THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 28, Issue of July 13, pp. 25929–25938, 2001

Printed in U.S.A.

Functional Organization of the GluR1 Glutamate Receptor Promoter*

Received for publication, October 5, 2000, and in revised form, April 25, 2001
Published, JBC Papers in Press, May 4, 2001, DOI 10.1074/jbc.M009105200

Karin Borges‡ and Raymond Dingledine

From the Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

The GluR1 glutamate receptor subunit is expressed in most brain areas and plays a major role in excitatory synaptic transmission. We cloned and sequenced 5 kilobase pairs of the rat GluR1 promoter and identified multiple transcriptional start sites between −295 and −202 (relative to the first ATG). Similar to other glutamate receptor subunit promoters, the GluR1 promoter lacks TATA and CAAT elements in that region but binds Sp1 proteins at two sites. Promoter activity of GluR1 fragments cloned into pGL3 was assessed by immunocytochemistry and by measuring luciferase activity after transfection into primary cultures of rat cortical neurons and glia. GluR1 promoter activity was stronger in neurons, with neuronal specificity appearing to reside mainly within the neuronal expression-enhancing regions, −1395 to −743 and −253 to −48. The latter region contains 4 sites that bound recombinant cAMP-response element-binding proteins and a glial silencing region between −253 and −202. In both neurons and glia, promoter activity was increased by a 64-base pair GA repeat upstream of the initiation sites and reduced by a 57-base pair region that contained an N box. In contrast to the GluR2 promoter the regulatory regions are mainly located outside of the GluR1 initiation region.

GluR1 is one of the most abundant glutamate receptor subunits of the AMPA[1] subtype in the brain. AMPA receptors are assembled from GluR1–GluR4 subunits and mediate fast excitatory responses at most synapses. AMPA receptor subunits, including GluR1, are expressed by neurons and glial cells *in vivo* and *in vitro* (for reviews see Refs. 1 and 2), but the density of functional receptors is much lower in astrocytes than neurons.

GluR1 expression is regulated by many different stimuli. For example, GluR1 protein levels are up-regulated by basic fibroblast growth factor in hippocampal neurons (3), and GluR1 transcription is increased by basic fibroblast growth factor and platelet-derived growth factor in oligodendrocyte precursor cells (4). In cultured cerebellar granule cells, high potassium (25 mM) induces GluR1 mRNA levels by 27-fold and protein levels by 11-fold (5). GluR1 is also induced during long term potentiation (6, 7) and repeated electroconvulsive shocks (8). In human epileptic tissues, increases of GluR1 and GluR2 mRNAs (9) as well as immunoreactivity (10) and [^{3}H]AMPA binding (11) have been reported. Furthermore, chronic treatment with cocaine or morphine up-regulates GluR1 specifically in the ventral tegmental area, an area known to be involved in addiction (12, 13), and GluR1 is up-regulated after treatment with haloperidol or clozapine in the medial prefrontal cortex (14).

On the other hand, GluR1 levels decline with maturation in the brain (15). Moreover, neurectomy of the hypoglossal nerve leads to total loss of GluR1 in the hypoglossal nucleus 14 days later, with recovery by 60 days after the transection (16). Deafferentiation in the dorsal horn produces down-regulation of GluR1 in laminae I and II and up-regulation in laminae III–V (17).

Thus, GluR1 expression is up- and down-regulated in multiple conditions, both normal and pathological, but the mechanisms regulating GluR1 expression have not been studied previously. As an initial step toward this goal, we have cloned the rat GluR1 promoter. We have identified Sp1- and CREB-binding sites near the transcriptional start sites and several regions that contribute to high expression in neurons.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Genomic Clones—Probes for genomic screening were radiolabeled using random primers, Klenow DNA polymerase, and [α-^{32}P]ATP (Megaprime kit, Amersham Pharmacia Biotech). About 1.35×10^6 phage plaques from a Wistar rat genomic library (in λ dash II vector, Stratagene) were screened on nitrocellulose filters using the 5′ 1587-bp *Sac*I-*Bam*HI fragment from rat GluR1 cDNA (18) (GenBank™ accession number X17184) as a probe. Five initially positive plaques were rescreened twice, prepared on a large scale, and restriction-site mapped in Southern blots. Out of the five phages, one phage insert hybridized to a PCR-amplified probe (bases 16–223 of the GluR1 cDNA) and contained mainly 5′-untranslated sequence, the first intron, and at least part of the second intron. A *Sac*I-*Sac*I fragment containing 5035-bp 5′ sequence, the first exon, and 593 bp of the first intron was subcloned into the pBluescript KS(−) vector. This construct and several deletion constructs derived from it were sequenced. Moreover, a 4.7-kb *Eco*RI-*Eco*RI fragment with the 5′ *Eco*RI site at −459 (relative to the first ATG) and containing the first intron and at least part of the second intron was cloned into pBluescript KS(−) and partially sequenced (see Fig. 1). The programs from the Genetics Computer Group (GCG) and MatInspector (19) were used for sequence analysis of the promoter.

Constructs—To obtain a control RNA for primer extension and RNase protection assays, a deletion construct from the original *Sac*I-*Sac*I construct was made by digestion with *Eco*RI and religation, resulting in a GluR1 construct from −459 to +675. By using this construct as a template after digestion with *Sac*I, T3 RNA polymerase, and the RNA transcription kit (Stratagene), a synthetic GluR1 cRNA was

* This work was supported in part by grants from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF302117.

‡ Supported by the Deutsche Forschungsgemeinschaft and the Markey Foundation. To whom correspondence should be addressed: Dept. of Pharmacology, Emory University School of Medicine, 1510 Clifton Rd., Atlanta, GA 30322. Tel.: 404-727-5635; Fax: 404-727-0365; E-mail: kborges@pharm.emory.edu.

[1] The abbreviations used are: AMPA, α-amino-3-hydioxy-5-methyl-4-isoxazole propionic acid; CREB, cAMP-response element-binding proteins; bp, base pair; PCR, polymerase chain reaction; kb, kilobase pair; PE, primer extension; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; GFAP, glial acidic fibrillary protein; TK, thymidine kinase; TLU, Turner light units; IBMX, isobutylmethylxanthine; N/G, neurons *versus* glia.

synthesized that included the GluR1 5′ sequence from −459 and 56-bp 5′ linker sequence (named control RNA-1). Control RNA-2 was made from GluR1 cDNA with a 5′ end 266 bp upstream of the ATG (Ref. 18 and see Fig. 2).

For transfection and luciferase assays, fragments of the 5′-flanking regions of the thymidine kinase (TK), GluR1, and GluR2 genes were cloned into the pGL3 basic vector (Promega) in front of the firefly luciferase reporter gene using restriction sites or PCR cloning with *Pfu* polymerase (CLONTECH). All resulting constructs were verified by sequencing. The *Bgl*II to *Hin*dIII fragment of the TK promoter was excised from the pRL-TK vector (Promega) and cloned into pGL3. The GluR2 promoter (−1253 to −111 relative to the first ATG or −822 to +320 relative to the main initiation site) was excised from the pGL2 construct (24) using *Nhe*I and *Bgl*II and inserted into pGL3. PCR-amplified GluR1 fragments were cloned using *Sac*I and *Bgl*II sites introduced in the primers. The 64-bp GA repeat in GluR1 was shortened during the PCR in some constructs to 62 bp (−743/+8, −459/−202, and −459/−48), 58 bp (−402/+8), or 36 bp (−459/+7deltaGA). No correlation between promoter activity and a 64- to 58-bp length of the GA repeat in different constructs was observed. In the −459/rev−48 construct, the region between −254 and −48 was inverted. This region was PCR-amplified with the upper primer containing a *Hin*dIII site and the downstream primer a *Bgl*II site. The PCR product was inserted in reverse orientation into the *Bgl*II and *Hin*dIII sites of the −459/−253 construct. For a list of constructs see Fig. 3.

Putative CRE sites were mutated by a PCR mutagenesis strategy (20) introducing different restriction sites as follows: 243 *Spe*I, −208 *Nsi*I, −151 *Nde*I, and −84 *Eco*RV. During this process −219A was mutated unintentionally to T. As a positive control for CREB activity, we used a vector that contained eight CRE sites from the intragenic inducible cAMP early repressor (ICER) promoter upstream of the interleukin 2 promoter and luciferase (21).

Poly(A)$^+$ RNA Preparation—For primer extension analysis and RNase protection assays, poly(A)$^+$ RNA was prepared from adult male rat brains according to Verdoorn and Dingledine (22).

Primer Extension Analysis—The following oligonucleotides were used for primer extension analysis (bottom strand, all relative to the first ATG, see Fig. 1): PE-A, −187 to −216; PE-B, −155 to −184; PE-C, −31 to −48; PE-D, +13 to −5; PE-E, −427 to −455. 100 ng of each HPLC-purified oligonucleotide was end-labeled using 30 μCi of [γ-^{32}P]ATP and T4 polynucleotide kinase (Life Technologies, Inc.). The radiolabeled primers (specific activity ~5×10^8 cpm/μg) were hybridized for 2 h at 65 °C to 50 μg of poly(A)$^+$ whole brain RNA (~3×10^6 cpm of primer) or 20 ng of synthetic GluR1 control RNA (~10^6 cpm of primer) with 10 μg of yeast total RNA in 150 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM EDTA. The mixture was slowly cooled to room temperature and precipitated with ethanol. Reverse transcription was carried out for 1 h at 42 °C with 200 units of Superscript II (RNase H-free, Life Technologies, Inc.) in first strand synthesis buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) using 625 μM of each dNTP and 5 ng/μl actinomycin D. After ethanol precipitation the reaction products were analyzed on a 5% acrylamide, 8 M urea sequencing gel using sequencing reactions as markers.

RNase Protection Assay—DNA templates for the antisense probes were cloned into pBluescript KS(−) (probe A, −870 to −88; probe B, −1398 to −184) and linearized with *Eco*RI at −454. Antisense RNA probe was synthesized from 0.5 μg of linearized template DNA by T7 polymerase and the Stratagene RNA transcription kit using the supplied transcription buffer, 500 μM ATP, UTP, and GTP each, 12 mM dithiothreitol, 33 units of RNAsin (Promega), 100 μCi of [μ-^{32}P]CTP (Amersham Pharmacia Biotech, 800 Ci/mmol) at 37 °C for 1 h. 2 units of DNase I (Epicentre) were added for 15 min at 37 °C to destroy the DNA template. Template A gave rise to a complementary GluR1 RNA from −454 to −88 (Fig. 2, *A* and *C*), and probe B covered −454 to −184. The probes were purified on a 5% acrylamide, 8 M urea gel and eluted at 37 °C for 2 h into the buffer supplied by the RPA 2 kit (Ambion). The specific activity of the probes was about 1.6×10^9 cpm/μg. 2×10^6 cpm probe was mixed with the different RNA samples (10 μg of poly(A)$^+$ rat adult whole brain RNA, 2 ng of GluR1 control RNA in 10 μg of total yeast RNA, or 10 μg of total yeast RNA alone) in hybridization buffer (80% formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, 1 mM EDTA), denatured for 3–4 min at 90 ± 5 °C, and hybridized overnight at 45 °C. The single-stranded RNA was then digested with 0.5 units of RNase A and 20 units of RNase T1 per reaction at 37 °C for 30 min in the supplied digestion buffer. The reaction was stopped with the supplied solution, precipitated with ethanol after adding 20 μg of additional yeast RNA, and analyzed on a 5% acrylamide, 8 M urea sequencing gel using sequencing reactions as size markers.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from embryonic rat forebrain cultures maintained for 5–9 days *in vitro* according to Harant *et al.* (23). Annealed oligonucleotides were end-labeled with [α-^{32}P]ATP using T4 polynucleotide kinase and were subsequently purified by PAGE. Gel slices were incubated overnight in TE buffer, and the probe was separated from the gel by centrifugation in microcon columns (Amicon) and elution in water. *In vitro* binding reactions were conducted for 20 min at room temperature in a 20-μl volume containing 10 mM Tris, pH 7.5, 10% glycerol, 50 mM NaCl (Sp1 and some c-Jun experiments), or 25 mM NaCl (CREB experiments), 1 mM dithiothreitol, 1 mM EDTA, 0.2 μg of poly(dI-dC)·poly(dI-dC), 2.5 μg of bovine serum albumin, and 20,000–40,000 cpm probe. Adding nuclear extracts in the Sp1 experiments increased the salt concentration up to 70 mM NaCl and 7 mM KCl. In some experiments competitor oligonucleotides (10-, 30- or 100-fold of the amount of labeled probe) were included. When needed, 4 μg of anti-Sp1 or anti-c-Jun polyclonal rabbit IgG antibodies (Santa Cruz Biotechnology, Inc.) were preincubated with nuclear extracts on ice for 30 min. 2 μg of nuclear extracts or one of the following recombinant proteins were added to start the reaction, 0.2 footprinting units (fpu) of human Sp1, 0.3 footprinting units of human c-Jun (both Promega), or 0.5 μg of human CREB1-bZIP corresponding to amino acids 254–327, which contained the DNA binding and dimerization domain (Santa Cruz Biotechnology, Inc.). The DNA-protein complexes were separated on non-denaturing 6% PAGE, 0.5× TBE gels (Fig. 5, assays concerning Sp1 and some c-Jun binding), or Tris glycine, 8% PAGE gels (Fig. 6, assays using CREB-bZip and some assays with c-Jun protein), run at 4 °C for 1.5–3 h. Dried gels were exposed to Kodak X-Omat films for visualization. Double-stranded oligonucleotide probes are as follows (top strand only): Sp1 consensus, ATTCGATCGGGGCGGGGCGAGC; CREB consensus, AGAGATTGCCTGACGTCAGAGAGCTAG; and AP1 consensus, CGCTTGATGAGTCAGCCGGAA.

The GluR1 oligonucleotides were named by their 5′ end, and sequences containing one or more putative SP1 sites are underlined: −332a, AACACGGGAGGGTGAGAGAGG; −296, TAGAGAAGAGGAGGAGAGCAGAGG; −275, AGGGAGAGGGGGAGCGAGCTAGCG; −244, CATGAGGACGGGCTGCTCAA; −232, CTGCTCCCGG**CTCA**GTTAATCTGGC (AP1 half-site is in boldface).

For CREB binding assays the following GluR1 oligonucleotides (putative CRE sites are underlined) were tested: −332b, AACACGGGAGGGTGAGAGAGGAGA; −250, TCCAAGCATGAGGACGGGCTGCTC; −218, GTTAATCTGGCTGTCAGTCGGTGTT; −193, ACGCTGCAGTTGAACTGCTCGGCTCCC; −165, CTTCCAAGAGAAACCTCACGGAAGGAA; −117, CAAGGAACTGCAGGAAGAAAAGAGCCG; −93, CCGGCAGAGCATCAAGAAGAATCGAAG; and −78 as a negative control, GAAGAATCGAAGGGAGGGGAGGGAAGA.

Cell Culture, Transfection, Immunostaining, and Luciferase Assays—The procedures were slightly modified from Myers *et al.* (24). Primary mixed neuronal-glial cell cultures (referred to as "forebrain cultures") were prepared from E18 to E19 rat cortex with striatum. For transfections, the dissociated cells were plated into 12-well culture dishes (Falcon or Costar) precoated overnight with 180 μg/ml poly-D-lysine and grown in defined serum-free DMEM supplemented with B27 (both Life Technologies, Inc.), which enhances neuronal survival. The cultures were transfected on days 4–7, when counting of immunolabeled cultures grown on glass coverslips revealed about 3% GFAP-positive cells and 65% MAP2-positive cells. Primary glial cultures were obtained from the primary forebrain cultures by changing the medium to DMEM with 10% fetal bovine serum, which induced neuronal cell death as described by Myers *et al.* (24). When glial cells were confluent they were trypsinized and replated into 12-well dishes for transfection. Immunolabeling showed about 90% GFAP-positive cells in these cultures. Visually, the contaminating cells appeared to be progenitor cells, microglia, and some oligodendrocytes.

For transfection, cells in each well were incubated with 1 μg of each construct in 6 μl of Plus reagent mixed with 4 μl of LipofectAMINE (Life Technologies, Inc.) and 0.5 ml of minimal serum-free medium (DMEM supplemented with 0.5 μM insulin, 100 μg/ml human apotransferrin, 0.03 μM selenium, and 60 μM putrescine, all Sigma) per well. After 3–5 h, 0.5 ml of fresh minimal serum-free medium was added to forebrain cultures, or DMEM with 10% fetal calf serum was added to glial cultures. After 24 h cells were harvested for luciferase assays in 150 μl of lysis buffer (Promega) per well or fixed and immunostained (as in Ref. 24). For experiments assessing CRE sites, cortical cultures were used, and the DNA-LipofectAMINE Plus mixture was replaced after 3–5 h with 1 ml of fresh minimal serum-free medium containing glutamate receptor blockers (30 μM 6-cyano-7-nitroquinoxaline-2,3-dione, 20 μM

MK801, 100 μM D(-)2-amino-5-phosphonovaleric acid). 24 h later, 100 μl of 10× drug solution in medium was added for 18 h until lysis. Luciferase activity was determined on 20-μl samples with a Turner Designs luminometer using 85 μl of luciferase assay reagent (Promega) per sample. At least three independent plasmid preparations for each construct were transfected in triplicates or quadruplets in at least three different primary cultures.

All luciferase activity values were normalized to the activity of the TK promoter after subtracting the activity of the promoter-less pGL3 basic vector, which were both transfected in parallel plates of the same culture preparation. Alternatively, when promoter activity of GluR1 constructs was expressed as a ratio to that of the empty pGL3 vector, similar results were obtained. Luciferase activity of the pGL3 vector was 16 times lower in glial cultures compared with forebrain cultures; luciferase driven only by the pGL3 vector gave an average of 12 ± 2.4 Turner light units (TLU) per well (mean ± S.E., $n = 34$) in glial cultures *versus* 194 ± 28.5 TLU ($n = 41$) in forebrain cultures. The TK promoter activity per well was 10,600 ± 2000 TLU for forebrain cultures ($n = 43$) and 538 ± 94.5 TLU for glial cultures ($n = 48$). To measure the neuronal specificity of GluR1 promoter constructs, which mainly expressed luciferase in neurons (see Table I), we assessed neuronal specificity (N/G) relative to the TK promoter by calculating the ratio of neuronal to glial expression after normalization to the TK promoter. This N/G ratio is only used as a tool to compare the neuronal specificity of different GluR1 promoter fragments, but it underestimates the actual neuronal specificity since the TK promoter itself was expressed at higher levels on a per cell basis in the forebrain cultures. By taking the average transfection efficiency of forebrain (0.75%) and glial (7%) cultures and an estimated 5 times higher cell density in the forebrain cultures into account, the "neuronal" to glial activity ratio for the TK promoter per cell is about 37.

Statistics—For all statistical comparisons GraphPad Prizm was used. An analysis of variance was performed followed by a post hoc Bonferroni test with selected pairs to compare neighboring constructs. To compare all constructs to each other a post hoc Tukey test was employed. When only two samples were in one group (−2349/+8 *versus* −2349/−253 and −459/−48 *versus* −459/rev−48) the unpaired *t* test was used.

RESULTS

Sequence and Transcriptional Start Sites—The sequence of the 5′-proximal region and 593 nucleotides of the first intron of the rat GluR1 gene is shown in Fig. 1. All numbering refers to the first ATG, and some important features are highlighted within the sequence. The exon-intron borders of the first two introns were determined from sequence comparison of the genomic clone and the published GluR1 cDNA (18). This comparison revealed a discrepancy between the two sequences in the 5′-untranslated region. In the rat genomic sequence, the CAAGAGAAA sequence (at −161 relative to the first ATG) only occurs once, whereas it is repeated in tandem in the mouse GluR1 gene (Celera data base) and the rat cDNA (Fig. 1, overscored). This difference might be due to genetic variation among rats. The first intron of about 2.8 kb is inserted after 82 bp of translated sequence, and the second intron is located after the 138-bp-long exon 2. In the 5′ region the rat GluR1 gene harbors a 96-bp ACAT tetranucleotide repeat at −4815 and at −4309 a B2-like repetitive sequence (25) of 210 bp including typical 15-bp terminal direct repeats. The function of a 64-bp GA repeat between −394 and −333 is examined further below.

The initiation region is GC-rich but lacks convincing TATA and CCAAT elements. Multiple initiation sites were identified by primer extension analysis using five different primers and poly(A)$^+$ brain RNA (Fig. 2*A*, *B*). Nonspecific termination of the reverse transcriptase was monitored using synthetic GluR1 control RNAs with known length (* in Fig. 2*B*). Primers PE-A, PE-B, and PE-D (PE-D not shown) gave rise to main DNA products identifying transcriptional start sites near −295 and −266 (Figs. 1 and 2; sites denoted as *A* and *B*). Extension of primers PE-B, PE-C, and PE-D (PE-D not shown) resulted in bands at −219 and −214 (*sites C* and *D*) and primers PE-C (Fig. 2*A*) and PE-D (not shown) labeled −202 (*site E*). The matching results from different primers confirmed the absence of a $^{-161}$CAAGAGAAA repeat in brain RNA of Harlan Sprague-Dawley rats. Another primer PE-E starting further upstream at −427 did not give rise to any DNA product (not shown); thus it was unlikely that there were more 5′-initiation sites.

RNase protection assays were performed to confirm primer extension analysis. Probe A revealed all the RNA ends found by primer extension (Fig. 2*C*), and probe B, which was located more upstream, reconfirmed sites A and B (not shown). Two faint bands found by RNase protection and primer extension assays with primers PE-A and -B, revealing possible start sites flanking the GA repeat at −333 and −394, were not labeled in Figs. 1 and 2. A number of other fainter bands appeared in these gels but were not consistently identified by the various primers and probes, so they were not considered further. Thus, RNase protection assays with two probes of different length confirmed all main initiation sites found by primer extension (−295, −266, −219, −214, and −202) and ruled out introns in the 5′-untranslated region.

GluR1 Promoter Activity—Promoter activity of different GluR1 and GluR2 fragments cloned into the pGL3 vector was assessed by measuring firefly luciferase activity after transfection into primary forebrain and glial cultures. To compare promoter activities across experiments, we normalized all luciferase activities to the TK promoter. GluR1 promoter activity was strongest in forebrain cultures for the construct that contained all transcriptional start sites and the 64-bp GA repeat but lacked the region with the first two upstream ATGs in the 5′-untranslated region (construct −459/−48, 3′-deletion series of Fig. 3). In glial cultures the C, D, and E initiation sites were not essential because highest promoter activity was produced by construct −459/−253. The shortest GluR1 promoter construct tested (−209/+8), with only one main transcriptional start site (E at −202), lacked activity in glial cultures and had one of the lowest activity of all constructs in forebrain cultures (18% of TK promoter activity).

Neuronal Specificity—All examined GluR1 promoter constructs showed higher activity in forebrain compared with glial cultures. To determine whether luciferase was expressed primarily in neurons or glial cells, and whether there is heterogeneity in the glial cultures, we transfected both forebrain and glial cultures with GluR1 promoter constructs on glass coverslips and double-immunostained with antibodies directed against luciferase and either the neuronal marker, microtubule-associated protein 2 (MAP2), or the astrocyte marker, glial acidic fibrillary protein (GFAP). In forebrain cultures, most luciferase-positive cells transfected with GluR1 constructs of different length were co-labeled with the anti-MAP2 antibody but never co-stained for GFAP (see Table I and examples of luciferase- and MAP2-positive double stainings in Fig. 4). In glial cultures, we found no cells that expressed luciferase in three different immunocytochemical experiments. The low luciferase activity in glial cultures might be below detection threshold. We only detected a few luciferase-positive cells when the luciferase gene was under control of the SV40 promoter, which results in higher enzymatic luciferase activity in glial cultures. These results demonstrate that in our glial and forebrain cultures, astrocytes uniformly show low levels of luciferase expression rather than a small proportion of glial cells showing high expression. Therefore, we conclude that the GluR1 promoter is mostly neuron-specific.

The immunolabeling results of transfected cells allow us to use the ratio of luciferase activity in forebrain cultures *versus* glial cultures (N/G, after normalization to the TK promoter, see "Experimental Procedures") as a measure to assess neuronal specificity of different GluR1 fragments. Different GluR1 pro-

FIG. 1. **GluR1 sequence.** The 5′ sequence of the rat GluR1 gene, part of the first two introns (*italics*), and important features are shown. Numbering is relative to the first ATG (*circled*). The main transcriptional start sites are marked by *closed triangles* and the *same letters* as in Fig. 2. All primers, PE-A to PE-E, used for primer extension are *underlined*. Repetitive sequences are shown in *bold*, and the sequence repeated in tandem in the cDNA sequence (18) is *overscored*. Restriction sites used for making the pGL3 constructs are *underlined*. The bases at the beginning or end of GluR1 sequences cloned into pGL3 are *bold* and *numbered*. Transcription factor binding sites for Sp1 and CREB and a putative N box (on lower strand) are *boxed*. The location of the exon/intron borders are shown with the intronic sequences in *italics*. The GenBank™ accession number of this sequence including the omitted sequence between −4035 and −1435 is AF302117.

moter fragments showed ratios of expression in neurons *versus* glia (N/G) ranging from 2.2 to higher than 49 (Fig. 3). The neuronal to glial expression ratio of the most neuron-specific GluR1 constructs (all constructs extending 5′ from −1395, N/G >49) was higher than that of GluR2 (N/G = 16.2), due to low GluR1 promoter activity in glia in contrast to GluR2. Inspection of functional promoter activities in the 5′- and 3′-deletion series (Fig. 3) revealed several regions responsible for significant effects on GluR1 promoter activity, often in a cell type-specific manner.

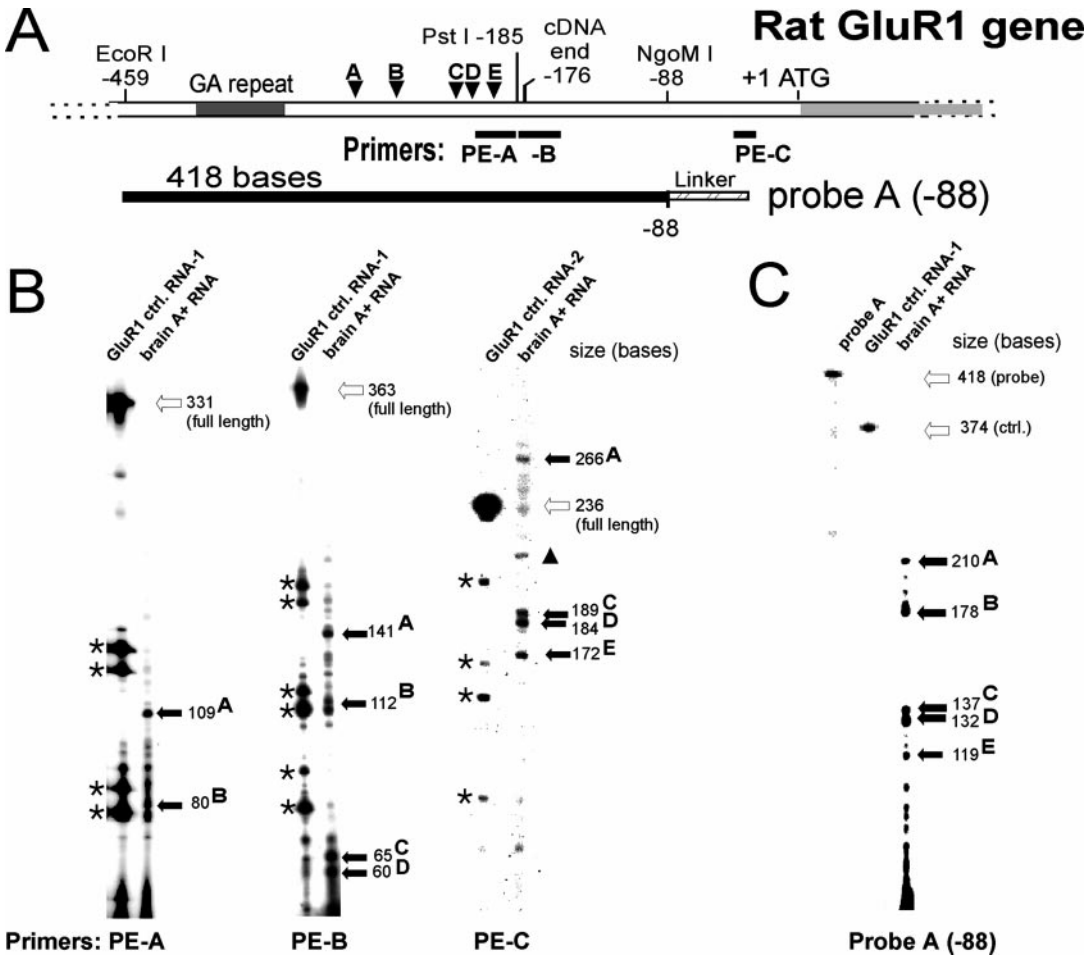

FIG. 2. **Mapping of transcriptional start sites.** *A,* schematic of the 5′-GluR1 region showing the location of three primers used for primer extension and RNase protection analysis. The identified main transcriptional start sites in *B* and *C* are depicted by the *same letters* (*A–E*). *B,* results from primer extension experiments with three different primers, PE-A, PE-B, and PE-C. Nonspecific drop off sites of the reverse transcriptase on control RNAs are marked by *asterisks.* The *solid arrows* mark the main specific DNA products and their length. Adding the first base number of the primer used (shown at the bottom gel) to the length minus 1 gives the position of the transcriptional start sites. The *open arrows* mark the full-length end of the control RNAs. Note that the autoradiogram of primer PE-C did not reveal a clear band for the initiation site labeled *B* but shows a band (*triangle*) that was not found in any other experiment and was therefore not regarded to indicate a real initiation site. *C,* results from a representative RNase protection assay. The length of the protected RNA probe is shown in DNA bases. The actual length of the RNA is about 5 bases (2–5%) shorter. Adding the nucleotide length to the beginning of the probes minus 1 and adjusting for the actual length matches the sites found by primer extension, depicted by the *same letters.* Probe and control RNA were treated the same way as the poly(A)$^+$ RNA.

Regions Conferring Neuronal Specificity—Within the regions tested, the major elements conferring neuronal specificity reside between −253 and −48, −686 and −457, and −1395 and −743. Deletion of the latter sequence, −1395 to −743, from construct −1395/+8 lowered the neuronal to glial expression ratio 4.9-fold. This deletion reduced promoter activity in neurons 2.3-fold, whereas activity in glia was very low for both constructs, suggesting a neuron-specific region increasing expression. In contrast, deletion of the region −686 to −459 reduced the N/G ratio 2-fold by increasing activity specifically in glia (2.3-fold, $p > 0.001$) but not neurons. These data suggest that a glial silencing region may exist between −686 and −459. Furthermore, deletion of −258 to +7 or +8 from constructs −2349/+8 and −459/+7 in the 3′-deletion series greatly reduced the neuronal to glial expression ratio, from >49 and 5.1 in the parent constructs to 2.1 and 2.2 in the deletion constructs. Moreover, the shortest constructs (−258/+7 and −209/+8) in the 5′-deletion series were still neuron-specific as judged from the neuronal to glial expression ratios of 8.9 and >49, consistent with the immunocytochemistry (see below).

The neuronal specificity of the region from −253 to +8 results from an orientation-dependent neuronal expression increasing region and a glial silencing region. Adding the sequence between −202 and −48 to the construct −459/−202 increased expression both in neurons (1.8-fold, $p < 0.001$) and in glia (3.3-fold, $p < 0.001$). However, the expanded region, −253 to +8 seems to be a neuron-specific positive region. In neurons, deleting the sequence between −253 and +8 from the long −2349/+8 construct reduced promoter activity by 2.1-fold ($p < 0.05$), whereas activity increased in glia ($p < 0.001$). Furthermore, in neurons the construct −459/−48 displayed 2.7-fold stronger promoter activity than −459/−253 ($p < 0.001$), whereas both constructs had similar activity in glia. The activity of the region between −253 and −48 was orientation-dependent, in that inverting this sequence reduced promoter activity in neurons by 3.2-fold ($p < 0.001$) and dramatically reduced neuronal selectivity by 5.5-fold (compare −459/−48 with −459/rev−48 in Fig. 3).

The presence of the glial silencing region was suggested by the results of deleting sequence between −253 and −202 from the 3′ end of the construct −459/−202, which specifically increased expression in glia by 4.1-fold ($p < 0.001$). Likewise, deleting this 3′ region (−253 to +7/8) in the context of either a long construct (−2349/+8) or a shorter construct (−459/+7) enhanced luciferase activity in glia (both $p < 0.001$). This effect was not observed when comparing construct −258/+7 with

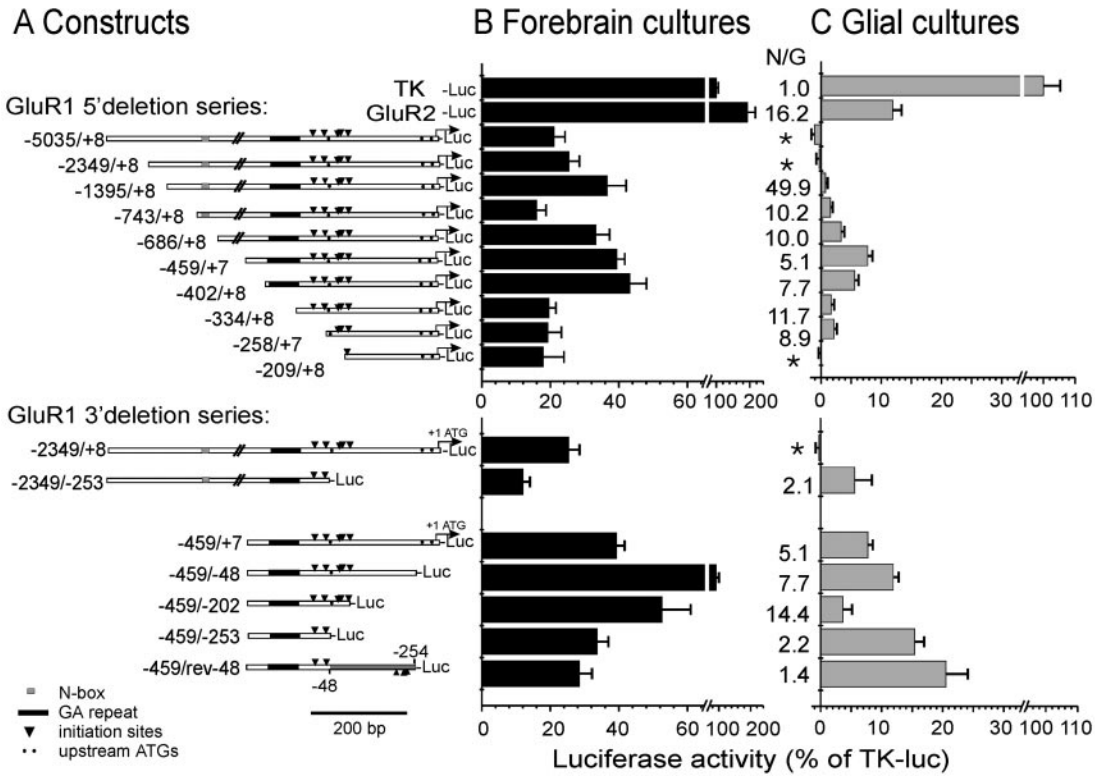

FIG. 3. **GluR1 promoter activity in rat forebrain and glial cultures.** *A* depicts the promoter constructs used to transfect primary forebrain and glial cultures derived from embryonic rat cerebral cortex and striatum (see "Experimental Procedures"). The initiation sites, the N box, the GA repeat, and the upstream ATGs are marked. *B* and *C*, the mean and S.E. of the luciferase activity of each construct is displayed as a percentage of the TK promoter activity for forebrain (*B*) and glial (*C*) cultures. For each construct at least three independent plasmid preparations in at least three different primary cultures were tested (*N* ranges from 9 to 67 for each construct). The numbers, N/G, denote the ratio of neuronal over glial expression; negative ratios due to lack of expression in glia are labeled by *asterisks* and are described in the text as being >49 (higher than the highest real ratio observed, which was 49).

TABLE I
Immunostaining of transfected cells in rat forebrain cultures

Forebrain cultures grown on coverslips were transfected with three different GluR1 promoter constructs. 24 h later cells were fixed and double-stained for expression of luciferase and MAP2 or GFAP, respectively. Some luciferase-positive cells (9%) appeared to be MAP2-negative, but their morphology was clearly neuronal, as observed by luciferase staining in several long (>400 μm) branching processes containing varicosities.

	Constructs		
	209/+7	−2349/+8	−459/−48
MAP2 staining			
Luciferase-positive cells	244	150	629
% MAP2-positive or clear neuronal morphology	96	96	92
GFAP-staining			
Luciferase-positive cells	76	150	141
% GFAP-positive	0	0	0

−209/+8 in the 5′-deletion series (Fig. 3) however, suggesting that the repressive effect required sequences between −459 and −258. In summary, the neuronal specificity of the GluR1 promoter appears to result from several regions that either increase expression in neurons or reduce activity in glia. One potential explanation for the low GluR promoter activity in glial cells could have been culturing in serum-containing medium before and after transfection. However, removing the serum from glial cultures had no effect on the neuronal specificity of the GluR1 promoter constructs (data not shown).

Regions Enhancing Expression, GA Repeat—Sequential 5′-deletions from −459 to −209 gradually reduced promoter activity in glia, suggesting the importance of the initiation region and modest general positive elements in this region. Deletion of the GA repeat had the largest effect in the 5′-deletion series in both neurons and glia (55 and 70% reduction, $p < 0.001$), although addition of a second GA repeat to the 5′ end of −402/+8 or of a GA repeat to the 5′ end of −258/+7 did not further increase promoter activity (not shown). Purine or pyrimidine repeats often vary in length in different alleles, *e.g.* between 24 and 62 bp in the 5′-untranslated region of human GluR3 (26) and between 72 and 86 bp in rat GAP-43 (27). Therefore, we tested whether the length of the GA repeat would affect promoter activity of GluR1. A construct −459/+7ΔGA with the GA repeat shortened to 36 bp had similar luciferase activity as the native −459/+7 construct with 64 bp in neurons ($n = 24$) but lost activity by 55% in glia ($n = 30$, $p < 0.05$).

Regions Reducing Expression—Deletion of several sequences enhanced promoter activity, pointing to possible negative regulatory regions in the GluR1 promoter. For example, in neurons and in glia, deletion of the sequence between −743 and −459 resulted in an increase of promoter activity (2.2- and 2.4-fold, both $p < 0.001$). The constructs −743/+8 and −686/+8 were specifically designed to test whether the 57-bp region containing the N box (CACNAG) had any silencing activity. By using a Student's *t* test, the increase in activity seen in both neurons and glia after deletion of the 57-bp region was significant ($p < 0.001$), suggesting that the N box reduces expression of GluR1.

Deletion of the sequence between −48 and +7 from the 3′ end of the −459/+7 construct increased promoter activity 2.6-fold in neurons ($p < 0.001$) and more modestly in glia (1.3-fold, $p < 0.05$). This region is transcribed and includes the GluR1 translational start ATG plus two out-of-frame upstream ATGs, so its repressive effect may have been at either the translational or transcriptional levels. The data, taken together, sug-

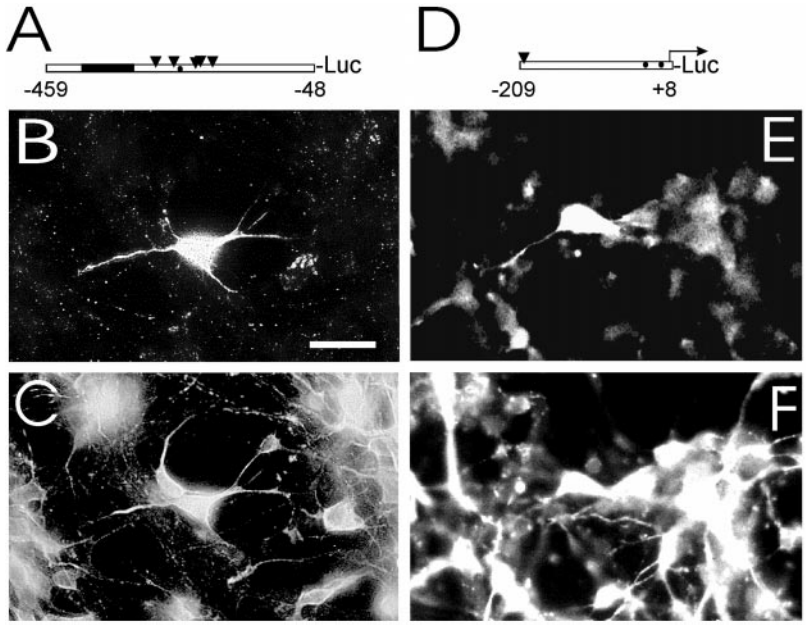

FIG. 4. **Luciferase expression driven by GluR1 promoters in MAP2-positive neurons.** Forebrain cultures were transfected with the GluR1−459/−48 (*A–C*) and −209/+8 (*D–F*) constructs, and 24 h later cultures were double-immunostained for firefly luciferase using a secondary Cyan Green-labeled antibody (*B* and *E*) and MAP2 using a Texas Red-labeled secondary antibody (*C* and *F*). Representative luciferase-positive cells co-stained for MAP2 were photographed (*E* and *F*) or videocaptured (*B* and *C*). *Scale bar* is 50 μm.

gest that multiple negative and positive regions regulate the activity of the GluR1 promoter in both neurons and glia.

Sp1 Sites—In most TATA-less promoters, including those for GluR2, NR1, NR2B, NR2A, and KA2 glutamate receptor subunits, Sp1 sites reside close to the initiation sites (28). A search of the GluR1 sequence around the initiation sites allowing one mismatch to the consensus sequences G/T G/A GGC G/T G/A G/A G/T (from tfsites data base in GCG) or G/T G/A GG C/A GG G/A (from the transfac data base used by MatInspector) revealed five putative Sp1 binding sites between −470 and −140. These sites were assessed for Sp1 binding by EMSAs. Two out of the five synthetic GluR1 oligonucleotides, with 5′ ends at −296 and −275, formed high molecular weight bands with human recombinant Sp1 protein as did an Sp1 consensus probe (Fig. 5*A, lanes 1, 3,* and *4*). The same probes formed similar complexes with nuclear extracts from forebrain cultures, which disappeared following pretreatment with anti-Sp1 antibodies (Fig. 5*B*, compare *lanes 8–10* with *lanes 2, 4,* and *5*) but not with c-Jun antibodies (not shown). Furthermore, −296 and −275 oligonucleotides competed for binding of nuclear extracts to the Sp1 consensus probe, whereas a nonspecific oligonucleotide did not (Fig. 5*C*). This experiment was repeated with labeled −296 and −275 probes and the respective competitors giving similar results. The Sp1 sites were assigned to the sites that best matched the consensus sequence and are located between the most upstream main initiation sites A and B (Figs. 1 and 5*D*). The site at −270 also resembles the GC box consensus sequence with one mismatch.

CRE Sites—The transcription factor CREB is thought to play a key role in long term potentiation and addiction, in both of which the GluR1 expression is up-regulated. Thus, we examined the GluR1 gene for putative CRE sites (consensus sequences, TGACG(T/A)(C/A)A and (A/T)CGT(A/C)AC and (G/T)(A/T)CGTCA), and we identified several promising regions near the initiation sites. Eight oligonucleotides spanning the region between −332 and −67 were examined for CREB binding in EMSAs, including one negative control sequence starting at −78. Four of those oligonucleotides bound recombinant CREB-bZIP (Fig. 6*A*, *lanes 4, 5, 7,* and *9*), although they all contained mismatches to CRE consensus sites. To assess the function of these sites, all four sites were mutated in construct −334/+8 as shown schematically in Fig. 6*B*. The effect of 10 μM of the adenylate cyclase activator forskolin and 500 μM of the phosphodiesterase inhibitor IBMX was compared between cortical cultures transfected with wild type and mutated constructs. To maximize a potential CREB effect on promoter activity, we grew the transfected cells in glutamate receptor blockers (30 μM 6-cyano-7-nitroquinoxaline-2,3-dione, 20 μM MK801, 100 μM D(-)2-amino-5-phosphonovaleric acid, Fig. 6, *B* and *C*) in order to lower any basal CREB activation. This protocol enhanced the effect of forskolin treatment (not shown), but we did not find any difference in expression between constructs that contained or lacked the four CREs (Fig. 6*C*). Another CREB-bZIP-binding site between −486 and −442 was found by EMSAs (not shown), and CRE consensus sites are located further upstream but were not tested for binding. However, the full-length GluR1 promoter did not give substantially higher responses to the forskolin/IBMX treatment, indicating that under the conditions tested proteins of the CREB family did not affect GluR1 promoter activity (Fig. 6*C*).

By computer search two putative AP1 sites were found around −219 and −205. However, in several independent EMSAs these AP1 sites or the other GluR1 oligonucleotides did not bind recombinant c-Jun, whereas the AP1 consensus oligonucleotide did (not shown). In addition, we did not observe any evidence for AP1 regulation of the GluR1 promoter when cortical cultures were transfected with different constructs (−5035/+8, −2349/+8, −459/+7) and treated with 30–100 nM PMA for 5–24 h (99–144% of non-treated parallel cultures; $n = 4$).

DISCUSSION

We have cloned and characterized 5 kb of the rat GluR1 promoter. The main findings of this study are as follows. 1) The GluR1 promoter organization is similar to other known glutamate receptor promoters (reviewed in Ref. 28) with multiple transcriptional start sites, Sp1-binding sites, and the lack of TATA and CAAT boxes. 2) The GluR1 promoter is neuron-specific, the neuronal specificity appears to reside mainly within the neuronal expression-enhancing regions, −1395 to −743 and −253 to +8, the latter also contains a glial silencing region. 3) Several CREB-binding sites, an expression-increasing GA repeat, and a silencing region containing an N box were identified (Fig. 7). Many neuronal promoters, including glutamate receptor promoters, contain an RE1 silencing element,

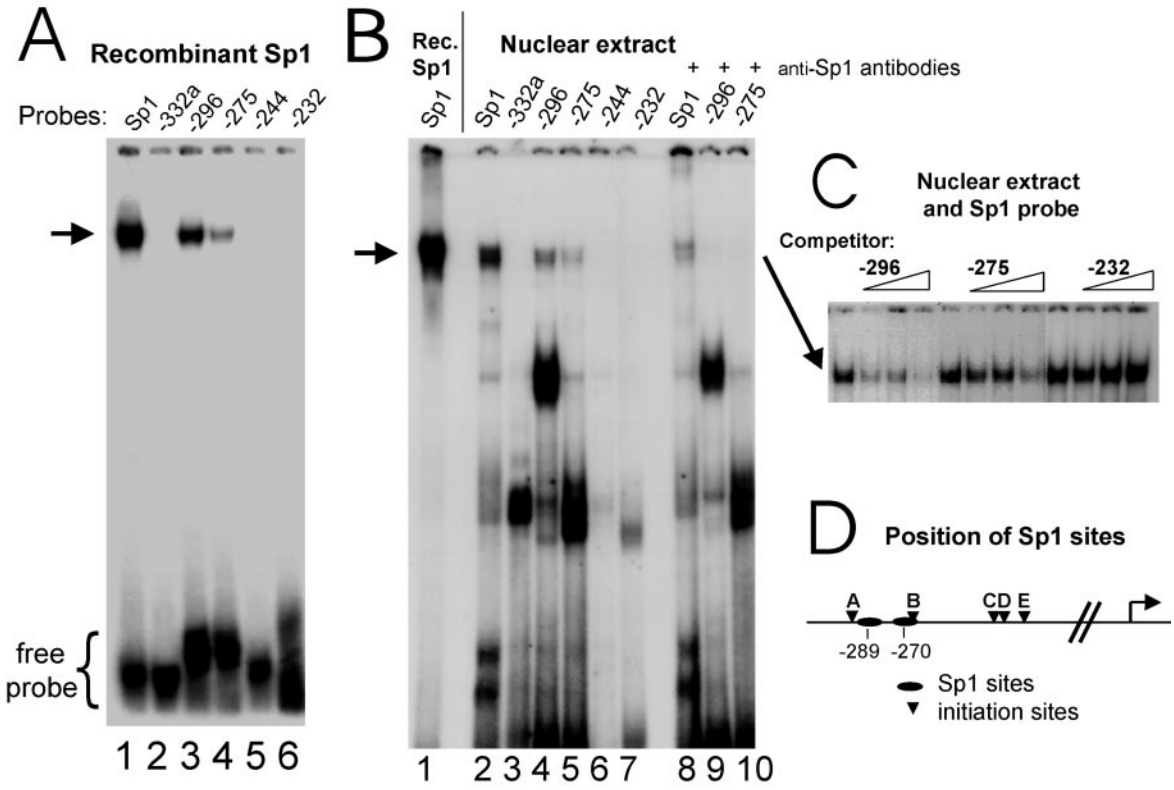

FIG. 5. **Sp1 binding around the initiation sites.** *A,* a representative EMSA using five different radiolabeled GluR1 oligonucleotides (named by their 5′ ends) and recombinant Sp1 protein ($n = 3$ independent experiments). The *arrow* identifies a specific Sp1 protein-probe complex. *B,* a similar experiment using recombinant Sp1 protein and nuclear extracts prepared from 5-day forebrain cultures, which gave rise to similar high molecular weight bands ($n = 3$). Preincubation of the extracts with anti-Sp1 antibodies reduced the band intensity, indicating that the highest band contained Sp1 protein ($n = 3$). *C,* a competition experiment using 0-, 10-, 30-, and 100-fold competing oligonucleotides verifying that the GluR1 oligonucleotides, −296 and −270 but not −232, can compete for binding of nuclear extracts from forebrain cultures to Sp1 probe ($n = 2$). *D,* the predicted location of the two identified Sp1-binding sites in the GluR1 promoter in relation to the initiation sites *A–E.*

which reduces expression in non-neuronal cells. However, no RE1-like sequences were found in the 5 kb of the GluR1 promoter. Because primary cortical cultures consist of a mixture of neuronal cell types, we cannot exclude that some promoter regions influence expression only in a subpopulation of cells.

Neuronal Selectivity of a Minimal Promoter—Luciferase assays and immunohistochemistry show that even short GluR1 promoter constructs express selectively in primary cultured neurons compared with GFAP-positive astrocytes. The shortest and the longest GluR1 constructs are the most neuron-specific because they display only background activity in glia. Even small GluR1 promoter fragments close to the transcriptional start sites retain substantial neuronal selectivity. This is also observed with other promoters of neuronal genes, such as GluR2 (24), rat β_2-nicotinic acetylcholine receptor subunit (29), the mouse neural adhesion molecule polysialic acid synthase (30), and rat synapsin II (31). The 5′- and 3′-deletion series reveal that no single region dominates or is essential for GluR1 promoter activity in neurons. Rather, transcriptional start sites and regulatory elements are distributed throughout a broad region, including transcribed regions and regions distant from the initiation sites.

Purine-rich Regions and Other Regulatory Elements—In this first description of the GluR1 promoter, we have identified several regions governing expression, some of which resemble other neuronal promoters: 1) a purine-rich region in the 5′-untranslated region similar to GluR3 and a GA repeat similar to GAP-43, both increase expression in GluR1; 2) a GAP-43-like element in the region reducing expression in glia; and 3) a 57-bp activity-reducing region containing an N box.

The 64-bp GA repeat and a purine-rich region (−147 to −47) reside in sequences that substantially increase GluR1 promoter activity. The human GluR3 gene has a similar purine-rich region in the 5′-untranslated region (26), and GA or TC repeats are found in many promoters, including neuronal promoters such as rat GAP-43 (32) and mouse neurofilament (33). In supercoiled plasmids and in chromatin, purine or pyrimidine repeats or enriched regions can form triplex DNA structures, which leaves one strand single-stranded (34, 35). These structural changes themselves might favor transcription by making the DNA more accessible or allow binding of transcription factors. The mechanism of the positive effect of the GluR1 GA repeat is unclear. However, the positive effect was dependent on the position of the GA repeat close to the initiation region, suggesting that the GA repeat might help recruit Sp1 or general initiation factors. Moreover, promoter activity was reduced by shortening the GA repeat in glia but not in neurons, suggesting that variations of repeat length in different alleles might affect neuronal specificity. In summary, purine-rich regions are prominent in the GluR1 promoter and influence promoter activity; however, their mechanism of action, whether by changing the conformation of the DNA or by binding transcription factors, remains largely unknown.

Silencing Regions—About half of the neuronal specificity of the GluR1 promoter was conferred by the region between −253 to −202, which reduces expression in glia. This region resembles the GAP-43 non-neuronal repressive element, which consists of two sequences separated by 9 or 10 bp (36, 37). The GluR1 Sp1 site at −289 outside of the identified silencing region is identical to the upstream GAP-43 half-site, and the sequence around the −242 thymidine with 4 and 5 purines on either side resembles the downstream half-site (37). Apart from

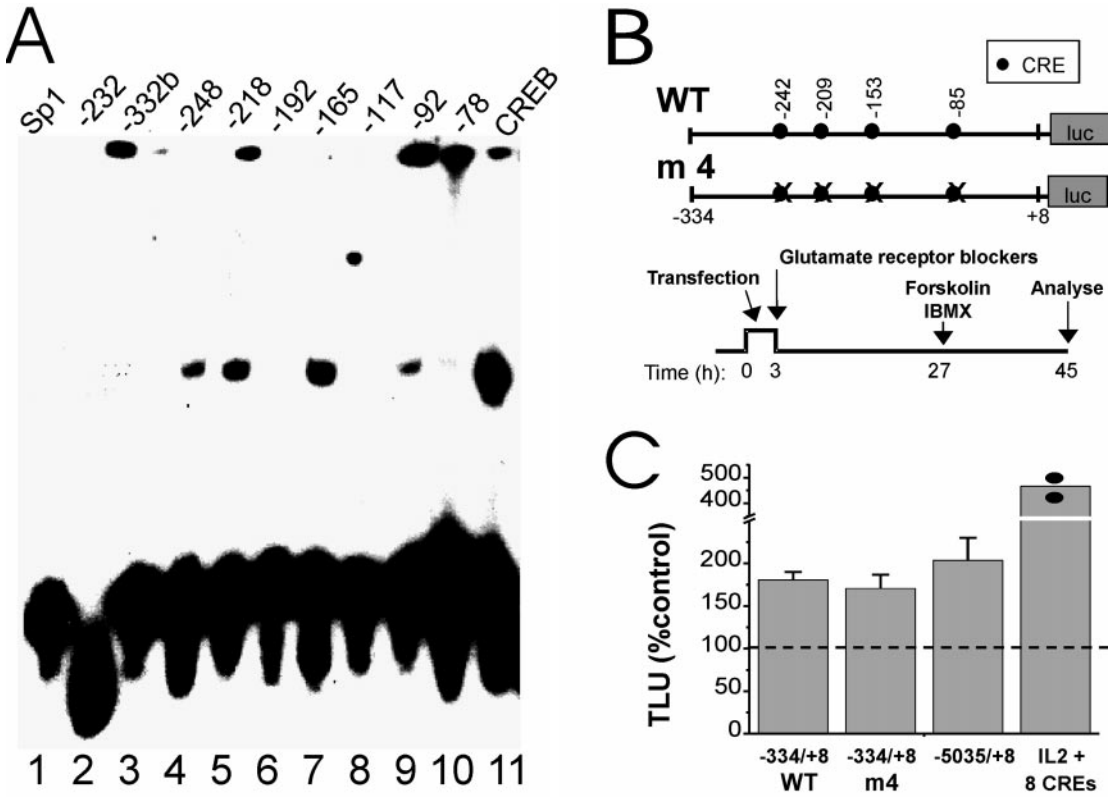

FIG. 6. **CREB binding to GluR1 sites.** *A,* binding of recombinant CREB-bZIP to various GluR1 oligonucleotides named by their 5′ ends was tested by EMSA. Data shown are representative of three experiments. *B*, the CRE sites identified in *A* (positions shown) were mutated in the −334/+8 construct. *B* and *C*, the function of CRE sites was assessed by transfection of cortical cultures with the wild type (*WT*, −334/+8) or mutated construct (*m4*) and the full-length GluR1 promoter (−5035 to +8), and stimulating with 10 μM forskolin and 500 μM IBMX (n = 5). Promoter activity was normalized to that of parallel cultures treated with 10 μM dideoxyforskolin (100%). No difference between the mutated and the wild type constructs was observed. The IL2 promoter with 8 CRE sites from the ICER promoter was stimulated to 436 and 506% of control under the same conditions.

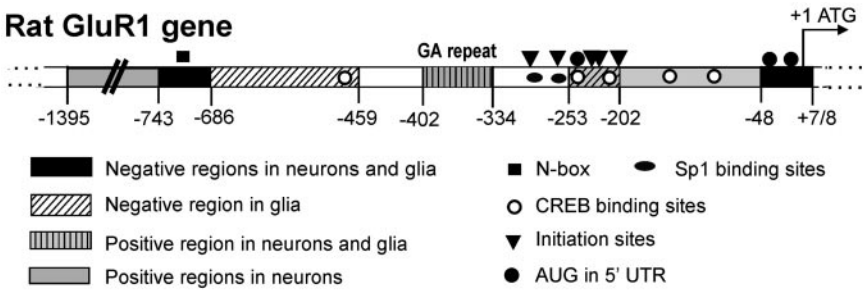

FIG. 7. **Main features of the rat GluR1 gene.** The main features of the GluR1 gene are shown. The ATGs, initiation sites, N box, the GA repeat, and the identified Sp1- and CREB-binding sites are shown. Cloning sites used for the promoter activity studies are marked. Positive and negative regions active in neurons or glia are shown. Note the neuron-specific positive elements between −1395 to −743 and −253 to −48 and the glial-specific negative elements −253 to −202 and −686 to −459. Also note the common positive regions, such as the GA repeat and −202 to −48, and the common negative elements, −48 to +7 and −743 to −686 containing the N box.

transcriptional regulation, cell-specific translational control mediated by this sequence in either glia or neurons is possible, since the glial silencing region is transcribed when the upstream GluR1 initiation sites are used.

A 57-bp region that inhibited expression in both forebrain and glial cultures contains an N box (CACNAG) located at −735. An N box was also found in a 1.4-kb negatively acting region of NR2B (38) and is found in the homologous region of the human GluR1 promoter (see also Fig. 7). N boxes can bind the Notch effectors Hes1 and Hes5, which are negative regulators of the basic helix-loop-helix family and regulate neuronal differentiation (39–41). Our finding that deletion of the short sequence containing the N box increases luciferase activity recalls the observation that binding of Hes1 to the N boxes of the *Hes*1 promoter suppresses transcription (40).

Taken together, our findings indicate that GluR1 silencing and activation elements are distributed over a wide region of the GluR1 promoter, as summarized in Fig. 7. Most features, including the N box, the upstream AUGs, and the three CREB-binding sites closest to the transcriptional start sites, are conserved in the human and mouse GluR1 promoter (GenBank™ accession number AC025156 (human); mouse promoter assembled from Celera data base). Indeed, the region between the GA repeat and the translational initiation codon shows 86 and 98% identity in human and mouse, respectively.

Translational control can play a prominent role in the regulation of other glutamate receptor subunits, such as NR1 (42), NR2A (43), and GluR2 (44). It would be worthwhile to investigate the possibility of translational control in GluR1. Our study mapped the regions conferring neuronal specificity and GluR1

expression and will lead the way to the identification of individual elements governing GluR1 expression.

Acknowledgments—We thank Nancy Ciliax for preparation of the forebrain primary cultures and Dr. T. J. Murphy for providing the CREB and AP1 control plasmids. We are grateful to Dr. Scott Myers for many useful discussions and encouragement during the study and to Dr. Edward Morgan and Dr. T. J. Murphy for comments on the manuscript.

REFERENCES

1. Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999) *Pharmacol. Rev.* **51,** 7–61
2. Gallo, V., and Ghiani, C. A. (2000) *Trends Pharmacol. Sci.* **21,** 252–258
3. Cheng, B., Furukawa, K., O'Keefe, J. A., Goodman, Y., Kihiko, M., Fabian, T., and Mattson, M. P. (1995) *J. Neurochem.* **65,** 2525–2536
4. Chew, L.-J., Fleck, M. W., Wright, P., Scherer, S. E., Mayer, M. L., and Gallo, V. (1997) *J. Neurosci.* **17,** 227–240
5. Condorelli, D. F., Dell'Albani, P., Aronic, E., Genazzani, A. A., Casabona, G., Corsaro, M., Balazs, R., and Nicoletti, F. (1993) *J. Neurochem.* **61,** 2133–2139
6. Nayak, A., Zastrow, D. J., Lickteig, R., Zahniser, N. R., and Browning, M. D. (1998) *Nature* **394,** 680–683
7. Cammarota, M., Bernabeu, R., deStein, M. L., Izquierdo, I., and Medina, J. H. (1998) *Eur. J. Neurosci.* **10,** 2669–2676
8. Naylor, P., Stewart, C. A., Wright, S. R., Pearson, R. C. A., and Reid, I. C. (1996) *Mol. Brain Res.* **35,** 349–353
9. Garcia-Ladona, F. J., Palacios, J. M., Probst, A., Wieser, H. G., and Mengod, G. (1994) *Mol. Brain Res.* **21,** 75–84
10. Mathern, G. W., Pretorius, J. K., Mendoza, D., Lozada, A., Leite, J. P., Chimelli, L., Fried, I., Sakamote, C., Assirato, J. A., and Adelson, P. D. (1998) *J. Neuropathol. Exp. Neurol.* **57,** 615–634
11. Zilles, K., Qü, M. S., Köhling, R., and Speckmann, E.-J. (1999) *Neuroscience* **94,** 1051–1061
12. Churchill, L., Swanson, C. J., Urbina, M., and Kalivas, P. W. (1999) *J. Neurochem.* **72,** 2397–2403
13. Fitzgerald, L. W., Ortiz, J., Hamedani, A. G., and Nestler, E. J. (1996) *J. Neurosci.* **16,** 274–282
14. Fitzgerald, L. W., Deutch, A. Y., Gasic, G., Heinemann, S. F., and Nestler, E. J. (1995) *J. Neurosci.* **15,** 2453–2461
15. Pelligrini-Giampietro, D. E., Bennett, M. V. L., and Zukin, R. S. (1992) *Neurosci. Lett.* **144,** 65–69
16. Yeo, J. F., Tang, F. R., and Leong, S. K. (1997) *Int. J. Neurosci.* **90,** 9–20
17. Carlton, S. M., Hargett, G. L., and Coggeshall, R. E. (1998) *Neurosci. Lett.* **242,** 21–24
18. Hollmann, M. O'Shea-Greenfield, A., Rogers, S. W., and Heinemann, S. (1989) *Nature* **342,** 643–648
19. Quandt, K., Frech, K., Karas, H., Wingender, E., Werner, T. (1995) *Nucleic Acids Res.* **23,** 4878–4884
20. Horton, R. M., Hunt, H. D., Ho, N. S., Pullen, K. J., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 61–68
21. Abbott, K. L., Robida, A. M., Davis, M. E., Pavlath, G. K., Camden, J. M., Turner, J. T., and Murphy, T. J. (2000) *J. Mol. Cell. Cardiol.* **32,** 391–403
22. Verdoorn, T., and Dingledine, R. (1988) *Mol. Pharmacol.* **34,** 298–307
23. Harant, H., de Martin, R., Andrew, P. J., Foglar, E., Dittrich, C., and Lindley, I. J. D. (1996) *J. Biol. Chem.* **271,** 26954–26961
24. Myers, S. J., Peters, J., Huang, Y., Comer, M. B., Barthel, F., and Dingledine, R. (1998) *J. Neurosci.* **18,** 6723–6739
25. Lueders, K. K., and Paterson, B. M (1982) *Nucleic Acids Res.* **10,** 7715–7729
26. Gécz, J., Barnett, S., Liu, J., Hollway, G., Donnelly, A., Eyre, H., Eshkevari, H. S., Baltazar, R., Grunn, A., Nagaraja, R., Gilliam, C., Peltonen, L., Sutherland, G. R., Baron, M., and Mulley, J. C. (1999) *Genomics* **62,** 356–368
27. Eggen, B. J. L., Nielander, H. B., Rensen-de Leeuw, M. G. A., Schotman, P., Gipsen, W. H., and Schrama, L. H. (1994) *Mol. Brain Res.* **23,** 221–234
28. Myers, S. J., Dingledine, R., and Borges, K. (1999) *Annu. Rev. Pharmacol. Toxicol.* **39,** 221–241
29. Bessis, A., Salmon, A.-M., Zoli, M., Le Novére, N., Picciotto, M., and Changeux, J.-P. (1995) *Neuroscience* **69,** 807–819
30. Yoshida, Y., Kurosawa, N., Kanematsu, T., Kojima, N., and Tsuji, S. (1996) *J. Biol. Chem.* **271,** 30167–30173
31. Chin, L.-S., Li, L., and Greengard, P. (1994) *J. Biol. Chem.* **269,** 18507–18513
32. Nedivi, E., Basi, G. S., Akey, I. V., and Skene, J. H. P. (1992) *J. Neurosci.* **12,** 691–704
33. Yaworski, P. J., Gardner, D. P., and Kappen, C. (1997) *J. Biol. Chem.* **272,** 25112–25120
34. Larsen, A., and Weintraub, H. (1982) *Cell* **29,** 609–622
35. Htun, H., and Dahlber, J. E. (1989) *Science* **243,** 1571–1576
36. Weber, J. R. M., and Skene, J. H. P. (1997) *J. Neurosci.* **17,** 7583–7593
37. Weber, J. R. M., and Skene, J. H. P. (1998) *J. Neurosci.* **18,** 5264–5274
38. Sasner, M., and Buonanno, A. (1996) *J. Biol. Chem.* **271,** 21316–21322
39. Akazawa, C., Sasai, Y., Nakanishi, S., and Kageyama, R. (1992) *J. Biol. Chem.* **267,** 21879–21885
40. Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S., and Kageyama, R. (1994) *J. Biol. Chem.* **269,** 5150–5156
41. Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., and Kageyama, R. (1999) *EMBO J.* **18,** 2196–2207
42. Sucher, N. J., Brose, N., Deitcher, D. L., Awobuluyi, M., Gasic, G. P., Bading, H., Cepko, C. L., Greenberg, M. E., Jahn, R., Heinemann, S. F., and Lipton, S. A. (1993) *J. Biol. Chem.* **268,** 22299–22304
43. Wood, M. W., VanDongen, H. M. A., and VanDongen, A. M. J. (1996) *J. Biol. Chem.* **271,** 8115–8120
44. Myers, S. J., Huang, Y., and Dingledine, R. (1998) *Soc. Neurosci. Abstr.* **23,** 923