Cellular/Molecular

Identification of a Smad4/YY1-Recognized and BMP2-Responsive Transcriptional Regulatory Module in the Promoter of Mouse GABA Transporter Subtype I (*Gat1*) Gene

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GABAergic dysfunction is implicated in a variety of neurodevelopmental and psychiatric disorders. The mechanisms underlying GABAergic differentiation, however, are not well understood. GABA transporter 1 (*Gat1*; *Slc6a1*) is an essential component of the GABAergic system, and its ectopic mRNA expression may be responsible for GABAergic malfunction under different pathological conditions. Thus, monitoring the transcriptional regulation of *gat1* may help to elucidate the mechanisms that govern the differentiation of GABAergic neurons. In this study, we identified a promoter region that is sufficient to recapitulate endogenous *gat1* expression in transgenic mice. A 46 bp *cis*-regulator in the promoter sequence was responsible for the stimulation of bone morphogenetic protein-2 (BMP2) on *gat1* expression in cortical cortex. Furthermore, our study demonstrated that Smad4 and YY1 are physically bound to the element and mediate both the negative and positive regulatory effects in which BMP2 can affect the balance. In summary, we have identified a Smad4/YY1-based bidirectional regulation model for GABAergic gene transcription and demonstrated a molecular cue important for the differentiation of GABAergic neurons.

Introduction

Aberrant development and dysfunction of GABAergic neurons are implicated in a variety of neurodevelopmental and psychiatric disorders, such as epilepsy (DeFelipe, 1999; Cossart et al., 2005), schizophrenia (Woo et al., 1998; Lewis et al., 1999, 2005; Volk and Lewis, 2002; Ruzicka et al., 2007), and anxiety disorders (Baraban, 2002; Heilig and Thorsell, 2002). Signaling molecules that regulate the acquisition and maintenance of GABAergic phenotype include bone morphogenetic proteins (BMPs) (Li et al., 1998; Mabie et al., 1999; Gulacsi and Lillien, 2003), Notch (de la Pompa et al., 1997; Kabos et al., 2002), and glial cell-derived neurotrophic factor (Pozas and Ibáñez, 2005), as well as basic helix-loop-helix (Casarosa et al., 1999; Bae et al., 2000; Fode et al., 2000; Miyoshi et al., 2004; Schuurmans et al., 2004; Nakatani et al., 2007) and homeodomain (Anderson et al., 1997; Sussel et al., 1999; Kroll and O'Leary, 2005) transcription factors. However, the molecular mechanisms regulating gene transcription resulting in GABAergic differentiation are still far from clear.

The GABAergic phenotype requires coordinated activation of glutamate decarboxylases (*Gad1-2*), the plasma membrane GABA transporters (*Gat1-4*), and the vesicular inhibitory amino acid transporter (*Vgat*). In *Caenorhabditis elegans*, transcription factor *unc-30* has been found to determine GABAergic phenotype by regulating the transcriptions of *unc-25/gad* and *unc-47/vgat* in a coordinated manner (Eastman et al., 1999; Westmoreland et al., 2001). It enlightens us to elucidate the mechanisms underlying the differentiation of GABAergic neurons by monitoring the transcriptional regulation of functional genes in GABAergic neurons.

GABA transporter 1 (*Gat1*; *Slc6a1*) is the major neural GABA transporter, and plays an important role in the termination of GABAergic transmission and the regulation of extracellular GABA concentration (Chiu et al., 2002). Studies of mice overexpressing, or deficient in, *gat1* suggest that *gat1* is associated with seizures (Ma et al., 2001; Zhao et al., 2003; Chiu et al., 2005) and emotional behaviors such as anxiety (Chiu et al., 2005; Liu et al., 2006). Alterations of *gat1* expression on transcription level play a key role in some GABAergic-related pathological circumstances such as epilepsy (Fueta et al., 2003; Sperk et al., 2003; Jiang et al., 2004), schizophrenia (Woo et al., 1998; Lewis et al., 1999; Volk et al., 2001; Volk and Lewis, 2002), and substance abuse (Peng and

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Simantov, 2003; Zink et al., 2004). Furthermore, polymorphism in the 5′-flanking region of *gat1* is highly associated with anxiety disorders (Thoeringer et al., 2009). A recent study demonstrated that 21 bp insertion polymorphism increases *gat1* promoter activity (Hirunsatit et al., 2009).

In this study, mouse *gat1* gene promoter was identified in transgenic mice and a 46 bp *cis*-regulatory element was found to regulate *gat1* transcription activity in the cerebral cortex. In addition, we found evidence suggesting that functional interaction between Smad4/YY1 and the 46 bp element mediates both the negative and positive regulatory effects in which morphogenetic protein 2 (BMP2) can affect the balance. To the best of our knowledge, this is the first report of bidirectional *in vivo* transcriptional regulation of *gat1*.

Materials and Methods

Rapid amplification of 5′ cDNA ends. Total RNA was isolated from mouse brain and subjected to 5′-race analysis (Invitrogen). The primer used for the first step of amplification was 5′-GCCTTCTTCTGCACCTTGACTACC-3′, located within exon 3. 5′-Race was performed by incubating with an aliquot of first-strand cDNA, a nested PCR primer positioned within exon 3 (5′-CAGGTGGGCGCGAGATGTC-3′), and an abridged anchor primer. The PCR conditions were: initial denaturation at 94°C for 5 min, 35 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C. The resulting products were cloned into pMD-18T vector (TaKaRa) and sequenced to determine the transcription initiation site(s).

Constructs and mutagenesis. A 5.7 kb fragment of mouse *gat1* promoter was generated by PCR with mouse genomic DNA as a template. The forward primer was from base 66540243 to 66540265 of the 5′-flanking region of *gat1* gene (GI:149255466) with an additional MluI site (italics) (5′-TTA*ACGCGT*GAGAGAGCACAACGCAGGAACAG-3′). The reverse primer was from base 66545546 to 66545570 of the downstream of the exon 1 with an additional XhoI site (underlined) (5′-TTA*CTCGAG*CGAACGAACTAGGACATAGACGGC-3′). The 5.7 kb MluI-XhoI fragment was cloned into pGL_3-basic (Promega). This construct is referred to as −5377/luc throughout this manuscript. The construct −3006/luc was assembled by deleting the KpnI-BamHI fragment from the −5377/luc construct. Constructs containing fragments of *gat1* gene promoter beginning at −2135, −2085, −1978, −1782, −1493, −1090, −891, −706, −333, −288, −241, −167, −93, or +109 from the transcription initiation site were prepared in a similar manner using forward primers with a MluI site at 5′ terminal (italics) 5′-TTA-*ACGCGT*TGCTTTGGTCACGGTGTCTCTTC-3′ (−2135/luc), 5′-TTA*ACGCGT*GAGGTCAAACAGATGCAAAG-3′ (−2085/luc), 5′-TTA*ACGCGT*CCTGGATTTGGTTCCCAGCAC-3′ (−1978/luc), 5′-TTA*ACGCGT*CTTCAGGCACAGCTGGATCAC-3′ (−1782/luc), 5′-TTA*ACGCGT*ATGAGACGTGGGGAGAAGACC-3′ (−1493/luc), 5′-TTA*ACGCGT*GTCTGGGCTCTCGAAAGGTTG-3′ (−1090/luc), 5′-TTA*ACGCGT*AGCCTAGATGCTTGTGGGAGG-3′ (−891/luc), 5′-TTA*ACGCGT*TGGGGAACATGGAAAAGGGAGAG-3′ (−706/luc), 5′-TTA*ACGCGT*GTGACAGAGCCAGAGAAACCAAG-3′ (−333/luc), 5′-TTA*ACGCGT*GAGGCCAGGAGACTGAAGGAG-3′ (−288/luc), 5′-TTA*ACGCGT*GGGAGCAGGGCTGGGAGAGAG-3′ (−241/luc), 5′-TTA*ACGCGT*GGCAAGGCGGGCAGGGCCTAG-3′ (−167/luc), 5′-TTA*ACGCGT*GAGGAGGCAGGCAGAGGGAGG-3′ (−93/luc), 5′-TTA*ACGCGT*CTAGAGAGCTGAGAGGTTGCAGG-3′ (+109/luc), respectively. The reverse primer was identical to that used to generate the construct −5377/luc. Restriction analysis and sequencing were used to verify the location of the promoter.

Deletion was introduced to construct −5377m/luc using a two-step PCR method with the −5377/luc DNA as the template. Two overlapping oligonucleotides were synthesized: −5377m/luc (forward) 5′-CTCCTGGCCTCATCTCTCTTGGAAGCATTGTGG-3′ and −5377m/luc (reverse) 5′-GAGAGATGAGGCCAGGAGACTGAAGGAG-3′. The first-step PCR was initiated using one of these primers and an appropriate primer which was used to generate the construct −5377/luc. The products of the first-step PCR were used as a template for the second-step PCR with a set of primers used to generate the construct −5377/luc. The resulting product was digested with MluI and XhoI and cloned into the vector pGL_3-basic. The resulting construct lacked fragment −333 to −288, and was verified with sequencing.

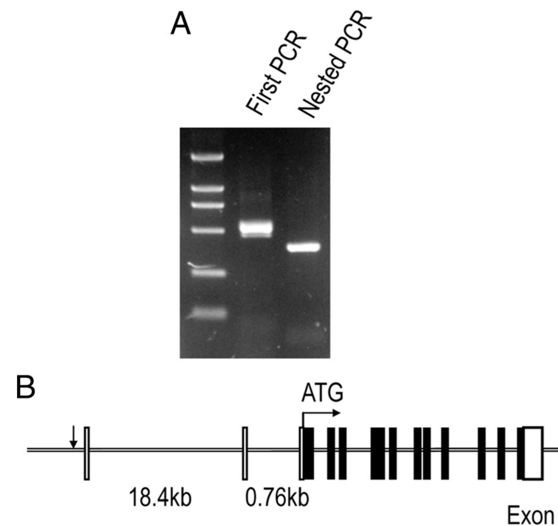

Figure 1. 5′-Race determination of transcription initiation site(s) in mouse *gat1* gene. ***A***, Gel analysis of the first PCR and nested PCR products from 5′-race with RNA derived from mouse brain. ***B***, Schematic representation of mouse *gat1* genomic structure. The transcription initiation site is indicated by arrow. Open boxes represent the noncoding region and black boxes represent the coding region. The start of translation occurs in exon 3, as indicated by ATG.

Table 1. Transgene expression in *gat1(5.7)lacz* transgenic mice

Transgenic mice tissue	*gat1(5.7)lacz* transgenic founder 88F ($n \geq 3$)	32F ($n \geq 3$)
Brain	100	100
Heart	0	11.4
Lung	2.9	5.4
Liver	5.6	4.4
Spleen	4.4	3.0
Kidney	0.3	5.1

LacZ mRNA expression in the brain of each line was artificially set at 100. Three replicates of each reaction were performed.

The sequence of the mouse *gat1* gene was analyzed for potential transcription factor binding sites with P-Match program (BioBase, Wolfenbüttel, Germany) using Transfac 6.0 Public database.

Short hairpin RNA (shRNA) oligonucleotides were designed to specifically target either *smad4* or *yy1*. Two different shRNA oligonucleotides of *smad4*: shRNA1 5′-CCAGCTACTTACCATCATA-3′ (Thuault et al., 2006) and shRNA2 5′-GCCATAGTGAAGGACTGTT-3′ (Rees et al., 2006). Two different shRNA oligonucleotides of *yy1*: shRNA1 5′-GAACTCACCTCCTGATTAT-3′ (Allouche et al., 2008) and shRNA2 5′-TGACAGGCAAGAAACTCCC-3′. Two nonspecific control shRNA oligonucleotides with a similar GC content as *smad4* shRNA and *yy1* shRNA were used. The inhibitory efficiency of each shRNA was determined by Western blot assay.

Generation of transgenic mice. The pSVβ-galactosidase was obtained from Promega. The 1690 bp HindIII-XbaI fragment was isolated from pGL_3-basic. The 3744 bp HindIII-XbaI fragment from pSVβ-galactosidase was inserted to pGL_3-basic at the HindIII site. The resulting fusion gene consisted of the *Escherichia coli* gpt gene fragment containing its translation initiation site, the *LacZ* gene encoding β-galactosidase from amino acid position 9 and the simian virus 40 (SV40) fragment with the polyadenylation signal. The 5.7 kb fragment, containing the 5′-flanking sequences, exon 1 and part of intron 1, was introduced to the plasmid carrying the *LacZ* gene at the XhoI and SmaI sites. The final fusion gene was referred to as *gat1(5.7)lacz*. *gat1(5.7m)lacz* was prepared in a

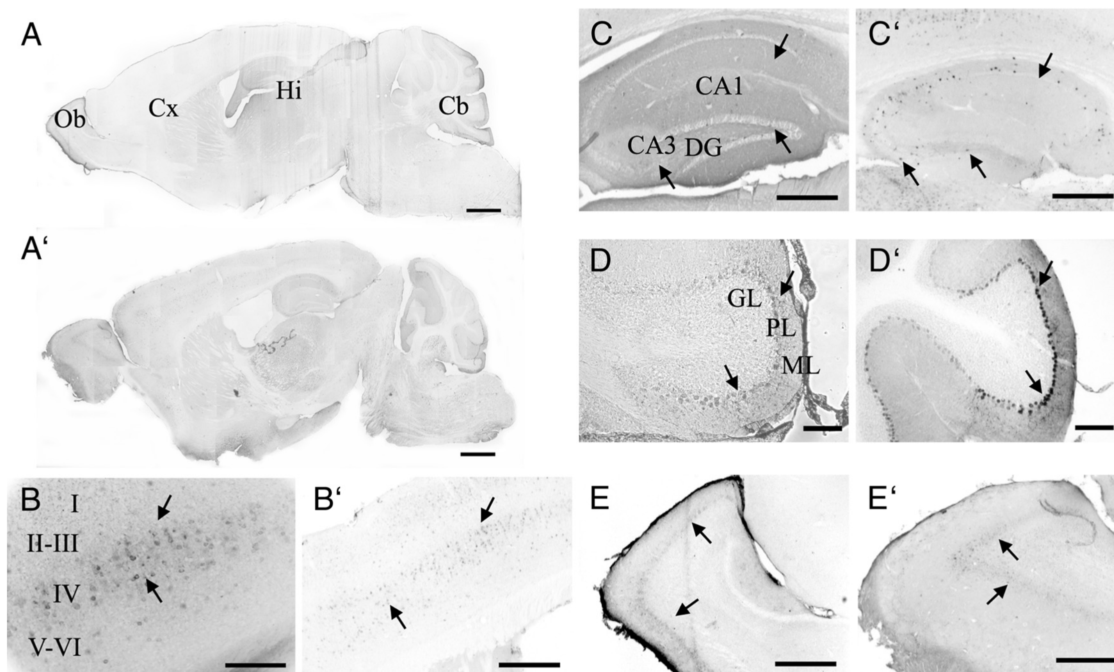

Figure 2. Histological detection of GAT1 and LacZ in the brain of adult *gat1(5.7)lacz* mice. ***A–E′***, Sagittal brain sections from *gat1(5.7)lacz* transgenic mice were stained by anti-GAT1 antibodies (***A***–***E***) or anti-LacZ antibodies (***A′***–***E′***). Shown are images of whole brain (***A***, ***A′***), cerebral cortex (Cx; ***B***, ***B′***), hippocampus (Hi; ***C***, ***C′***), cerebellum (Cb; ***D***, ***D′***), and olfactory bulb (Ob; ***E***, ***E′***). Abbreviations for this and subsequent figures: CA1, CA3, Fields of the hippocampus; DG, dentate gyrus; GL, ML, and PL are granule, molecular, and Purkinje cell layers, respectively. In all panels, arrows denote immunopositive cells. Scale bars: ***A***, ***A′***, 1 mm; ***D***, ***D′***, 100 μm; ***B***, ***B′***, ***C***, ***C′***, ***E***, ***E′***, 400 μm.

similar manner to construct −5377m/luc. All clones were verified by sequencing.

gat1(5.7)lacz and *gat1(5.7m)lacz* constructs were linearized with ScaI and subsequently purified with the Qiaex II Gel Extraction kit (Qiagen). The constructs were microinjected into fertilized eggs of C57BL/6J×DBA/2J hybrid mice. Founders were identified by PCR analysis of tail genomic DNA with primers that amplify a 470 bp region spanning the junction between the mouse *gat1* promoter and the *lacZ* cDNA. Primers for PCR were (forward) 5′-AGCCCCG-GCCGCAGGTAGGAA-3′ and (reverse) 5′-GCTGGCGAAAGGGG-GATGTGCT-3′.

Quantitative real-time PCR. Total RNA was extracted from mouse tissues (brain, heart, lung, liver, spleen, and kidney) using Trizol (Invitrogen). RNA samples were treated with RNase-free DNase I (TaKaRa) for 30 min at 37°C to eliminate DNA contamination. Reverse transcription was performed with M-MLV (Promega). Fluorescent signals were generated using SYBR Green PCR Master Mix (Applied Biosystems). PCRs for each gene of interest were run in triplicate on Rotor-Gene 3000 as follows: 10 min at 95°C and 40 cycles of 15 s at 94°C, 15 s at 66°C, and 30 s at 72°C. Primer sequences were: for *lacZ* (the target gene), (forward): 5′-TCAATCCGCCGTTTGTTCCCAC-3′, and (reverse): 5′-TCCA-GATAACTGCCGTACTCCAGC-3′; and for *gapdh* (the internal control), (forward): 5′-TGATGACATCAAGAAGGTGGTGAAG-3′, and (reverse): 5′-TCCTTGGAGGCCATGTGGGCCAT-3′. A melting curve analysis was performed at the end of the PCR cycle. Electrophoresis with 2% agarose gel was used to verify the amplification a single product. Experimental controls included non-reverse-transcribed RNA samples. Data were analyzed by Rotor-Gene software to determine the threshold cycle (CT) above the background for each reaction. Normalization was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The $\triangle$CT variability calculation revealed a slope value close to zero in a cDNA dilution over a 100-fold range in three independent experiments.

Tissue processing and immunocytochemistry. Heterozygous transgenic mice (2 months old) were deeply anesthetized and perfused with 4% paraformaldehyde in 0.1 M PB. Whole brains were removed, postfixed in the same fixative for 4 h, and cryoprotected in 20% glycerol/PB overnight at 4°C. Sagittal sections (30 μm) were cut and then stored in an ethylene glycol based cryoprotective solution at −20°C.

Immunohistochemical staining of free-floating sections was performed using an immunoperoxidase kit (VECTA ABC Kit, Vector Laboratories). The sections were incubated in 0.3% H_2O_2 in PBS for 30 min at room temperature to quench endogenous peroxidase. Sections were then incubated in 10% normal goat serum for 1 h at room temperature to block nonspecific binding. Sections were incubated with rabbit anti-GAT1 (1:100 dilution; Millipore Bioscience Research Reagents) or rabbit anti-LacZ (1: 2000 dilution; Abcam) in 1% normal goat serum overnight at 4°C. After rinsing, the sections were incubated for 30 min each with an appropriate biotinylated goat secondary antibody (ProteinTech Group) and an avidin–biotin complex solution. Staining was developed for 10 min in nickel-DAB solution (0.3%). No staining was observed in the control experiments, in which primary antibodies were omitted. Non-transgenic controls failed to be stained by anti-LacZ antibody under these conditions.

For immunofluorescence staining, free-floating sections were washed twice in PBS and then blocked with 10% normal goat serum in PBS. Double-labeling studies were performed using non-cross-reacting secondary antibodies after primary antibody incubation. The sections were examined under a Nikon Eclipse TE 2000-U fluorescence microscope with filters suitable for selectively detecting the fluorescence of FITC (green) and Cy3 (red) or under a light microscope. For colocalization, images from the same section but showing different antigen signals were overlaid.

The number of positively labeled cells and the intensity of the immunopositive signal were estimated by a blinded observer using Image-Pro Plus program (Media Cybernetics). For an unbiased determination, every 20th serial section at ∼1.2–4.2 mm from the midline were selected. For each brain, five sagittal sections were analyzed, and the average was used to calculate the group means (n = 5 brains).

Cell culture, transfections, and reporter gene assays. NIH 3T3 fibroblasts and Neuro 2a (mouse neuroblastoma) cell lines were grown in DMEM (Invitrogen) with 10% newborn calf serum supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen). The mouse embryo teratocarcinoma P19 cells were cultured in DMEM/F12 (Invitrogen) containing 10% fetal bovine serum.

Primary cortical neuron cultures were obtained from embryonic day 18 (E18) mouse embryos, dissociated with 0.125% trypsin (Invitrogen) for 15 min at 37°C, and then dissociated mechanically with a glass Pas-

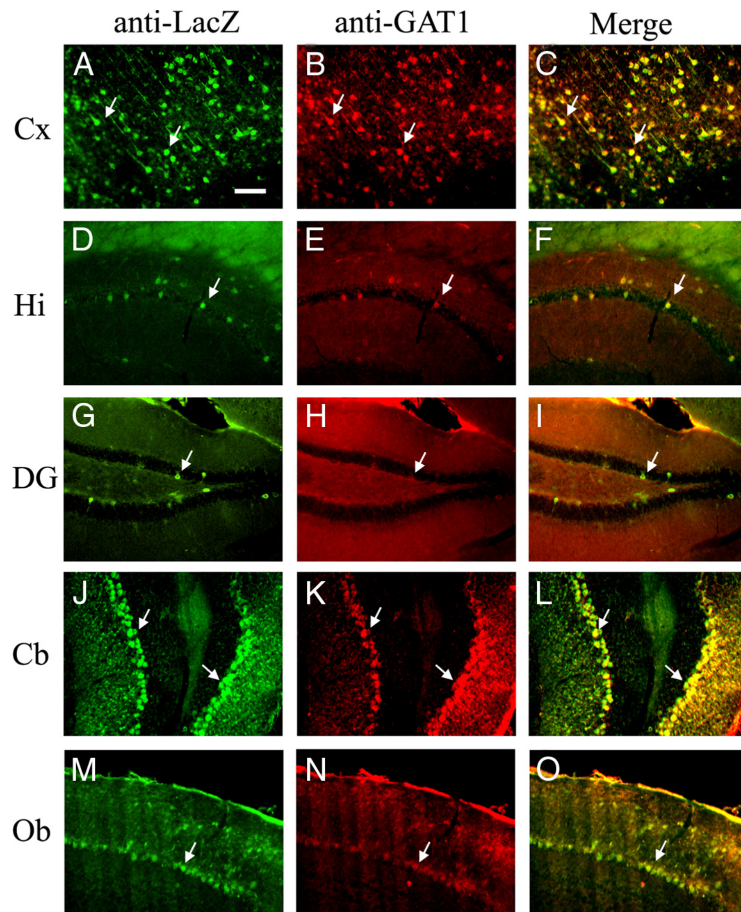

Figure 3. Colocalization of LacZ and GAT1 in the brain of adult *gat1(5.7)lacz* mice. ***A–O***, Sagittal brain sections from *gat1(5.7)lacz* transgenic mice were stained by anti-LacZ antibodies (green) and anti-GAT1 antibodies (red). Shown are images of cerebral cortex (Cx; ***A–C***), hippocampus (Hi; ***D–F***), dentate gyrus (DG; ***G–I***), cerebellum (Cb; ***J–L***), and olfactory bulb (Ob; ***M–O***). The third window in each row represents the merging of the red and green channels. Yellow staining indicates colocalization of LacZ and GAT1. ***A–O***, Arrows denote immunopositive cells. See legend of Figure 2 for abbreviations not used in the text. Scale bar, 100 μm.

teur pipette. Cells were plated in Neurobasal medium (Invitrogen) containing 0.5 mM L-glutamin (Invitrogen), and 2% B-27 supplement (Invitrogen) on poly-L-lysine-coated plates. On day 4 *in vitro*, one-quarter of the media was replaced with fresh media containing cytosine arabinoside (final concentration, 2 μM) to eliminate non-neuronal cells.

Primary neural stem cell (NSC) cultures were obtained from E14 mouse embryos. Dissected whole brain was transferred to ice-cold Hank's balanced salt solution (Invitrogen) and mechanically dissociated into a single-cell suspension with a fire-polished Pasteur pipette. Cells were seeded in noncoated T-25 culture flask in DMEM/F12 (Invitrogen) containing 2% B-27, 20 ng/ml epidermal growth factor (Peprotech) and 20 ng/ml basic fibroblast growth factor (Peprotech) at a density of 100,000 cells/ml. Primary neurospheres were dissociated by incubation with Accutase (Millipore Bioscience Research Reagents) and reseeded in fresh media at 50,000 cells/ml until secondary spheres were generated. All spheres used for experiments were passaged at least once. All cell lines were kept at 37°C in a humidified atmosphere containing 5% CO_2.

Cells were transfected in Opti-MEM (Invitrogen) with Lipofectamine-2000 (Invitrogen). The pRL-SV40 or pRL-TK vector (Promega) was used as internal control. P19 cells and NSCs were treated with bone BMP2 (Peprotech) at 20 and 10 ng/ml, respectively (Lee et al., 2000). Cells were harvested 48–72 h post-transfection and assayed for reporter gene activity with a Dual-Luciferase Reporter Assay System (Promega).

Nuclear extract preparation and electrophoretic mobility shift assay. Nuclear extracts were prepared according to the method of Jiang et al. (2008). Biotin-labeled double-stranded oligonucleotide from mouse *gat1* gene promoter region (probe A 5′-GTGACAGAGCCAGAGAAAC-CAAGAGACCAATTAAGGTAGACCTTT-3′) or reported smad4-binding site (probe SBE 5′-AGACAGACAATGTCTAGTCTATTTGA-AATGCCTGA-3′) was used as a probe. LightShift chemiluminescent EMSA kit (PIERCE) was used for the binding reactions. Before the addition of biotin-labeled probe, 2 μg of nuclear extracts was incubated for 10 min at room temperature in 10 μl of reaction buffer. Biotin-labeled probe was then added, and the incubation was allowed to proceed for 20 min at room temperature. Protein–DNA complexes were separated on nondenaturing polyacrylamide gels. In competition experiments, the nuclear extracts were preincubated with excess unlabeled double-stranded oligonucleotides for 10 min. The sequences of the competitor A_{SBE3m} and SBEm oligonucleotides were: A_{SBE3m} 5′-GTGACAGAGCCAGAGAAACCAATTT-TTCAATTAAGGTAGACCTTT-3′, SBEm 5′-AGACAGACAATGTTTATTCTATTTG-AAATGCCTGA-3′, and their complementary strands.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed by using a ChIP assay kit (Millipore Biotechnology). Briefly, P19 cells or primary cultured neurons were cross-linked for 10 min at 37°C by the addition of formaldehyde (final concentration, 1%). After washing with cold PBS, cells were resuspended in SDS lysis buffer supplemented with protease inhibitors and incubated on ice for 10 min. Cell lysate was subsequently sonicated seven times with 3 s bursts at 40 W in a Sonifier (JY92–2D, Ningbo Scientz Biotechnology) to yield input DNA enriched with fragments between 200 and 1000 bp in size. A small proportion of the lysate was immediately heated at 65°C for 4 h in the presence of 5 M NaCl to reverse the cross-links, and was later used for monitoring equal DNA amounts for ChIP (Input). Sonicated lysate obtained from $\sim 1 \times 10^6$ cells was reconstituted in 2 ml of ChIP dilution buffer with protease inhibitors. To reduce nonspecific background, the lysate was treated with salmon sperm DNA/protein A-agarose beads for 30 min at 4°C. The precleared lysate was immunoprecipitated with 5 μg of rabbit anti-YY1 (Santa Cruz Biotechnology), anti-Smad4 (Santa Cruz Biotechnology), or the same amount of normal rabbit IgG at 4°C overnight. Immune complexes were collected with salmon sperm DNA/protein A-agarose. After elution of immune complexes, cross-linking was reversed as described above, and the DNA was then purified by a typical phenol/chloroform procedure and ethanol precipitation. Real-time PCR analysis of ChIP DNA was conducted in three independent experiments, quantified using the standard curve method on ABI 7300 thermocycler, and normalized to Smad4 bound to the region from −333 to −288 of mouse *gat1* gene (set at 100%). The primers used were: (forward) 5′-ACACATCCTCCAA-GACCAATCCT-3′ and (reverse) 5′-GGCCTCCACCCTCCTTCA-3′.

Statistics. Results are expressed as the mean ± SD for at least three independent experiments. Differences between two samples were assessed by a two-tailed Student's *t* test. Differences among multiple means were assessed by one-way ANOVA followed by Bonferroni correction. *p* values of ≤0.05 were considered statistically significant.

Results

Identification of the mouse *Gat1* 5′-flanking region

Rapid amplification of 5′ cDNA ends was performed to map the transcription initiation site(s) of mouse *gat1* gene. The major PCR product was cloned into pMD-18T vector and then se-

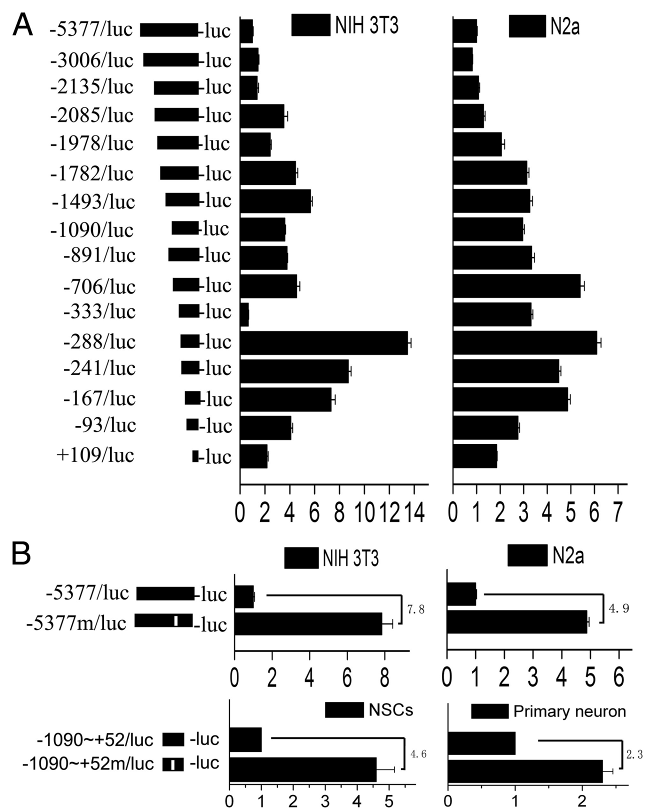

Figure 4. Transient transfection assays define a *cis*-regulator in mouse *gat1* gene promoter. ***A***, Schematic structure of the luciferase constructs used in this study. The boundaries of the promoter constructs are defined relative to the transcription initiation site and are indicated on the left. Independent constructs were transfected into NIH 3T3 or Neuro 2a cells. Luciferase activity was normalized to Renilla luciferase activity encoded by cotransfected control plasmid pRL-SV40 and then normalized to that of −5377/luc construct. ***B***, In the −5377m/luc or −1090∼+52m/luc construct, a 46 bp fragment, from −333 to −288, is indicated by an open rectangle and was deleted. Independent constructs were transfected into NIH 3T3, Neuro 2a cells, primary cortical neurons, or NSCs. Luciferase activity was normalized to Renilla luciferase activity encoded by cotransfected control plasmid, pRL-SV40, or pRL-TK. The promoter activity was normalized to −5377/luc or −1090∼+52m/luc. Induction of promoter activity in different cell lines is indicated as fold increase. Results are shown as the mean ± SD for three independent experiments ($n = 3$ in each independent experiment).

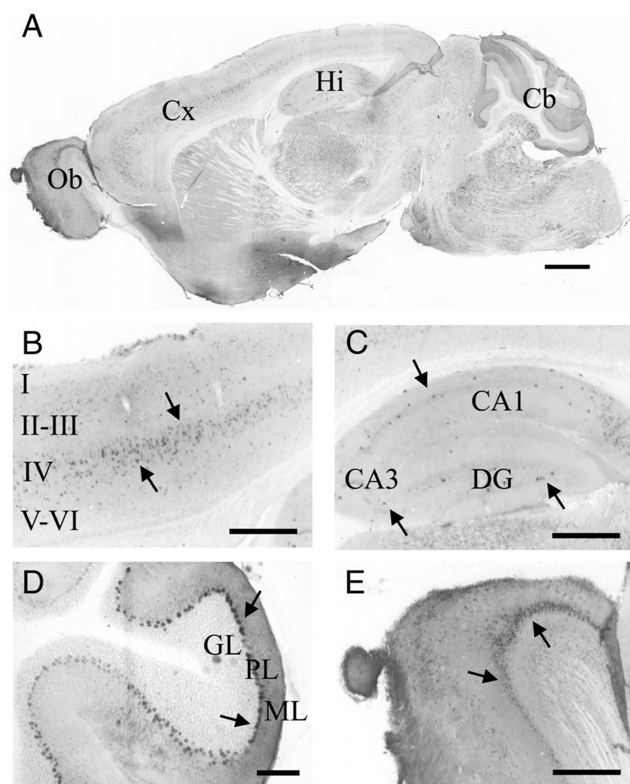

Figure 5. Histological detection of LacZ in the brain of adult *gat1(5.7m)lacz* mice. ***A–E***, Sagittal brain sections from *gat1(5.7m)lacz* transgenic mice were stained by anti-LacZ antibodies. Shown are images of whole brain (***A***), cerebral cortex (Cx; ***B***), hippocampus (Hi; ***C***), cerebellum (Cb; ***D***), and olfactory bulb (Ob; ***E***). ***A–E***, Arrows denote immunopositive cells. Scale bars: ***A***, 1 mm; ***D***, 100 μm; ***B***, ***C***, ***E***, 400 μm.

Table 2. Transgene expression in *gat1(5.7m)lacz* transgenic mice

	gat1(5.7m)lacz transgenic founder	
Transgenic mice tissue	2F ($n \geq 3$)	3F ($n \geq 3$)
Brain	100	100
Heart	7.7	15.5
Lung	5.7	18.0
Liver	12.2	7.4
Spleen	3.8	2.3
Kidney	12.4	5.1

LacZ mRNA expression in the brain of each line was artificially set at 100. Three replicates of each reaction were performed.

quenced (Fig. 1*A*). From the sequences of 12 independent clones, a major transcription initiation site (+1) corresponding to an A residue was identified.

Using the sequence information for mouse *gat1* gene and flanking sequence (GI:149255466) as a guide for primer design, a 5752 bp genomic fragment was cloned from mouse genomic DNA. The fragment begins inside intron 1 of mouse *gat1* gene at the position +375 bp (using transcription initiation site as +1) and extends to −5377 bp upstream of the transcription initiation site. Because the translation initiator ATG sequence is located inside exon 3 of mouse *gat1* gene and separated from exon 1 by two introns, the isolated 5′-flanking region of mouse *gat1* gene did not contain the translation initiation site (Fig. 1*B*). The identity of the genomic DNA fragment was established by sequencing.

Gat1 promoter activity in transgenic mice

Transgenic mice with the *gat1(5.7)lacz* construct harbored a fusion gene consisting of 5.7 kb *gat1* 5′-flanking region extending 375 bp into the 5′-untranslated leader sequence, a nuclear LacZ expression cassette, and one polyadenylation site derived from the SV40 gene. Five founder lines were obtained, and all expressed the transgene as confirmed by reverse transcriptase PCR assay.

Quantitative reverse transcriptase PCR assay demonstrated almost exclusive distribution of the transgene in the brain, with minimal level of expression in peripheral tissues (Table 1). This pattern is consistent with previous results of endogenous mouse *gat1* gene expression (Liu et al., 1993), indicating that the DNA fragment containing the promoter region is sufficient to confer tissue specificity of mouse *gat1* gene expression.

In the two lines analyzed, GAT1 (Fig. 2*A–E*) and LacZ (Fig. 2*A′–E′*) immunoreactivity were detected throughout the entire cerebral cortex, hippocampus, cerebellum, and olfactory bulb. These results match the distribution of endogenous mouse GAT1 (Borden, 1996). At higher magnification, GAT1 and LacZ immunoreactivity were strong in layers II and IV and moderate in other layers of the cerebral cortex (Fig. 2*B*,*B′*). CA1, CA3, and the dentate gyrus of hippocampus show immunoreactivity for LacZ and GAT1 (Fig. 2*C*,*C′*). Immunoreactivity for LacZ and GAT1

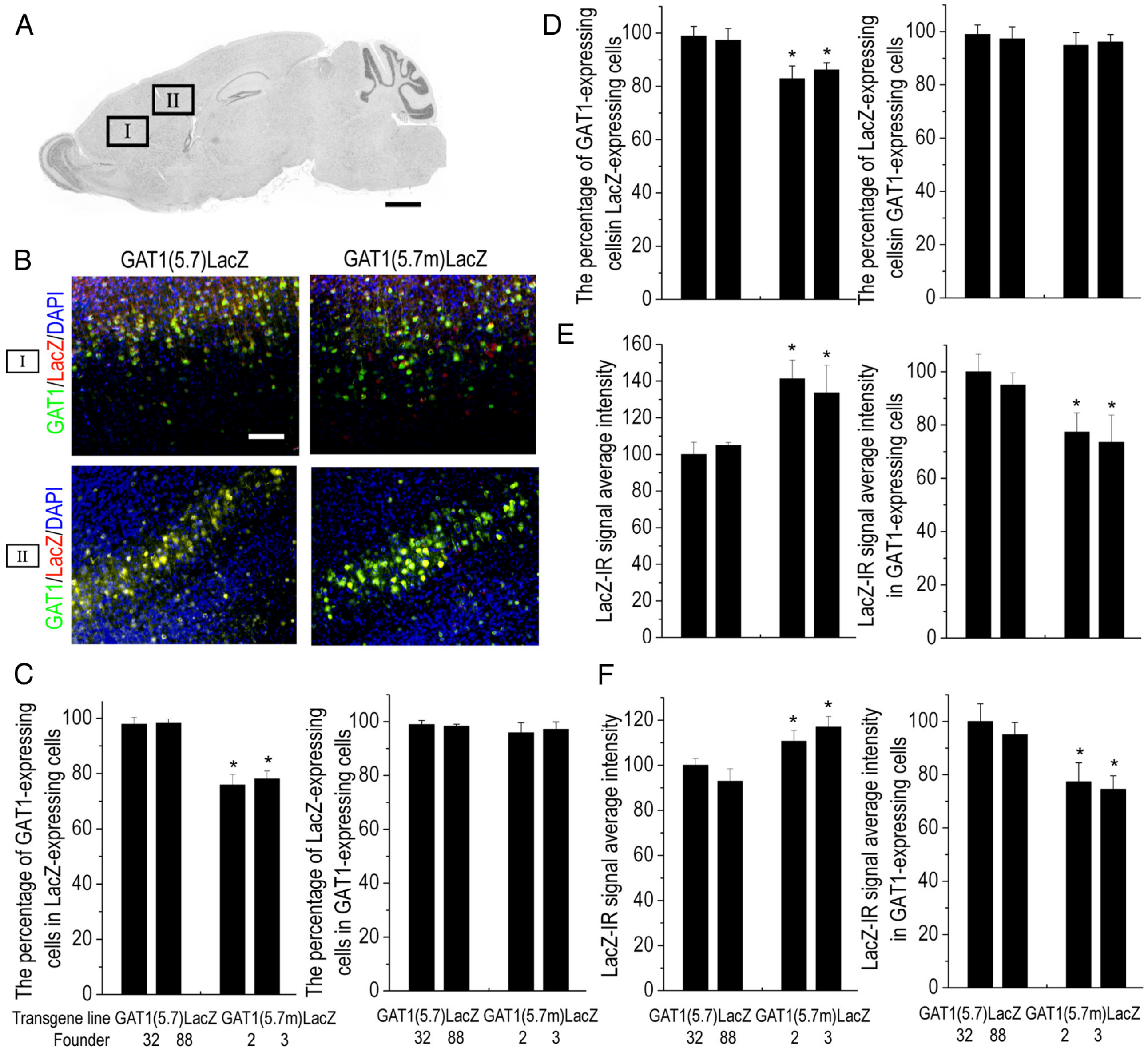

Figure 6. Quantitative analysis of LacZ or GAT1 immunopositive cells in cerebral cortex of *gat1(5.7)lacz* and *gat1(5.7m)lacz* mice. ***A***, Schematic representation of a sagittal brain section showing image locations. ***B***, Representative images of LacZ and GAT1 immunopositive signal on sagittal brain sections from *gat1(5.7)lacz* or *gat1(5.7m)lacz* mice. ***C***, ***D***, The percentage of LacZ-expressing cells in GAT1-expressing cells or the percentage of GAT1-expressing cells in LacZ-expressing cells of region I (***C***) or region II (***D***). ***E***, ***F***, Intensity of LacZ-immunoreactive signal in all cells or in GAT1-expressing cells of region I (***E***) or region II (***F***). Scale bars: ***A***, 1 mm; ***B***, 100 μm. Data represent mean ± SE ($n \geq 5$ mice per line). *$p < 0.05$ significantly different from *gat1(5.7)lacz* group (ANOVA followed by Bonferroni correction). DAPI, 4′,6′-Diamidino-2-phenylindole dihydrochloride.

was strong in the Purkinje cell layer, moderate in the molecular layer, and very faint or not detected in the granular layer (Fig. 2*D*,*D*′). Finally, GAT1 and LacZ immunoreactivity were present in the olfactory bulb (Fig. 2*E*,*E*′). LacZ was colocalized with GAT1 in double-staining experiments (Fig. 3). These results further indicate that the 5.7 kb 5′-flanking region of mouse *gat1* gene harbors most, if not all, of the *cis*-regulatory information required for *gat1* gene expression *in vivo*.

Definition of regulatory elements within *gat1* promoter *in vitro*

To localize the *cis*-regulatory elements that negatively regulate *gat1* gene expression, reporter gene assays were performed in two cell lines that do not have endogenous *gat1* gene expression (NIH 3T3 and Neuro 2a) (Fig. 4*A*). In both cell lines, a strong gene expression suppression effect was observed in the constructs with 5′-terminal deletion up to nt −333, ∼300 bp upstream from the transcription initiation site. A further deletion of 46 bp (deletion up to nt −288) abolished this effect. Moreover, an internal deletion of the 46 bp element, from −333 to −288, resulted in an increase of full-length promoter activity in both cell types (Fig. 4*B*). Similar results were achieved both in NSCs and primary cortical neurons (Fig. 4*B*). Notably, induction of *gat1* promoter activity observed in primary cortical neurons was significantly less than that in NSCs, NIH 3T3, and Neuro 2a. These results support the contention that the 46 bp element is an essential requirement for the negative transcriptional regulation of mouse *gat1* gene.

Identification of the *cis*-regulatory element in *gat1* promoter *in vivo*

Transgenic mice with the *gat1(5.7m)lacz* construct, bearing a deletion of the 46 bp element, were established. The overall trans-

gene expression pattern of *gat1(5.7m)lacz* mice was not significantly different from that of *gat1(5.7)lacz* mice (Table 2) (two-tailed Student's *t* test). These findings suggest that silencer elements other than the 46 bp element are required to prevent expression of *gat1* in most non-neural tissues. Alternatively, non-neuronal tissues may lack transactivators that are critical for neuronal gene expression.

Similar to that of *gat1(5.7)lacz* mice, a high level of LacZ expression was present in the cerebral cortex, hippocampus, cerebellum, and olfactory bulb in two independent lines of *gat1(5.7m)lacz* mice (Fig. 5). However, the percentage of GAT1-expressing cells in LacZ-expressing cells was significantly lower in brains of *gat1(5.7m)lacz* mice than those of *gat1(5.7)lacz* mice, while the percentages of LacZ-expressing cells in GAT1-expressing cells were not significantly different between two transgenic mice (Fig. 6*A–D*). These results indicate that deletion of the 46 bp element results in an increased number of LacZ-expressing cells, part of which are GAT1 nonexpressing. It is notable that although the overall LacZ-immunoreactive signal intensity of *gat1(5.7m)lacz* mice was higher than that of *gat1(5.7)lacz* mice (Fig. 6*E*,*F*), in GAT1-expressing cells the LacZ-immunoreactive signal intensity of *gat1(5.7m) lacz* mice was lower than that of *gat1(5.7) lacz* mice ($p < 0.05$, ANOVA and Bonferroni correction). These results indicate that the 46 bp element has an opposite effect on *gat1* gene promoter activity depending on the cellular context.

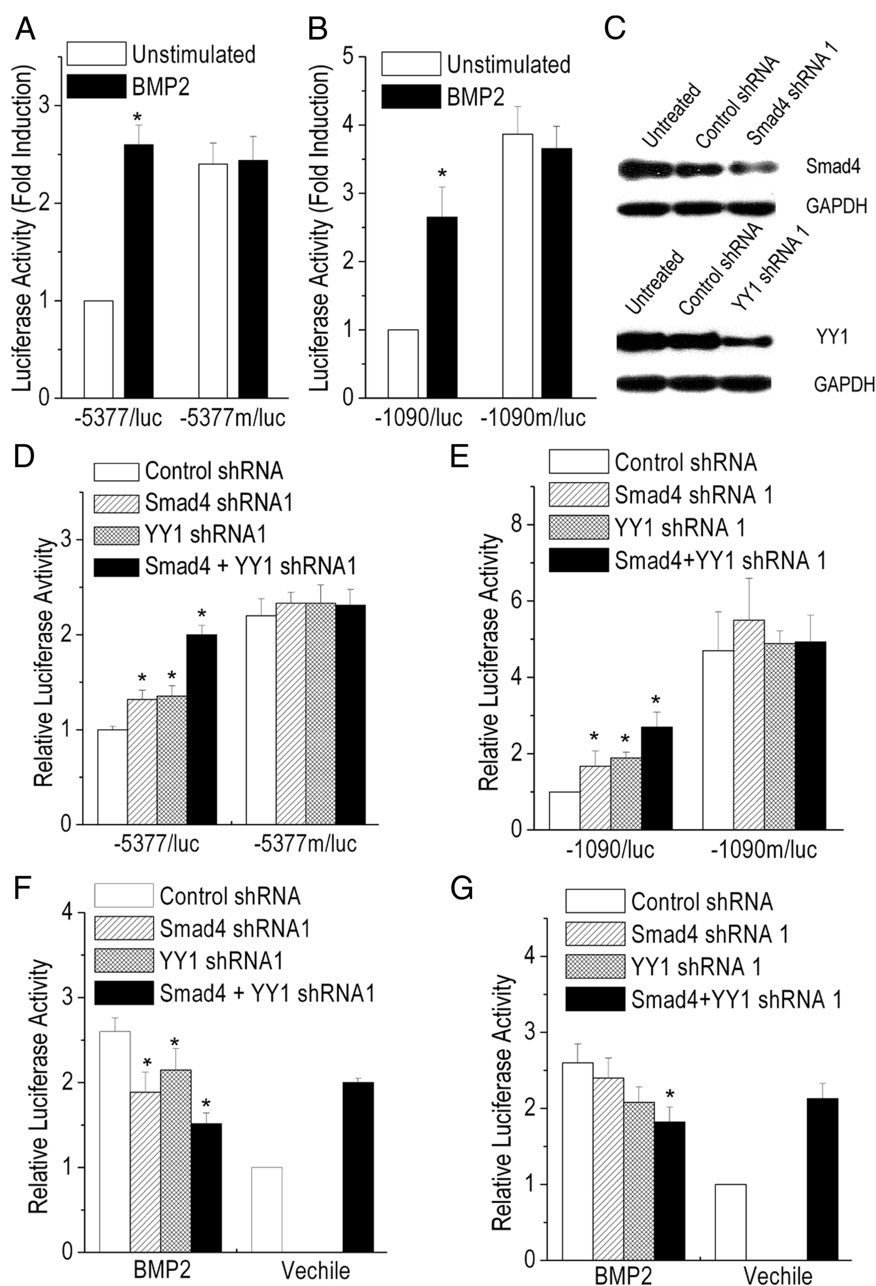

Figure 7. BMP2-induced *gat1* promoter activity mediated by Smad4 and YY1. ***A***, ***B***, P19 cells (***A***) or neural stem cells (***B***) were transiently transfected with either wild-type or mutant *Gat1* gene promoter construct, grown in the presence or absence of BMP2. The activity of wild-type *Gat1* promoter construct in the absence of BMP2 was set at 1. Results are expressed as the mean ± SD for three experiments. $*p < 0.05$, compared with the unstimulated wild-type construct control (Student's *t* test). ***C***, The inhibitory efficiency of the shRNA directed against *smad4* or *yy1* evaluated by Western blot analysis. At 48 h after shRNA transfection, total cell lysate was prepared and normalized for protein concentration. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Results shown are representative of three independent experiments. ***D–G***, P19 cells (***D***, ***F***) or neural stem cells (***E***, ***G***) were transfected with either wild-type or mutant *gat1* gene promoter construct, together with the indicated shRNA expression constructs. Transfected cells were then treated with BMP2 (***F***, ***G***) or not (***D***, ***E***), as indicated. The activity of *gat1* promoter construct cotransfected with control shRNA obtained in the absence of BMP2 has been set equal to 1. Results are expressed as the mean ± SD for three experiments. $*p < 0.05$, compared with control shRNA (ANOVA followed by Bonferroni correction).

BMP2 induction of *gat1* gene expression mediated by Smad4 and YY1

BMP2 is reported to regulate cortical GABAergic neuron differentiation (Mabie et al., 1999; Yung et al., 2002). BMP2 increased the transcriptional activity of wild-type but not mutant *gat1* gene promoter construct with the 46 bp element deleted (Fig. 7*A*,*B*). These findings indicate that the BMP2 response depends on the region in the mouse *gat1* gene promoter between −333 and −288.

Sites for Smad4, the major intracellular signaling effector for BMP signals, and YY1, a reported Smad4-interacting protein (Kurisaki et al., 2003), were found in the 46 bp element (P-Match). Each two shRNA oligonucleotides for *smad4* or *yy1* were designed and their effects were studied (Fig. 7*C*; supplemental Fig. S1*A*, available at www.jneurosci.org as supplemental material). Inhibiting the expression of *smad4* or *yy1* with shRNA in P19 cells or NSCs resulted in an increase of *gat1* gene promoter construct transcription activity, and this effect was further accentuated by a combination of Smad4 shRNA and YY1 shRNA (Fig. 7*D*,*E*; supplemental Fig. S1*B*, available at www.jneurosci.org as supplemental material). Neither Smad4 shRNA nor YY1 shRNA had an effect on the transcriptional activity of the mutant *gat1* gene promoter construct. However, in the presence of BMP2, knockdown of *smad4* or *yy1* expression resulted in a decrease of *gat1* gene promoter construct transcription activity (Fig. 7*F*,*G*; supple-

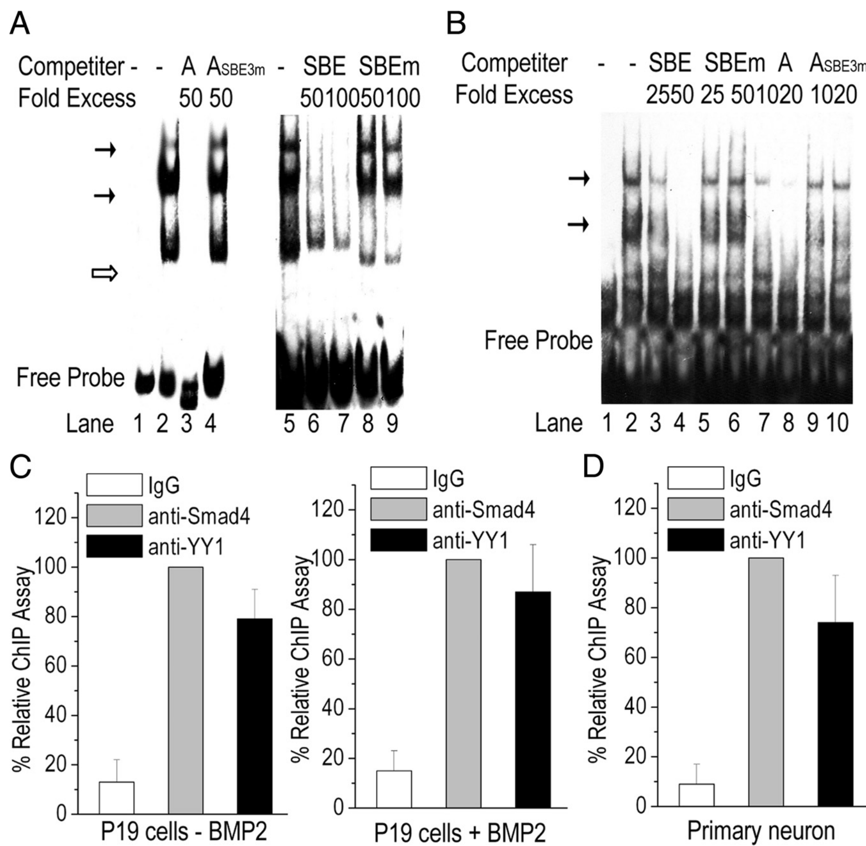

Figure 8. Traditional EMSA and ChIP localize Smad4 and YY1 bindings to *gat1* gene promoter. ***A***, EMSA competition experiments. Nuclear protein was isolated from primary cortical neurons, and EMSA was performed using biotin-labeled oligonucleotide from *gat1* gene promoter from −333 to −288 as a probe. Lane 1, free probe; lanes 2 and 5, no competitor; lanes 3 and 4, unlabeled wild-type probe (***A***) or mutant probe (A_{SBE3m}) as competitor; lanes 6–9, unlabeled wild-type or mutant SBE oligonucleotides as competitor. Indicated are the specific complexes (solid arrow) or nonspecific complexes (open arrows). Results are representative of at least three independent experiments. ***B***, EMSA competition experiments using biotin-labeled SBE oligonucleotides as a probe. Lane 1, free probe; lane 2, no competitor; lanes 3 and 4, wild-type SBE oligonucleotides as competitors; lanes 5 and 6, mutant SBE oligonucleotides as competitors; lanes 7 and 8, wild-type A oligonucleotides as competitors; lanes 9 and 10, mutant A_{SBE3m} (with the functional SBE mutant) oligonucleotides as competitors. Results are representative of at least three independent experiments. ***C***, ***D***, Protein interactions at the region from −333 to −288 of mouse *gat1* gene in P19 cells (***C***) or primary cortical neurons (***D***) were determined by ChIP assay. IgG was used as control. Chromatin immunoprecipitated DNA was analyzed by real-time PCR.

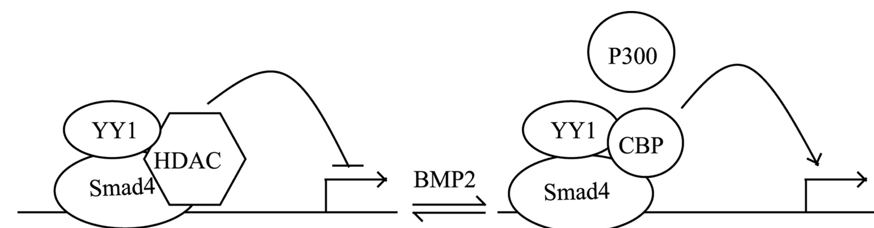

Figure 9. A model depicting the convergence of Smad4 and YY1 in BMP2-mediated gene transcription. Smad4 and YY1 bind to target gene promoter. In the absence of BMP2, Smad4/YY1 recruits HDACs and represses target gene transcription. In the presence of BMP2, Smad4/YY1 recruits histone acetyltransferases (P300/CBP) and activates target gene transcription.

mental Fig. S1*C*, available at www.jneurosci.org as supplemental material). Together, these data strongly suggest that Smad4 and YY1, at least in part, mediate BMP2 regulation of *gat1* gene expression.

DNA-binding activity of the 46 bp element on *gat1* gene promoter

The biotin-labeled 46 bp oligonucleotide derived from mouse *gat1* gene promoter, from −333 to −288, was used as a probe (probe A) in EMSA. Extracts from the primary cortical neurons interacted with probe A and revealed multiple bands on gel electrophoresis (Fig. 8*A*). The bands were greatly diminished by an excess of unlabeled oligonucleotide probe or unlabeled SBE oligonucleotide containing a canonical Smad4 site. In contrast, a mutant unlabeled probe (A_{SBE3m}) with a mutation in functional Smad4 site (supplemental Fig. S2*A*, *B*, available at www.jneurosci.org as supplemental material) failed to inhibit the binding activity. An unlabeled SBEm oligonucleotide containing the mutant Smad4 site also failed to inhibit the binding activity. Similar results also were observed in EMSA studies with extracts of NSCs (supplemental Fig. S3*A*, available at www.jneurosci.org as supplemental material) and P19 cells (supplemental Fig. S3*B*, available at www.jneurosci.org as supplemental material). The binding of Smad4 to the 46 bp element was further confirmed by EMSA experiments using a biotinylated SBE as a probe and an unlabeled wild-type or mutant probe A as a competitor (Fig. 8*B*).

Unlabeled canonical YY1-binding sites cannot inhibit the binding activity of probe A in competitive EMSAs (data not shown). It is possible that YY1 is tethered to DNA through interaction with another transcription factor (e.g., Smad4) *in vivo*, instead of binding directly to DNA. This possibility is supported by our observation that YY1 shRNA can induce the *gat1* promoter construct activity, but not the mutant lacking Smad4-binding site (supplemental Fig. S2*C*, available at www.jneurosci.org as supplemental material).

The ChIP assay showed that Smad4 and YY1 are bound to the 46 bp element of *gat1* gene promoter (Fig. 8*C*). Most interestingly, the Smad4-DNA and YY1-DNA interaction were detected in the chromatin from BMP2-treated P19 cells as well as in lysates derived from untreated cells. Similar results were achieved in primary cortical neurons (Fig. 8*D*).

Discussion

This study identified a promoter region in mouse *gat1* gene with sufficient regulatory information to recapitulate endogenous *gat1* expression. Deletion of the 46 bp BMP2-responsive element resulted in an increase in the number of LacZ-expressing cells and the overall LacZ-immunoreactive signal intensity. But, in GAT1-expressing cells, deletion of the 46 bp element resulted in a decrease in LacZ-immunoreactive signal intensity. Transcription factors Smad4 and YY1 were found to bind to the 46 bp element and potentiate or inhibit *gat1* gene transcription activity depending on the BMP2 signal in the cellular environment.

BMPs and their receptors are abundantly expressed in the brain from early embryogenesis throughout adult life (Mehler et al., 1997; Zhang et al., 1998). Endogenous BMP signaling has been reported to influence the migration of GABAergic neurons (Li et al., 1998; Mabie et al., 1999). BMP2 has been reported to promote the terminal differentiation of striatal (Hattori et al., 1999) and cortical (Mabie et al., 1999; Yung et al., 2002) GABAergic neurons in culture. Loss of *bmpr1a*, a high-affinity receptor for BMP2, increases the number of calbindin-expressing GABAergic interneurons (Samanta et al., 2007). These observations collectively suggest that BMP2 plays a fundamental role in many aspects of GABAergic neurogenesis and regulation of GABAergic neuron-specific gene expression. Results from this study demonstrated that stimulation of the BMP2 signal could induce *gat1* promoter constructs activity via a BMP2 response element.

Smad4 requires partners for regulating transcription of target genes (Attisano and Wrana, 2000). P-Match analysis showed that YY1-binding sites are adjacent to Smad4-binding sites in the 46 bp element of *gat1* promoter. YY1 can physically interact with Smad4 both *in vitro* and *in vivo*, and functionally cooperate with Smad4 in response to BMP2 signals in epithelial and myoblastic cells (Kurisaki et al., 2003). These previous reports together with our data provide strong evidence that Smad4 and YY1 are synergistic in the regulation of *gat1* promoter activity in response to BMP2 stimuli. However, more study is still needed to elucidate whether Smad4 and YY1 are required for BMP2 regulation of endogenous *gat1* expression and function.

Smad4–YY1 complex binding to the *cis*-regulatory element had opposite effects on *gat1* promoter constructs transcription activity in the absence versus presence of BMP2, suggesting the involvement of other cofactors in different cellular environment. Both Smad4 and YY1 can recruit histone acetyltransferases (e.g., P300/CBP) to form a transcriptional activation complex or histone deacetylases (HDACs) to form a transcriptional repressor complex (Austen et al., 1997; Thomas and Seto, 1999; Wotton et al., 1999; Yao et al., 2001). YY1 has been reported to repress target promoters by recruiting HDACs during oligodendrocyte differentiation (He et al., 2007). In this context, we hypothesized that the Smad4–YY1 complex recruits HDAC repressing *gat1* expression in the absence of BMP2, whereas it recruits P300/CBP activating *gat1* expression in the presence of BMP2 (Fig. 9). Support for this hypothesis comes from the observations that inhibition of HDACs by trichostatin A increased the *Gat1* promoter constructs activity (supplemental Fig. S4, available at www.jneurosci.org as supplemental material) and that inhibition of endogenous *p300* or *cbp* with shRNA abolished BMP2 induction of *gat1* promoter constructs activity (supplemental Fig. S5*A*,*B*, available at www.jneurosci.org as supplemental material). The response of CBP–DNA interaction to BMP2 signals was confirmed by ChIP analysis on a 46 bp sequence in P19 cells (supplemental Fig. S5*C*, available at www.jneurosci.org as supplemental material).

Proper neuronal function requires orchestrated regulation of many genes that contribute to neurotransmitter synthesis, vesicular packaging, release, and termination. In *C. elegans*, the *unc-25/gad* and the *unc-47/vgat* were regulated by transcription factor unc-30 (Eastman et al., 1999; Westmoreland et al., 2001). So, it is reasonable to think that in mammals several common signaling molecules and transcription factors may also regulate expression of GABAergic neuron-specific genes. The promoters of *gad* (Makinae et al., 2000; Kobayashi et al., 2003) and *vgat* (Ebihara et al., 2003; Oh et al., 2005), two essential components of GABAergic neurons, have already been investigated in transgenic and transfection experiments. However, little was known about the upstream regulators, including signaling molecules and transcription factors. It is notable that adjacent Smad4 and YY1 consensus motifs were also found in the promoter regions of other GABAergic neuron-specific genes, such as *gat4*, *gad1-2*, and *vgat*. So, our results also provide a new idea to understand other GABAergic neuron-specific gene expression regulation.

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