studies revealed that a proximal region of the *N*-methyl-D-aspartate receptor 1 (*NMDAR1*) promoter is important for cell-type-specific expression. We have now explored the contributions of several regulatory elements to this specificity. Deletion of the neuron-restrictive silencer element partially relieved the suppression of promoter activity in C6 glioma and HeLa cells. An overlapping G(C/G)G/tandem Sp1-containing region crucial for both basal and nerve growth factor (NGF)-regulated promoter activity specifically bound nuclear proteins on its purine-rich sense strand. A faster migrating complex, single-stranded binding protein complex 1 (SBPC1), was highly enriched in HeLa cells, whereas a slower migrating complex, SBPC2, was enriched in PC12 cells. A high ratio of 2/1 complex correlated with a high level of promoter activity. NGF treatment of PC12 cells reduced SBPC1 but increased SBPC2. Competition experiments showed that the SBPC1 binding required a dG<sub>4</sub> sequence and the SBPC2 needed a core of TG<sub>3</sub>A plus a 5'-flanking sequence. Single-stranded DNA encompassing TG<sub>3</sub>A and/or dG<sub>4</sub> specifically suppressed cotransfected *NMDAR1* promoter activity. UV cross-linking studies indicated that a 31.5-kDa protein mainly formed SBPC1, whereas SBPC2 contained several larger proteins. Our results suggest that neuron-restrictive silencer factor and single-stranded DNA-binding proteins may both play a role in cell-type specificity of the *NMDAR1* gene, and the latter may also be involved in basal and NGF-regulated activity.

The NMDA<sup>1</sup> subtype of glutamate receptor plays important roles in voltage-dependent Ca<sup>2+</sup> influx, synaptic plasticity, and excitotoxic neuronal death in the mammalian central nervous system (1–3). Functional NMDA receptors contain a key *NMDAR1* subunit in combination with one or more members of the *NMDAR2*(A–D) subunits (4–7). The expression pattern of

the *NMDAR1* gene is widespread in the brain and restricted to neuronal cells (8–10). A great deal of data suggest that interactions of cis elements in a gene with tissue-specific transacting factors play crucial roles in determining tissue-specific expression of the gene (11–14). We previously isolated a 3-kilobase pair promoter of the rat *NMDAR1* gene and observed that a proximal region with 356 bp is sufficient to confer to the promoter a cell-type specificity in neuronal-like PC12 cells compared with C6 glioma and HeLa cervical cancer cells (15–17). This proximal promoter region includes a 5'-untranslated region that contains a neuron-restrictive silencer element (NRSE)/RE1-like element. Several neuronal genes contain this element, and a neuron-restrictive silencer factor/REST, expressed in nonneuronal tissues, may recognize this element and restrict gene expression in nonneuronal cells (11, 18).

Recently, several factors were identified that preferentially bind transcriptional elements on single-stranded DNA and function as suppressors or activators (19–31). For example, heterogeneous nuclear ribonucleoprotein K specifically binds to a cytosine-rich single-stranded DNA of the human *c-MYC* promoter and transactivates a heterogeneous promoter containing this binding sequence only when this promoter is present in a circular, supercoiled conformation and not in a linearized construct (19). The supercoiled structure is thought to provide a single-stranded region of DNA due to negative supercoiling of the circular plasmid. Another example is a cellular nucleic acid binding protein (22). This cellular nucleic acid binding protein is a ~19–20-kDa zinc finger protein that recognizes a purine-rich single strand sequence GTGCGGTG in the sterol regulatory element (21) or a sequence TGGGGAGGG in the CT element of the human *c-MYC* promoter and acts as an activator of gene expression (22). Recently, Taira and Baraban (20) identified a protein complex that recognizes a G-rich strand of an NGFI/Egr binding element (20). Proteins in this complex are enriched in the rat brain. Their results suggested that the NGFI/Egr element may be a convergence point for double and single-stranded binding transcription factors.

The proximal 356-bp promoter of the *NMDAR1* gene contains a GC-rich sequence encompassing GSG and Sp1 elements. The sense strand of this region also is purine-rich (15). Our previous studies showed that a disruption of these sequences eliminating GSG and Sp1 binding decreased the basal and growth factor-regulated promoter activities (17). In this study, we examined the role of an NRSE and the interactions of nuclear proteins with a purine-rich single (sense) strand of GSG/Sp1 region in the control of cell-type-specific expression of the *NMDAR1* promoter.

## EXPERIMENTAL PROCEDURES

**Plasmid Construction**—Plasmids of the *NMDAR1* promoter/luciferase reporter gene were constructed as described previously (16, 17). Deletion of the NRSE in the *NMDAR1* promoter construct, pNRL356,

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<sup>1</sup>The abbreviations used are: NMDA, *N*-methyl-D-aspartate; *NMDAR1*: *NMDA* receptor 1; bp, base pair(s); NRSE, neuron-restrictive silencer element; REST, repressor element 1-silencing transcription factor; PCR, polymerase chain reaction; NGF, nerve growth factor; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; SBPC1, single-stranded binding protein complex 1; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; NGFI/Egr, NGF-induced early response gene/early growth reaction factor, GSG, G(C/G)G.

was prepared using deletion PCR (32). Primers were designed and used as follows. For the upper fragment in the first round of PCR, primer GB46 (17) was paired with a downstream primer GB126, 5'-GAGCGCGGGCGGATG|GCGTGT|TTGGCGCT. The lower fragment was generated with GB40 (17) with an upstream primer, GB125, 5'-AGCGCCAAACACGC|CATCCGCGCGCTC. A “|” indicates a deletion of a 17-bp NRSE-containing sequence in both GB126 and GB125. The second round of PCR was performed with agarose gel-purified products from the first round of PCR under the conditions as described before (17). The final PCR products were cloned into pGEM-T and subcloned into a luciferase expression vector as reported previously (17).

**Cell Culture and Reporter Gene Assay**—PC12, C6, and HeLa cells were maintained as described before (16). Methods for transfection of the NMDAR1 promoter constructs were described previously (16). A plasmid containing the cytomegalovirus promoter-driven  $\beta$ -galactosidase gene was used to correct for transfection efficiency. In some experiments, G-25 Sephadex-purified oligonucleotides were added to transfection mixtures together with promoter/reporter plasmids.

**Interaction of DNA with Nuclear Proteins**—Crude nuclear extracts were prepared from cultured PC12, C6, and HeLa cells using a modified Dignam method (16). In some experiments, PC12 cells were treated with 100 ng/ml NGF for different times before harvesting for nuclear extraction.

DNA probes or competitors used in electrophoretic mobility shift assays (EMSA) are as follows. Single strand DNA probes encompassing the GSG/Sp1 element are GB59 (sense), 5'-GCGTCAGGAAGCGGGGGCGGTGGGAGGGGTAGAACGCGTAGGT, and GB60 (antisense), 5'-ACCTACGCGTTCTACCCCTCCCACCGCCCCGCTTCCTGACGC; double-stranded oligonucleotide probe for the NMDAR1 promoter was formed by annealing GB59 and GB60. Sequences of all competitors are listed in Fig. 5, A and C. Single-stranded sense and antisense Sp1 consensus oligonucleotides (22-mer) were synthesized following the sequence of Briggs *et al.* (33). EMSA experiments were done as described before (16). Briefly, crude nuclear proteins at 4.5–9  $\mu$ g/reaction were preincubated with poly(dI:dC) and in some experiments with the addition of competitors at 50-fold excess of probe. Synthesized double or single-stranded oligonucleotides were labeled at the 5' end with [ $\gamma$ - $^{32}$ P]ATP and T<sub>4</sub> kinase. Radiolabeled probes were purified by G-25 Sephadex chromatography, and single-stranded probes were freshly denatured by heating for each experiment. 10–50 fmol of probe were used in each reaction. In supershift EMSAs, a polyclonal Sp1 antibody purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) was added to reaction mixtures 20 min after the addition of the probe. DNA-protein complexes were separated on 5% native polyacrylamide gels (PAGE), and dried gels were exposed to x-ray film or quantitatively analyzed on a PhosphorImager SI system as described before (17).

UV cross-linking experiments were carried out using a method described by Chodosh (34). A modified sense strand probe, GB59–2, was synthesized by replacing the deoxyguanine at position 23 in GB59 with a 8-bromo-deoxyguanine and purified by a reverse-phase chromatographic column at Paragon Biotech, Inc. (Baltimore, MD). Radioactive  $^{32}$ P labeling of GB59–2 and its reaction with nuclear extracts were performed as described for EMSAs. Reaction mixtures were then irradiated with UV light at 254 nm for 15 min on ice. Irradiated samples were mixed with one volume of 2  $\times$  sample buffer and denatured at 85  $^{\circ}$ C for 3 min. DNA/protein cross-linked products were fractionated on SDS-PAGE (Novex, San Diego, CA) and visualized by exposing dried gels to x-ray film. The apparent molecular weight of each complex was estimated based on protein molecular weight markers, SeeBlue pre-stained standard (Novex), run simultaneously on the same gel.

## RESULTS

**Neuron-restrictive Silencer Factor/REST Participates in the Control of Cell-specific Expression of the NMDAR1 Promoter**—Using a luciferase reporter gene assay in a transient transfection system, we observed that activity of the 356-bp NMDAR1 promoter showed a 309-fold induction over a promoter-less plasmid in PC12 cells but only 2-fold and 8.5-fold induction in HeLa and C6 glioma cells respectively (Table I). This reporter activity is consistent with the endogenous NMDAR1 mRNA levels that are easily measurable by RNase protection assay in PC12 cells but not detectable in C6 and HeLa cells (16). A similar activity for the 3-kb promoter was seen in each cell line (16). These results strongly suggest that elements important for the cell-type-specific expression reside in this proximal pro-

TABLE I  
Activity of the NMDAR1 promoter in different cells

Cells were cultured and transfected with indicated NMDAR1 promoter luciferase constructs as described under “Experimental Procedures.” The relative luciferase activity was normalized to cotransfected  $\beta$ -galactosidase activity. Promoter activity was then calculated based on the -fold increase in the relative luciferase activity over a promoter-less vector, pGL2Basic. Numbers are mean values  $\pm$  standard error.

	Promoter activity relative to vector		
	pNRL3029	pNRL356	pNRL239
PC12 cells	305.7 $\pm$ 8.3	307.9 $\pm$ 8.5	8.9 $\pm$ 1.1
C6 cells	5.6 $\pm$ 0.1	8.5 $\pm$ 0.6	1.7 $\pm$ 0.1
HeLa cells	1.7 $\pm$ 0.1	2.0 $\pm$ 0.2	1.1 $\pm$ 0.2

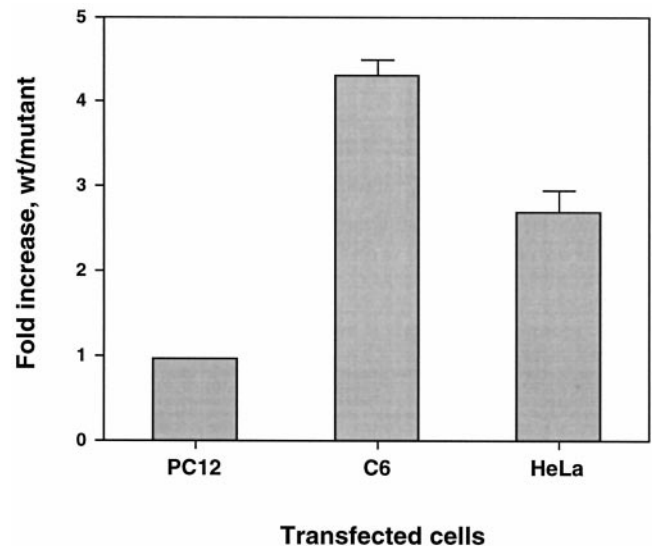
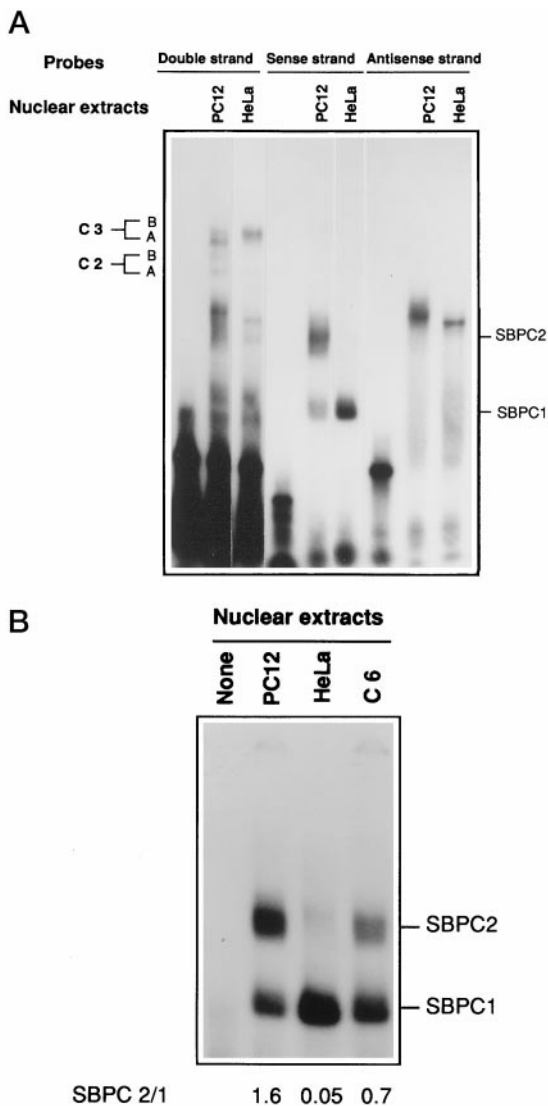


FIG. 1. Effect of deletion of the NRSE/RE1 element on NMDAR1 promoter activity. A construct carrying the deleted NRSE/RE1 element in the NMDAR1 promoter, pNRL356 $\Delta$ NRSE, was transfected into the indicated cells. Wild-type construct, pNRL356, was used as a control in the same experiment. The relative luciferase activity was obtained as described in Table I, and fold increase in mutant over wild-type construct was calculated for each cell line. wt, wild type.

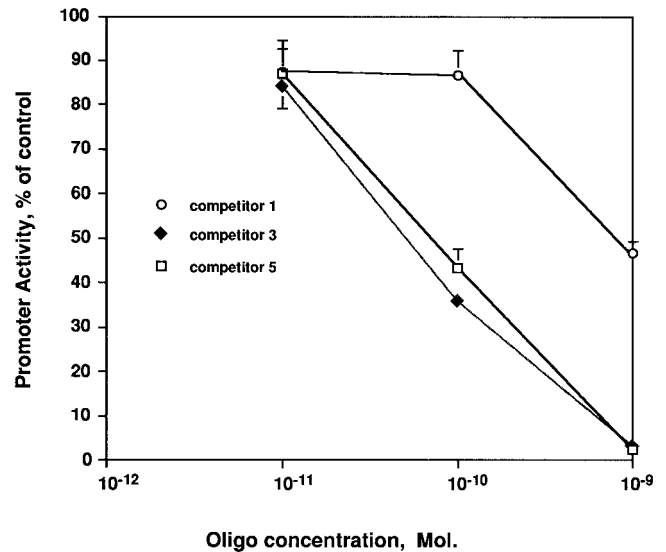
moter region. As reported previously (12, 18), a NRSE/RE1-like sequence was found in the NMDAR1 5'-untranslated region encoded by exon 1 and is part of the 356-bp promoter construct (16). To test its functional role in silencing NMDAR1 promoter activity in nonneuronal cells, we deleted the 17-bp core sequence of the NRSE in the 356-bp promoter and examined the activity of the mutated promoter in both HeLa and C6 cells. Fig. 1 shows that deletion of the NRSE had no effect on activity in PC12 cells but increased activity in C6 cells 4.6-fold and in HeLa cells 2.7-fold when compared with the wild-type promoter activity in each cell line. This result strongly suggested that NRSE participates in the cell-type-specific expression of the NMDAR1 gene. However, after the NRSE/RE1 deletion, the fold increase in activity of the NMDAR1 promoter over promoter-less plasmid was only 40-fold in C6 cells and 5.4-fold in HeLa cells. This is in contrast to a 309-fold induction over promoter-less plasmid in PC12 cells. This lack of a similar fold induction in C6 and HeLa cells suggests the possibility that additional factors, particularly activators, may participate in the control of the NMDAR1 promoter in PC12 cells. This assumption prompted us to search for other nuclear factors involved in this control.

**Novel Purine-rich Single-stranded DNA-binding Proteins May Participate in the Regulation of the NMDAR1 Promoter in PC12 Cells**—As shown in Table I, deletion of a 117-bp 5' sequence (–356/–240) from the 356-bp promoter abolished activ-

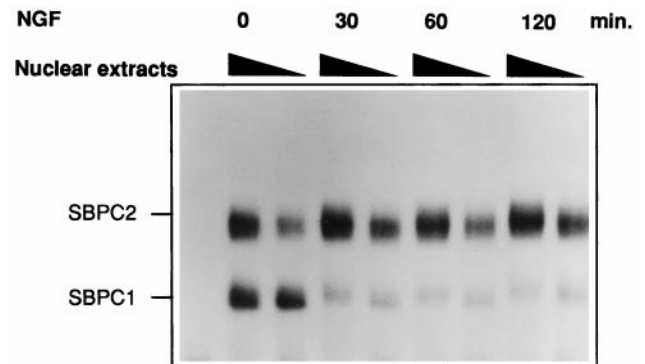


**FIG. 2. EMSA of double- and single-stranded NMDAR1 promoter oligonucleotides with nuclear proteins.** *A*, interaction of the GSG/tandem Sp1 element of the NMDAR1 promoter with nuclear proteins. EMSA was performed as described under "Experimental Procedures." Radiolabeled probes for the *NMDAR1* gene including double- and single-stranded oligonucleotides were incubated with 4.5  $\mu$ g of nuclear extracts and fractionated on 5% nondenaturing PAGE. Autoradiograms were obtained by exposing the dried gel to x-ray film for 8 h at  $-80^{\circ}\text{C}$ . *B*, interaction of single-strand GSG/Sp1 element of the NMDAR1 promoter with different nuclear extracts. Sense strand probe, GB59, was labeled with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and incubated with 4.5  $\mu$ g of nuclear extracts from PC12, HeLa, or C6 cells. The dried gel was exposed to a PhosphorImager plate for quantitation before exposure to an x-ray film. Quantitated radioactivity in each complex was used to calculate the ratio of SBPC 2 to 1. The ratios are listed for each cell line on the bottom of autoradiogram shown in the figure.

ity of the reporter gene. An overlapping GSG/tandem Sp1 element in this deleted sequence was previously shown to be important for promoter activity (17). Also, it is well known that the ubiquitously expressed transcription factor Sp1 and inducible NGFI/Egr proteins interact with this element. Therefore, we tested the binding activity of this sequence in an EMSA. Results in Fig. 2A show that a 43-bp double-stranded DNA encompassing the GSG/Sp1 region formed complexes with nuclear extracts similar to those reported previously using a 112-bp fragment as probe (16). However, we also observed single-stranded DNA binding activity with both sense and antisense oligonucleotides of the same region as the 43-bp



**FIG. 3. Effect of cotransfected sense strand oligonucleotides on the NMDAR1 promoter activity in PC12 cells.** Different amounts of 22-mer oligonucleotides were cotransfected with the NMDAR1 promoter luciferase construct, pNRL356, into PC12 cells as described under "Experimental Procedures." The sequences of each oligonucleotide competitor are listed in Fig. 5A. The luciferase activity was measured and used to calculate promoter activity as a percentage of control (pNRL356 activity transfected without oligonucleotide). The luciferase of the control was  $17,131 \pm 176.7$  fg (mean  $\pm$  S.E.).



**FIG. 4. Differential effects of NGF treatment on SBPC1 and 2 binding in PC12 cells.** PC12 cells were treated with 100 ng/ml NGF for 30, 60, and 120 min, and nuclear extracts were prepared for EMSAs. Nuclear extracts from nontreated PC12 cells served as a control. Two different amounts of nuclear extracts, 4.5 and 9  $\mu$ g/reaction, were used. Binding activities in SBPC1 and 2 were quantitated as in Fig. 2B and expressed as a percentage of control.

dsDNA. One complex was formed on the antisense strand and showed more binding in PC12 nuclear extracts than in HeLa extracts. The nuclear extracts of HeLa cells formed only one band with the sense strand. We named this band single-stranded binding protein complex 1, SBPC1. Interestingly, in addition to SBPC1, PC12 cell extracts formed a second, slower migrating band. We named this band as SBPC2. We quantified the binding activities of SBPC1 and SBPC2 by phosphorimaging. Interestingly, a high SBPC2/SBPC1 ratio as shown in Fig. 2B correlated with a strong promoter activity in PC12 cells, whereas a low ratio was observed in HeLa cells corresponding to a low promoter activity. This suggests that both SBPC1 and SBPC2 may participate in the regulation of NMDAR1 promoter activity. To confirm this possibility, we performed two types of experiments: cotransfecting single-stranded DNA as competitor with the NMDAR1 luciferase reporter gene and testing the effect of NGF treatment of PC12 cells on the formation of these complexes.



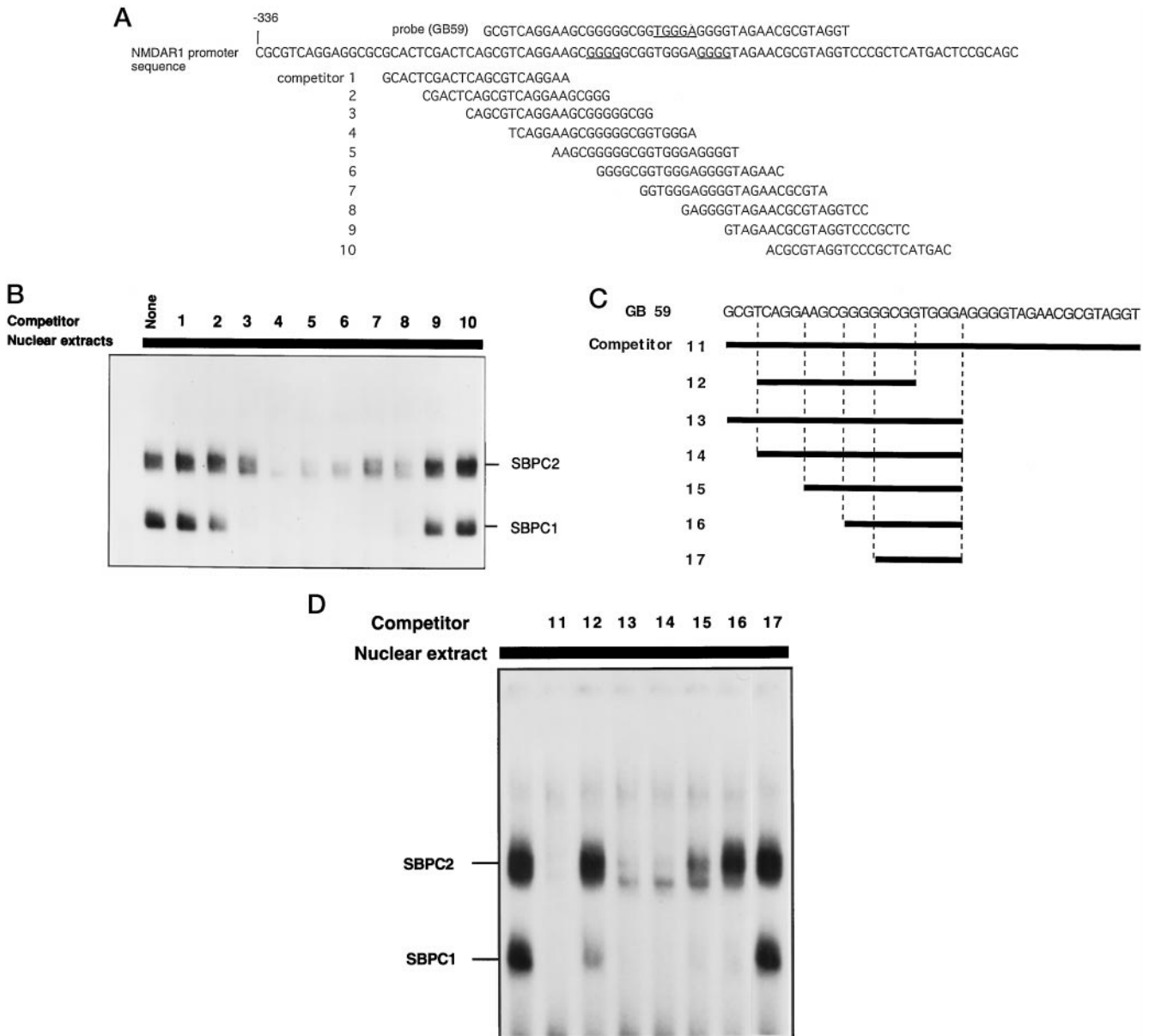
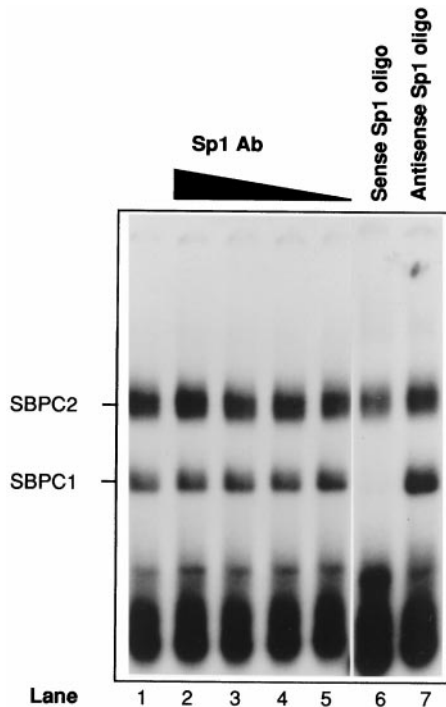


FIG. 5. **Sequence specificity of SBPC1 and SBPC2 formation.** *A*, sequences of sense strand probe and competitors. A 43-mer probe (GB59) and 22-mer oligonucleotide competitors are listed *above* and *below* the NMDAR1 promoter sequence (numbering according to Ref. 15). TG<sub>3</sub>A and dG<sub>4</sub> elements are *underlined*. *B*, competition of SBPC1 and SBPC2 formation on the sense strand of the NMDAR1 promoter. PC12 cell nuclear extracts were preincubated with the indicated competitors at a 50-fold molar excess over probe before the addition of radiolabeled sense probe, GB59. *C*, sequence GB59 and shorter competitors. The sense probe (GB59) and various competitors are listed. *D*, SBPC1 and SBPC2 are competed by different oligonucleotides. EMSA experiments were done as described in *B* with competitors listed in *C*.

22-mer oligonucleotides were cotransfected with pNRL356 into PC12 cells. Those single-stranded oligonucleotides known to compete with formation of SBPC (see below results) dramatically reduced reporter activity in a dose-dependent manner. In contrast, an oligonucleotide known not to compete for SBPC complex formation did not have an inhibitory effect (Fig. 3). PC12 cells treated with 100 ng/ml NGF for 30, 60, and 120 min showed a significant ~65% reduction in SBPC1 and an ~80% increase in SBPC2 complex formation (Fig. 4). These results suggest that single-stranded binding proteins may contribute to both basal and trophic factor-induced NMDAR1 promoter activities.

**SBPC1 Binding Involves a dG<sub>4</sub> Core, Whereas SBPC2 Binding Requires a Core Plus Flanking Sequence**—In an experiment to define the binding elements of SBPC1 and SBPC2, EMSAs were performed with multiple competing 22-mer oligonucleotides overlapping the sense strand probe and flanking sequences shown in Fig. 5A. A typical binding pattern is shown in

Fig. 5B. Using phosphorimaging, we quantitated SBPC1 and SBPC2 formation in the presence of each competitor and compared the remaining complex to a control without competitor. Oligonucleotides containing dG<sub>4</sub> sequence effectively reduced SBPC1 binding to <15% control (competitors 3–7), whereas competitor 4 containing a core sequence of TG<sub>3</sub>A with a 15-nucleotide-flanking region reduced SBPC2 binding to only 23% control. Competitor oligonucleotides at the 3' end of the probe (competitors 7–10), resulting in the loss of 5' sequence, reduced the competition compared with competitors 4–6. This competition study suggests that SBPC1 and SBPC2 protein complexes may recognize slightly different elements located within this region. This concept was further supported by experiments with other oligonucleotide competitors (Fig. 5, C and D). Competitor 12 containing dG<sub>4</sub> but lacking TG<sub>3</sub>A sequence competed SBPC1 but not SBPC2. Competitor 14 containing TG<sub>3</sub>A in addition to all of the sequence of competitor 12 was able to

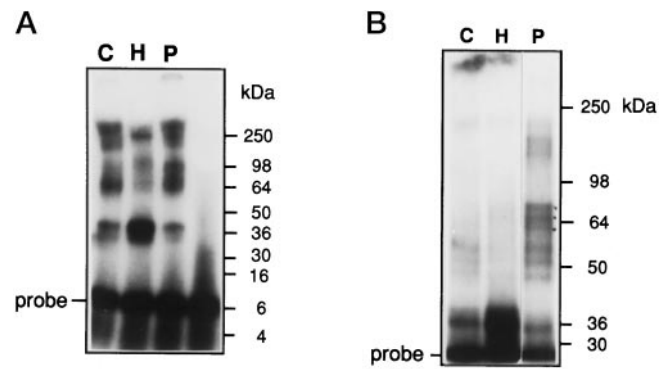


**FIG. 6. Sp1 protein is not involved in the formation of SBPC1 and SBPC2 complexes.** EMSAs were performed as detailed under "Experimental Procedures" using PC12 nuclear extracts. Increasing amounts of polyclonal antibody against Sp1 protein were added to reaction mixtures 20 min after the addition of probe. Single-stranded sense or antisense consensus oligos of Sp1 elements were used as competitors.

compete for both SBPC1 and SBPC2. However, removing the sequence 5' to TG<sub>3</sub>A (competitors 14–17) resulted in a loss of SBPC2 competition. Competitor 17 lacking dG4 failed to compete any complexes although it contains a TG<sub>3</sub>A core sequence.

**Sp1 Binding Is Not Involved in the Formation of SBPC1 and SBPC2**—Considering that the Sp1 element (TGGGAGGGG) in the NMDAR1 promoter overlaps the apparent SBPC1 and SBPC2 binding sequence, we performed experiments to clarify whether these complexes contained Sp family members. In an EMSA, neither SBPC1 nor SBPC2 was changed by a Sp1 antibody, which we previously showed was able to supershift Sp1 binding on the double-stranded probe (Fig. 6). A 70-fold excess of double-stranded Sp1 consensus oligonucleotide failed to compete for the SBPC1 binding. Only the sense strand of Sp1 consensus competed for SBPC1 binding. This sense consensus contains a multiple dG sequence, also present in oligonucleotides that competed for SBPC1 binding (Fig. 5). Furthermore, both SBPC1 and SBPC2 showed a faster migration than complexes detected with either a 24-bp Sp1 consensus double-stranded oligonucleotide (data not shown) or a 43-bp double-stranded probe of the NMDAR1 promoter (Fig. 2A). Therefore, these data suggest that the Sp1 protein is not involved in the single strand binding complexes seen on the NR1 promoter.

**SBPC1 Contains a 31.5-kDa Protein, and SBPC2 Consists of Several Larger Proteins**—To obtain evidence of direct nuclear protein interactions with the sense strand of the GSG/Sp1 region in the NMDAR1 promoter, we modified the oligonucleotide probe GB58 shown in Fig. 5A by replacing residue 23, guanidine, with a bromo-guanidine. After 5'-radiolabeling of this modified probe and incubating it with nuclear extracts, reaction mixtures were irradiated with UV light to cross-link the DNA-protein complexes. As shown in the right-most lane of Fig. 7A, the DNA probe itself migrated as approximately 6.5 kDa in a SDS-PAGE gel. HeLa cells showed a major band with



**FIG. 7. UV cross-linking of nuclear proteins with sense strand of the GSG/tandem Sp1 element in the NMDAR1 promoter.** Cross-linking of nuclear proteins with radiolabeled GB59–2 oligonucleotide encompassing the GSG/tandem Sp1 element was performed as described under "Experimental Procedures." Cross-linked products in 10  $\mu$ g of protein were fractionated on 10–20% Tricine-SDS/PAGE for panel A and on 8% Tris-glycine SDS/PAGE for panel B. Autoradiograms were obtained after exposing dried gels to x-ray film. A control of probe alone was included to locate its migrating position. C, H, and P represent nuclear extracts from C6 glioma, HeLa, and PC12 cells, respectively.

an apparent molecular mass of 38 kDa, corresponding to a 31.5-kDa protein after subtracting the contribution of the probe (6.5 kDa) based on one probe per protein molecule stoichiometry. This band is relatively weak in PC12 cells and C6 cells. The same band disappeared after adding oligonucleotide that removed only SBPC1 in both PC12 or HeLa nuclear extracts (data not shown). These results along with those in Fig. 2B suggest that this 31.5-kDa protein may be the major component of the SBPC1 complex. The SBPC2 complex contains several proteins with larger molecular masses (P, Fig. 7B).

#### DISCUSSION

Neurons express many genes in common with other cell types and in addition express their own unique set of genes important in determining neuron-specific functional characteristics (11, 14, 18, 35). One of these genes is the NMDAR1 gene, which is an important subunit of all NMDA receptors (4–9). It is expressed widely in the brain but exclusively in neurons. A factor important in determining the distribution pattern of proteins is the transcriptional regulation of gene expression. Our previous work and the results reported here suggest that the NMDAR1 gene proximal promoter is able to confer neuronal-specific expression. This region of the promoter contains GSG and overlapping tandem Sp1 elements that we previously showed were important for both basal and neurotrophic factor-induced promoter activities. Strong activity was observed in neuronal-like PC12 cells but not in C6 glioma and HeLa cells. In addition, the NMDAR1 gene contains a consensus NRSE in the 5'-untranslated region. Deletion of this element relieved the suppression of promoter activity in C6 and HeLa cells without having any effect on PC12 cell activity, suggesting that this element plays an important role in neuron-specific expression of the NMDAR1 gene. Nonneuronal cells express a factor, neuron-restrictive silencer factor/REST, that binds to NRSEs located in several neuronal-specific genes and represses their expression (11, 12). Neuronal cells, including PC12 cells, do not express this repressor, thus allowing cell-specific gene expression. Deletion of the NRSE in the NMDAR1 gene relieved suppressed activity in C6 and HeLa cells (Fig. 1), but the resulting elevation in activity of the deleted construct was between 8 and 60 times less than that observed with wild-type promoter in PC12 cells. This is similar to results reported by Li *et al.* (36) with the synapsin I gene promoter and suggested to us that other factors, present in PC12 cells, may be responsible

for the more efficient expression in this cell line.

We showed previously that a GC-rich region of the NMDAR1 promoter encompassing a GSG and overlapping Sp1 sites was protected in DNase footprinting experiments and was able to form several complexes with double-stranded oligonucleotides in EMSAs (16). Since this region is also critical for efficient expression of activity, we further examined this region for other interacting factors that might be important in transcriptional regulation. Until recently, the importance of single-stranded DNA-binding proteins in regulating gene expression has been under-appreciated. Several groups now have identified proteins that selectively bind to single-stranded DNA transcriptional elements (19–31). We found that nuclear extracts from several cell types were able to form complexes with single-stranded oligonucleotides derived from the proximal GC-rich region of the NMDAR1 promoter. Complexes were formed on both sense and antisense oligonucleotides (Fig. 2A). These complexes were different from those that formed when a double-stranded probe from the same region was used (Fig. 2A). PC12 cell extracts contained more of a slower migrating complex, SBPC2, on the sense strand than C6. In contrast HeLa cell extracts lacked this complex but contained more of the faster migrating complex, SBPC1. An approximately 20-nucleotide G-rich region of the promoter sequence appeared to be critical for complex formation, since a series of competitors containing at least a G<sub>3</sub> sequence effectively competed for the binding at both sites (Fig. 5). In UV cross-linking studies, an ~31.5-kDa protein formed the faster migrating complex and larger, >~64-kDa proteins formed the slower complex. Although the components of these complexes are unknown, the SBPC1 complex may be somewhat similar to the GS1 (complex binding selectively to the G-rich strand of the Egr response element) protein complex recently described by Taira and Baraban (20). They reported that this complex, enriched in rat brain extracts, contains proteins of 36 and 30 kDa and is effectively competed by G-rich DNA and, less effectively, by RNA oligonucleotides. The composition of the SBPC2 complex is not known.

Our data suggest that these single-strand complexes may be important in modifying the activity of the NMDAR1 promoter. When single-stranded oligonucleotides known to compete with complex formation (competitors 3 and 5, See Fig. 5A) were cotransfected with reporter plasmid, the reporter activity was dose-dependently reduced (Fig. 3). Another single-stranded oligonucleotide (competitor 1, see Fig. 5A) derived from the NMDAR1 gene promoter 5' of the GC-rich region that did not compete for complex formation did not interfere with reporter activity. In separate experiments, SBPC2 complex was increased when PC12 cells were treated with NGF, a treatment known to increase NMDAR1 promoter activity (17), whereas SBPC1 was dramatically decreased. Thus two treatments, one inhibiting reporter activity (competitive oligonucleotide) and one increasing reporter activity (NGF), also influence the amount of single-stranded complex formation. We have yet to determine the exact relationship between the changes in complex 1 and 2 upon NGF treatment and changes in promoter activity. In any case, this GC-rich region of the NMDAR1 promoter apparently plays an important role in regulating gene expression.

Several genes lacking a TATA-box motif in the proximal promoter region contain a GC-rich region similar to the NMDAR1 gene. These types of gene promoters were thought to be responsible for constitutive expression. It is now becoming apparent that these promoters also may be under regulatory control and induced by various treatments (37–40). It is possible that multiple transcription factors may be competing for the same DNA elements and that activation of transcription

may involve displacement of one group of factors in favor of another that leads to more efficient transcription (41, 42). Likely candidates on the NMDAR1 promoter might be Sp1 and Egr transcription factors, which efficiently bind to GC-rich double-stranded DNA elements. In addition, the current work suggests that other distinct factors interacting exclusively with single-stranded oligonucleotides of the same region also may form complexes and play a role in regulation. In fact, we showed by competition experiments and supershift EMSAs that Sp1 protein does not interact with the single-stranded probes. Thus the GC-rich region of the NMDAR1 gene promoter may be a target for multiple transcription factors whose interactions with both double-stranded and single-stranded forms of the promoter region ultimately determine the transcriptional activity.

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**Single-stranded DNA-binding Proteins and Neuron-restrictive Silencer Factor Participate in Cell-specific Transcriptional Control of the *NMDAR1* Gene**

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