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Chromatin Structure and Expression of the AMPA Receptor Subunit GluR2 in Human Glioma Cells: Major Regulatory Role of REST and Sp1

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ABSTRACT

It has been suggested that reduced glutamate receptor expression protects glioma cells from glutamate toxicity. GluR2 is the critical subunit of the GluR2 subtype of AMPA glutamate receptors as this subunit determines the Ca^{2+} permeability of the receptor. The gene encoding the GluR2 subtype of AMPA receptors has been described as a target gene for the transcription repressor REST. However, we recently showed that the GluR2 gene is not regulated by REST in several neuronal and neuroendocrine cell lines, due to a repressive chromatin environment. Here, we show that the GluR2 gene has an open chromatin configuration in human glioma cells. Overexpression of REST reduced GluR2 mRNA levels while shRNA-mediated depletion of REST or expression of a REST mutant, that contained a transcriptional activation domain, enhanced GluR2 expression. Incubation with trichostatin A (TSA), a histone deacetylase inhibitor, induced acetylation of histone 4 of the GluR2 locus in glioma cells, leading to an upregulation of GluR2 expression. Together, these data suggest that REST is responsible for the reduced expression of GluR2 in glioma cells. The transcription factor Sp1 additionally binds under physiological conditions to the GluR2 gene in human glioma cells and expression of a dominant-negative mutant of Sp1 reduced expression of GluR2. Thus, the regulation via Sp1 represents a further control point for GluR2 expression in glioma cells. Together, we show that the GluR2 gene is embedded into an open chromatin configuration in glioma cells and expression of GluR2 is controlled by REST and Sp1. J. Cell. Biochem. 113: 528–543, 2012.

KEY WORDS: GluR2; GFAP, REST (RE-1 SILENCING TRANSCRIPTION FACTOR); HISTONE METHYLATION, HISTONE METHYLATION; HISTONE ACETYLATION; Sp1; U87MG CELLS

Glioma cells secrete large amount of glutamate through the X_C^- amino acid antiporter system [Savaskan et al., 2011] that results in excitotoxic cell death of neighboring neurons [Takano et al., 2001], thus facilitating tumor expansion. Glioma cells express AMPA receptors, a subtype of glutamate receptors, although at low levels in comparison to primary astrocytes [Savaskan et al., 2011]. It has been suggested that transcriptional downregulation of AMPA receptor expression protects the glioma cells against the high glutamate microenvironment of the tumor [van Vuurden et al., 2009; Savaskan et al., 2011].

Functional AMPA receptors are tetramers consisting of combinations of the four AMPA receptor subtypes termed GluR1–4. The GluR2 subunit determines the conductance properties of the receptor. In the absence of GluR2, AMPA receptors are Ca^{2+} permeable channels, while the presence of the GluR2 subunit generates AMPA receptors that are Ca^{2+} impermeable. This ability of the GluR2 receptor subunit is the result of RNA editing where a glutamine residue in the M2 pore region is exchanged with a positively charged arginine residue, thus preventing the passage of divalent cations such as Ca^{2+} [Tanaka et al., 2000; Isaac et al., 2007].

Abbreviations used: AMPA, 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid; ChIP, chromatin immunoprecipitation; ER, estrogen receptor; GFAP, glial fibrillary acidic protein; NRSE, neural-restrictive silencer element; 4OHT, 4-hydroxytamoxifen; REST, RE-1 silencing transcription factor; TSA, trichostatin A.

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The key role of the GluR2 subunit in AMPA receptor function focuses attention on the regulation of GluR2 gene transcription, as downregulation of GluR2 expression may function as a "molecular switch" to increase the formation of Ca^{2+} permeable AMPA receptors [Pellegrini-Giampietro et al., 1997]. The analysis of the 5′-flanking region of the rat GluR2 gene showed the presence of a neural restrictive silencer element (NRSE)-like sequence, the binding site for the transcriptional repressor REST [Myers et al., 1998]. Deletion mutagenesis of the rat GluR2 promoter, in vitro protein- and DNA-binding assays and overexpression experiments using REST expression vectors supported the conclusion that the NRSE is functional [Myers et al., 1998]. Both REST and GluR2 are expressed in glioma cells, suggesting that REST reduces GluR2 gene transcription. In this context, REST would play a prominent role in shaping the tumor environment of glioma cells. This hypothesis would fit very well to recent observations that REST may function as either tumor suppressor or tumor promoter in different tumor cells [Majumder, 2006]. However, we recently showed that the GluR2 subunit of AMPA receptors is not regulated by REST in neuronal, neuroendocrine, and endocrine cells, due to a repressive chromatin environment [Hohl and Thiel, 2005]. Thus, the cell type-specific microenvironment, in particular the cell type-specific structure of the chromatin, is crucial for the ability of REST to control GluR2 gene transcription.

Given the importance of glutamate signaling for neuronal cell death and tumor protection, we decided to analyze the chromatin structure and expression of GluR2 in glioma cells. Here, we show that the nucleosomal environment of the GluR2 gene in U87MG glioma cells contains di- and trimethylated forms of lysine residue 4 of histone 3 (H3K4), markers of an open configuration of actively transcribed genes. Accordingly, GluR2 gene expression was upregulated in human glioma cells following treatment of the cells with a histone deacetylase (HDAC) inhibitor, via expression of an activating mutant of REST that bound to the GluR2 gene under physiological conditions, or following shRNA-mediated depletion of REST. The fact that REST regulates GluR2 expression in glioma cells suggests that REST is responsible for the reduced expression of GluR2 in these tumor cells. In addition, we show that the GluR2 gene is a *bona fide* target for the zinc finger transcription factor Sp1 in human glioma cells. Expression of a dominant-negative mutant of Sp1 reduced GluR2 expression in glioma cells. Thus, the regulation of the GluR2 gene by Sp1 represents a further control point for GluR2 expression in glioma cells.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

The human brain glioma cell line U87MG was obtained from the European Collection of Cell Cultures (ECACC #89081402). Human SH-SY5Y neuroblastoma cells and HepG2 hepatoma cells were purchased from ATCC. HaCaT keratinocytes were kindly provided by N.E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The cells were cultured as described elsewhere [Kaufmann and Thiel, 2001, 2002; Cibelli et al., 2002; Rössler and Thiel, 2004; Bauer et al., 2007]. Trichostatin A (TSA, # T8552; Sigma–Aldrich, Steinheim, Germany) was used at a concentration of 100 ng/ml dissolved in DMSO. 4-Hydroxytamoxifen (4OHT, # H7904; Sigma–Aldrich) was dissolved in ethanol and used at a concentration of 1 μM.

RETROVIRAL GENE TRANSFER

The retroviral vector encoding a positive-dominant mutant of REST, fused to the ligand-binding domain of the estrogen receptor (ER), has been described elsewhere [Hohl and Thiel, 2005]. Plasmid pMSCV-FLAG-REST was constructed by inserting the coding region of FLAG-REST [Lietz et al., 2003] into pMSCVpac [Hawley et al., 1994]. The retroviral packaging cell line Phoenix-Ampho was obtained from Gary Nolan (Stanford University). Cells were transfected with retroviral vectors using the calcium coprecipitation procedure. Retroviral infection was performed as described [Rössler and Thiel, 2004]. U87MG and HepG2 cells were selected with 0.4 and 0.75 μg puromycin/ml, respectively. Mass pools of stable transfectants were selected and used for all experiments in order to eliminate the possibility of specific clonal effects.

LENTIVIRAL GENE TRANSFER AND REPORTER ASSAY

The lentiviral transfer vectors used in this study are derivatives of plasmid pFUW [Lois et al., 2002]. The lentiviral transfer vectors pFUWSp1ΔN and pFUWluc2 have been described elsewhere [Ekici et al., 2008b; Mayer et al., 2008]. To generate a GAL4-REST fusion protein, we cloned the coding region of GAL4-REST9 [Thiel et al., 1998], encompassing amino acids 953–1097 of REST, into plasmid pFUW. The lentiviral transfer vector pFUW-GAL4-NK10 was made via subcloning of the GAL4-NK10 coding region derived from plasmid pGAL4-NK10 [Thiel et al., 2001] into pFUW. Plasmid pPacSp1 was a kind gift of R. Tjian. The plasmid was cut with *Eco*RI and *Bam*HI. The fragment was isolated and inserted into the *Bam*HI and *Eco*RI sites of plasmid pM2 [Sadowski et al., 1992], generating plasmid pGAL4-Sp1. The coding region was excised with *Bgl*II and *Bam*HI and cloned into the *Bam*HI site of plasmid pFUW, thus generating the lentiviral transfer vector pFUW-GAL4-Sp1 that encodes for the human Sp1 sequence from amino acid 88 to 620, fused to the GAL4 DNA-binding domain. To generate the luciferase reporters pFWUAS^{5}Sp1^{2}luc and pFWUAS^{5}SV40luc, we excised the regulatory regions from plasmids pUAS^{5}Sp1^{2}luc and pUAS^{5}SV40-luc [Thiel et al., 2001] with Acc65I/*Hin*dIII and cloned the fragments upstream of the luciferase reading frame in a lentiviral transfer vector. The lentiviral transfer vector pFW(NRSE)^{2}SV40luc was generated via cloning of the (NRSE)^{2}SV40 promoter regulatory region derived from plasmid pSyINRSE^{2}SV40luc [Lietz et al., 2003] as *Pac*I/*Bam*HI fragment into a lentiviral vector upstream of the luciferase open reading frame. To generate plasmid pFWHIVLTRluc, a lentiviral transfer vector containing the HIV LTR sequence (sequence from −120 to +83) upstream of the luciferase open reading frame, we exchanged the ubiquitin C promoter from plasmid pFUWluc2 with the HIV LTR sequence derived from plasmid pGL3-HIV-1 LTR [Bauer et al., 2007]. The human GluR2 promoter was amplified by PCR with PCR Phusion™ Hot Start Polymerase (New England Biolabs) using the primers 5′-GGC GGG TTA ATT AAT CTA CAC AGC AGC CGG AGA T-3′ and 5′-GGG CGG GAT CCC TCT CTC GCG CTC TCT TCT C-3′. The PCR conditions were: denaturation (98°C, 10 s), annealing (63°C, 30 s), primer extension (72°C, 30 s) for

35 cycles. The amplified product was digested with *Pac*I and *Bam*HI, purified by polyacrylamide gel electrophoresis and cloned upstream of the luciferase coding region in the *Pac*I and *Bam*HI sites of plasmid pFUWluc2, thus generating the lentiviral transfer vector pFWGluR2luc. The viral particles were produced as previously described [Stefano et al., 2006] by triple transfection of 293T/17 cells with the *gag-pol-rev* packaging plasmid, the *env* plasmid encoding VSV glycoprotein and the transfer vector. Cell extracts were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and analyzed for luciferase activities [Thiel et al., 2000]. Luciferase activity was normalized to the protein concentration (relative luciferase activity, RLU/μg protein).

LENTIVIRAL EXPRESSION OF SHORT HAIRPIN RNAs (shRNAs)

The lentiviral vector pLentiLox3.7 (pLL3.7) was purchased from American Type Culture Collection (Manassas, VA). The sequences used to knock down human REST have been described (#1, Greco et al., 2007; #2, Yang et al., 2008). The oligonucleotides for creating RNAi stem loops for pLL3.7. were designed as described (http://mcmanuslab.ucsf.edu/protocols/ll37stemloop_design.pdf). In this study, we show the data obtained following expression of shREST #1, although expression of shREST #2 gave similar results. The lentiviral transfer vectors encoding p53 or Pdx-1-specific shRNAs, used as a negative control, will be described elsewhere.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR was performed as previously described [Bauer et al., 2007]. The primers used to detect GluR2 mRNA are listed in Table I. The PCR conditions were: denaturation (95°C, 45 s), annealing (60°C, 45 s), primer extension (72°C, 1 min) for 30 cycles. Amplified products were resolved by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. The experiments were performed at least three times with consistent results. Quantification was performed using programm XnView from Pierre e.Gougelet to scan at least three independent photographs for each experiment. The data were quantified using the programm LabWorks 4.6 from UVP BioImaging Systems. Data presentation was done with program GraphPad Prism 3.02 (Prism), showing the standard error of the means (SEM) from at least three independent experiments. The statistical difference was analyzed using the Student's *t*-test. A *P*-value of <0.05 was considered significant.

CHROMATIN IMMUNOPRECIPATION (ChIP)

Chromatin immunoprecipation (ChIP) experiments were performed as described elsewhere [Ekici et al., 2008a]. The ChIP primers are listed in Table I. The antibodies used for immunoprecipitation were anti-di-methyl H3K4 (#07-030; Upstate Biotechnology, Lake Placid, NY), anti-tri-methyl H3K4 (#ab8580; Abcam, Cambridge, UK), anti-di-methyl H3K9 (#ab7312; Abcam), anti-acetyl H4 (#06-866, Millipore), anti-HDAC1 (#0S-614; Upstate Biotechnology), anti-NRSF (#sc-25398; Santa Cruz, Heidelberg, Germany), and anti-Sp1 (#sc-59; Santa Cruz). To detect binding of FLAG-tagged REST or DP-REST:ER to DNA under physiological conditions, we used M2 agarose (#A2220; Sigma–Aldrich) that interacted with the FLAG epitope of the REST mutant as described [Hohl and Thiel, 2005].

ANTIBODIES AND IMMUNOBLOT ANALYSIS

Nuclear extracts and whole cell extracts were prepared as described [Kaufmann and Thiel, 2002]. Protein (10 μg) was separated on 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membrane (0.2 μm pore size, Schleicher & Schuell, Dassel, Germany). Membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat dry milk at 37°C for 1 h, and incubated for 2 h at room temperature with the M2 monoclonal antibody directed against the FLAG epitope (#F3165; Sigma–Aldrich), at 1:3,000 dilution in TBS. To detect expression of GAL4 fusion proteins, we used a monoclonal antibody (#sc-510; Santa Cruz). Secondary antibodies (goat anti-mouse peroxidase conjugated antibody, Jackson Immuno Research Laboratories, West Grove, PA) were incubated at room temperature for 1 h and were used at a dilution of 1:10,000. Immunoreactive bands were detected via enhanced chemiluminescence using a 1:1 solution of solution 1 (100 mM Tris–HCl, pH 8.5, 5.4 mM H_2O_2) and solution 2 (2.5 mM Luminol, 400 μM *p*-coumaric acid, 100 mM Tris–HCl, pH 8.5).

RESULTS

CELL TYPE-SPECIFIC EPIGENETIC CONFIGURATION OF THE GLuR2 GENE

A recent analysis of the epigenetic configuration of the GluR2 gene in immortalized neurons and neuroendocrine cells revealed that the GluR2 gene is embedded in an environment characterized by an epigenetic marker for silenced genes. Accordingly, GluR2 expression could not be induced by treatment of the cells with TSA or by forced expression of a dominant-positive mutant of REST that

TABLE I. Sequence of the PCR Primers Used for RT-PCR and ChIP

Gene	Forward primer	Reverse primer	Product size (bp)	Gene accession number
List of gene-specific primers for RT-PCR				
GluR2	CAC ACT GAG GAG TTT GAA GAT GGA	TTA GTA CTG CGA GGT TAA CCG CAT	600	BC01574.1
GFAP	CTG TTG GCC AGA GAT GGA GGT T	TCA TCG CTC AGG AGG TCC TT	382	BC013596
GAPDH	TTC CAG GAG CGA GAT CCC	CAC CCA TGA CGA ACA TGG G	175	BC83511.1
REST	TTT GAA GTT GCT TCT ATC TGC TGT	GAA TCT GAA GAA CAG TTT GTG CAT	626	NM_005612
List of gene-specific primers for ChIP-PCR				
GluR2	CCA GGT TGG AGC ATC TCC GCA GC	TAG CCG CTG TCC CTC CGC GAG A	150	AC 112240
GFAP	GAG AGG GTC CTC TTG CTT CAG	TGA AGG AGT GGG CTA GAC TGG	238	M 67446.1

activates NRSE-containing genes [Hohl and Thiel, 2005]. To test the hypothesis that REST is responsible for the reduced glutamate receptor expression in glioma cells we assessed the epigenetic configuration of the human GluR2 gene in human U87MG glioma cells using markers that differentiate between actively transcribed or silenced genes. An epigenetic marker for actively transcribed genes is methylation of H3K4 [Santos-Rosa et al., 2002]. In contrast, methylation of H3K9 functions as an epigenetic marker for silenced genes [Rea et al., 2000]. As a control, we analyzed GluR2 expression in neuroblastoma and hepatoma cells and in keratinocytes. The histone H3 methylation status of the GluR2 gene was analyzed by ChIP using antibodies directed against either the di-methylated and tri-methylated form of histone H3K4 or the di-methylated form of histone H3K9. The precipitated DNA was analyzed with primers that amplified the region of the GluR2 gene encompassing the NRSE, the binding site for REST (Fig. 1A). The results show that in U87MG glioma cells the GluR2 gene is mainly embedded in a nucleosomal context having histone H3 molecules carrying di-methylated and tri-methylated lysine residue 4 (Fig. 1B), markers that are linked to open chromatin and actively transcribed genes [Ruthenburg et al., 2007]. Additionally, methylation of H3K9 was also observed, but to a lesser extent (Fig. 1B, panel "U87MG"). As a control, the epigenetic profile of the GFAP gene was analyzed. GFAP as a marker for astrocytes is expressed in glioma cells, suggesting that the GFAP locus is embedded into an open chromatin structure in these cells. The location of the primers used to amplify the proximal region of the GFAP regulatory region is depicted in Figure 1A. ChIP experiments showed that in U87MG glioma cells the GFAP gene is embedded into a chromosomal context with histone H3 molecules methylated on lysine residue 4. Together, these results show that the GFAP as well as the GluR2 gene are embedded into an open chromatin configuration in U87MG glioma cells.

Next, we compared the epigenetic profiles of the GluR2 and GFAP genes obtained in the analysis of U87MG glioma cells with those in neuroblastoma and hepatoma cells and in keratinocytes. The GluR2 gene was located in a chromatin environment characterized by histone 3 molecules carrying methylated lysine 9 residues in hepatoma cells and in keratinocytes (Fig. 1B, panels "HaCaT" and "HepG2"), a marker that is linked to a condensed form of chromatin and gene silencing. In neuroblastoma cells, the GluR2 gene locus was found in nucleosomes carrying both methylated H3K4 and H3K9 (Fig. 1B, panel "SH-SY5Y"). These data are in accordance with a Western blot analysis showing expression of GluR2 in SH-SY5Y cells [Brené et al., 2000]. The GFAP gene was silenced in SH-SY5Y, HepG2, and HaCaT cells.

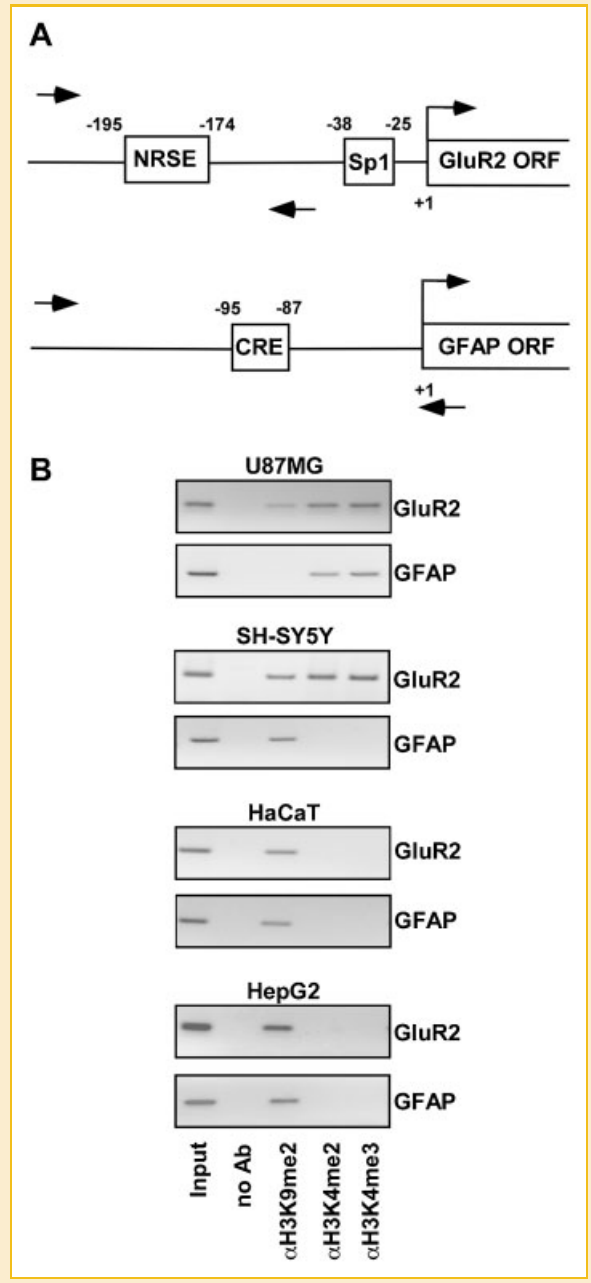

Fig. 1. Epigenetic modification of the GluR2 gene in human cell lines. A: Schematic representation of the 5′-portion of the human GluR2 and GFAP genes. The start sites of transcription are shown. The binding site for the transcriptional repressor REST (termed NRSE) and the Sp1 consensus binding site are depicted within the GluR2 regulatory region. The location of the cyclic AMP response element (CRE) within the GFAP promoter is shown. The arrows symbolize the primers used for PCR. B: Cross-linked and sheared chromatin prepared from U87MG glioma, SH-SY5Y neuroblastoma, HepG2 hepatoma cells, and HaCaT keratinocytes was immunoprecipitated with antibodies directed against di-methylated and tri-methylated H3K4 or dimethylated H3K9. Immunoprecipitated chromatin fragments were amplified with primers encompassing the proximal regulatory region of the GluR2 gene. As a negative control, no primary antibody was added. 1% of the total input was also examined by PCR. Each experiment illustrated here and in all subsequent figures was repeated a minimum of three times with consistent results.

UPREGULATION OF GluR2 PROMOTER ACTIVITY AS A RESULT OF HISTONE DEACETYLASE INHIBITION

The level of histone acetylation is increased when HDAC activity is blocked by TSA, a member of the hydroxamate class of HDAC inhibitors. TSA inhibits classes I and II HDACs, whereas class III is insensitive to TSA [Sengupta and Seto, 2004]. We recently showed that treatment of SN56 neurons or neuroendocrine cells derived from the pituitary or the pancreas with TSA failed to activate GluR2 gene transcription [Hohl and Thiel, 2005]. In contrast, an upregulation of GluR2 expression and promoter activity has been

reported for TSA-treated C6 glioma cells [Huang et al., 1999]. These authors also reported—without showing data—that TSA enhanced GluR2 promoter activity in cultured astrocytes. Thus, the TSA-inducibility of the GluR2 promoter was assessed in U87MG glioma cells. We implanted a GluR2 promoter/luciferase reporter gene into the chromatin of U87MG cells using lentiviral gene transfer. A schematic representation of the integrated provirus encoding the GluR2 promoter/luciferase reporter gene is depicted in Figure 2A. U87MG glioma cells were infected with lentiviruses encoding a GluR2 promoter/luciferase reporter gene (GluR2luc). The cells were subsequently stimulated with TSA and luciferase activities were measured 24 hours later. Figure 2B shows that treatment of U87MG cells with TSA significantly increased the transcriptional activity of the GluR2 promoter/luciferase reporter gene.

CELL TYPE-SPECIFIC ENHANCEMENT OF HISTONE ACETYLATION OF THE GluR2 GENE LOCUS FOLLOWING TREATMENT WITH TSA

Inhibition of HDACs by TSA shifts the balance between histone acetylation/deacetylation towards acetylation. We analyzed the acetylation status of the GluR2 gene in TSA-treated glioma and hepatoma cells by ChIP, using antibodies directed against the acetylated form of histone H4 to precipitate the chromatin. Figure 2C shows that the TSA treatment led to an upregulation of histone H4 acetylation of the GluR2 locus in U87MG glioma cells, while no increase in H4 acetylation was observed in human HepG2 hepatoma cells (Fig. 2D).

UPREGULATION OF GluR2 EXPRESSION AS A RESULT OF HISTONE DEACETYLASE INHIBITION

We tested whether an inhibition of HDACs is sufficient to induce GluR2 gene transcription in glioma, neuroblastoma, hepatoma cells, and in keratinocytes. Cells were treated for 24 h with TSA, cytoplasmic RNA was prepared, reverse transcribed into cDNA, and analyzed by RT-PCR. Figure 3A,B shows that expression of GluR2 was upregulated as a result of HDAC inhibition in U87MG glioma and SH-SY5Y neuroblastoma cells, suggesting that the GluR2 gene is regulated by the balance of histone acetylation and deacetylation in these cells. The epigenetic profile of the GluR2 gene correlates perfectly with the TSA inducibility in human glioma cells. TSA-induced inhibition of HDAC activity did not induce expression of GluR2 in HepG2 and HaCaT cells, indicating that the regulation of this gene is independent of histone acetylation and deacetylation. These expression data of GluR2 in U87MG cells are in agreement with the observed upregulation of histone H4 acetylation following TSA treatment. Likewise, the fact that no increase in H4 acetylation was observed in TSA-stimulated human HepG2 hepatoma cells fits very well to the results, showing that TSA treatment do not induce GluR2 expression in this cell line.

BINDING OF REST AND HDAC1 TO THE GluR2 GENE IN HUMAN GLIOMA CELLS

The GluR2 gene of the rat has been described as a target gene for REST [Myers et al., 1998; Huang et al., 1999]. However, we were unable to show that the GluR2 gene is regulated by REST in SN56 cells and neuroendocrine cells [Hohl and Thiel, 2005]. REST contains two "active" transcriptional repression domains on its

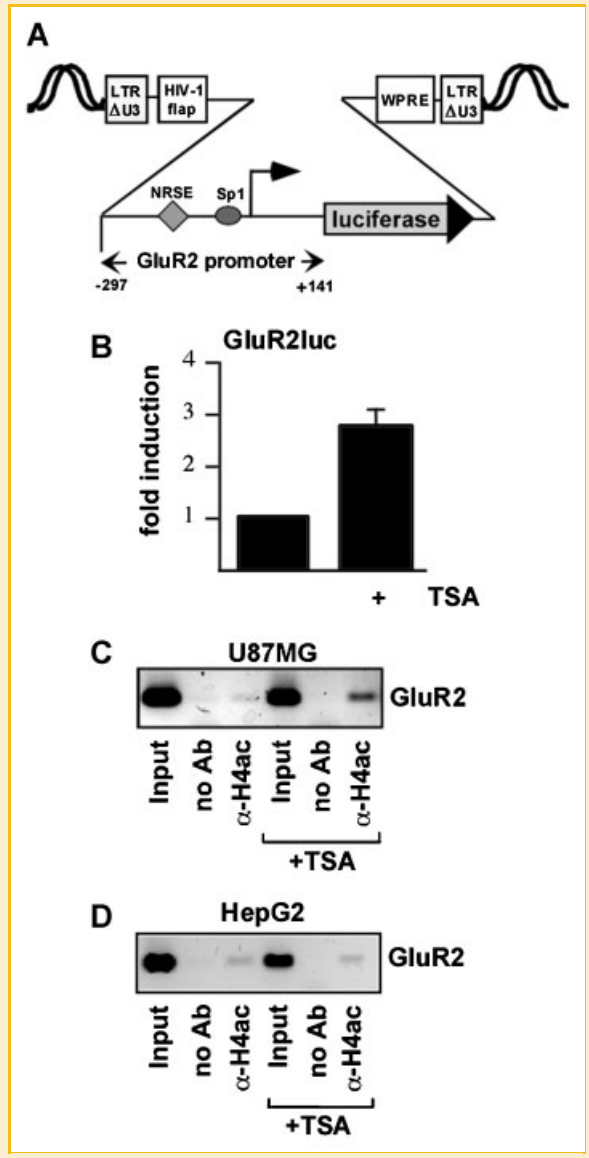

Fig. 2. GluR2 promoter activity and histone acetylation following TSA-induced inhibition of histone deacetylase. A: Schematic representation of integrated provirus encoding a GluR2 promoter/luciferase reporter gene. The transfer vector pFWGluR2luc contained the sequence from −297 to +141 derived from the human GluR2 gene. B: U87MG cells were infected with a recombinant lentivirus encoding a GluR2 promoter/luciferase reporter gene. The infected cells were stimulated with TSA as indicated. Cell extracts were prepared and analyzed for luciferase activities which were normalized to the protein concentrations. C,D: ChIP experiments were performed with chromatin isolated from either U87MG (C) or HepG2 cells (D), respectively, using antibodies detecting acetylated histone 4. The cells had been treated with TSA, as indicated, or with vehicle. Immunoprecipitated chromatin fragments were amplified with primers encompassing the proximal promoter region of the human GluR2 gene as depicted Figure 1. As a negative control, chromatin immunoprecipitation was performed with protein A-sepharose, without addition of antibodies (*no Ab*). As a positive control an aliquot of the total chromatin in the absence of immunoprecipitation was analyzed by PCR (*input*).

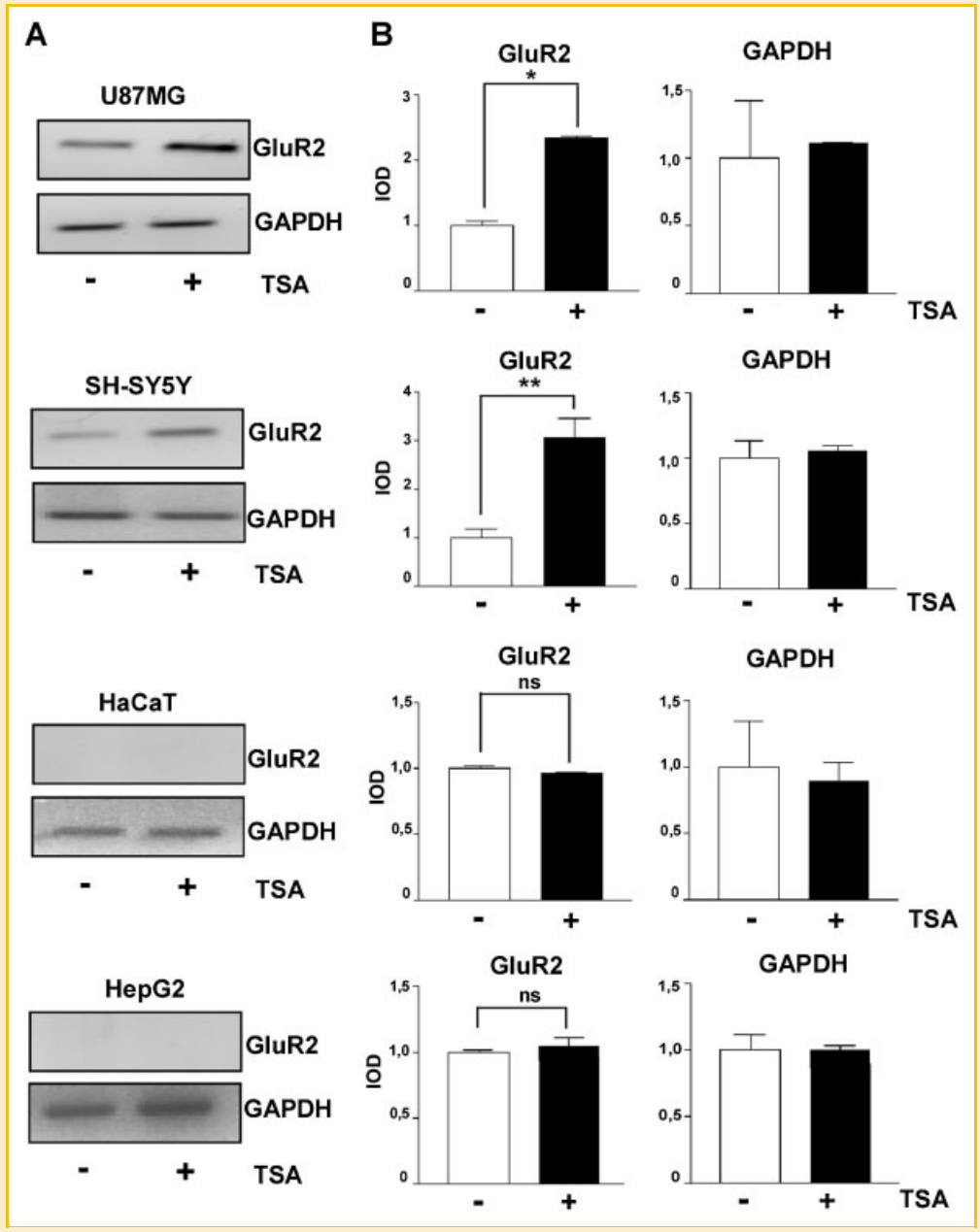

Fig. 3. Inhibition of histone deacetylase activity by TSA upregulates GluR2 expression in glioma and neuroblastoma cells, but not in heptoma cells and keratinocytes. A: U87MG glioma cells, SH-SY5Y neuroblastoma cells, HaCaT keratinocytes, and HepG2 hepatoma cells were treated for 24 h with the histone deacetylase inhibitor TSA (100 ng/ml) or with the vehicle DMSO. RNA from DMSO-treated (denoted "−") and TSA-treated (denoted "+") cells was isolated and analyzed by RT-PCR. The expression of GAPDH served as a control. The ethidium bromide stained gels (A) and the quantification and statistical analysis of these results (B) are depicted ($^{*}P < 0.05$; $^{**}P < 0.01$; n.s., not significant). IOD, integrated optical density.

N-and C-termini [Thiel et al., 1998], both of which recruit HDACs to the REST-bound transcription units. We analyzed the binding of REST to the GluR2 gene under physiological conditions using ChIP. We also analyzed the binding of HDAC1 to the GluR2 gene, as it is known that REST recruits HDAC1 to its target genes. Figure 4 reveals that REST and HDAC1 interacted with the GluR2 gene in glioma, neuroblastoma, hepatoma cells, and keratinocytes. Thus, binding of REST and HDAC1 to the GluR2 gene was observed in all cells analyzed, despite differences between the chromatin configuration and the TSA inducibility. In contrast no binding of REST or HDAC1 to the GFAP gene was observed.

REST ACTIVITY IN DIFFERENT CELL TYPES

The ChIP data revealed binding of REST to the GluR2 gene in different cell types, but did not reveal any evidence about the biological activity of REST. Thus, we established a REST activity assay to measure REST activity in glioma, neuroblastoma, hepatoma cells, and keratinocytes. We used two luciferase

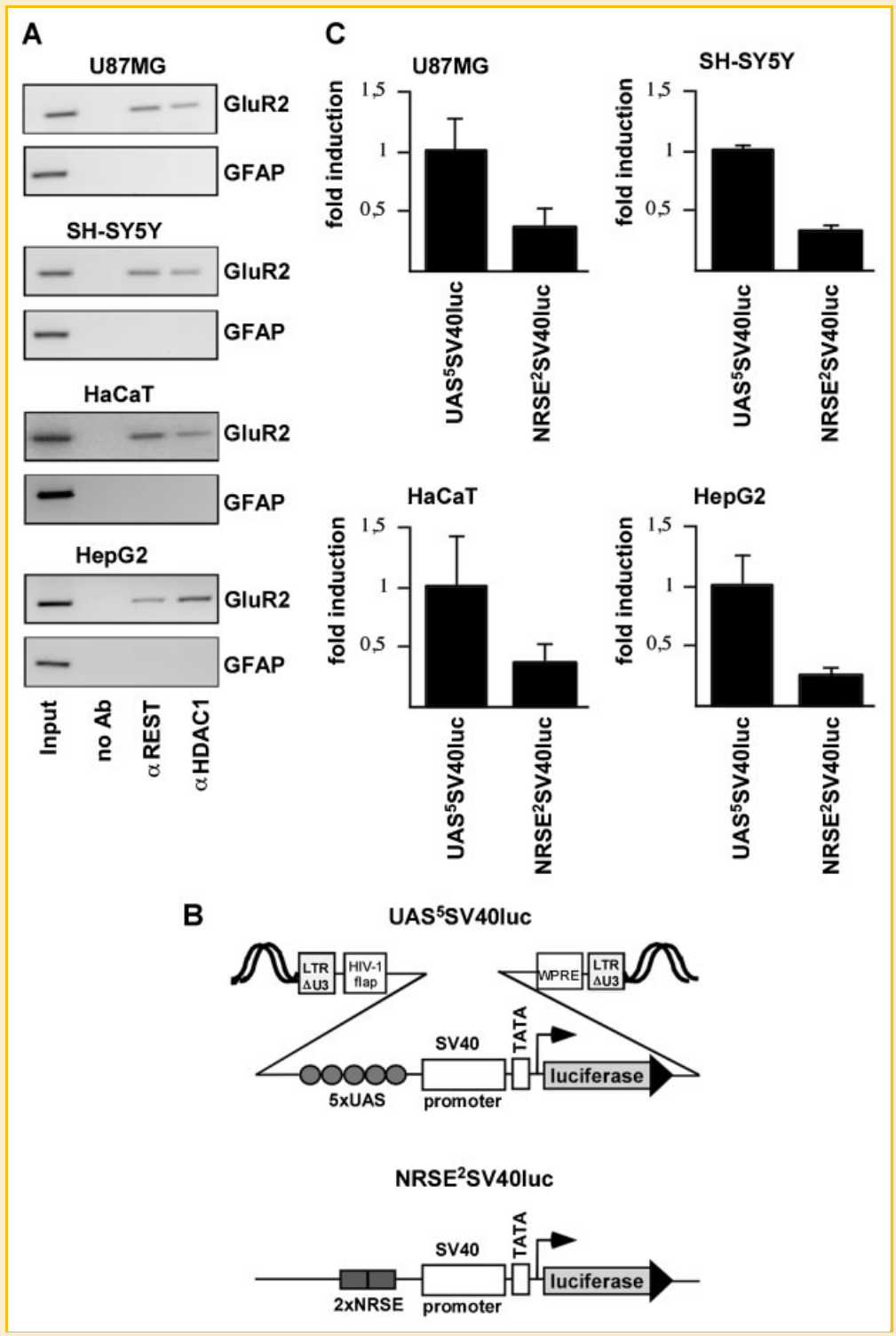

Fig. 4. REST binding to the GluR2 gene and REST activity in different cell types. A: ChIP experiments were performed with anti-REST and anti-HDAC1 antibodies. Immunoprecipitated chromatin fragments were amplified with primers encompassing the proximal promoter region of the human GluR2 and GFAP genes as depicted in Figure 1. As a negative control, chromatin immunoprecipitation was performed with protein A-sepharose, without addition of antibodies (*no Ab*). As a positive control an aliquot of the total chromatin in the absence of immunoprecipitation was analyzed by PCR (*input*). B: Schematic representation of integrated proviruses encoding a luciferase reporter gene under the control of the SV40 promoter. Upstream of the SV40 promoter, either five copies of the GAL4-binding site UAS (UAS^{5}SV40luc) or two copies of the REST binding site NRSE (NRSE^{2}SV40luc) were inserted. C: U87MG, SH-SY5Y, HaCaT, and HepG2 cells were infected with lentiviruses encoding SV40 promoter/luciferase reporter genes. The reporter genes contain either GAL4 (UAS, upstream activating sequence) or REST (neural-restrictive silencer element) binding site 5′ of the SV40 promoter. Cell extracts were prepared 48 h later and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

reporter genes containing the SV40 promoter under the control of either GAL4 binding sites (UAS^5SV40luc) or REST binding sites ($NRSE^2$SV40luc; Fig. 4B). The UAS^5SV40luc served as a control, since there are no mammalian transctiption factors known to bind to GAL4 binding sites. In contrast, REST interacts with the NRSEs in the $NRSE^2$SV40luc reporter and should repress SV40 promoter activity. Figure 4C shows that REST activity could be detected in U87MG glioma cells, SH-SY5Y neuroblastoma cells, HepG2 hepatoma cells, and HaCaT keratinocytes.

EXPRESSION OF A DOMINANT-POSITIVE MUTANT OF REST IN HUMAN GLIOMA AND HEPATOMA CELLS

The previous experiments revealed that the GluR2 gene is embedded into an open configuration in U87MG glioma cells and that GluR2 expression could be stimulated with TSA. We then asked whether we can directly activate GluR2 gene expression in U87MG cells via expression of a mutant form of REST. This inducible dominant-positive mutant of REST termed DP-REST:ER activates transcription of REST-responsive genes following stimulation with 4OHT [Hohl and Thiel, 2005]. The modular structure of REST and the REST mutant DP-REST:ER is depicted in Figure 5A. DP-REST:ER contains the DNA-binding domain of REST, and therefore binds to the identical site on DNA. The N- and C-terminal repression domains of REST are deleted. Instead, the activation domain of the herpes simplex virus protein VP16 was fused to the DNA-binding domain of REST along with the hormone-binding domain of the ER and an immunological tag used for detection of the protein. The hormone-binding domain of the ER confers regulation by 4OHT as described [Hohl and Thiel, 2005; Ekici et al., 2008ab]. DP-REST:ER is expressed in an inactive state in the absence of 4OHT, but can be activated by the addition of 4OHT. We infected human U87MG glioma cells and—as a control—human HepG2 hepatoma cells with recombinant retroviruses encoding DP-REST:ER. As an additional control, cells were infected with recombinant retroviruses encoding the selection marker puromycin acetyltransferase (U87MGpac and HepG2pac cells). The expression of the transgene that occured under control of the murine stem cell virus long terminal repeat was verified in Western blot experiments using antibodies specific for the FLAG epitope (Fig. 5B). The DP-REST:ER fusion protein could be immunologically detected in both infected human U87MG glioma and HepG2 hepatoma cells, but not in cells expressing puromycin acetyltransferase (pac).

CHIP EXPERIMENTS REVEAL CELL TYPE-SPECIFIC BINDING OF DP-REST:ER TO THE GluR2 GENE

ChIP experiments were performed to test the binding of DP-REST:ER to the GluR2 gene. Cross-linked and sheared chromatin prepared from unstimulated U87MG-DP-REST:ER and HepG2-DP-REST:ER cells and cells that had been stimulated with 4OHT was immunoprecipitated with M2-agarose that interacted with the FLAG tag of the fusion proteins. Figure 5C shows that in U87MG-DP-REST:ER cells, the GluR2 gene was accessible for the DP-REST:ER mutant in 4OHT-stimulated cells. We did not detect binding of DP-REST:ER to the GluR2 gene in non-stimulated U87MG glioma cells, suggesting that the ER ligand-binding domain not only masks the VP16 activation domain within the DP-REST:ER molecule, but furthermore blocked the DNA binding of the fusion protein. DP-REST:ER did not bind to the GluR2 gene in HepG2 cells engineered to express this mutant of REST (Fig. 5C). Thus, DP-REST:ER binds to the GluR2 gene in U87MG cells, where the GluR2 gene is mainly embedded into an open chromatin configuration. DP-REST:ER fails to bind to the GluR2 gene in HepG2 cells, where the GluR2 gene is embedded into a closed chromatin structure.

ACTIVATION OF A DOMINANT-POSITIVE MUTANT OF REST ENHANCES GluR2 PROMOTER ACTIVITY AND GluR2 EXPRESSION IN GLIOMA BUT NOT IN HEPATOMA CELLS

To test the biological activity of DP-REST:ER expressed in U87MG glioma cells, the activity of the GluR2 promoter was analyzed. U87MG glioma cells expressing DP-REST:ER were infected with lentiviruses encoding either a GluR2 promoter/luciferase reporter gene (GluR2luc), or, as a control, an ubiquitin C promoter/luciferase reporter gene (Ubcluc). The cells were subsequently stimulated with 4OHT and luciferase activities were measured 24 h later. Figure 5D shows that treatment of U87MG cells expressing DP-REST:ER increased the transcriptional activity of the GluR2 promoter/luciferase reporter gene on the order of 2.8-fold. In contrast, no stimulation of reporter gene transcription was observed in U87MG cells that expressed the luciferase gene under the control of the ubiquitin C promoter. These results show that the DP-REST:ER mutant is biologically active in U87MG cells.

Next, we assessed whether DP-REST:ER is able to enhance expression of GluR2 in U87MG and HepG2 cells. U87MG and HepG2 cells expressing DP-REST:ER were treated with vehicle (−) or 4OHT (+) for 24 h. Cytoplasmic RNA was prepared, and analyzed by RT-PCR. Figure 5E shows that expression of GluR2 was upregulated in 4OHT-treated U87MG cells expressing DP-REST:ER. This experiment underlines the importance of REST as a key regulator of GluR2 expression in human glioma cells. In contrast, no stimulation of GluR2 expression was observed in 4OHT-treated HepG2 cells that expressed DP-REST:ER (Fig. 5F). Thus, DP-REST:ER bound under physiological conditions to the GluR2 gene in glioma cells and transactivates the GluR2 gene following 4OHT treatment. DP-REST:ER was unable to bind to the regulatory region of the GluR2 gene in HepG2 cells and therefore failed to transactivate the GluR2 gene.

OVEREXPRESSION OF REST REDUCED GluR2 EXPRESSION IN U87MG CELLS BUT RETAINED THE TSA INDUCIBILITY

The previous experiment showed that REST is an important regulator of GluR2 expression in human glioma cells. These results were corroborated in U87MG cells expressing FLAG-tagged REST. The expression of the transgene was verified in Western blot experiments using antibodies that detected the FLAG epitope (Fig. 6A). ChIP experiments showed that FLAG-tagged REST bound to the GluR2 gene in U87MG glioma cells under physiological conditions (Fig. 6B). Expression analysis by RT-PCR revealed that GluR2 expression was reduced in U87MG cells overexpressing REST (Fig. 6C). However, the ability of TSA to induce GluR2 transcription was still retained in U87MG cells overexpressing REST.

EXPRESSION OF A REST-SPECIFIC SMALL-HAIRPIN RNA STIMULATED GluR2 PROMOTER ACTIVITY AND GluR2 EXPRESSION IN U87MG CELLS

The previous experiments showed that overexpression of REST reduced GluR2 expression in glioma cells. To assess whether reduced levels of REST enhance GluR2 expression, we knocked down REST expression by infecting glioma cells with a lentivirus encoding a shRNA against REST. As a control, cells were infected with a recombinant lentivirus that has been generated using plasmid pLL3.7 as lentiviral transfer vector. To validate the biological function of the shRNA, we infected U87MG cells that expressed FLAG-tagged REST with lentiviruses encoding an shRNA directed

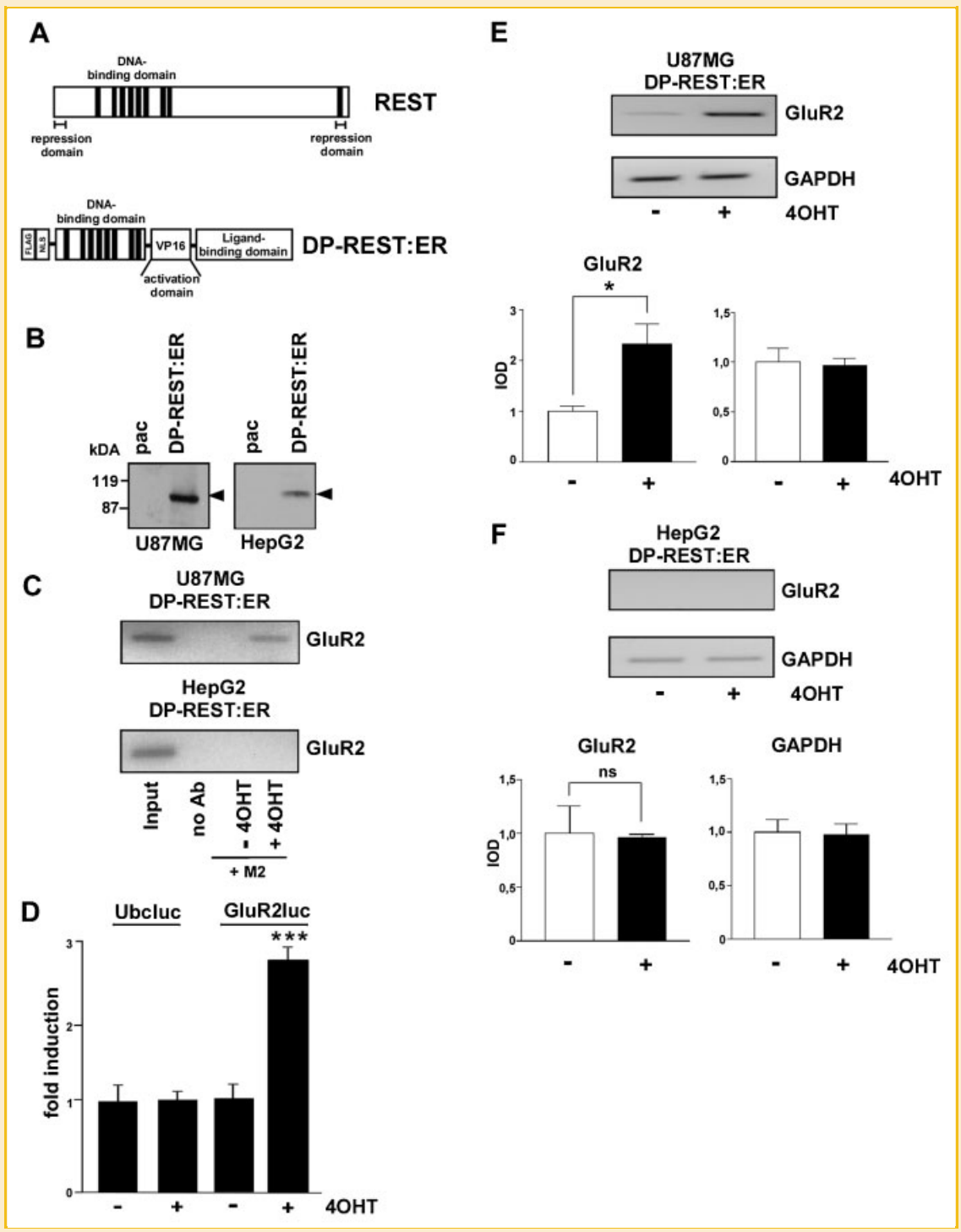

Fig. 5.

against REST. Cell extracts were prepared and analyzed for FLAG-REST immunoreactivity. Figure 6D shows that expression of REST was significantly reduced in cells infected with the shREST-encoding lentivirus, indicating that expression of this shRNA induced a downregulation of REST expression. Furthermore, significantly reduced REST mRNA levels were detected in U87MG glioma cells, expressing the REST-specific shRNA (Fig. 6E). Expression of shRNAs specific for the transcription factors p53 or Pdx-1 did not change the REST expression level (data not shown). We used U87MG cells expressing the REST-specific shRNA to further assess the role of REST in controlling GluR2 expression. Figure 6F shows that GluR2 promoter activity was increased threefold in cells expressing the REST-specific shRNA. Likewise, GluR2 expression was significantly upregulated as a result of downregulation of REST expression (Fig. 6G). These data, together with the previous data, show that the GluR2 gene is a *bona fide* target of REST in human glioma cells.

Sp1 REGULATES GluR2 EXPRESSION IN U87MG CELLS

The proximal promoter of the human GluR2 gene contains a GC-rich consensus site for the zinc finger transcription factor Sp1. The location of this binding site is depicted in Figure 1A. ChIP experiments performed with antibodies directed against Sp1 revealed that Sp1 bound under physiological conditions to the GluR2 gene promoter (Fig. 7A). To analyze the functional impact of Sp1 binding to the GluR2 gene, we expressed a dominant-negative mutant of Sp1 termed Sp1ΔN that lacked the N-terminal transcriptional activation domain (Fig. 7B). Sp1ΔN additionally contains a FLAG epitope for immunological detection and a nuclear localization signal (NLS). Nuclear proteins of mock-infected U87MG cells or cells infected with a Sp1ΔN encoding lentivirus were fractionated by SDS–PAGE. Expression of Sp1ΔN was identified by Western blot analysis using antibodies targeting the FLAG epitope (Fig. 7C). To verify the biological activity of Sp1ΔN, we implanted a luciferase reporter gene under the control of the HIV LTR into the chromatin of U87MG cells using lentiviral gene transfer. The LTR contains three binding sites for Sp1 and has been shown to be regulated by Sp1 [Al-Sarraj et al., 2005]. A schematic representation of the integrated provirus encoding the HIV LTR/luciferase reporter gene is depicted in Figure 7D. Expression of Sp1ΔN significantly reduced reporter gene transcription in infected U87MG cells (Fig. 7E). Next, we analyzed expression of GluR2 in U87MG cells expressing Sp1ΔN. Figure 7F shows that Sp1ΔN reduced expression of GluR2 in U87MG cells. These data, together with the ChIP analysis, reveal that the GluR2 gene is a *bona fide* target gene of Sp1 in U87MG cells.

INHIBITION OF Sp1 ACTIVITY DOES NOT INTERFERE WITH THE UPREGULATION OF GluR2 EXPRESSION IN TSA-TREATED U87MG CELLS

A transcriptional cross-talk between REST and Sp1 has been proposed [Plaisance et al., 2005]. We therefore tested TSA-regulated GluR2 expression in U87MG and U87MG FLAG-REST cells in the absence or presence of Sp1ΔN. As a control, we show that expression of Sp1ΔN reduced GluR2 promoter/luciferase reporter gene activity to a similar extent in U87MG cells and in U87MG cells overexpressing REST (Fig. 8A). GluR2 expression was upregulated by TSA in the presence or absence of Sp1ΔN expression (Fig. 8B), suggesting that TSA-mediated de-repression in U87MG glioma cells does not rely on the level of biologically active Sp1.

THE TRANSCRIPTIONAL ACTIVITIES OF REST AND Sp1 ARE INDEPENDENT FROM EACH OTHER IN U87MG GLIOMA CELLS

It has been suggested that exogenous introduction of REST blocks Sp1-mediated transcriptional activity [Plaisance et al., 2005]. We therefore analyzed the transcriptional activation potential of Sp1 in U87MG glioma cells. We expressed the N-terminal activation domain of Sp1 as fusion protein with the DNA-binding domain of GAL4 (Fig. S1A) in U87MG and U87MG FLAG-REST cells using lentiviral gene transfer. Since GAL4 does not bind to any known mammalian gene promoter element, interference by other transcriptional regulatory proteins was avoided. Expression of the GAL4-Sp1 fusion protein was verified by Western blot analysis using antibodies targeting the GAL4 DNA-binding domain (Fig. S1B). To measure the biological activity of the GAL4-Sp1 fusion protein we used a model promoter with engineered GAL4 binding sites (upstream activating sequence, UAS) upstream of a minimal promoter consisting of two Sp1-binding sites, a TATA box and an initiator element. We implanted this reporter gene into the chromatin of U87MG and U87MG FLAG-REST cells to ensure that the reporter gene is packed into an ordered nucleosomal structure. Figure S1C shows a schematic depiction of the integrated provirus. U87MG and U87MG FLAG-REST cells were infected with a lentivirus encoding the reporter gene together with a lentivirus that

Fig. 5. Cell type-specific upregulation of GluR2 expression in glioma and hepatoma cells following activation of a dominant-positive mutant of REST. A: Schematic representation of the modular structure of REST and the REST mutant DP-REST:ER. The functional domains for DNA-binding, transcriptional repression, and activation are indicated. The activation domain present in the REST mutant DP-REST:ER is derived from the herpes simplex virus protein VP16. DP-REST:ER also contains the ligand-binding domain of the murine estrogen receptor (ER). B: Expression of DP-REST:ER in infected U87MG and HepG2 cells. Nuclear extracts of DP-REST:ER expressing cells cultured in the presence or absence of 4OHT were prepared and analyzed by immunoblotting for FLAG immunoreactivity. As a control, cells expressing the selection marker puromycin acetyltransferase (pac) were analyzed. Molecular-mass markers in kDa are shown on the left. C: ChIP experiments reveal cell type-specific binding of DP-REST:ER to the GluR2 gene. ChIP was performed with M2-agarose as described in the Materials and Methods section, to selectively precipitate the FLAG-tagged DP-REST:ER mutant. Immunoprecipitated chromatin fragments were amplified with primers encompassing the REST-binding site of GluR2 gene. As a negative control, ChIP was performed with protein A-sepharose, without adding M2-agarose (*no Ab*). As positive control an aliquot of the total chromatin in the absence of immunoprecipitation was analyzed by PCR (*input*). D: U87MG-DP-REST:ER cells were infected with a recombinant lentivirus generated with the lentiviral transfer vector pFWGluR2luc. The infected cells were stimulated with 4OHT as indicated. Cell extracts were prepared and analyzed for luciferase activities which were normalized to the protein concentrations ($n = 4$, $^{***}P < 0.001$). E,F: U87MG (E) and HepG2 cells (F) expressing DP-REST:ER were incubated for 24 h with 4OHT or ethanol (vehicle). Total RNA from ethanol-treated (denoted "−") and 4OHT-treated (denoted "+") cells was isolated and analyzed by RT-PCR. The expression of GAPDH served as a negative control. The ethidium bromide stained gels (upper panels) and the quantification and statistical analysis of these results (lower panels) are depicted ($^{*}P < 0.05$; n.s., not significant).

encoded the GAL4-Sp1 fusion protein. As a control, cells were infected with a lentivirus produced with the lentiviral transfer vector pFUW (mock). The results, depicted in Figure S1D, reveal that the transcriptional activation potential of Sp1 is similar in U87MG and U87MG FLAG-REST cells, indicating that overexpression of REST does not interfere with the biological function of Sp1 to activate gene transcription.

Next, we analyzed the transcription repression potential of REST. We expressed a GAL4-REST fusion protein in U87MG glioma cells that contained the C-terminal transcriptional repression domain of

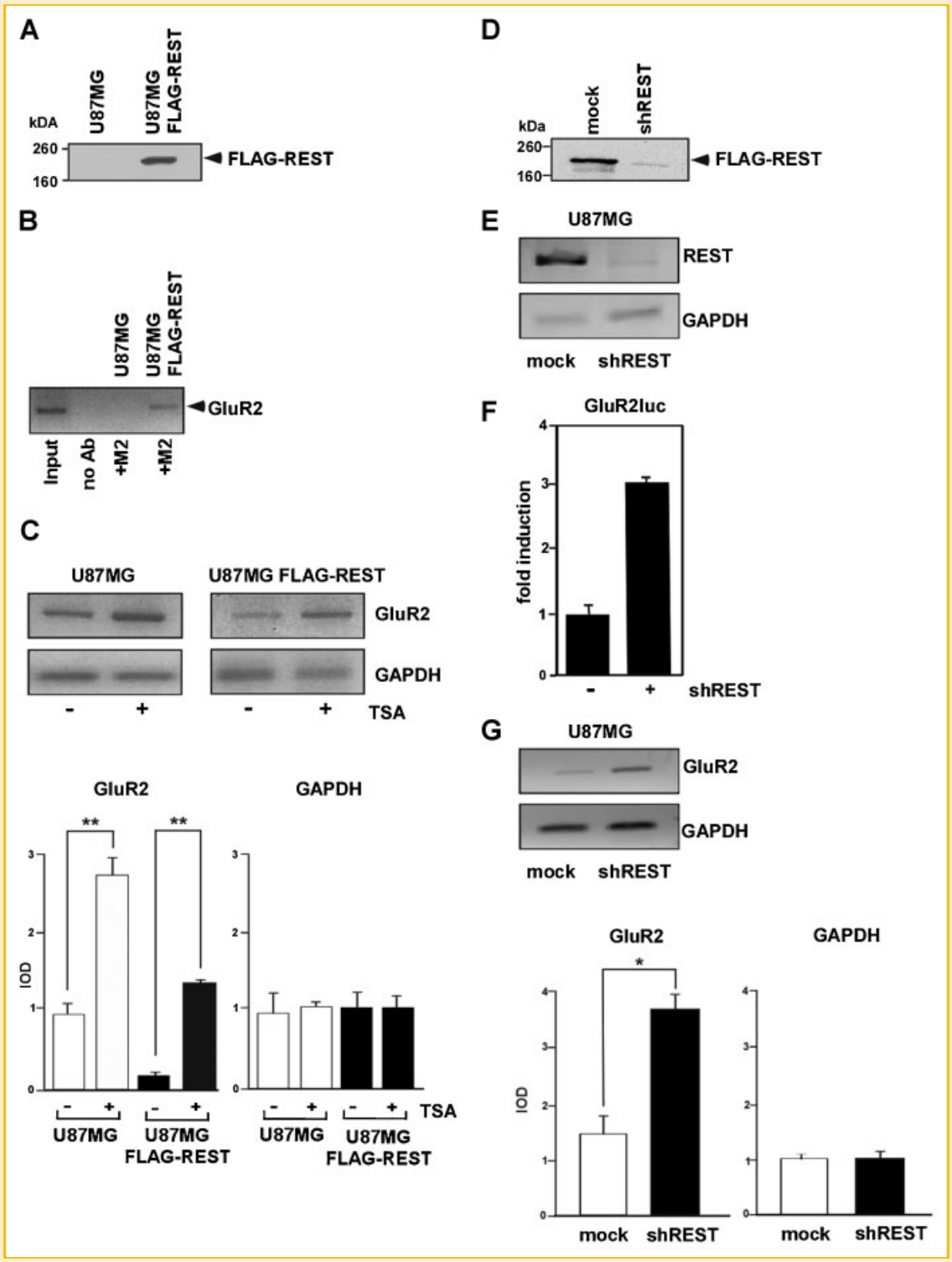

Fig. 6.

REST fused to the GAL4 DNA-binding domain (Fig. S2A). As a control, we expressed the repression domain of the zinc finger domain of NK10 (Thiel et al., 2001) as GAL4 fusion protein. The expression of the fusion proteins was verified by Western blot analysis in infected U87MG cells using antibodies specific for the DNA-binding domain of GAL4 (Fig. S2B). The apparent molecular weights of the fusion proteins were as predicted from their sequences. To measure transcriptional repression, we inserted a transcription unit into the chromatin of U87MG cells consisting of five copies of the GAL4-binding site (UAS), the SV40 promoter, and the luciferase reporter gene. A schematic depiction of the integrated provirus is depicted in Figure S2C. Next, we infected U87MG cells with a lentivirus encoding the $UAS^5SV40luc$ reporter gene together with a lentivirus that encoded either GAL4-REST or GAL4-NK10. As a control, cells were infected with either a lentivirus produced with the lentiviral transfer vector pFUW (mock) or with a lentivirus encoding the dominant-negative mutant of Sp1, Sp1ΔN, as indicated. Figure S2D shows that GAL4-REST and GAL4-NK10 repressed expression of the luciferase reporter gene independently of Sp1ΔN expression, indicating that the repression activity of REST does not rely on biologically active Sp1 in the glioma cell.

DISCUSSION

The transcription factor REST regulates many neuronal genes and is important for the establishment of the neuronal phenotype [Bruce et al., 2006; Thiel and Hohl, 2006]. In addition, recent data suggest a role of REST in tumor suppression or tumor promotion. An RNAi-based genetic screen revealed a role of REST as tumor suppressor in human mammary epithelial cells. Moreover, reduced REST expression in colorectal cancer cells induced a transformation phenotype, including an anchorage-independent growth, involving an upregulation of the PI3 kinase signaling pathway [Westbrook et al., 2005]. Likewise, in human non-small cell lung carcinoma cells, a derepression of REST target gene transcription was correlated with enhanced tumorigenicity [Watanabe et al., 2006]. In contrast, an oncogenic role of REST has been proposed for medulloblastoma [Majumder, 2006], suggesting that the cellular origin of the tumor cells is crucial for REST to function either as tumor suppresor or as a tumor promoter.

So far, there are no data available concerning the role of REST in glioma tumor development. However, REST may control the expression of GluR2, a critical subunit of AMPA ionotropic receptors, leading to reduced expression of GluR2 in glioma cells as observed by others [Savaskan et al., 2011]. In fact, a functional binding site for REST in the proximal promoter region of the rat GluR2 gene has been described [Myers et al., 1998; Huang et al., 1999]. However, a recent analysis of REST target gene expression in neuronal and neuroendocrine cells revealed that the GluR2 gene was not regulated by REST in these cells [Hohl and Thiel, 2005]. ChIP analysis showed that the lack of GluR2 expression was due to a repressive chromatin environment. The objective of this study was to analyze the chromatin structure and regulation of the GluR2 gene in glioma cells.

RT-PCR analysis showed that GluR2 is expressed at low levels in U87MG glioma cells and could be upregulated by treating the cells with TSA. In line with this, inhibition of HDACs increased GluR2 promoter activity and the level of histone acetylation of the GluR2 locus in C6 and U87MG glioma cells [Huang et al., 1999; this study]. In contrast, no incremental increase in histone acetylation was monitored in cortical neurons or hepatoma cells [Huang et al., 1999; this study], indicating that TSA treatment increased GluR2 promoter activity in a cell type-specific manner. The data obtained by ChIP experiments with antibodies directed against the methylated lysine residues 4 or 9 revealed that in glioma cells the GluR2 is mainly embedded in nucleosomes that carry a marker for actively transcribed genes, methylated H3K4. In human neuroblastoma cells, the GluR2 gene also carried markers of both open and repressed chromatin. In contrast, in keratinocytes and hepatoma cells the GluR2 gene is embedded into a chromatin environment characterized by methylation of lysine residue 9 of histone 3, a marker of repressed genes. Likewise, the GFAP gene contains an open chromatin structure in glioma cells, but a compact chromatin architecture in keratinocytes and hepatoma cells. The analysis of these epigenetic marks clearly shows major differences between different cell types, supporting the hypothesis that chromatin environment is of major importance for REST target gene expression. In contrast, REST bound to the GluR2 gene in glioma, neuroblastoma, hepatoma cells, and keratinocytes, providing no discrimination between REST-responsive genes in different cell types. We like to emphasize that ChIP experiments show protein-

Fig. 6. Expression of REST and expression of a REST-specific shRNA modulate GluR2 mRNA levels in glioma cells. A: Nuclear extracts of FLAG-tagged REST expressing U87MG cells were prepared and analyzed by immunoblotting for FLAG immunoreactivity. As a control, wild-type U87MG cells were analyzed. Molecular-mass markers in kDa are shown on the left. B: ChIP experiments reveal binding of FLAG-tagged REST to the GluR2 gene. ChIP was performed with M2-agarose to selectively precipitate the FLAG-tagged REST. Immunoprecipitated chromatin fragments were amplified with primers encompassing the REST-binding site of GluR2 gene. As a negative control, ChIP was performed without adding M2-agarose (*no Ab*). As positive control an aliquot of the total chromatin in the absence of immunoprecipitation was analyzed by PCR (*input*). C: Total RNA from U87MG cells expressing FLAG-tagged REST was isolated and analyzed by RT-PCR. The expression of GAPDH served as a negative control. The ethidium bromide stained gels (upper panel) and the quantification and statistical analysis of these results (lower panel) are depicted ($^{**}P < 0.01$). D: U87MG FLAG-REST cells were either mock-infected or infected with lentiviruses that encoded for a REST-specific shRNA. In addition, cells were infected with a recombinant lentivirus that has been generated using plasmid pLL3.7 as lentiviral transfer vector (mock). Nuclear cell extracts were prepared and analyzed for the expression of FLAG-REST, using antibodies directed against the FLAG tag. E: U87MG glioma cells were either mock-infected or infected with lentiviruses that encoded for a REST-specific shRNA. In addition, cells were infected with a recombinant lentivirus that has been generated using plasmid pLL3.7 as lentiviral transfer vector (mock). RNA was isolated and analyzed by RT-PCR using REST-specific primers. The expression of GAPDH served as a control. F: U87MG-glioma cells were infected with a recombinant lentivirus encoding a GluR2 promoter/luciferase reporter gene. In addition, cells were mock infected or infected with a lentivirus that encoded a REST-specific shRNA. Two days later, cell extracts were prepared and analyzed for luciferase activities which were normalized to the protein concentrations. G: U87MG glioma cells were either mock-infected or infected with lentivirus that encoded for a REST-specific shRNA. RNA was isolated 2 days later and analyzed by RT-PCR. The expression of GAPDH served as a control. The ethidium bromide stained gels and the quantification and statistical analysis of these results are depicted. IOD, integrated optical density ($^{*}P < 0.05$).

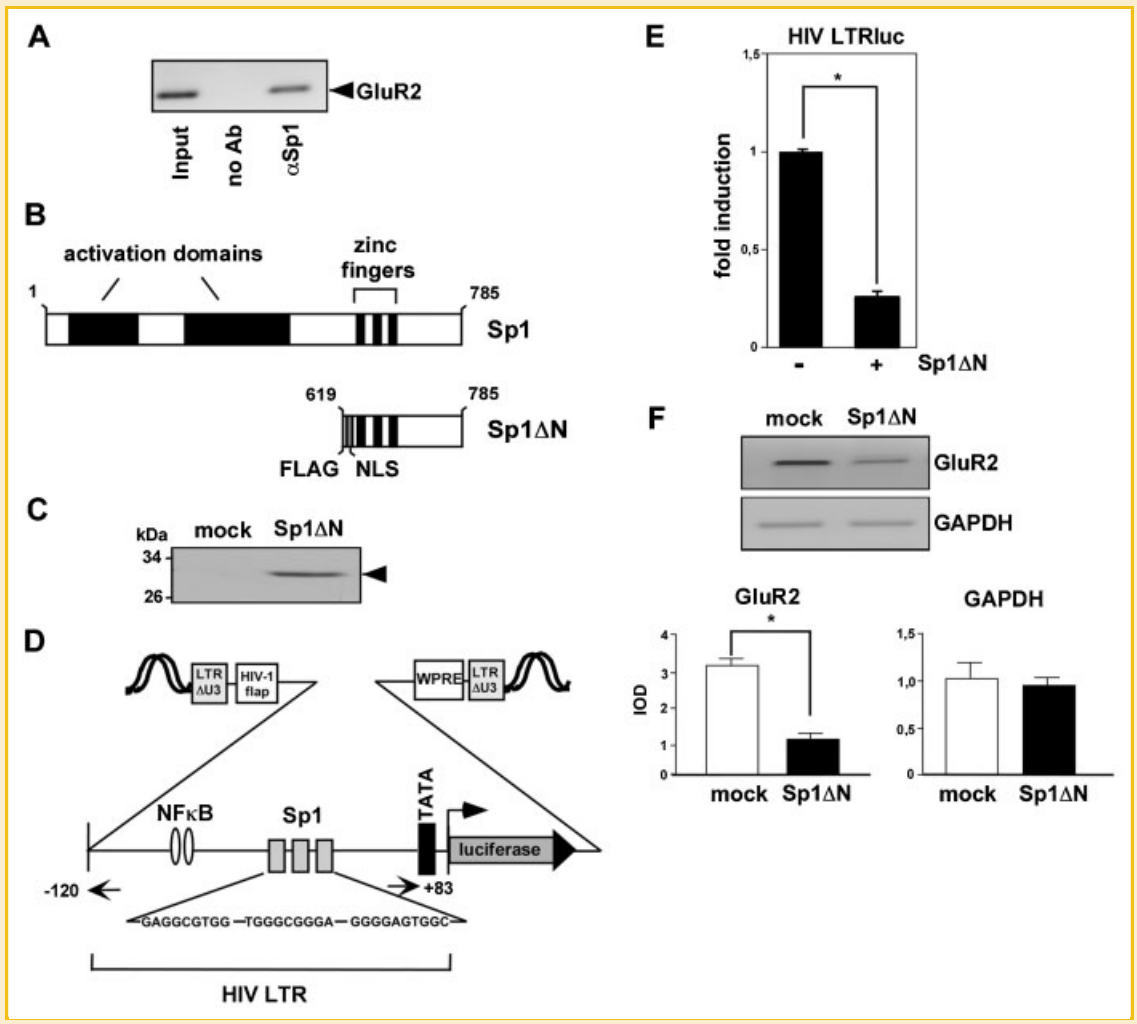

Fig. 7. The GluR2 gene is a *bona fide* target gene of Sp1 in glioma cells. A: ChIP experiments were performed with anti-Sp1 antibodies. Immunoprecipitated chromatin fragments were amplified with primers encompassing the proximal promoter region of the human GluR2 gene. As a negative control, ChIP was performed with protein A-sepharose, without addition of antibodies (*no Ab*). As a positive control an aliquot of the total chromatin in the absence of immunoprecipitation was analyzed by PCR (*input*). B: Schematic representation of the modular structure of Sp1 and Sp1ΔN. The N-terminal activation domains of Sp1 are depicted. The Sp1 mutant Sp1ΔN includes the DNA-binding domain, but lacks the Q-rich activation domains. Sp1ΔN also contains a triple FLAG tag and a nuclear localization signal (NLS). C: Western blot analysis of U87MG cells either mock infected or infected with a recombinant lentivirus encoding Sp1ΔN. The Western blot was probed with an antibody against the FLAG tag. D: Schematic representation of the integrated provirus encoding a luciferase reporter gene under the control of the proximal LTR derived from HIV (HIV LTRluc). The location of the NF-κB and Sp1 binding sites are depicted. E: U87MG glioma cells were double-infected with a lentivirus encoding a HIV LTR/luciferase reporter gene and a lentivirus encoding Sp1ΔN. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration ($^*P < 0.05$). F: U87MG glioma cells were infected with a lentivirus encoding Sp1ΔN. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). RNA was isolated and analyzed by RT-PCR. The expression of GAPDH served as a control. The ethidium bromide stained gels (upper panel) and the quantification and statistical analysis of these results (lower panel) are depicted. IOD, integrated optical density ($^*P < 0.05$).

DNA binding and do not give information about the biological activity of a transcription factor. Therefore, we designed a REST activity assay and showed that REST is active in glioma, neuroblastoma, hepatoma cells, and keratinocytes. We conclude that the analysis of epigenetic marks such as of histone methylation and acetylation correlates with GluR2 gene expression and discriminates between different cell types concerning GluR2 expression and accessibility of the GluR2 locus.

To directly investigate the impact of REST on GluR2 gene transcription in U87MG cells, we expressed the mutant of REST, DP-REST:ER. These experiments enabled us to test whether expression of a transcriptional activator, that binds to NRSE of the GluR2 gene in its natural chromosomal context, is sufficient to induce REST-target gene transcription. ChIP experiments confirmed that DP-REST:ER interacted under physiological conditions with the GluR2 gene in U87MG cells following 4OHT stimulation, indicating that the GluR2 promoter was accessible for the REST mutant in glioma cells. In contrast, DP-REST:ER did not bind to the GluR2 gene in hepatoma cells. Thus, the REST mutant could only interact with the GluR2 gene in those cells where the GluR2 gene is embedded into an open

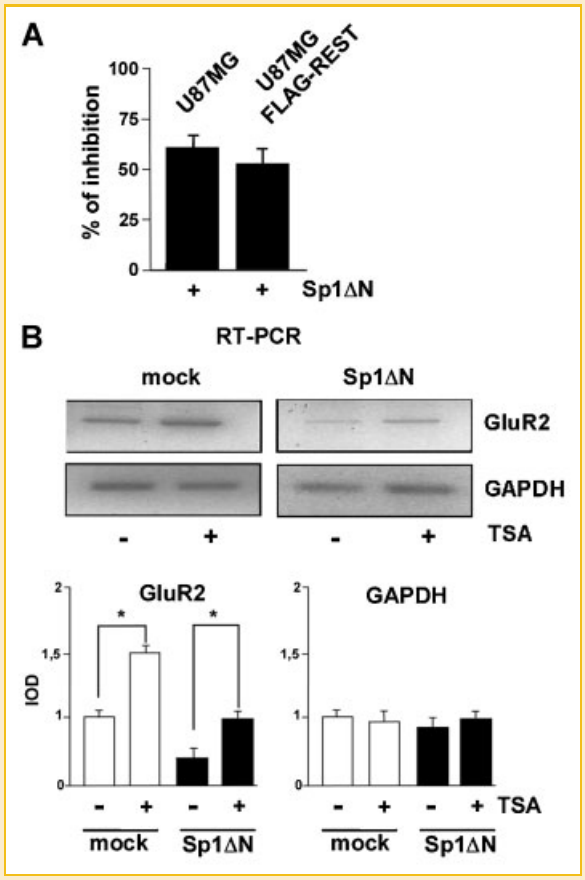

Fig. 8. Role of Sp1 in TSA-induced upregulation of GluR2 expression in glioma cells. A: U87MG glioma cells and U87MG glioma cells expressing FLAG-tagged REST were double-infected with lentiviruses encoding a GluR2 promoter/luciferase reporter gene and a lentivirus encoding Sp1ΔN. As a control, cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data are shown as the % of inhibition of GluR2 promoter/reporter gene activity in Sp1ΔN expressing cells relative to the activity of the reporter gene in mock-infected cells. B: U87MG glioma cells were infected with a lentivirus encoding Sp1ΔN. As a control cells were infected with a lentiviral stock prepared with the lentiviral transfer vector pFUW (mock). Cells were treated for 24 h with TSA (100 ng/ml) or with DMSO. RNA from DMSO-treated (denoted "−") and TSA-treated (denoted "+") cells was isolated and analyzed by RT-PCR. The expression of GAPDH served as a control. The ethidium bromide stained gels (upper panel) and the quantification and statistical analysis of these results (lower panel) are depicted. IOD, integrated optical density ($^{*}P < 0.05$).

chromatin structure. In fact, GluR2 promoter activity and GluR2 expression was upregulated in U87MG cells following activation of DP-REST:ER by 4OHT. Together, these data indicate that the GluR2 gene is a genuine target for REST in human U87MG glioma cells. In support of this conclusion, we have shown here that FLAG-tagged REST bound under physiological conditions to the GluR2 gene and triggered a downregulation of GluR2 expression. Likewise, depletion of REST using a lentivirally encoded shRNA increased GluR2 promoter activity and GluR2 expression in U87MG glioma cells.

Activation of DP-REST:ER failed to stimulate GluR2 transcription in HepG2 cells. ChIP experiments showed that DP-REST:ER is unable to bind under physiological conditions to the NRSE of the GluR2 gene in HepG2 cells. This observation explains why DP-REST:ER is unable to transactivate the GluR2 gene in this cell type. Collectively, these data shed light on the fact that the concentration of REST is not the only determinant that controls REST target gene expression. Rather, cell type-specific modifications of the chromatin structure are critical for DP-REST:ER to gain access to the REST-binding sites in neuronal genes. Based on recent results [Hohl and Thiel, 2005; Ekici et al., 2008a] and the data presented here, we assume that REST targets are silenced in differentiated cell types via histone acetylation/deacetylation-independent mechanisms that do not permit access of DP-REST:ER to REST target genes. Thus, expression of an activating REST mutant is not sufficient to induce a de-repression of REST target genes and to induce transdifferentiation of non-neuronal cells to neurons. REST has been characterized as a dual-specific repressor [Thiel et al., 2004], that induces transcriptional repression via recruiting of HDACs and promotes gene silencing by recruitung MeCP2, HP-1, G9a histone methyltransferase, and C-terminal binding proteins CtBP1 and CtBP2 [Ballas and Mandel, 2005; Ooi and Wood, 2007]. Hence, not only the cellular concentration of REST is a key factor in determining whether REST target genes are transcribed but also the configuration of the chromatin. The GluR2 gene is thus only a REST-regulated gene in those cells where the chromatin configuration of the GluR2 locus is open. This assumption directs future investigation towards the analysis of cell type-specific variations of the chromatin structure. It has been suggested that transcriptional downregulation of AMPA receptor expression protects the cells against the high glutamate microenvironment of the tumor [van Vuurden et al., 2009]. In the light of the data presented here we suggest that expression of REST in glioma cells is, at least in part, responsible for the downregulation of GluR2 expression in glioma cells.

In addition to the NRSE, a binding site for Sp1 has been described in the GluR2 promoter of the rat, based on promoter deletion mutants and in vitro protein/DNA-binding assays [Myers et al., 1998]. The functional role of Sp1 in controlling GluR2 expression has not been defined. In the human GluR2 gene, a similar GC rich sequence was detected that may function as a binding site for Sp1. ChIP experiments verified that Sp1 bound under physiological conditions to the human GluR2 gene. We went a step further and showed that expression of a dominant-negative mutant of Sp1 reduced GluR2 expression, indicating that the GluR2 gene is a *bona fide* target gene for Sp1 in U87MG glioma cells.

Recently, cross-talk between Sp1 and REST has been proposed. It has been suggested that exogenous introduction of REST blocked Sp1-mediated transcriptional activity. Furthermore, inhibition of Sp1 was required to silence REST target genes outside neuronal or β cells [Plaisance et al., 2005]. The GluR2 gene is controlled by both REST and Sp1 and thus represents a model to investigate whether a connection exists between these transcription factors. Using U87MG cells that overexpress REST we showed that the biological activity of Sp1 is not changed as a result of elevated concentrations of REST, indicating that overexpression of REST does not block Sp1-mediated transcriptional activity. Likewise, we show that transcriptional repression of REST occurs either in the presence or absence of a dominant-negative mutant of Sp1, indicating that the level of

biologically active Sp1 plays no role in the control of the transcription repression potential of REST. Moreover, we show that the upregulation of GluR2 expression by TSA is independent of Sp1, indicating that transcriptional repression by REST and TSA-mediated de-repression does not rely on the level of biological active Sp1 in U87MG glioma cells. We conclude that there is no cross-talk between Sp1 and REST in U87MG cells and that REST represses GluR2 gene transcription in an activator-unspecific manner as described earlier [Lietz et al., 2001].

In summary, we have shown that the GluR2 gene is embedded into an open chromatin structure in U87MG glioma cells, with nucleosomes carrying di- and trimethylated H3K4. A similar epigenetic configuration was found for the GFAP gene. GluR2 gene expression is upregulated in U87MG glioma cells following treatment with TSA, expression of a mutant of REST (DP-REST:ER) that contains a transcriptional activation domain, or shRNA-mediated downregulation of REST expression. A direct binding of the REST mutant to the GluR2 gene could be demonstrated. These data implicate that REST is a likely candidate to downregulate GluR2 expression in human glioma cells. In hepatoma cells and keratinocytes, the GluR2 gene is embedded into a repressed chromatin environment. Accordingly, GluR2 expression could not be induced in these cells following expression of an activating REST mutant. Thus, the compact chromatin structure prevents access of transcription factors to the GluR2 gene. In addition, we showed that Sp1 regulates GluR2 expression in U87MG glioma cells independently of REST.

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