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Activity of the rat GluR4 promoter in transfected cortical neurons and glia

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Abstract

AMPA (alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate) receptors are assembled from four subunits, GluR1-4. Although GluR4 is widely expressed in brain its abundance is less than GluR1-3. We have isolated ~5 kb of the rat GluR4 promoter region and analyzed its capacity to drive expression of a luciferase reporter gene in transfected rat cortical neurons and glia, and C6 glioma cells. Multiple transcriptional start sites were identified in a GC-rich region lacking TATA-boxes between −1090 and −1011 bp from ATG. In transfected mixed cortical cultures, luciferase expression driven by GluR4 promoter segments were found predominantly in TuJ1-positive neurons, indicating neuronal preference of GluR4. The GluR4 promoter fragments were 6–12-fold more active in neurons than glia, compared with a 30-fold neuronal selectivity of GluR2. Deletion of the GluR4 transcriptional initiation region decreased luciferase activity in neurons, but increased activity in C6 cells, suggesting that regulatory elements governing neuronal expression reside in this region. An intron within the 5′-untranslated region and Sp1, IK2 and E-box sites are conserved in the rat, mouse and human GluR4 promoters. The relative activity of GluR4 and GluR2 promoters in transfected cells correlates with their expression in brain, and in both promoters regulatory elements for neuronal expression reside near the initiation sites.

Keywords: astrocyte, GluR1, GluR2, glutamate receptor, *GRIA4*, neuronal expression.

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The AMPA (alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate) receptor family of glutamate receptors mediates fast neurotransmission at most excitatory synapses in the brain. AMPA receptors are assembled from a combination of one or more of the four subunits, GluR1, 2, 3 and 4, to form a homomeric or heteromeric structure, most likely containing four subunits (Mansour *et al*. 2001; Schorge and Colquhoun 2003). Through activity-dependent phosphorylation, both GluR4 and GluR1 seem to play a key role in the regulated insertion of AMPA receptors into synapses, which is important for synaptic plasticity (Esteban *et al*. 2003). The permeation properties of AMPA receptor channels, such as calcium permeability, polyamine blockade and rectification are dominantly controlled by the relative abundance of the GluR2 subunit (Geiger *et al*. 1995; Washburn *et al*. 1997; reviewed in Dingledine *et al*. 1999). The desensitization rate, however, is mainly controlled by alternative splicing of the flip and flop exons, especially in the GluR4 subunit (Mosbacher *et al*. 1994). Other properties of AMPA receptors are dependent on mRNA editing at the R/G site (recovery from desensitization) and alternative splicing of the C-terminus, which binds to different cytoplasmic signalling and anchoring molecules and contains unique phosphorylation sites that, in GluR4 and GluR1, control insertion into the synaptic membrane (reviewed in Dingledine *et al*. 1999; Sheng and Pak 1999; Ruberti and Dotti 2000; Lee *et al*. 2002). The distribution of AMPA receptor mRNAs and protein varies across brain regions with GluR4 being the least abundant subunit in neurons (e.g. Petralia and Wenthold 1992; Sato *et al*. 1993; Tsuzuki *et al*. 2001). In astrocytes and microglia, however, GluR4 is expressed at a similar level as other AMPA receptors subunits (e.g. Martin *et al*. 1993; Noda *et al*. 2000; review by Seifert and Steinhauser 2001),

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Abbreviations used: 5' UTR, 5' untranslated region; AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate; HDAC, histone deacetylase; LINE, long interspersed element; MRE, metal responsive element; RPA, Rnase protection assay; TIR, transcription initiation region; TSA, trichostatin A.

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whereas its expression in oligodendrocytes varies during development (Itoh *et al*. 2002). Furthermore, GluR4 expression is regulated by metabotropic glutamate receptor activation in Bergman glial cells (Lopez *et al*. 1998). These regulated changes and cell-specific expression profiles are likely to be largely due to transcriptional regulation. The promoters of the GluR1 and GluR2 AMPA receptor subunits have been functionally investigated by transfection into mixed neuronal-glial cultures and enriched glial cultures (Myers *et al*. 1998; Borges and Dingledine 2001) and the sequence of the human GluR3 gene (*GRIA3*) has been described (Gecz *et al*. 1999). Transcriptional regulatory elements have been identified in several glutamate receptors (reviewed in Myers *et al*. 1999), including the RE1 silencer which controls neuronal expression of GluR2 (Myers *et al*. 1998; Huang *et al*. 1999, 2002; reviewed in Roopra *et al*. 2001). Because of the low abundance of neuronal GluR4 in brain relative to that of GluR1-3, it is anticipated that the GluR4 promoter may harbour unique transcriptional regulatory elements controlling expression in neurons and glial cells. Here, we have isolated the rat GluR4 gene and investigated the transcriptional capacity of the GluR4 promoter region by transfection into cultured cortical neurons and glia in order to identify regulatory regions that control expression.

Materials and methods

Isolation and characterization of the proximal promoter region of the rat GluR4 gene

We screened a Wistar rat genomic library (in λ dash II vector, Stratagene Inc., La Jolla, CA, USA) on nitrocellulose filters. DNA probes were radiolabelled with α^{32}P-dCTP, random primers and Klenow DNA polymerase according to the manufacturer's instructions (Megaprime kit, Amersham Biotech., Piscataway, NJ, USA). An initial library screen using a fragment from rat GluR4c cDNA (Gallo *et al*. 1992) revealed the presence of two introns, the first in the GluR4 5′UTR and the second within the protein coding region, but no sequence 5′ to the original rat cDNA end (acc. number M85037; Bettler *et al*. 1990). We re-screened the λ dash II rat genomic library with a second probe, − 21 to + 217 relative to the ATG, containing 129 bp of the first intron in the coding region, synthesized by PCR amplification from rat genomic DNA, and identified two overlapping clones. The purified plaques were rescued into pBluescript and sequenced revealing 4.9 kb of 5′ flanking GluR4 genomic sequence (− 4885 to + 195 bp, relative to the translational start ATG). This sequence was deposited in GenBank (acc. number AY158020).

The human GluR4 promoter (GenBank, acc. no. AP001561.4; base 81 359 is the translational start site) and the mouse 5′-gene sequence from the Celera database (GA × 6K02T2NR0T, base 222 505 is ATG) and in GenBank (accession number NW_000350) were aligned with the rat sequence for comparison, and analyzed with the programs from the Genetics Computer Group (GCG) and MatInspector (Quandt *et al*. 1995).

Plasmid constructs

All constructs are defined by their first and last true GluR4 base relative to the ATG and sequences were confirmed first by restriction analysis then by DNA sequencing. For Rnase protection assays (RPA) two plasmids were constructed in pKS3/4 Bluescript. These template constructs were generated by PCR and included the GluR4 sequences − 1518 to − 966 (template for probe A) and − 1169 to − 880 (template for probe B).

For promoter reporter assays, a series of GluR4 fragments were cloned into the pGL2-Basic luciferase reporter vector (Promega Inc., Madison, WI, USA). GluR4 fragments were amplified from the pBluescript construct by PCR introducing an *Nhe*I or *Kpn*I restriction site at the 5′ end (bases: −4637, − 4427, −3429, − 2476, − 1518) and a *Bgl*II site at the 3′ end (bases: − 686, − 584 and − 13), respectively. The region containing the transcription sites (TIR, − 970 to − 1147) was deleted in two constructs GluR4(− 1518/ − 686)*luc* and GluR4(− 1513/− 13)*luc* by digesting these constructs with *Nco*I and *Pst*I followed by a fill-in reaction with Klenow DNA polymerase and blunt end ligation. Internal deletion of the 5′UTR intron, bases − 149 to − 644, was achieved by a two-round PCR mutagenesis protocol (Cormack and Somssich 1997), which removed 496 bp (83%) of the intron while retaining sequences necessary for splicing.

Analysis of transcriptional start sites

PolyA$^+$ RNA was prepared from adult male rat cerebellum according to Verdoorn and Dingledine (1988). Primer extensions were performed as described in Borges and Dingledine (2001) using 31mer oligonucleotides. Primer 1 (CTCAGTCTTGCTAAAGGCTT TTCAGAGAGGC) anneals to − 43 through − 73 from the GluR4 ATG and primer 2 (GGATTTTGCGTACACTCCAGTATCTGAT CCC) anneals to bases − 736 to − 766 (Figs 1 and 2). Rnase protection assays (RPAs) were performed as described (Borges and Dingledine 2001) using the RPA kit from Ambion Inc. (Woodward Austin, TX, USA), two different probes, and a GluR4 cRNA as a positive control. Probe A spanning the GluR4 promoter region from − 1411 to − 966 was transcribed by T3 RNA polymerase from the *Afl*III linearized GluR4 plasmid (− 1518/− 966 in pBluescript). Probe B included GluR4 sequence from − 1169 to − 880 and was synthesized by T3 RNA polymerase from the *Sac*I digested − 1169/ − 880 plasmid. Control GluR4 cRNA was complementary to probe B and was synthesized from the same plasmid but using *Kpn*I for linearization and T7 RNA polymerase (Fig. 2).

Cell culture, transfection, immunostaining and luciferase assays

The procedures are according to Myers *et al*. (1998) with slight modification. Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and conducted in accordance with its guidelines. Primary cultures were prepared from E18 rat cortex and grown in defined serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented as described (Myers *et al*. 1998) or in Neurobasal media supplemented with B27 (Invitrogen, Carlsbad, CA, USA). Cells were plated into 12-well culture dishes (Falcon or Costar) pre-coated with poly D-lysine (180 μg/mL), and then 20% serum. For immunolabelling studies, cells were plated onto glass coverslips pre-coated with poly D-lysine and laminin (20 μg/mL; 2 h) to enhance survival on the glass surface, and grown in Neurobasal

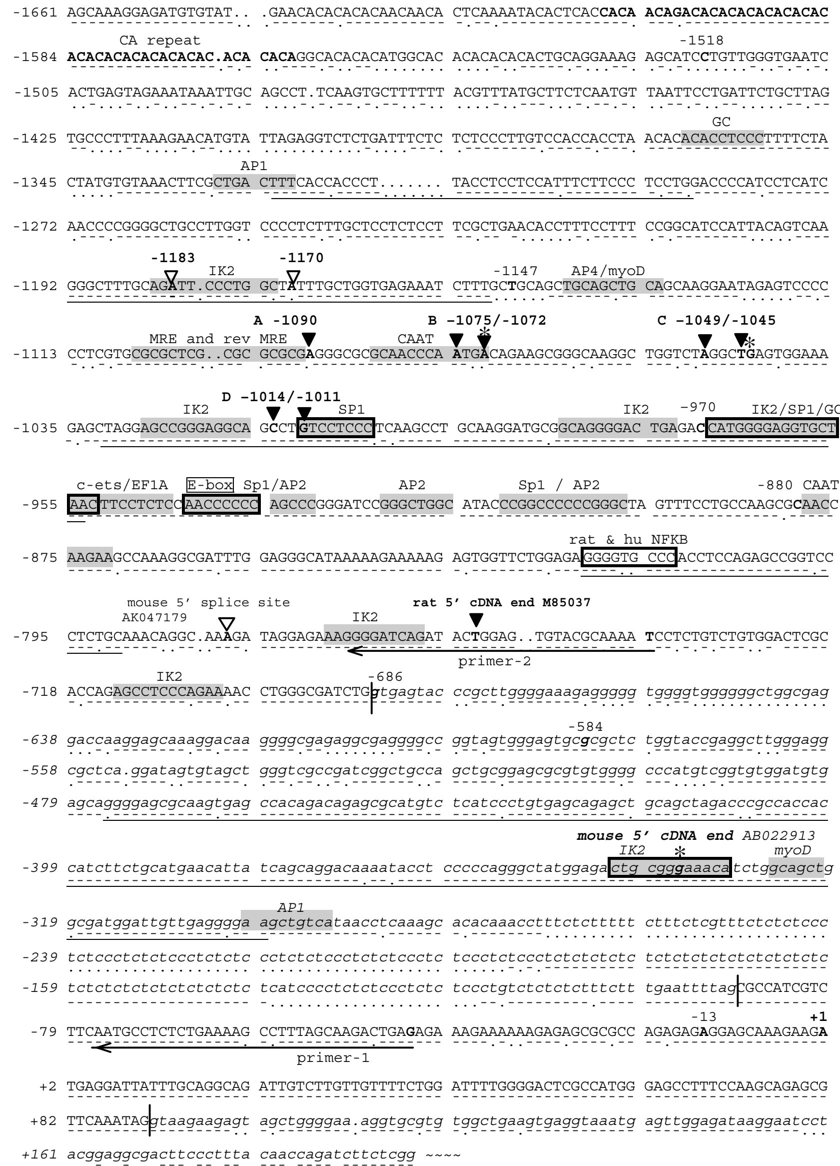

Fig. 1 Rat GluR4 promoter sequence and homology to the mouse and human sequences. The 5′ sequence of the rat GluR4 gene, including the first intron (italics, lower case) and beginning of the second intron (italics, lower case) are shown. Numbering is relative to the translational start ATG (bold +1). Identical (dashes) and non-identical bases or gaps (dots) in the mouse GluR4 sequence are shown below the rat sequence. The mouse promoter sequence shown here corresponds to the bases between 1720 022 (5′ end) to 1718 301 (3′ end) in GenBank accession number NW_000350. Areas that were more than 80% identical between rat and human (GenBank AP001561.4) are underlined. Transcriptional start sites identified in the rat by both primer extension and ribonuclease extension assays are indicated by bold letters A-D and filled triangles (see also Fig. 2). Other potential start sites found only by one strategy are indicated by open triangles. Putative transcriptional start sites in the mouse GluR4 promoter corresponding to cDNA ends are marked by asterisks. The location of primers 1 and 2 used for primer extension assays are indicated by arrows. Putative binding sites for transcription factors are shaded when found in rat and mouse, boxed when found in rat and human, or boxed and shaded when found in all three species. The locations of cloning sites are numbered in bold.

medium. Counting of immunolabelled cultures at 5–6 days *in vitro* revealed about 65% MAP2-positive, 64% TuJ1-positive cells and 3% GFAP-positive cells in B27 supplemented Neurobasal media ($n = 3$).

Primary glial cultures containing about 90% GFAP-positive cells were obtained from the primary cortical cultures by changing the medium and replating in DMEM supplemented with 10% fetal bovine serum. C6 glioma cells were grown in DMEM with 10% fetal bovine serum. Immunostaining was performed as described in Myers *et al.* (1998) using monoclonal mouse anti-βIII tubulin antibodies (Tuj1; Graff *et al.* 1997) and rabbit anti-firefly luciferase antibodies (both Promega).

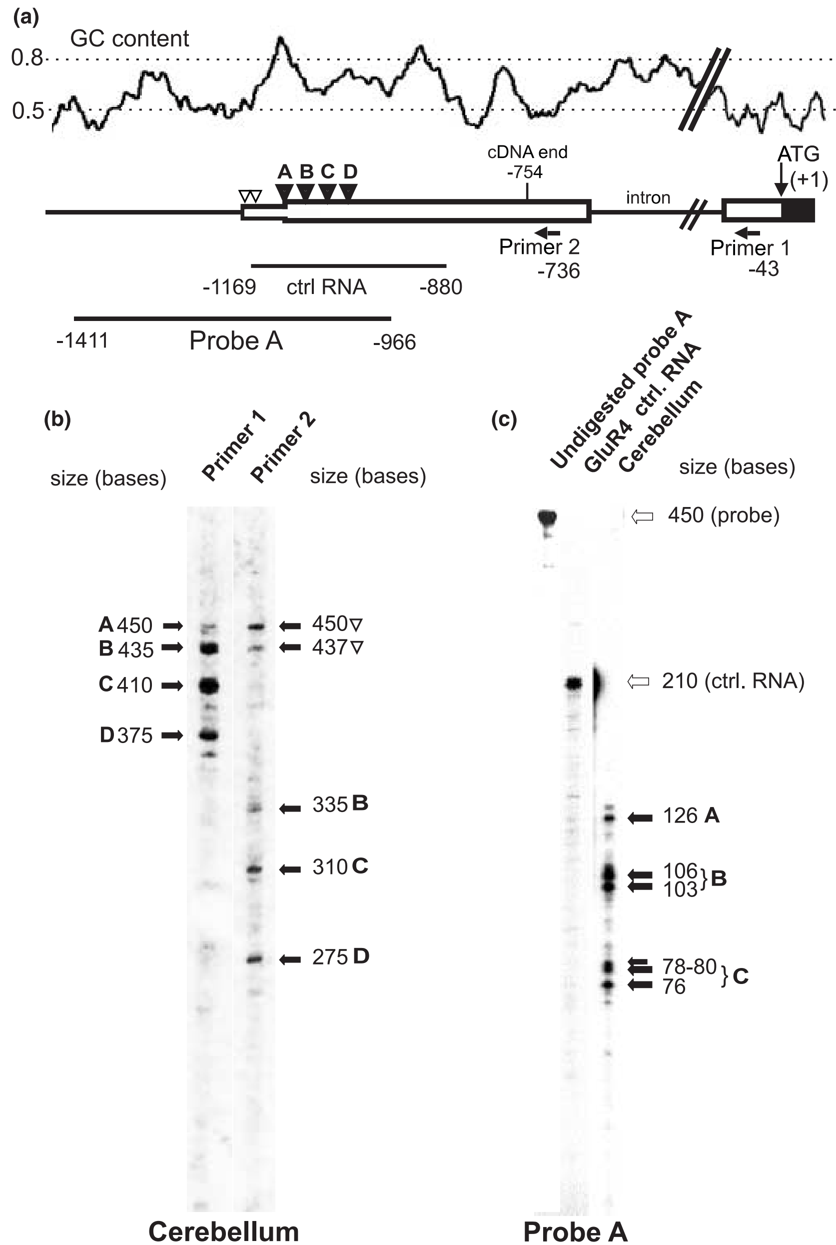

Fig. 2 Mapping of transcriptional start sites. (a) GC-content and schematic of the rat GluR4 gene, RPA probe, control cRNA and primers 1 and 2 used for the primer extension experiments. The main transcriptional start sites identified in primer extension assays (b) and RPAs (c) were assigned to four different nucleotides or regions (A-D, filled triangles, see also Fig. 1) taking into account the broad bands obtained and allowing an error up to four bases. For RPAs and for primer extension experiments, the bands were compared with a sequencing reaction (not shown); adding their nucleotide length to the position of the beginning of the probe (or the primer) and subtracting 1 gives the position of the RNA end. (b) Results from primer extension experiments with two different primers annealed with adult cerebellar polyA$^+$ RNA. The solid arrows mark the main specific DNA products and their length. Note that primer 2 did not identify site A, but repeatedly gave rise to bands indicating initiation sites even further upstream (open triangles in panel a and b and Fig. 1). (c) Results from a representative Rnase protection assay. The lane marked 'undigested probe A' shows the length of the RNA probe as calculated from a sequencing reaction (not shown). Probe and control RNA were treated the same way as the polyA$^+$ RNA. The bands marked by open arrows represent the full-length probe and the end of the control RNA. Filled arrows indicate bands identifying the most prevalent ends of cerebellar GluR4 polyA$^+$ RNA.

Primary cortical cultures were transfected on days 4–7 *in vitro*. For promoter-gene reporter assays, plasmid DNA (1 µg/well) was mixed with Lipofectamine Plus according to the manufacturer's instructions (Invitrogen) in serum-free medium (DMEM supplemented with 0.5 µM insulin, 100 µg/mL human apo-transferrin, 0.03 µM selenium and 60 µM putrescine, all Sigma, St Louis, MO, USA) and placed on cells. After 4 h, transfection media was removed and replaced with normal growth media. Cells were harvested 20 h post transfection and luciferase activity measured in a Turner TD-20e luminometer. In some experiments, both neurons and C6 glioma cells were pre-treated with 100 nM Trichostatin A (TSA, Sigma) prior to transfection and TSA was maintained in the culture medium until harvest as described (Huang *et al*. 1999). For each construct, at least three independent plasmid preparations (Qiagen, Chatsworth, CA, USA) were transfected in triplicates in two to four different culture preparations for each cell type. All luciferase values were normalized to the activity of the GluR2(−302/+320)*luc* promoter construct after subtracting the activity of the promoter-less pGL2-Basic vector; both plasmids were transfected into parallel wells in all experiments.

To estimate the neuronal selectivity of the GluR4 promoter fragments (Figs 3 and 5) we calculated luciferase activity of each GluR4 promoter fragment per transfected cell, assuming the transfection efficiencies were similar to those measured in the same culture systems for the GluR2(−302/+320) promoter (Myers *et al*. 1998). This assumption appears valid because the activities of GluR2(−302/+320)*luc* relative to SV40*luc* in our experiments (680–770% in mixed cortical and 4% in glial cultures) were similar to those by found by Myers *et al*. (1998) (710 and 2%, respectively).

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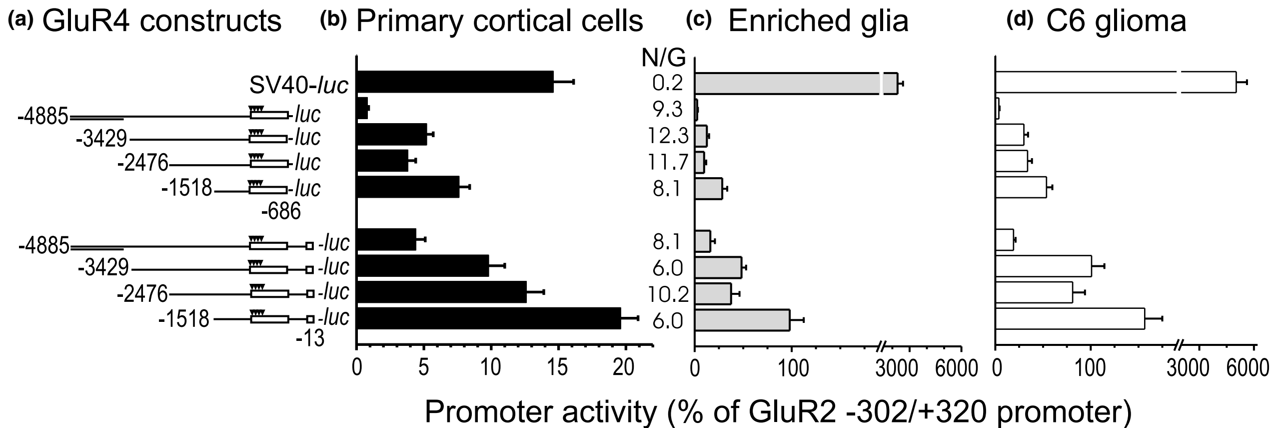

Fig. 3 GluR4 promoter activity in transfected cells. (a) The promoter constructs used for transfections in (b) mixed neuron-glial primary cultures of rat cortex (c) enriched glial cultures, and (d) C6 glioma cells. Exons in (a) are shown by open rectangles, the transcriptional start sites are indicated by triangles, and the upstream region that contains part of a long interspersed element (LINE) is underlined. (b–d) The mean and SEM of the luciferase activity of each construct is expressed as a percentage of GluR2(– 302/+ 320)*luc* promoter activity (Myers *et al.* 1998) for each culture system. For each construct, at least three independent plasmid preparations in at least three different cultures were tested ($n = 11–12$ for each construct). The ratio, N/G, denotes the ratio of expression of each construct in primary cortical (N) and enriched glial (G) cultures on a per transfected cell basis. The N/G ratio for the GluR2 promoter is 30 (see Myers *et al.* 1998). Note the high activity of SV40*luc* in glial cultures.

Statistical analysis

Graphpad Prizm was used to perform ANOVAS followed by a *post-hoc* Tukey test to compare all constructs with each other. A *post-hoc* Bonferroni test with selected pairs was employed when neighbouring constructs or selected pairs were compared.

Results

Rat GluR4 Promoter: exon–intron structure and transcriptional start sites

Comparison of our cloned genomic GluR4 sequence to the rat GluR4 cDNA (acc. no. M85037 Bettler *et al.* 1990) revealed the presence of a 597-bp intron (from – 90 to – 686) in the 5′-UTR. The presence of this intron was confirmed by matching results in primer extension assays using two different primers that flank the 5′-end (primer 2) and 3′-end (primer 1) of the intron (Figs 1 and 2). The main bands from the primer extension assays suggested that 5’ RNA ends reside upstream of – 1000, thus we designed two RPA probes to test for these sites specifically. As primer extension and RPAs often gave rise to broad bands, or clusters of bands (Fig. 2), we assigned initiation regions as well as initiation sites. We identified four main transcriptional start regions in rat cerebellum, A-D, all of which were found by overlapping results from at least one primer extension experiment and at least one probe in Rnase protection experiments (Figs 1 and 2, filled triangles). RPAs with both probes and primer extensions using primer 1 identified the most 5′ initiation site, termed A, at – 1090 from the translational start ATG in the genomic sequence. Three additional main initiation regions – 1072 to – 1075 (B), – 1045 to – 1049 (C) and – 1011 to – 1014 (D) were found by primer extensions with both primers 1 and 2. Initiation regions A, B, and C were confirmed by RPAs with probe A (Fig. 2c) and initiation regions B, C, and D were confirmed using probe B (not shown). Two additional 5′ potential start sites at – 1183 and – 1170 (Figs 1 and 2; open triangles) were only consistently identified with primer 2 but not with primer 1, nor with probe A in RPAs, therefore the validity of these upstream sites remains questionable. In Fig. 1 we labelled the closest adenosines within the regions as the transcriptional start sites. The confirmed initiation sites A through D are flanked by three GC-rich regions (Fig. 2a), – 1082 to – 1117 (89% GC content), – 978 to – 1026 (71%) and – 898 to – 941 (84%). No TATA-boxes were located near the initiation sites identified (Fig. 1). From these findings, mature GluR4 mRNAs will include long 5′-untranslated leaders of 413–485 bp in length, but we cannot exclude other potential GluR4 mRNAs with 5′UTR leaders shorter than 90 or longer than 815 bases.

Comparison of rat, mouse, and human GluR4 sequences

The rat genomic GluR4 sequence (from – 1661 to + 196) was aligned to the mouse (GenBank acc. no. NW_000350 and Celera) and human GluR4 5′ (AP001561.4) sequences and to mouse cDNAs (GenBank acc. numbers AK031568, AK047179, AK032103, AB022913) for comparison using the bestfit alignment protocol in GCG (Fig. 1). The cDNA ends of three mouse cDNAs found in GenBank overlapped with the transcriptional start regions identified by us in rat

(site B at − 1072 found in AK032103 and site C at − 1044 found in AK031568 and AK047179, Fig. 1, stars). Curiously, another 5′ mouse cDNA end (AB022913) resides within the rat 5′UTR intron (Fig. 1, star). The mouse cDNA, AK047179, indicated an alternative 5′ end splice site at − 778, which effectively shortens the 5′UTR by 91 bp (Fig. 1). However, identical bases were found in all three species at the exon/intron borders, indicating the common presence of an intron in the GluR4 5′ UTR. Thus, two mouse cDNAs confirmed the 5′ intron splice junction and three mouse cDNAs confirmed the 3′ intron splice junction. Identical bases between mouse and rat are indicated by dashes in Fig. 1, non-identical bases or gaps by dots. When gaps are not taken into account, the two rodent sequences are 86% identical between the translation ATG and − 3440 in rat; for comparison, the GluR4 protein coding sequences are 94% identical. The more distal rat sequence contains part of a long interspersed element, LINE, which was not found in mouse or human. The sequence upstream of the most 5′-transcriptional start site A diverged the most (84% identity) and includes a CA repeat in rat (45 bp at 608) and mouse, but not human. The sequence identity between mouse and rat downstream of transcription start site A, disregarding the intron, is 90%. However, even the intron sequences are conserved between mouse and rat (86% identity), when the 18, 22 and 75 bp gaps in the aligment are excluded from the analysis. The comparison of the human and rat GluR4 promoter sequence reveals an overall 73% sequence identity from the translation ATG to − 1969 in rat, and another region of 83% identity between − 3041 and − 3099. Areas with more than 80% identity between rat and human are underlined in Fig. 1. Other conserved features are three GC-rich areas, at − 1082 to − 1117 (89% GC in rat, 87% mouse, the sequence is absent in human), − 978 to − 1026 (70–71% in all three species) and − 898 to − 941 (84% rat and mouse, 87% rat and human). These conserved regions flank the major transcription initiation sites identified in rat (Fig. 1).

In addition, we searched for putative transcriptional elements which, if present in at least two species, are indicated in Fig. 1. In rat and mouse, we identified several common putative elements including metal response elements (MRE), AP1, AP2, AP4, IK2, Sp1, c-ets, EF1A, and myoD sites. Some of these putative transcriptional binding sites are conserved among all three species, i.e. one E-box, two Sp1 and two IK2 sites. A putative NFKB site at − 821 is present in rat and human, but not mouse.

The GCGCGCTCGCGCG site overlapping the putative MRE site (− 1091 to − 1106) closely resembles an NRF-1 element identified previously as a functional enhancer in the rat GluR2 promoter (Myers *et al*. 1998). However, when the GluR2 and putative GluR4 NRF-1 elements were tested for binding recombinant NRF-1 in an electrophoretic mobility shift assay, only the GluR2 site showed a gel shift. No binding was detected using the GluR4 sequence (data not shown), thus the GluR4 sequence is unlikely to be a functional NRF-1 element but may instead be recognized by other transcription factors.

Promoter activity and neuronal specificity

Promoter activity of different GluR4 fragments was assessed by measuring firefly luciferase activity following transfection of the reporter plasmids into primary cortical, primary enriched glial and C6 glioma cultures. GluR4 promoter activity was found in all three culture preparations. Table 1 compares the raw measurements of luciferase activity for selected GluR2, GluR4 and SV40 promoters in the three cell types. It can be seen that the GluR4(− 1518/− 13) promoter has similar activity to the GluR2 reference promoter in enriched astroglia and C6 glioma cultures, but substantially less activity than the GluR2 promoter in primary cortical cultures. As expected from previous studies, SV40 shows a strong preference to glia. In order to compare the relative strength of GluR4 and GluR2 promoters in the three cell types, we normalized the activity of all GluR4 promoter fragments to that of the GluR2 reference promoter, which was transfected into parallel wells in each experiment.

Our initial GluR4 promoter constructs were designed to evaluate (i) 4.9 kb of the proximal GluR4 promoter in ∼1 kb increments, including the LINE sequence between − 4885 and − 3610, (ii) the activity of these constructs either with or without the 5′UTR intron, and (iii) the functional role of the region containing transcriptional start sites in neuronal and glial cells. Construct GluR4(− 4885/− 686)*luc,* with the most distal 5′ end, produced weak promoter activity in all three cell types (0.8–3.7% of the GluR2 promoter, Fig. 3). When this construct was shortened from the 5′ end, a progressive increase in promoter activity was observed in all cell types regardless whether the 5′-UTR intron was present or absent. The construct GluR4(− 1518/− 13)*luc* produced the strongest luciferase activity in each cell type examined.

Our primary cortical cultures contain both neurons and glia. To determine whether luciferase was expressed in neurons or glial cells in these cultures, we performed double-immunolabeling of cultures transfected with the GluR4 (− 1518/− 13)*luc* construct using an anti-luciferase antibody and a neuronal marker antibody (Tuj1). Ninety-six per cent of 167 luciferase-positive cells were co-labelled with Tuj1 (example in Fig. 4, $n = 3$ culture preparations), indicating that the GluR4(− 1518/− 13)*luc* promoter was most active in neurons in these mixed cultures. This finding is similar to that found for GluR1 (Borges and Dingledine 2001) and GluR2 (Myers *et al*. 1998) promoters in similarly prepared cortical cultures. Although the cell in Fig. 4 is unusually bright, the staining for luciferase driven by the GluR4 promoter was generally much weaker per cell than that for luciferase expression driven by the GluR2 promoter in the same experiment. This observation is consistent with the

Table 1 GluR4 and GluR2 promoter activity in three cell types

Promoter construct	Cortical neuronal cultures	Astroglia cultures	C6 glioma
	TLU/well, mean ± SEM (*n*)		
SV40*luc*	519 ± 78 (13)	50 ± 13 (12)	31 900 ± 5,240 (13)
GluR4(− 1518/− 13)*luc*	910 ± 250 (10)	2.27 ± 0.80 (7)	1570 ± 437 (10)
GluR2(− 302/+ 320)*luc*	4840 ± 1340 (15)	3.05 ± 0.66 (13)	928 ± 176 (16)
GluR2(− 302/+ 320)*luc* TLU/transfected cell*	0.66 ± 0.14 (4)	0.022 ± 0.002 (4)	0.034 ± 0.019 (4)

For each construct, the table shows the luciferase activities per well averaged among different culture preparations (*n*) for three different cell types. Within each culture preparation the activity for individual constructs was determined as an average of several plasmid preparations each transfected into triplicate wells. *From Myers *et al.* (1998).

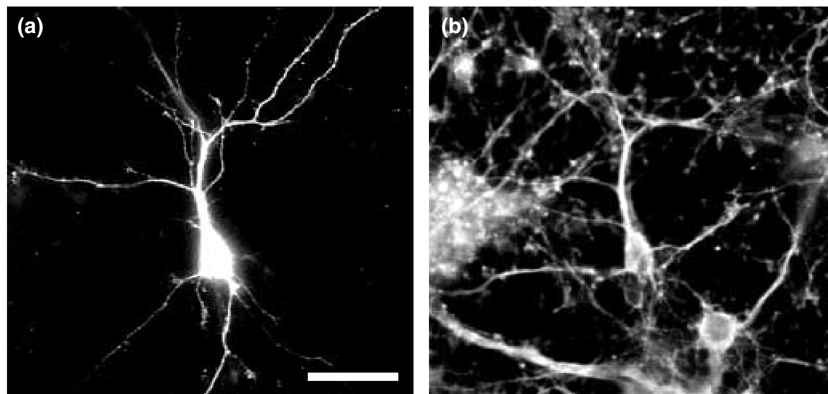

Fig. 4 GluR4 promoter drives luciferase expression in Tuj1-positive neurons. Mixed cortical cultures were transfected with the GluR4(− 1518/− 13)*luc* construct and 24 h later cultures were double–immunostained for firefly luciferase using a secondary Cyan green-labelled antibody (a) and Tuj1 using a Texas Red-labelled secondary antibody (b). A representative cell co-labelled with both antibodies was photographed. Scale bar: 50 μm.

finding that the GluR4 promoter is weaker than the GluR2 promoter when measured on a population of cells in transfected cortical cultures (Fig. 3).

When luciferase activity was estimated per transfected cell (N/G in Fig. 3), the GluR4 promoter fragments exhibited 6–12-fold higher activity in cortical neuronal cultures than enriched glial cultures. For comparison the GluR2(− 302/ + 320) promoter is 30-fold more active in cortical cultures than glial cultures (Myers *et al*. 1998). Taken together, these data indicate that GluR4 promoter fragments are not as strong in neuronal cultures as the GluR2 promoter, but can be approximately the same strength as GluR2 in non-neuronal cells.

Interestingly, when we deleted the region containing the main transcriptional start regions A-D found in cerebellar tissue (transcription initiation region, TIR, − 970 to − 1147) in construct GluR4(− 1513/− 13ΔTIR)*luc*, luciferase activity was significantly decreased in primary cortical cultures but increased in C6 glioma cells ($p < 0.05$; Figs 5a–c). With the shorter construct GluR4(1513/− 686 ΔTIR)*luc* the same trends were observed, although the differences were not significant. Note that the neuronal selectivity estimated by the ratio of promoter activity in primary cortical cultures and C6 glioma cells decreased with deletion of the transcription initiation region (Fig. 5d). Thus, it seems that neurons, but not glia, may use the transcriptional start sites mapped in Figs 1 and 2. Moreover, regulatory elements that govern activity selectively in neurons appear to reside in the transcriptional initiation area. The increase of luciferase activity after deletion of TIR in GluR4(− 1513/− 13ΔTIR)-*luc* in C6 glioma (Fig. 5c) suggests that the mapped transcriptional start sites are not necessary for glial expression and that glial cells may predominantly use different transcriptional start sites.

Control of promoter activity: upstream silencing region in LINE, histone deacetylase activity and splicing

In all three culture systems, promoter activity increased when the GluR4 fragment was shortened at the 5′ end from − 4885 to − 1518, or lengthened at the 3′ end from − 686 to − 13 (Fig. 3). Comparing the effect of 5′ deletions, the largest effect on promoter activity (2.2- to 5.3-fold; $p < 0.05$) was observed by removal of the sequence between − 4885 and − 3429 from construct GluR4(− 4885/− 13)*luc*. The sequence − 4885 to − 3610 is part of a long interspersed element (LINE). In experiments designed to further narrow the silencing region, we found that deletions near − 4885, within the LINE sequence itself, had the most pronounced effect on promoter activity (Fig. 6). In primary cortical cultures, deleting the 248 bp sequence between − 4885 to − 4637

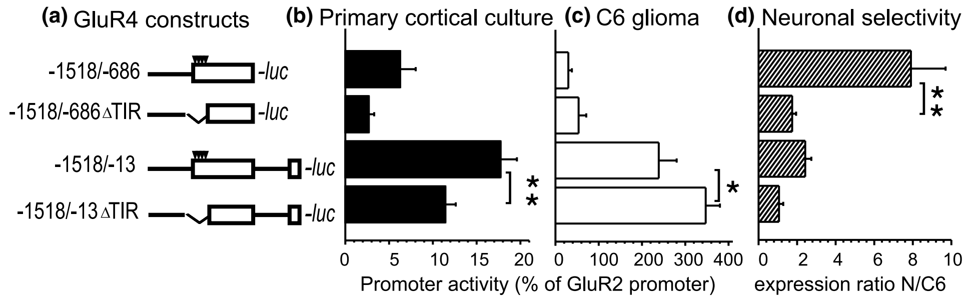

Fig. 5 Neuron-specific loss of promoter activity after deletion of the transcription initiation region (TIR). (a) The promoter constructs and their nucleotide boundaries, which were transfected into primary cortical (b) and C6 glioma cells (c), are shown. The constructs with the internal deletion of the transcription initiation region (ΔTIR) lack the sequence between – 1147 and – 970. (b, c) shows the mean and SEM of the luciferase activity of each construct expressed as a percentage of the GluR2(– 302/+ 320)*luc* promoter activity for each cell population. Two or three independent plasmid preparations of each construct were tested in two or three independent experiments ($n = 6$ for each construct). (d) The ratio, N/C6, denotes the ratio of expression of each construct in primary cortical (N) and C6 glioma (C6) cultures on a per transfected cell basis. The stars indicate significant differences by ANOVA followed by a Bonferroni test using selected pairs (*$p < 0.05$; **$p < 0.01$).

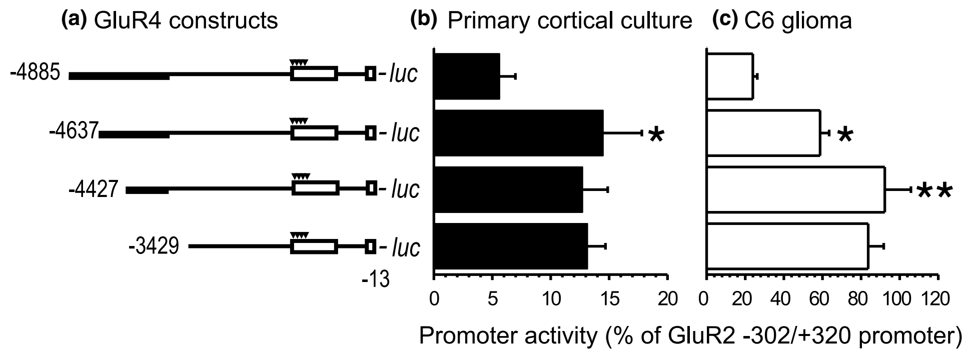

Fig. 6 Silencing activity within a 248–458 bp fragment. (a) Constructs shown were designed to narrow down a silencing activity originally found between – 3429 to – 4885 which contains part of a long interspersed element (underlined, see Fig. 3). The mean and SEM of the luciferase activity of each construct is expressed as a percentage of the R2(– 302/+ 320)*luc* promoter activity after transfection into primary cortical cultures (b) and C6 glioma (c). Three independent plasmid preparations of each construct were tested in three independent experiments ($n = 9$ for each construct). From these data, the extreme 5′ 458 bp and 248 bp are required for silencing of promoter activity in C6 glioma and neurons, respectively. The stars indicate significant differences when comparing luciferase activity of each construct with that of the longest construct GluR4(– 4885/– 13)*luc* by ANOVA followed by a Bonferroni test with selected pairs.

restored promoter activity to the level of the GluR4(– 3429/ – 13)*luc* construct ($p < 0.05$). This deletion increased promoter activity by a similar extent in C6 glioma cells ($p < 0.05$), thus it does not contain a neuron-selective silencer. In C6 cells luciferase activity was restored to the level of the GluR4(– 3429/– 13)*luc* construct only after deletion of a 458-bp fragment between – 4885 and – 4427 ($p < 0.01$). To assess whether the silencing activity of the sequence at the very 5′ end of GluR4(– 4885/– 13)*luc* was context-dependent we attached bp – 4885 to – 4427 to the 5′ end of the GluR4(– 1518/– 13)*luc* construct and the 5′ end of GluR2(– 302/+ 320)*luc*-ΔB construct, a GluR2 construct lacking its natural RE1 silencer element (Myers *et al.* 1998). In the context of these short promoters no consistent reduction of promoter activity by the GluR4 458 bp sequence was found (data not shown), indicating that the silencing activity is dependent on its context within the GluR4 promoter region.

Histone deacetylase (HDAC), which regulates gene activity in many genes, can be blocked by trichostatin A (TSA). A TSA-sensitive silencer element resides in the GluR2 gene and represses GluR2 promoter activity specifically in glial cells by recruiting HDAC (Myers *et al.* 1998; Huang *et al.* 1999; Huang *et al.* 2002). Thus, to investigate whether HDAC may control GluR4 promoter activity, we evaluated the effect of TSA on GluR4 promoter activity in C6 glioma and primary cortical cultures. TSA treatment caused no significant change in GluR4 promoter activity in cultured neurons, whereas TSA increased GluR4 promoter activity in C6 glioma cells by 3.5–10.5 fold (Fig. 7). Deletion of the

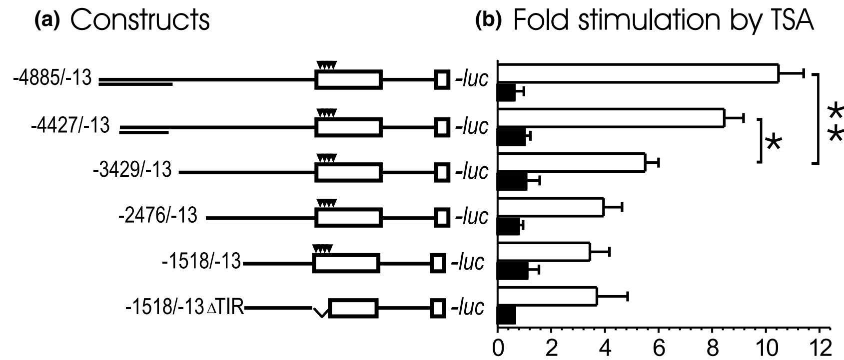

Fig. 7 Sensitivity to trichostatin A (TSA). (a) Shows the constructs that were transfected in the presence and absence of 100 nM TSA (see methods). (b) The mean and SEM of the fold stimulation of luciferase activity by TSA in primary cortical cultures (black bars) and C6 glioma cultures (white bars) is depicted ($n = 3$–4 for each construct in C6 glioma cells, $n = 2$–3 in primary cortical cultures). Stars indicate significant differences by ANOVA and a Bonferroni test using selected pairs.

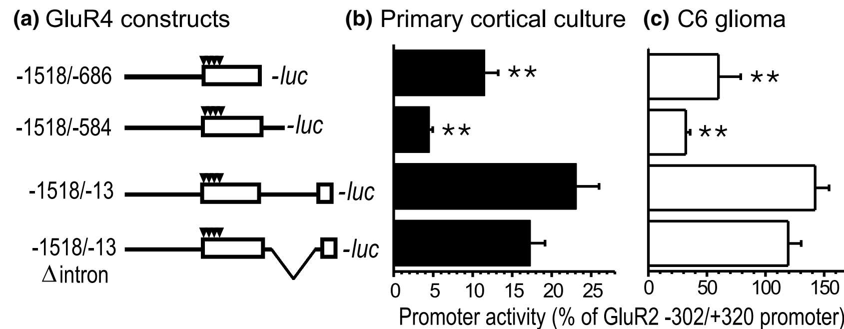

Fig. 8 Splicing and promoter activity. (a) The constructs transfected into primary cortical (b) and C6 glioma cells (c), and the mean and SEM of the luciferase activity of each construct is expressed as a percentage of the R2(−302/+320)*luc* promoter activity for each culture. Three independent plasmid preparations of each construct were tested in two to three independent experiments ($n = 8$–9 for each construct). $^{**}p < 0.01$ for comparisons to GluR4 (−1518/−13).

GluR4 sequence between −4885 and −3429 decreased TSA-sensitivity two-fold ($p < 0.01$), suggesting that this region of the GluR4 promoter may recruit HDAC activity in glial cells. Deletion of the 458 bp silencing region from −4885 to −4427 did not significantly alter the TSA effect, suggesting that the upstream silencing region probably does not recruit HDAC. In contrast to the effect of TSA on the GluR2 promoter, deletion of the transcription initiation region in GluR4 (−1513/−13 ΔTIR)-*luc* did not reduce TSA sensitivity, suggesting the increase in C6 cell promoter activity observed by the ΔTIR deletion in Fig. 5 is not due to loss of recruited HDAC activity to the proximal promoter.

Another set of constructs was designed to evaluate the effect of the 5′UTR intron on GluR4-directed luciferase synthesis. When promoter constructs were extended from −686 to −13 at the 3′ end, to include the complete 5′UTR intron, a significant increase of promoter activity was observed for most constructs (1.9- to 3.8-fold, for GluR4 (−1518/−13) *luc* $p < 0.05$) in all three culture systems (Fig. 3). These observations may be due to the inclusion of additional transcriptional regulatory elements in the longer constructs or enhanced stability of the resultant pre-mRNA via inclusion of the 5′-and 3′ splice site junctions. Two constructs were designed to address this question (Fig. 8), GluR4(−1518/−584)*luc,* which retains the 5′ splice site but lacks the 3′ splice site, and GluR4(−1518/−13)*luc*-Δintron, which lacks 496 bp of the intron sequence but retains both 5′-and 3′ splice sites junctions. As shown in Fig. 8, when the majority of the intron sequence was deleted in R4(−1518/−13)*luc*-Δintron, no significant change in promoter activity was observed in either primary cortical cultures or C6 gliomas. However, when one or both splice site junctions were removed in GluR4(−1518/−584)*luc* and GluR4(−1518/−686)*luc*, respectively, a significant drop in promoter activity was observed when compared with the parent construct GluR4(−1518/−13)*luc* ($p < 0.01$). Based on these results, we suggest that the intron sequence may contribute to GluR4 expression through a mechanism related to the splicing process rather than through direct regulation

of transcriptional activity by binding of a transcription factor to the intron nucleotide sequence.

Discussion

We have cloned and sequenced 5 kb of the rat GluR4 promoter and characterized the promoter by identifying transcriptional start sites, characterizing the activity of a number of promoter regions in neuronal and glial cells, and comparing promoter sequence to that of mouse and human. Our main findings are as follows. First, the GluR4 promoter is similar to other glutamate receptor promoters with multiple transcriptional start sites near GC-rich regions, conserved SP1 sites and lack of TATA boxes. In contrast to GluR1 and GluR2 promoters (Myers *et al*. 1999), the GluR4 promoter contains an intron in the 5′UTR. Secondly, the area containing transcriptional start sites contributes to promoter activity in neurons but not in glia, suggesting that the mapped transcription start sites are used in neurons and that regulatory elements governing expression in neurons reside in this area. Thirdly, the most distal 248–458 bp of the LINE sequence at −4885 to −3610 contains a non-cell selective silencing activity. Fourthly, the intron in the GluR4 5′-UTR did not appear to harbour strong transcriptional regulatory elements under basal growth conditions, unlike the first intron in the rat KA2 gene (*GRIK5*; Huang and Gallo 1997), because in luciferase reporter assays deletion of the majority of the intron sequence, while retaining splice site consensus signals, did not significantly alter promoter activity. Fifthly, the transcriptional start regions B and C are conserved in rat and mouse, and several putative transcription factor binding sites are conserved between rat, mouse and human, including an E-box, IK2 and SP1 sites. Finally, the GluR4 promoter is about 6–12-fold more active in mixed neuronal cultures than in glial cultures, but exhibits less neuronal selectivity compared with the GluR2 promoter, which shows a 30-fold neuronal selectivity (Myers *et al*. 1998).

Promoter activity and neuronal specificity

In neurons, GluR4 is the least abundant among the AMPA receptor subunits (Petralia and Wenthold 1992; Sato *et al*. 1993; Tsuzuki *et al*. 2001), whereas glial cells express the GluR4 subunit to a similar extent as other AMPA receptor subunits (Martin *et al*. 1993; Seifert and Steinhauser 2001). Similar to GluR1 and GluR2 promoters, immunolabelling showed that the GluR4 promoter prefers neurons in mixed cortical cultures that contain both neurons and glia. All GluR4 promoter fragments were less neuron-preferring than the GluR2 promoter, which is highly active in neurons. The activities of the GluR2 and GluR4 promoters in glia and C6 cultures are nearly equivalent except for the longest construct (Fig. 3). The relative activity of the GluR4 and GluR2 promoters in neuronal and non-neuronal cells thus recapitulates the generalized *in vivo* expression patterns of these genes, namely that GluR4 receptor expression is much lower than GluR2 in neurons. Neuronal specificity of a promoter can be achieved by the presence of neural-selective enhancers and/or the activity of silencing elements that restrict expression in non-neurons (reviewed in Roopra *et al*. 2001). Because deletion of the 177 bp region, including the transcription initiation sites (TIR, Fig. 5), reduced activity in cortical mixed cultures but increased activity in C6 glioma, this region might contain key control elements regulating GluR4 expression. The conserved AP4, MRE, CAAT and Sp1 sites are candidate sites to govern expression in neurons, whereas the conserved IK2 site, which binds transcription factors of the Ikaros family that can act as activators or repressors (reviewed in Georgopoulos 2002), might act as a negative regulator in glia. No other known silencing elements were found within this region and the mechanism for the putative glial silencing activity does not appear to be due to recruitment of TSA-sensitive HDAC activity (Fig. 7). Another region, 248–458 bp of the LINE segment, was found within the rat GluR4 promoter that might contain non-cell-type-specific silencing elements, because, when deleted, promoter activity was reduced in all cell types tested (Fig. 3). LINEs have been discovered close to other promoter sequences in several human genes and contained either silencing activity (Rothbarth *et al*. 2001; Kiyoshima *et al*. 2002) as found here, or functional enhancers as in the apolipoprotein(a) gene (Yang *et al*. 1998). The LINE segment flanking the 258–458 bp silencing region showed the largest TSA-sensitive component of the GluR4 promoter, raising the possibility that it may recruit HDAC activity to limit productive transcription initiation events in C6 glioma cells. Inspection of the GluR4 sequence did not reveal the presence of either an RE1 element like that found in GluR2 and NMDA-R1 (Myers *et al*. 1998; Huang *et al*. 1999) nor a sequence similarity to the KA2 silencer (Huang and Gallo 1997). Thus, similar to the GluR2 promoter, but different from the GluR1 promoter, some of the neuronal preference of the GluR4 promoter seems to reside within regions close to the initiation sites. In support of short core promoter regions driving strong expression in neuronal cells, a randomly constructed synthetic promoter library driving EGFP in Neuro2A cells was used to identify a number of short elements with sequences similar to those found in the GluR4 proximal promoter including SP1 and MAZ (TCCCCCCT, at −1108 in GluR4 TIR; Edelman *et al*. 2000).

Conserved features and elements among rat, mouse and human

Comparisons of promoter regions from several species are a useful tool for identifying potential conserved regulatory regions. For example, within the locus control regions of mammalian β-globin genes, many regulatory sites were found to be conserved among five species, some of which were shown to bind the cognate transcription factors and

were functional in *in vivo* or *in vitro* assays (review by Hardison *et al*. 1997). Although we have not identified individual elements in the GluR4 promoter, one can speculate that some of the regulatory sites conserved in several species may play a role for neuronal expression. These include an SP1 element downstream of the most 3′-initiation site conserved in rat, mouse and human, and elements conserved in rat and mouse, namely two overlapping MRE elements, one overlapping AP4 and myoD element, one CAAT box and two IK2 elements. The MRE elements are promising sites for further investigation because they reside immediately 5′ of the transcription initiation region and share high sequence identity to an active NRF-1 site in an analogous position in the GluR2 promoter (Myers *et al*. 1998).

IK2 regulatory elements can bind to several members of the zinc finger Ikaros transcription factor family, which are mostly confined to the hematopoetic system and are essential factors for normal lymphocyte development. The family member Eos is predominantly expressed in the developing nervous system (Honma *et al*. 1999; Perdomo *et al*. 2000) and within the enkephalin promoter a 17-bp region containing an IK2 element was identified by Dnase I footprint analysis of proteins present in the developing thalamus (Dobi *et al*. 1997). Ikaros family transcription factors interact with each other and can act both as activators or repressors, interacting with a variety of proteins that play a role in chromatin remodelling including histone deacetylases (reviewed in Georgopoulos 2002). However, despite the high conservation of the GluR4 intron and the IK2 element suggesting a functional role, deletion of the IK2-containing intronic sequence in construct GluR4(– 1518/– 13)*luc* had no effect on luciferase activity under basal culture conditions. This contrasts with the finding of a negative regulatory element in a similarly positioned intron of the KA2 promoter.

Other regions that were highly conserved between all three species, and thus may warrant further investigation in functional assays, are found just upstream of the initiation region or around the initiation site D and upstream of the rat 5′-cDNA end at – 753 (underlined in Fig. 1). Interestingly, most putative transcriptional elements that are conserved between rat, mouse and human are downstream of the initiation sites mapped in rat, indicating that other sites play a role in transcription initiation or that the basal promoters of human and rodent GluR4 are not conserved.

Comparison to other glutamate receptor promoters

Similar to other glutamate receptor promoters, the GluR4 promoter lacks a TATA box and contains several initiation sites. In contrast to GluR1 and GluR2, but similar to the promoters of the NMDA receptors NR2B and NR2C, and the kainate receptor KA2, the GluR4 gene contains an intron in the 5′ UTR. Strikingly, all ionotropic glutamate receptor promoters evaluated thus far (NR1, NR2b, NR2c, GluR1, GluR2, and KA2) contain Sp1 sites near the transcriptional start sites that either were shown to bind Sp1 *in vitro* or were functional by mutation or deletion (see Myers *et al*. 1999 and references therein). The GluR4 sequence contains only two conserved Sp1 sites downstream from initiation region D, but no other obvious Sp1 binding sites were found in the initiation region by inspection of the sequence.

In summary, the rat GluR4 promoter is a TATA-less promoter with multiple initiation sites dispersed over 77 bp of the proximal promoter region. The GluR4 promoter region was found to be selective for expression in neurons over glial cells. However, GluR4 promoter activity was less than the GluR2 promoter when compared directly in neurons. Splicing and two regions containing potential regulatory domains, the transcription initiation region and a 248–458 bp sequence within a LINE at – 4.9 kb, guide expression in both neurons and glia. Moreover, highly conserved Sp1, IK2 and E-box elements reside in the rat, mouse and human GluR4 promoters.

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