Exonic Sp1 sites are required for neural-specific expression of the glycine receptor β subunit gene

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The gene encoding the β subunit of the inhibitory glycine receptor (GlyR) is widely expressed throughout the mammalian central nervous system. To unravel the elements regulating its transcription, we isolated its 5' non-coding and upstream flanking regions from mouse. Sequence analysis revealed significant differences between the 5' region of the β subunit gene and the corresponding regions of the homologous GlyR α subunit genes; it also identified a novel exon (exon 0) that encodes most of the 5'-untranslated portion of the GlyR β mRNA. Primer extension experiments disclosed multiple transcriptional start sites. Transfection experiments with luciferase reporter gene constructs showed that sequences encompassing 1.58 kb of upstream flanking region and 180 bp of exon 0 displayed high promoter activity in two neuroblastoma cell lines but not in non-neural cells. Analysis of various deletion constructs showed that the 5'

flanking region preceding the transcriptional start sites silences expression in non-neural cells but is not essential for general promoter activity. In contrast, the deletion of sequences within exon 0 drastically decreased or abolished transcription; the removal of sequences harbouring Sp1 consensus sequences within exon 0 decreased expression specifically in a neuroblastoma cell line. Band-shift assays confirmed the binding of Sp1 to sites within the deleted sequence. Our results indicate that neural-specific expression of the GlyR β subunit gene might depend on a direct interaction of Sp1 transcription factors with *cis* elements located downstream from transcription initiation sites.

Key words: *cis*-acting sequences, gene structure, neuron-specific gene regulation, transcription factor Sp1.

INTRODUCTION

A precise regulation of neurotransmitter receptor number is of crucial importance for determining the functional characteristics of the postsynaptic membrane. So far, the transcriptional control of ionotropic receptor genes in the nervous system has been investigated for genes encoding nicotinic acetylcholine receptor [1–3], GABA_A (γ -aminobutyric acid) receptor [4–6] and various glutamate receptor subunits (reviewed in [7]). These studies have shown that the respective promoters share several characteristics, including the lack of TATA and CAAT boxes and multiple transcriptional start sites within CpG-rich genomic regions. In several cases, neuron-restrictive silencer elements seem to be important for neuron-specific expression, and among others the transcription factors Sp1, SCIP and ETS [1,2] have been found to be crucial for the regulation of such genes. Here we present an attempt to identify *cis*-acting sequences and *trans*-acting transcription factors that are implicated in the transcriptional regulation of the gene encoding the β subunit of the inhibitory glycine receptor (GlyR).

The GlyR is a pentameric postsynaptic chloride channel protein that mediates neuronal inhibition in the spinal cord, the brain stem and other regions of the central nervous system [8]. It is composed of two types of membrane-spanning subunits of

48–49 kDa (α) and 58 kDa (β) [9–11] respectively. The agonist-binding α subunit exists in four isoforms ($\alpha 1$ – $\alpha 4$) encoded by different genes that display distinct regional and developmental expression patterns [12–14]. In contrast, only a single β subunit gene [15] generates different splice variants [16] of this structural subunit. This β gene is widely expressed in the developing and adult central nervous system [13]. The β subunit is thought to be essential for the synaptic localization of the GlyR, because its cytoplasmic domain interacts with the receptor-associated protein gephyrin [17,18], which anchors the GlyR to the subsynaptic cytoskeleton [19–21]. In addition, the β subunit contributes to the regulation of ion flux through the GlyR channel [22]. A crucial role of the β subunit also emerges from the molecular analysis of GlyR mutations causing hereditary neuromotor diseases. In the mouse mutant *spastic*, an intronic insertion of a long interspersed element-1 ('LINE') into the GlyR β subunit gene results in low synaptic content of GlyR and thus a decreased inhibition of motor and sensory neurons [15,24–26].

Here we report an attempt to unravel the genetic basis for the widespread expression of the GlyR β gene. After isolating the 5' exonic and flanking regions of the murine GlyR β gene, we identified putative promoter elements by introducing luciferase reporter gene constructs into both neuronal and non-neuronal cell lines. Unexpectedly, important regulatory sequences con-

Abbreviations used: GlyR, glycine receptor; RT-PCR, reverse-transcriptase-mediated PCR; SV40, simian virus 40.

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ferring promoter activity in neuroblastoma cell lines, including consensus binding sites for Sp1 *trans*-acting factors were found within a novel 5' exon (exon 0) of the β subunit gene. Thus the GlyR β gene displays an unusual organization of its regulatory sequence elements, suggesting novel mechanisms for its transcriptional control.

EXPERIMENTAL

Isolation of genomic clones and DNA sequencing

A BALB/c mouse genomic library constructed in the λ -FixI vector (Stratagene) was screened with two 32 P-labelled fragments of the rat GlyR β subunit cDNA clone 58-6 [11]. The first probe was a *XhoI*-*EcoRV* fragment (814 bp) spanning the 5' region of the rat β subunit cDNA. In addition, an adjacent 1026 bp cDNA fragment was amplified by PCR. For the isolation of genomic clones, 1.2×10^6 λ phages were screened with a mixture of both 32 P-labelled probes under stringent conditions $\{4 \times \text{SET}$ [75 mM Tris/HCl (pH 7.5), 0.7 M NaCl, 12.5 mM EDTA, $5 \times$ Denhardt's solution and 200 $\mu\text{g/ml}$ herring sperm DNA] containing 50% (w/v) formamide, at 42 °C} in accordance with standard procedures [27]. Filters were washed at 65 °C in $2 \times \text{SSC}$ (0.30 M NaCl/0.03 M sodium citrate) containing 0.1% SDS. Fourteen hybridization-positive phages were isolated and rescreened with 25-mer oligonucleotides deduced from different regions of the rat β subunit cDNA sequence [$4 \times \text{SET}$ containing 20% (v/v) formamide, at 42 °C]. Washing was in $2 \times \text{SSC}/0.1\%$ SDS at 42 °C. After being subcloned into plasmid vectors, all exons and their flanking regions were sequenced. Sequences were determined by the dideoxy chain-termination method with a T7 sequencing kit (Pharmacia). Because of band compressions, GC-rich stretches in exon 0 were sequenced with a DyeDeoxyTM Terminator Cycle sequencing kit (Applied Biosystems) and the Deaza G/A T7 sequencing kit (Pharmacia). All sequences were confirmed by analysing both DNA strands.

Primer extension

An oligonucleotide corresponding to bp 159–189 of exon 0 was end-labelled with [γ - 32 P]ATP and T4 polynucleotide kinase, purified on a Biogel P6 column (Bio-Rad) and recovered by precipitation of the eluate with ethanol. Mouse brain or liver total RNA (20 μg) was incubated with 10^7 c.p.m. of the labelled oligonucleotide for 10 min at 70 °C, then cooled to room temperature and mixed with 20 μl of a Reverse Transcription Mix in accordance with the SuperScript reverse transcriptase protocol (Gibco). Elongation was performed for 1 h at 45 °C. A 4 μl aliquot of the reaction was mixed with polyacrylamide gel loading dye, heated for 2 min at 95 °C and subjected to electrophoresis on a 6% (w/v) polyacrylamide/7 M urea sequencing gel in parallel with the products of a double-stranded sequencing reaction.

Generation of reporter gene constructs

All constructs made for transfection analysis were derivatives of the 'GeneLight' vector pGL2-Basic (Promega). The vector pGL2-Basic contains a luciferase reporter gene with an upstream multiple cloning site for introducing putative promoter fragments. Constructs pGLa and pGLb were made by subcloning restriction fragments from a 7 kb *EcoRI* genomic fragment of the GlyR β subunit gene cloned into pBluescript (Stratagene). Construct pGLa was prepared by subcloning a 1.1 kb *EcoRI* (filled in)-*BglII* fragment into the *SmaI/BglII* sites of pGL2-Basic. The 3' end of construct pGLb was generated by taking

advantage of a *PvuII* site in exon 0 and subcloning this site into the filled *HindIII* site of pGL2-Basic. The insert's 5' ends of constructs pGLb to pGLe were subcloned into a suitable site of the pGL2-Basic polylinker, or alternatively, after performing a fill-in reaction, into its *SmaI* site. Additional deletion constructs were created either by digestion of construct pGLe with exonuclease III (generating constructs pGLf and pGLg) or by subcloning PCR-amplified fragments into the *BglII/HindIII* sites of pGL2-Basic (constructs pGLh to pGLk).

Transient transfections and luciferase assays

N18 mouse neuroblastoma cells (N18TG2; A. T. C. C. no. DSMACC1039) and 3T3 cells (A. T. C. C. no. CLL92) were cultured at 37 °C in a humidified air/CO₂ (9:1) atmosphere in Dulbecco's modified essential medium (Gibco). Human embryonic kidney (HEK-293) cells (A. T. C. C. no. CRL1537) and LA-N1 cells [28] were maintained in an air/CO₂ (19:1) atmosphere in minimal essential medium (Gibco). Media were supplemented with 50 i.u./ml penicillin, 2 mM L-glutamine and 10% (v/v) fetal calf serum. Cells were grown on six-well dishes to 80% confluence and transiently transfected with the indicated plasmids (2 μg per well), with the use of the calcium phosphate co-precipitation method [29] followed by a glycerol shock 5 h after transfection [30]. Transfection efficiencies were assessed by co-transfecting each well with equal amounts of a pSV40 β -galactosidase vector (Promega). After 2 days, cells were washed with cold PBS and scraped into 150 μl of lysis buffer (Promega). Luciferase assays were performed in accordance with the Promega application guide by using a Berthold chemoluminometer; transfection efficiencies were determined by a colorimetric β -galactosidase assay (Promega) in accordance with the manufacturer's instructions. All luciferase expression values obtained with the different reporter gene constructs were normalized to that obtained by parallel transfection of a construct in which the luciferase gene was driven by simian virus 40 (SV40) promoter elements (pGL-Pro).

Preparation of RNA

Total RNA from tissue was prepared by the caesium trifluoroacetate extraction method [31]. Pelleted RNA was resuspended in 250 μl of water previously treated with diethyl pyrocarbonate, and stored at -70 °C.

In situ hybridization and PCR

In situ hybridization of brain sections from adult mice was performed as described by Wisden et al. [32], with an anti-sense oligonucleotide probe that was complementary to positions 210–242 of exon 0 and 1643–1656 of exon I respectively (5'-AGCAGCTCAATCGCACGCGCCCCCTCAGCCCTCCGC-CGAGGTGAC-3') (see Figure 1). In addition, another oligonucleotide that overlapped the boundary between exons 8 and 9, and a third oligonucleotide complementary to exon L, were used (results not shown). Corresponding sense oligonucleotides served as negative controls. Sections were exposed to Kodak X-Omat AR film for 4 weeks. For analysing the presence of exon 0 in mouse brain mRNA by reverse-transcriptase-mediated PCR (RT-PCR), a cDNA from mouse brain (Marathon-ReadyTM; Clontech) was employed with the Advantage 2 PCR enzyme system (Clontech) by following the instructions of the user manual for PCR conditions. As the primer pair for exon 0 and the exon 8/9 boundary, we used 5'-CGCCGCCGTACCT-CGGCGGAGGGCTGA-3' with 5'-CTGGTCTACCAACCTGCAAAGTGCTGA-3'; as the primer pair for exon 0 to exon

Figure 1 Structure of the murine GlyR β subunit gene

(A) Schematic representation of the intron/exon structure. Exon numbering corresponds to that used by Mülhardt et al. [15]. A newly identified alternatively spliced exon encoding an insertion of 16 amino acid residues in the large intracellular loop (exon L, indicated by a stippled box) is located between exons 8 and 9. Structural features are highlighted as follows: large squares, coding regions; small squares, non-coding regions; black box, signal peptide; striped boxes, putative transmembrane regions 1–4; S, position of codons for extracellular cysteine residues implicated in subunit folding. The length of each exon (in bp) is indicated under the corresponding exon number. (B) Nucleotide sequence of the 5' region and alignment with the rat GlyR β cDNA. Nucleotide numbering is given at the left: nt 1–242 code for exon 0; nt 1646–1696 code for exon I. The sequences of the 5'-untranslated exon 0 and of exon I are indicated by the use of capitals; lower-case letters indicate upstream regions or intronic sequences. Transcriptional start sites determined by primer extension analysis are marked by arrows. Putative Sp1-binding sites are underlined. The translation start codon ATG at position 1675 is also underlined.

9 amplification we used 5'-CCACCTGCGCCGCGTCACC-TC-3' and 5'-GTCGATGCGCTTGGCTGCTGTTGGG-3'.

Preparations of nuclear extracts and band-shift analysis

Nuclear extracts were prepared from cultured N18TG2 cells with the 'micropreparation technique' described by Andrews and Faller [33]. Band-shift analysis was done as follows: binding reactions (20 μ l) contained 20 mM Hepes/KOH, pH 7.9, 10% (w/v) glycerol, 40 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 μ g of poly(dI·dC) (Pharmacia), 1 μ g of BSA, 0.002 pmol of 32 P-end-labelled probe and 5–10 μ g of nuclear protein. After incubation on ice for 15 min, the samples were loaded on a 4% (w/v) non-denaturing polyacrylamide gel that was pre-run for 30 min at 10 V/cm. In supershift experiments, binding reactions were incubated for an additional 15 min on ice with 1 μ g of a monoclonal Sp1 (PEP 2) antibody (Sigma) before gel electrophoresis. To examine protein binding,

the following double-stranded DNA probes were used: P1, 5'-GACCTCCGCGCCCGGTCCCCGAGTTGT-3'; P2, 5'-GCGGAGCAGGCGGCTCAAACCTCGGCGG-3'; P3, 5'-GGCGCGGTTGGCGGCGGTGGCGGGCGGCGGCGG-3'. The sequence of the Sp1 consensus oligonucleotide used was 5'-ATTCGATCGGGGCGGGGCGAGC-3'.

RESULTS

Identification of two novel exons in the GlyR β subunit gene

Previous work in our laboratory has established the genomic organization of the coding regions of the GlyR β subunit gene [15] and of three different GlyR α subunit genes [14]. All these genes are split into nine exons (I to IX), whose boundaries are highly conserved within the GlyR gene family. In the present study we extended the analysis of the β gene to its 5'-untranslated sequence and upstream regions. Sequence comparison of a mouse genomic subclone and the cDNA sequence of the rat β subunit

Figure 2 Expression analysis of GlyR β subunit transcripts in mouse brain

(A, B) *In situ* hybridization of horizontal (A) and sagittal (B) sections of adult mouse brain probed with an anti-sense oligonucleotide specific for the exon 0/exon I boundary of the GlyR β subunit gene. Abbreviations: Bo, olfactory bulb; Cb, cerebellum; Cx, cortex; H, hippocampus. Exposure on X-Omat AR film was for 4 weeks. (C) Agarose-gel electrophoresis of PCR amplification products obtained with cDNA from mouse brain by using oligonucleotides specific for exon 0 and the exon 8/9 boundary (lane 2), as well as for exons 0 and 9 (lane 4). In lanes 3 and 5, control reactions performed without cDNA were loaded. The sizes of DNA markers (lanes 1 and 6) were 0.5, 1, 1.5, 2 and 2.5 kb.

[11] revealed that the 5' non-coding cDNA sequence aligned to two different regions of the genomic DNA fragment (Figure 1B). A cDNA stretch of 29 bp upstream of the translation initiation codon was recovered in the 5' direction from the genomic sequence of exon I, whereas an additional 196 bp of the rat cDNA 5' end shared approx. 90% identity with a distant upstream sequence of the mouse genomic clone. Both regions are separated by a putative intronic DNA segment of 1.4 kb, with splice donor and acceptor sites matching the mammalian consensus sequences. Thus most of the 5' non-coding region of the GlyR β subunit gene seems to be transcribed from an additional exon, which we term here exon 0. Analysis of further genomic clones revealed an eleventh exon (exon L) of 48 bp located between exons 8 and 9 (Figure 1A), which encodes an insertion of 16 amino acid residues that others [16] have also described previously as an alternative β subunit splice variant.

GlyR β transcripts generally start with exon 0

To confirm that exon 0 of the GlyR β subunit gene is a transcribed exonic sequence, we designed anti-sense oligonucleotides overlapping the exon 0/exon I (probe 1) and the exon 8/exon 9 boundaries (probe 2) respectively as well as another oligonucleotide complementary to exon L; we used them for *in situ* hybridization on brain slices from adult mouse. Previous studies in our laboratory have shown that the β subunit is expressed throughout most regions of the rat brain [13]. Similar results were obtained in the present study with mouse brain slices with the use of probe 2, indicating that the general expression pattern of the GlyR β subunit gene is very similar between rat and mouse (results not shown). Macroscopic transcript distributions in adult mouse brain obtained with the oligonucleotide from exon 0/exon I (probe 1) are shown in Figures 2(A) and 2(B); the pattern of hybridization revealed an mRNA distribution that was identical with that with the use of probe 2 specific for exons 8 and 9 of the mouse GlyR β gene and very similar to the

Figure 3 Mapping of transcription initiation sites by primer extension analysis

Right panel: electrophoretic separation of reaction products with the use of either mouse brain (B) or liver (L) RNA as template. Five bands were detected with brain mRNA only, indicating multiple transcriptional start sites. Their sizes (in bp) are indicated at the right; the transcriptional start site corresponding to the band of 192 bp was designated +1. Left panel: products of a sequencing reaction serving as size markers (MS).

expression pattern obtained previously with rat brain slices [13]. The expression pattern seen with the oligonucleotide specific for exon L was very similar to the result obtained with probe 2 but was much weaker. No signals were observed with complementary sense oligonucleotides (results not shown). In addition, we performed PCR on mouse brain cDNA with oligonucleotides corresponding to exon 0 and to the probe used for *in situ* hybridization, to demonstrate the presence of both sequences within the same transcript (Figure 2C). Sequence analysis of a major amplification product confirmed the predicted β -mRNA sequence. The use of a second oligonucleotide primer pair specific for exons 0 and 9 resulted in a major amplification product that contained exons 0 to 9 without exon L, indicating that exon L is present only in a mRNA that occurs in low abundance, as already suggested by the *in situ* hybridization experiments. These results therefore demonstrate that the exon 0 sequence is present in the major fraction of GlyR β transcripts.

Transcription of the GlyR β gene starts at multiple sites

To determine the transcriptional start sites of the GlyR β subunit gene, primer extension analysis was performed. For this purpose we designed an anti-sense oligonucleotide complementary to positions 163–192 (Figure 1). Total RNA isolated from mouse brain was annealed to the 32 P-labelled oligonucleotide; specific

Figure 4 Promoter analysis of the murine GlyR β subunit gene

(A) Left panel: map of the 5' end of the GlyR β subunit gene, showing schematically the fragments used for generating the deletion constructs tested in transfection experiments. Transcriptional start sites are marked by arrows; the restriction sites used for generating different fragments are indicated as follows: E, *EcoRI*; EV, *EcoRV*; H, *HindIII*; B, *BglII*; P, *PvuII*. Right panel: luciferase activities obtained after transfection of the different reporter gene constructs into different cell lines. (B) Left panel: enlarged map of exon 0 and schematic representation of constructs containing deletions within the exon. Numbers indicate 5' and 3' nucleotide positions of promoter fragments. Right panel: corresponding luciferase activities of the different deletion constructs in the transfected N18TG2 and HEK-293 cell lines. Transfection and luciferase assays were performed as described in the Experimental section. Values are the ratios of luciferase activities normalized to that obtained with the SV40-promoter-driven luciferase gene. Values were corrected for transfection efficiency by determining the β -galactosidase activity generated by a co-transfected expression construct in each experiment. The background activity obtained after transfection with vector pGL2-Basic containing no insert upstream of the luciferase reporter gene is also shown (—). Results are means $\pm$ S.E.M. for at least three independent experiments.

templates were reverse transcribed. As a control for specificity, the same reaction was performed with RNA isolated from mouse liver. Analysis of the products of this reaction on sequencing gels revealed five bands with brain but not liver RNA of 192, 176, 175, 173 and 172 bp in length respectively, which are indicative of multiple transcriptional start sites of the GlyR β gene (Figure 3). The transcriptional start site nearest to the 5' end was defined as +1, followed by start sites at positions +16, +17, +19 and +20. Thus exon 0 had a length of between 242 and 222 bp, depending on the start site used.

A 1.76 kb genomic 5' fragment of the GlyR β gene confers neural-specific gene expression

To study the putative promoter function of an isolated genomic fragment and to identify potential regulatory elements in the 5'

flanking region of the GlyR β gene, we generated a series of reporter gene constructs. This was done by cloning a 1.76 kb 5' genomic fragment of the GlyR β gene or the respective deletion fragments into an expression vector containing a luciferase reporter gene. The resulting plasmids were then transfected into two neuroblastoma cell lines, N18TG2 (mouse) and LA-N1 (human), both of which were found by RT-PCR (results not shown) to express the endogenous GlyR β gene, and into two non-neural cell lines (3T3 and HEK-293). After 2 days the cell extracts were assayed for luciferase activity. In initial experiments we used a construct that included 180 bp of exon 0 and an additional 1.58 kb of the 5' upstream sequence (pGLb). This construct displayed about 1.5-fold higher promoter activity in the mouse neuroblastoma cell line N18TG2 than a SV40 promoter construct and was also effective in the human neuroblastoma cell line LA-N1 (Figure 4A). In contrast, expression

Figure 5 Binding of transcription factors to exon 0 sequences

(A) Sequence of the genomic promoter fragment in construct pGLg. Arrows indicate the mapped transcriptional start sites. Thick lines delineate the sequences of oligonucleotides P1, P2 and P3 used in gel-retardation and supershift experiments. The sequence that was deleted in construct pGLi is highlighted as a filled box; putative Sp1-binding sites are boxed. (B, C) Supershift (B) and gel-shift (C) experiments with nuclear extracts from N18TG2 cells. (B) Nuclear extracts were incubated with the end-labelled double-stranded DNA fragments P1, P2 and P3 with (+) or without (–) monoclonal antibody against Sp1 as described in the Experimental section. Note the supershifted band in lanes 2, 4 and 8 (arrowheads). (C) Oligonucleotide P2 was incubated with extracts from N18TG2 cells; specific binding was inhibited by adding an increasing molar excess (50-fold and 100-fold) of unlabelled oligonucleotide P2 (lanes 2 and 3) or Sp1 consensus oligonucleotide (lanes 4 and 5). To examine the sequence specificity of binding, a large excess (2.5 µg) of unlabelled poly(dG-dC) was added to another aliquot of the reaction mixture before PAGE (lane 6).

was almost undetectable in the non-neural cell lines 3T3 and HEK-293. As a negative control, a construct containing an upstream 1.1 kb *EcoRI*–*Bam*HI fragment (pGLa; Figure 4A, left panel) was assayed for promoter activity and found to be inactive in all cell lines tested (Figure 4A).

Three other constructs, in which the promoter fragment was consecutively truncated at the 5' end by 282 and 611 bp

(in constructs pGLc and pGLd respectively) and by 1096 bp (in construct pGLe) (Figure 4A, left panel), all displayed promoter activity with N18TG2 and LA-N1 cells (Figure 4A). With the shortest construct tested in these experiments (pGLe), normalized activities in extracts were similar in N18TG2 cells or were even increased approx. 3-fold in LA-N1 cells in comparison with the activities obtained with the full-length fragment (pGLb). In addition, a significant increase in reporter gene expression was seen with all three constructs in 3T3 cells and with constructs pGLd and pGLe in HEK-293 cells (Figure 4A). We conclude that the isolated genomic fragment (pGLb) of the GlyR β subunit gene contains a functional promoter that displays neural specificity.

GlyR β promoter is functional without upstream sequences

To characterize further the minimal promoter region required for transcriptional activity, additional deletion constructs (pGLf to pGLh) were generated and transfected into N18TG2 and HEK-293 cells. Three representative results from a total of six different constructs are shown in Figure 4(A, right panel). Unexpectedly, a further 5' shortening of the promoter fragment did not abolish reporter gene expression. In the shortest construct tested (pGLh), the 5' sequence upstream of the transcription initiation site +16 was deleted; however, this DNA fragment still displayed a promoter activity in N18 TG2 cells similar to that of the SV40 promoter. We therefore conclude that important elements of the GlyR β promoter must be located downstream of the transcriptional start sites.

Sequences within exon 0 determine GlyR β promoter activity

To identify potential regulatory sequences downstream of the transcriptional start sites, we created internal deletions within exon 0. These constructs were generated to eliminate three potential Sp1-binding sites that lay within this GC-rich exonic sequence (Figure 4B). In the first construct, we deleted 115 bp, including all three putative Sp1-binding sites, to generate construct pGLi. On transfection of this construct into N18TG2 and HEK-293 cells, a drastic decrease in promoter activity was observed (Figure 4B). Interestingly, in N18TG2 cells transcription was completely abolished, whereas in HEK-293 cells some remaining luciferase activity could be detected (Figure 4B). Thus the deleted sequence might have been required for efficient expression, particularly in neuronal cells. Consistent with this view was our observation that deletions of the Sp1-binding site at the 3' end of exon 0 in construct pGLj decreased promoter activity by approx. 70% in N18TG2 cells but by less than 50% in HEK-293 cells (Figure 4B). Another internal deletion, removing the Sp1 site nearest the 5' end (construct pGLk), displayed significantly decreased promoter activity in N18TG2 cells, whereas expression levels were unaltered in HEK-293 cells. Taken together, these results show that regulatory sequences, including putative binding sites for Sp1-related transcription factors, are localized within exon 0 of the GlyR β gene. These sequences seem to be particularly important for efficient expression in neuronal cells.

SP1 binds to sequences within exon 0 that are crucial for neural-specific promoter function

To corroborate the functional significance of the putative regulatory sequences deleted in construct pGLi, we designed serial

double-stranded oligonucleotide probes from the β subunit gene sequence to perform bandshift experiments with nuclear extracts from N18TG2 cells (P1, positions 46–74; P2, positions 101–128; P3, positions 130–162; Figure 5A). Oligonucleotide P1 included the first putative Sp1-binding site, deleted in constructs pGLi and pGLk. The SP1 consensus sequence nearest the 3' end was included in oligonucleotide P3; the P2 probe covered a region between both sequences and did not contain a full-length consensus Sp1 site (Figure 5A).

Incubation of oligonucleotides P1, P2, P3 and an Sp1 consensus oligonucleotide with nuclear extracts from N18TG2 cells led to the formation of specific DNA–protein complexes, as demonstrated by retardation of the 32 P-labelled DNA fragments in non-denaturing polyacrylamide gels (Figure 5B). Oligonucleotides P1, P3 and Sp1 were bound in two major differentially migrating DNA–protein complexes (Figure 5B), whereas oligonucleotide P2 was retarded in a single labelled band (Figures 5B and 5C).

Incubation of the DNA–protein complexes generated with extracts from N18TG2 cells by using an Sp1-specific antibody revealed the formation of a high-molecular-mass complex, as indicated by the appearance of a 'supershift' of the labelled oligonucleotides (Figure 5B). Our results show that the corresponding sequences of the oligonucleotides Sp1, P1 and partly P3, but not P2, bound Sp1 factors present in the extract of N18TG2 cells (Figure 5B).

The complex formed with oligonucleotide P2 was not recognized by the Sp1 antibody. Retardation of oligonucleotide P2 by the extract could be efficiently competed for by a 50-fold excess of the unlabelled oligonucleotide but not by the Sp1 oligonucleotide (Figure 5C); this is consistent with the formation of a sequence-specific DNA–protein complex. In conclusion, the sequence deleted in construct pGLi contained binding sites for both Sp1 transcription factor and for one or more other as yet unidentified DNA binding proteins, as revealed by the sequence-specific retardation of oligonucleotide P2.

DISCUSSION

The major finding of this study is the identification of regulatory sequences of the GlyR β subunit gene that are important for its efficient neural-specific expression. Analysis of the 5' region of the GlyR β gene from mouse revealed significant differences in structure in comparison with the corresponding regions of the homologous GlyR α subunit genes. In particular, a novel exon was identified that encodes most of the 5'-untranslated portion of the GlyR β mRNA. As shown by PCR, this exon is present in most, if not all, β gene transcripts. Transfection of the luciferase reporter gene construct pGLb comprising 1.58 kb of 5' flanking upstream sequence and 180 bp of this first exon of the GlyR β gene into the neural cell lines N18TG2 and LA-N1 resulted in efficient luciferase expression; however, almost no promoter activity was seen in two non-neural cell lines. These experiments define a functional promoter region of the GlyR β gene that displays neural specificity.

The difference in luciferase expression between neural and non-neural cell lines seen with construct pGLb decreased on progressive 5' deletion in constructs pGLc to pGLE owing to increasing expression levels in non-neural cell lines. This suggests that neural-specific expression of the GlyR β gene might be determined in part by 5' upstream sequences that suppress expression in non-neural cells. Interestingly, neuron-specific silencer elements (NRSEs) have been identified in a number of genes including the type II Na⁺ channel [34,35], synapsin I [36]

and the m4 muscarinic acetylcholine receptor [37]. NRSEs and the binding protein recognizing the NRSE of the Na⁺ channel gene (neuron-restrictive silencer factor, NRSF) have been shown to confer neural-specific expression by repressing transcription in non-neural cells [38–40]. It remains to be established whether the increased activity in non-neural cells of the GlyR β promoter seen on the deletion of upstream sequences reflects the presence of silencer-like elements in this fragment.

To identify positively acting *cis*-elements of the GlyR β gene, additional deletions of its 5' flanking region were analysed; however, no significant decrease in luciferase activity was found. Surprisingly, even a construct from which all 5' flanking sequence (construct pGLh) had been deleted still displayed promoter activity. This construct included only nt +15 to +180 of exon 0. We therefore reasoned that potential binding sites for Sp1 and other putative transcription factors downstream of the transcriptional start sites might be important for GlyR β gene expression. Indeed, a significant decrease in reporter gene expression levels was obtained on the deletion of sequences within exon 0. Removal of a 115 bp fragment within exon 0 in plasmid pGLi abolished expression in N18TG2 cells completely and decreased expression in HEK-293 cells to approx. 30%. The deleted sequence contained three putative Sp1-binding sites (Figure 1B), from which the site nearest either the 3' or the 5' end was removed in additional constructs. Both produced a significant decrease in promoter activity on expression in N18TG2 cells, whereas in HEK-293 cells deletion of the 3' Sp1 site resulted only in a moderate decrease in promoter activity, and deletion of the 5' Sp1 site had no effect in this non-neural cell line. Thus these Sp1 consensus sequences in concert with their flanking regions are likely to contribute to the neural-specific expression of the GlyR β gene.

The capacity of the deleted sequences to bind transcription factors was examined by gel retardation assays. This revealed binding of Sp1 transcription factors to an oligonucleotide (P1) containing the 5' Sp1 consensus sequence, as deduced from the supershift of the complex formed between P1 and a nuclear protein from N18TG2 cells. We therefore propose that the binding of Sp1 transcription factors within exon 0 contributes to the neural-specific regulation of the GlyR β subunit gene. Other, as yet unidentified, transcription factors might also bind within exon 0 because oligonucleotide P2, which did not contain a complete Sp1 consensus sequence, was also specifically retained. Retardation and supershift experiments with oligonucleotide P3, containing the putative Sp1-consensus binding site nearest to the 3' end, resulted only in a weak supershift signal, suggesting that only a small fraction of the complexes formed contained bound Sp1 protein.

The transcription factor Sp1 is thought to be expressed ubiquitously, a fact that is difficult to reconcile with the neural-specific effect of deletion of the Sp1-binding site observed in our experiments. However, more recently, functional SP1 sites were found in a number of neural-specific promoters, including the $\alpha 3$ and $\beta 4$ genes of the neuronal nicotinic acetylcholine receptor [1,2,41,42] and the genes encoding the *N*-methyl-D-aspartate receptor subunits NR1 and NR2c [43,44]. In addition, the homologous transcription factor Sp3 has been shown to be crucial for the expression of the gene encoding the nicotinic acetylcholine receptor $\beta 4$ subunit [42]. Thus distinct members of the Sp1 family of transcription factors might be generally required for the neural-specific expression of genes encoding ligand-gated ion channel subunits.

The binding of Sp1 to regulatory sequences within the promoter region of genes is known to be important for start site selection [45]. In addition, co-operative activation of transcrip-

tion by a number of clustered Sp1-binding sites has been reported for several genes lacking a TATA box [46]. However, in the promoters described so far, these sites usually are located in the 5' flanking regions of the respective genes. Furthermore, although there is a large number of genes in which *cis*-acting sequences serving as enhancers or silencers are dispersed throughout the entire gene or are localized in distal 5' and 3' flanking regions, only in a few cases have downstream elements been identified that constitute part of a core promoter structure. One such element named MED-1 (for 'multiple start site element downstream') has been shown to be important for start site selection in some TATA-box-deficient promoters [47]. Another example is the cell-type-specific expression of the von Willebrand factor gene that involves a GATA-factor-binding site localized in the first exon [48]. More recently, neural expression of the rat M_1 muscarinic acetylcholine receptor gene was shown to be regulated by elements in the first exon. Interestingly, also in this case the first exon contains SP1-binding sites [49]. Further studies will be required to elucidate which specific regulatory function the different sequence stretches of the GlyR β 5' exon 0 identified here might have *in vivo*.

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