An initiator element mediates autologous down-regulation of the human type A γ-aminobutyric acid receptor β1 subunit gene

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The regulated expression of type A γ-aminobutyric acid receptor ($GABA_AR$) subunit genes is postulated to play a role in neuronal maturation, synaptogenesis, and predisposition to neurological disease. Increases in GABA levels and changes in $GABA_AR$ subunit gene expression, including decreased β1 mRNA levels, have been observed in animal models of epilepsy. Persistent exposure to GABA down-regulates $GABA_AR$ number in primary cultures of neocortical neurons, but the regulatory mechanisms remain unknown. Here, we report the identification of a TATA-less minimal promoter of 296 bp for the human $GABA_AR$ β1 subunit gene that is neuron specific and autologously down-regulated by GABA. β1 promoter activity, mRNA levels, and subunit protein are decreased by persistent $GABA_AR$ activation. The core promoter, 270 bp, contains an initiator element (Inr) at the major transcriptional start site. Three concatenated copies of the 10-bp Inr and its immediate 3′ flanking sequence produce full neural specific activity that is down-regulated by GABA in transiently transfected neocortical neurons. Taking these results together with those of DNase I footprinting, electrophoretic mobility shift analysis, and 2-bp mutagenesis, we conclude that GABA-induced down-regulation of β1 subunit mRNAs involves the differential binding of a sequence-specific basal transcription factor(s) to the Inr. The results support a transcriptional mechanism for the down-regulation of β1 subunit $GABA_AR$ gene expression and raises the possibility that altered levels of sequence-specific basal transcription factors may contribute to neurological disorders such as epilepsy.

transcription | neural specific | epilepsy

GABA (γ-aminobutyric acid) is the major transmitter at inhibitory chemical synapses; yet little is known about the mechanisms underlying the genetic regulation of type A GABA receptor ($GABA_AR$) number and composition. $GABA_AR$ number and subunit mRNA levels are down-regulated in primary brain cultures as a response to persistent activation of the $GABA_AR$ (1–3). Moreover, down-regulation of subunit mRNAs precedes down-regulation of receptor number, indicating that GABA-induced $GABA_AR$ down-regulation is a consequence of decreased mRNA levels (4).

Whereas the effects of GABA are inhibitory in the adult brain, they are excitatory during embryogenesis and early postnatal life (5). A variety of observations indicate that $GABA_AR$ subunit gene expression is regulated by receptor activation (6, 7) regardless of age. $GABA_ARs$ may play a critical role in the development of the central nervous system and so determine its future susceptibility to seizures (8). Extracellular GABA is increased during paroxysmal hippocampal activity in epileptic patients (9), and GABA uptake is inhibited in genetic absence epilepsy rats (10). Both of these changes may lead to increases in the activity of cell surface $GABA_ARs$ and subsequent changes in $GABA_AR$ function through down-regulation of subunit specific gene expression. A decrease in the levels of α1 and β1 $GABA_AR$ subunit mRNAs precedes the onset of spontaneous seizures in pilocarpine-induced status epilepticus (11).

The β1 subunit gene is located in the β1-α4-α2-γ1 gene cluster on chromosome 4 (12) and is most highly expressed in the adult rat hippocampus. Seizure activity decreases hippocampal β1 mRNA levels by about 50% while increasing β3 levels (11). Because the subtype of β subunit influences the sensitivity of the $GABA_AR$ to GABA and to allosteric modulators such as etomidate, loreclezole, barbiturates, and mefenamic acid (13–17), a change in β subunit composition may alter receptor function and pharmacology *in vivo*. Modulation of receptor function by phosphorylation is also influenced by β subunit composition and contributes to increased inhibition during states of neuronal excitability (18).

To determine whether a transcriptional mechanism controls autologous regulation of $GABA_AR$ subunit gene expression, we have begun by identifying the minimal β1 promoter and the genomic mechanism that controls inhibition of promoter activity in primary neocortical cultures exposed to persistent $GABA_AR$ activation.

Materials and Methods

Genomic Walking by Using PCR. Inverse PCR (19) was carried out using *Msp*I and *Nco*I with (5′-CCATGGTCTGTTGTGCACACA G-3′) and (5′-ACTGTCCACATTACTAACTCTGAT3-′) primers. Additional upstream sequence was obtained by performing primer extension with *Taq* polymerase at 55°C for 3 min, yielding a single-stranded cDNA that was recovered by nick column chromatography (Pharmacia). The cDNA (15.4 μl) was tagged at the 3′ end with a cytosine homopolymeric tail, using 2.0 μl of terminal transferase (Pharmacia) in 2.2 μl of 10× phosphate buffer (Pharmacia) and 0.4 μl of 100 mM dGTP for 15 min at 37°C, and then inactivated at 75°C for 10 min. After ethanol precipitation, cDNAs were amplified by PCR using a sense primer that was complementary to the tail (5′-ATAATGGTACCGGGGGGGGGGGGG-3′) and a gene-specific antisense primer directed upstream of the original primer used for the first extension reaction, yielding 112 bp of additional upstream sequence.

Mapping of Transcriptional Start Sites. The locations of transcriptional initiation sites were determined by primer extension, S1 nuclease protection (20), and single-stranded ligation of complementary ends (SLIC) (21) using human brain total RNA (CLONTECH). We modified the SLIC procedure by synthesizing a ligation primer (5′-CTCATACATAGTACGAATTCGG-

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Abbreviations: GABA, γ-aminobutyric acid; $GABA_AR$, type A GABA receptor; Inr, initiator element; β-gal, β-galactosidase; EMSA, electrophoretic mobility-shift analysis; β1-P, β1 promoter; Sv40-P, Sv40 promoter; Sv40-E, Sv40 enhancer; Mt, mutation; SLIC, single-stranded ligation of complementary ends.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF285168).

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TACTGATTAC-3′) that contained an amino group at its 3′ end. This amino group prevents excess ligation primer from initiating a polymerase extension reaction. An antisense primer (5′-TCACTTCGGAGACCATGTCTATGC-3′) that recognizes a sequence 300 bp downstream from the end of the β1 cDNA was used with avian myeloblastosis virus reverse transcriptase to produce a DNA copy of the 5′ end of β1 subunit mRNAs. A second round of modified SLIC was performed by using an antisense primer that contained sequence immediately upstream of the most 5′ end of the first SLIC products.

Transfection Assays. Primary hippocampal, neocortical, muscle, and fibroblast cells were derived from 18-day-old rat embryos and grown in defined media (22). Neuronal cultures were exposed to 1 μM cytosine arabinoside on days 2 and 3 *in vitro*. Cells were transfected 1 wk after dissociation.

PCR products were cloned into Promega GeneLight plasmids (pGL) and into PNASSβ (Clonetech), for fluorescence imaging of β-galactosidase (β-gal) activity in intact neurons (Imagene Green; Molecular Probes). For fluorescence imaging of β-gal activity in hippocampal neurons, on 35-mm dishes, cells were incubated with fresh media (1 ml) containing 2.0–2.5 μl of fresh lipophilic fluorescein di-β-galactopyranoside (FDG) (Imagene Green; Molecular Probes). Internal deletions and site-directed mutagenesis constructs were generated by using the Kunkel method (23). Mt(−1,+2) contains mutation of "C" at −1 to "T" and "G" at +2 to "C." Inserts for luciferase constructs containing one or three copies of the initiator (Inr) and flanking sequence, 5′-TGCGCAGGTCCATTCGGGAATTAC-3′, were produced by using oligonucleotide synthesis. Cells were transfected by using the calcium phosphate technique (24), or by bombardment using the Bio-Rad Biolistic Particle Delivery System, both yielding 5%–8% efficiency. Chemiluminescence (Tropix, Bedford, MA) was used to monitor both luciferase activity driven by β1-P, and β-gal activity driven by the phosphoglucokinase (PGK) promoter in cotransfected cultures. Unless otherwise indicated, promoter activity is expressed as a percentage of the full-length β1 promoter region (β1-P; −436/+105) transfected into neocortical cells.

To control for difference in transfectional efficiency, β1-P activity was compared with that of the Sv40 promoter, with its downstream enhancer (Sv40-P) in sister cultures and with background activity as measured by the pGL2 promoterless vector. Activity was normalized to the amount of protein per dish determined by the Lowry method. Cotransfection with Sv40-P was not used because it decreased β1-P activity, possibly reflecting competition for transcription factors.

GABA Treatment. Fresh GABA solutions were used for each experiment. GABA (500 μM) and SR95531 (250 μM) were dissolved in DMEM. GABA treatment was initiated on day 7. Twenty-nine hours later, cells were transfected for 1 h in DMEM solution containing GABA and/or SR95531, and then DMEM was replaced with conditioned media containing fresh GABA and/or SR95531 solution. Cells were returned to the incubator for 18 h. Cells were exposed to GABA for 48 h.

RNA, Protein, and Ligand Binding. β1 subunit RNA was measured by RNase protection (20) using 20 μg of total RNA hybridized to a 201-bp probe containing sequence specific to the intracellular loop region; Western blot analysis was performed using sc-7361 and sc-7363 (Santa Cruz Biotechnology). Quantitation of enhanced chemiluminescence (ECL; Amersham) signals was done by densitometry with normalization to β-actin expression. [^{3}H]flunitrazepam binding was performed according to Roca *et al.* (1).

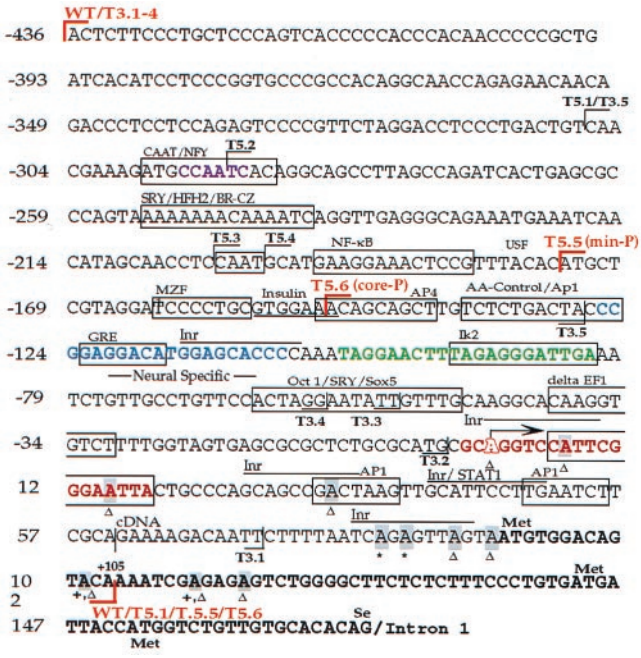

Fig. 1. Nucleotide sequence for the 5′ end of the human β1 subunit gene. Transcriptional start sites mapped by S1 nuclease protection are indicated by +, primer extension by *, and modified SLIC by △ for round one and ◇ for round two. The first nucleotide, A, of the most 5′ transcriptional initiation site is designated as +1. Positions of Inr elements are indicated by overlining. Half brackets mark the 5′ and 3′ ends of β1-P (−436/+105), T5.1–T5.6, and T3.1–T3.5 constructs. The 5′ end of the human cDNA sequence (25) is indicated by a bracket.

Electrophoretic Mobility-Shift Analysis (EMSA) and DNase 1 Footprinting. DNase 1 footprinting (20) was performed by using a 5′ end-labeled *Hin*cII/*Mse*I fragment of the β1 gene. Double-stranded oligonucleotides for EMSA contained the following sequences, where lowercase letters represent mutations: radiolabeled probe 5′-GCATGCGCAGGTCCATTCGGGAATTACTGC-3′; wild-type competitor 5′-TGCGCAGGTCCATTCGGGAATTAC-3′; mutant 1 competitor (M1) 5′-TGCattccTCCATTCGGGAATTAC-3′; M2 competitor 5′-TGCGCAttcttATTCGGGAATTAC-3′; and M3 competitor 5′-TGCGCAGGTCATTCttt-tAtaTAC-3′. Nuclear extracts were prepared (26) and used for gel shift analysis after concentration (Microcon no. 10 columns, Amicon, MA). Quantitation was performed on EMSAs under conditions that yield a standard curve for band intensity.

Statistical Analysis. Unless otherwise indicated, results are reported as mean ± SEM (n), where n is the number of independent experiments. Significance testing is by two-tailed t test.

Results

Identification of the 5′ End of the Human β1 Subunit Gene. The 5′ flanking sequence of the β1 gene was identified by using PCR genomic walking techniques. Results of S1 nuclease protection, primer extension, and modified SLIC identify multiple potential start sites in the 5′ flanking region (−436 to + 105) that are positioned downstream of the 5′ end of the human β1 cDNA (Fig. 1). Start sites at + 1, +7, +15, +33, + 82, +84, +88, and + 91 lie within or adjacent to Inr consensus sequences (27). The major transcriptional initiation site inferred from modified SLIC (second round) corresponds to the most upstream site and is therefore designated as position +1.

Additional potential start sites at +7, +15, and +33 produce transcripts that would code for a signal peptide of 26 aa (using the ATG at +92). The potential sites, at +82, +84, +88, and +91

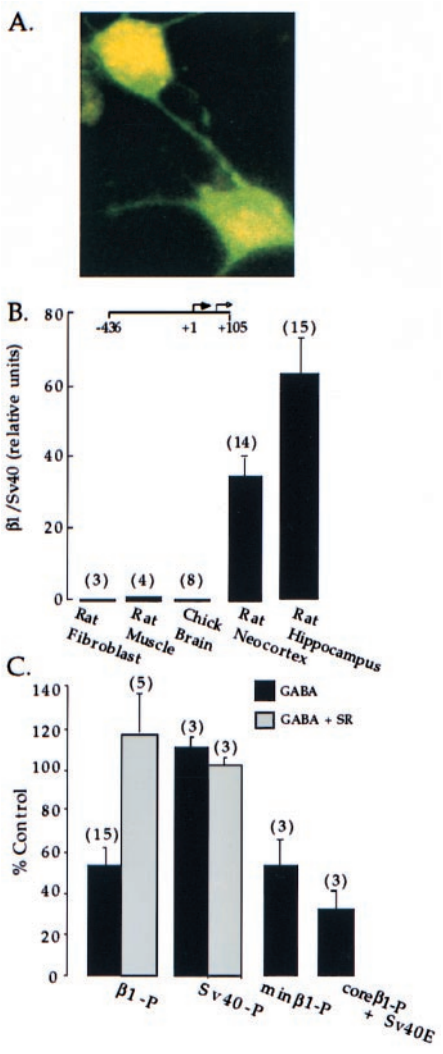

Fig. 2. The immediate 5′ flanking region of the human β1 gene directs neuron-specific expression that is subject to autologous regulation by GABA. (*A*) A β-gal reporter construct containing 541 bp of the β1 subunit gene and incubated with fluorescein di-β-galactopyranoside (a lipophilic substrate for β-gal that yields a fluorescent product on hydrolysis) exhibits functional promoter activity in hippocampal neurons after DNA bombardment. (*B*) Rat muscle, fibroblast, neocortical, and hippocampal cells and chicken brain cells were transfected by Ca^{2+} phosphate with β1-P, and luciferase reporter activity is expressed relative to Sv40-P activity in sister dishes. (*C*) Neocortical neurons were chronically treated with GABA or GABA + SR95531 and transfected with β1-P, Sv40-P, minimal (min) β1-P, or core β1-P + Sv40-E.

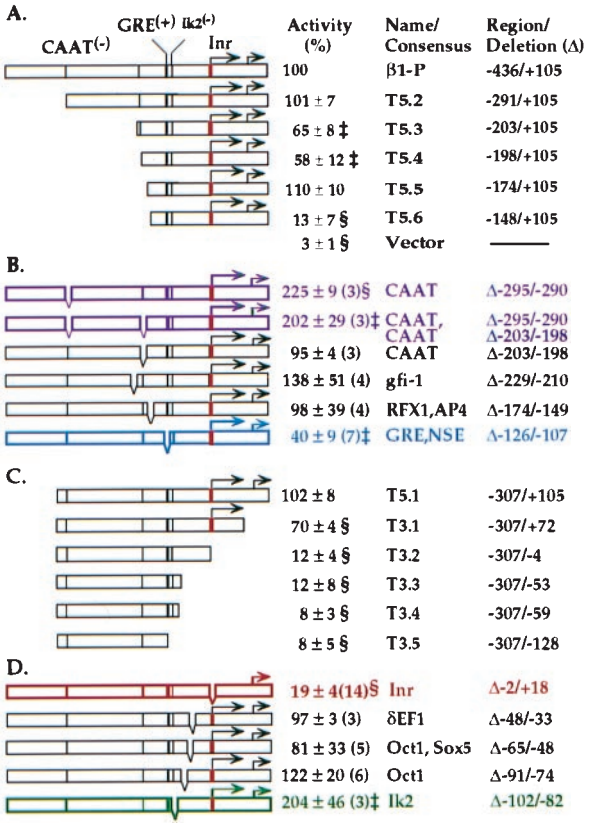

Fig. 3. A minimal promoter and core regulatory elements are revealed by truncation analysis and internal deletions. (*A*) Promoter activity of 5′ truncations (T5.2–T5.6) in transfected primary neocortical neurons is displayed as the percentage of activity relative to β1-P. A red bar denotes the Inr containing the major start site. (*B*) Internal deletions removed or interrupted consensus sequences identified by using Matinspector public and professional services (matrix setting, 0.90; core setting, 0.85; http://www.gsf.de/cgi-bin/matsearch.pl). Purple box and text indicate removal of possible negative control region whereas blue indicates removal of possible positive control region. (*C*) Promoter activity of 3′ truncations (T3.1–T3.5) in transfected neurons is displayed as the percentage of activity relative to β1-P. (*D*) The red box and text indicate loss of the presence of the major Inr whereas the green box and text indicate removal of a possible negative control region. Data represent the means ± SEM of five independent experiments, except where indicated by *n*. ‡, $P < 0.05$; §, $P < 0.01$, Student's *t* test.

are unlikely to be relevant because such transcripts would contain a very small (≤10 bp) untranslated region.

Identification of a Neural Specific Promoter in the 5′ Flanking Region of the β1 Subunit Gene. To study promoter activity relevant to central nervous system function, we developed techniques for transient transfection of primary neurons in culture derived from rat hippocampus and neocortex (Fig. 2). Hippocampal neurons become intensely fluorescent when transfected with a β-gal reporter construct containing β1-P (Fig. 2*A*). To quantitate activity, rat hippocampal, neocortical, muscle, and fibroblast cells and chicken brain cells were transiently transfected with a construct containing β1-P cloned upstream of a luciferase reporter gene. β1-P activity, expressed relative to Sv40-P activity in sister dishes, is greatest in hippocampal cells and parallels the tissue- and region-specific distribution of β1 mRNAs *in situ* (Fig. 2*B*) (28). Differences in transfectional efficiency cannot explain these results because β1-P activity in phosphoglucokinase-cotransfected neocortical neurons (3.1 ± 0.5 cpm luciferase/cpm β-gal, $n = 3$) is 31-fold greater than in fibroblasts (0.10 ± 0.03 cpm luciferase/cpm β-gal, $n = 3$). Furthermore, β1-P activity in the forward orientation (31.95 ± 3.3 × 10^6 cpm/μg protein, $n = 4$) is about 17-fold greater than in the reverse orientation (2.2 ± 0.4 × 10^6 cpm/μg protein, $n = 4$) or with the promoterless construct (1.93 ± 0.3 × 10^6 cpm/μg protein, $n = 4$), consistent with the hypothesis that the region flanking the major rapid amplification of cDNA ends (RACE) product contains a functionally relevant promoter.

Autologous Regulation of Human β1-P Activity by GABA. Persistent exposure of neocortical cells to a saturating concentration of GABA (500 μM, for 48 h) decreases β1-P activity ($P < 0.002$) (Fig. 2*C*). The selective $GABA_AR$ antagonist SR95531 (250 μM) blocks down-regulation, indicating that β1-P activity is mediated by the $GABA_AR$. Similarly, GABA decreases β1-P activity in hippocampal cells to 48% ± 10% of control ($P < 0.02$) and

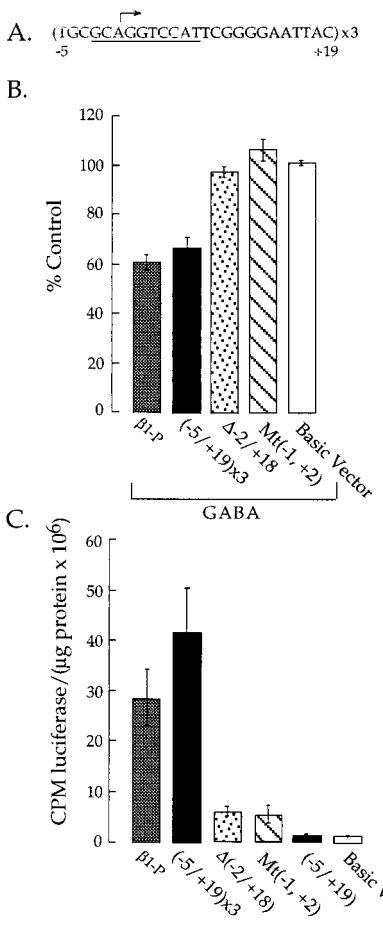

Fig. 4. Importance of the Inr sequence for GABA regulation and promoter activity. (*A*) Sequence of the Inr and downstream flanking region. (*B* and *C*) Primary cultures of rat neocortex were transfected with either β1-P, $(-5/+19)_3$, (−5, +19), Δ(−2/+18), Mt(−1,+2), or a promoterless vector. (*B*) Activity is expressed as the mean percentage of vehicle control ± SEM, $n = 4$. Neurons were exposed to 500 μM GABA for 48 h. (*C*) $n = 3$.

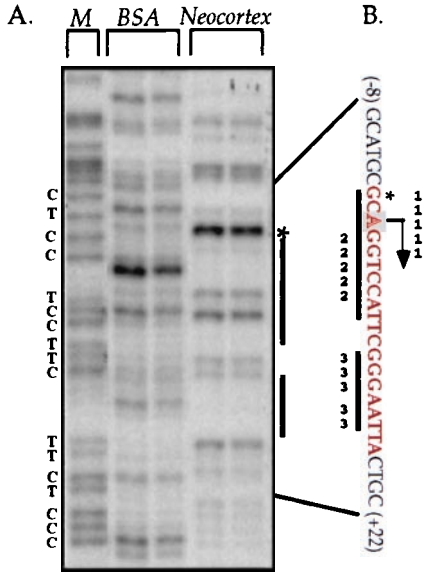

Fig. 5. Nuclease digestion of DNA/protein complexes reveals protection in the region corresponding to the β1 Inr. (*A*) DNase I footprinting identifies nucleotide interactions with putative transcription factors in the vicinity of the major transcription initiation site for the β1 gene [only the region of interest is displayed; lane 1, marker; lanes 2 and 3, BSA (60 μg); and lanes 4 and 5, nuclear extracts from neocortical cultures (60 μg)]. (*B*) Diagram depicts the Inr sequence with reference to protein binding sites and to promoter flanking sequence. The position of a hypersensitive site for nuclease digestion is marked *. Vertical bars indicate region in sequence corresponding to protected region in footprint. Mutations to wild type in M1, M2, and M3 are marked 1, 2, or 3 (cf. Fig. 6; see *Materials and Methods* for sequence).

SR95531 antagonizes the effect of GABA (78% ± 7% control ($P < 0.05$)). Thus, the 5′ flanking region contains the elements necessary to account for the observed down-regulation of β1 mRNA (see Fig. 7*C*). In contrast, GABA is without effect on the Sv40-P activity (Fig. 2*C*), demonstrating that GABA does not decrease the efficiency of transfection.

Identification of a Minimal Promoter. Placing the Sv40 enhancer (Sv40-E) downstream of the luciferase gene increases Sv40-P activity 10-fold but does not change β1-P activity in neocortical cells (data not shown). This observation strongly suggests that β1-P contains the positive regulatory elements necessary for maximum activity, because the powerful Sv40-E can stimulate the activity of all core promoters (29).

To identify the minimal promoter and to begin to locate regulatory sequences, the activities of 5′ truncated (T5.1–T5.6) and full-length β1-P (−436/+105) were determined in neocortical cells (Fig. 3 *A* and *C*). T5.2 (−291/+105) produces full activity (102% ± 8%, $n = 4$) that is orientation-specific (data not shown), and activity is reduced by about 37% on 5′ truncation of an 88-bp region yielding T5.3 (−203/+105). A negative regulatory sequence may be located in the 24 bp region between −174 and −198, as activity returns to control in T5.5 (−174/+105). The minimal promoter region (T5.5) is clearly defined by the next truncation of 26 bp, yielding T5.6 (−148/+105). T5.6 displays only a basal level of activity (13% ± 7% of β1-P, $n = 12$) even though it contains the full complement of potential transcriptional start sites. T5.6 should therefore represent the core promoter.

Identification of a Core Promoter. To test the hypothesis that T5.6 contains a core promoter that depends on an upstream activating sequence, the orientation nonspecific Sv40-E, which is known to act at a distance from core promoters, was cloned downstream of the luciferase gene in T5.6. Sv40-E increases promoter activity from 7.6% ± 3.4% of β1-P to 28% ± 4.5% ($n = 3$), demonstrating that T5.6 contains the elements necessary to support core promoter activity but requires the concerted action of additional positive regulatory sequence for activation.

The fact that Sv40-E does not increase activity of the core promoter to that of β1-P suggests that an additional upstream region is necessary for full tissue-specific promoter activation. As T5.1 (−307/+105, Fig. 3C) is fully active but T5.6 (−148/+105, Fig. 3A) exhibits little activity, sequence(s) yielding net positive regulation should be located upstream of T5.6 in the region between −148 and −307. Cloning of −307/−128 downstream of the luciferase gene in T5.6 failed to increase activity (13 ± 1% β1-P, $n = 4$), indicating that −128 to −307 contains a positionally dependent positive regulatory sequence(s) and is not an enhancer as traditionally defined.

In fibroblasts, core promoter activity (3.8% ± 0.3% of β1-P activity in neurons) is 5-fold greater than minimal promoter (0.76% ± 0.04%) or β1-P activity (0.54% ± 0.04%). This result suggests that β1 promoter activity is suppressed in fibroblasts by the binding of nonneuronal factors to upstream sequence(s). Because core promoter activity in neurons is also low (13 ± 7%), it is clear that upstream regulatory sequences are necessary for full promoter activity. A downstream Sv40-E partially increases

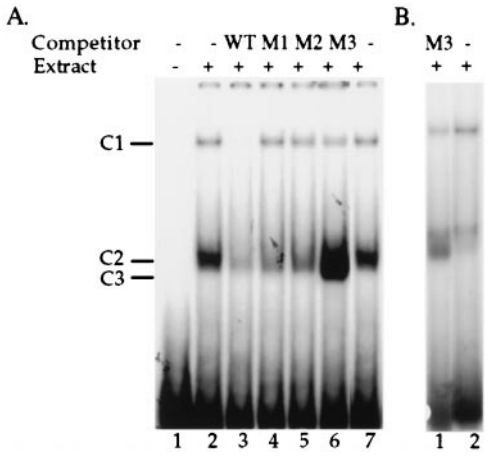

Fig. 6. Sequence-specific binding of nuclear factors to the Inr in the β1 promoter. EMSA using 30 μg of nuclear extracts from neocortical cultures reveals the formation of two complexes, C1 and C2. (*A*) Competition assays containing a 100-fold excess of cold oligonucleotides with mutations are indicated by M1, M2, and M3, and wild type by WT. Competition with M3 produces a new complex, C3. (*B*) C3 can be easily visualized on short exposure to film.

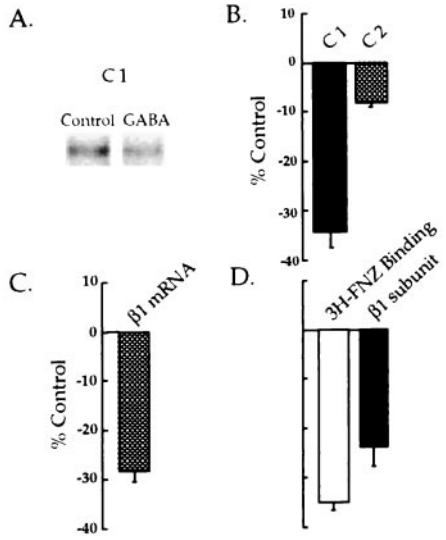

Fig. 7. Decreased formation of protein/DNA complex C1 correlates with down-regulation of β1 mRNA, [^{3}H]flunitrazepam binding, and β1 subunit protein. Neocortical cells were exposed to 500 μM GABA. (*A* and *B*) EMSA using 30 μg of nuclear extracts shows decreased formation of C1 (n = 3), (*C*) RNase protection shows decreased levels of β1 mRNAs (n = 3), and (*D*) radioligand binding using [^{3}H]flunitrazepam and quantitative Western blot analysis of β1 subunit protein levels, n = 3, show down-regulation of functional receptor and subunit. Quantitation was by densitometry (*D*) or phosphorimaging (*B* and *C*).

core promoter activity (25% ± 0.6%, n = 3) by about 6.5-fold but is without effect on β1-P activity in fibroblasts. This observation again suggests that β1-P activity is suppressed in fibroblasts through sequence(s) upstream of −148.

Internal Deletions Reveal the Presence of Potential Regulatory Elements and a Functional Inr. Internal deletion of CAAT (−295/−290) but not CAAT (−203/−198) (Fig. 3*B*, purple) increases promoter activity, indicating that CAAT (−295/−290) can serve as a negative regulatory element in the context of β1-P. Internal deletion of −107 to −126, which contains glucocorticoid receptor element (GRE) and neural-specific element consensus sequences, decreases activity (Fig. 3*B*, Blue), whereas deletion of −102 to −82, which contains consensus sequence for the Ik2 element, increases activity (Fig. 3*D*, green), suggesting that these regions contain positive and negative regulatory elements, respectively. The 26-bp sequence in T5.5, while essential for the activity of the minimal promoter, is not required in the context of β1-P (Fig. 3*B*, Δ−174/−149), indicating that an upstream positive regulatory sequence can compensate for the loss of the 26-bp region.

Deletion of the most 3′ initiation sites reduces activity by 30%, and deletion of the most 5′ initiation sites virtually eliminates activity (Fig. 3*C*). Moreover, an internal deletion of 20 bp (−2/+18, Fig. 3*D*, red), which spans an Inr at the major initiation site identified by SLIC (Fig. 1), dramatically reduces activity.

The β1 Core Promoter Mediates GABA-Induced Down-Regulation. To determine whether GABA-induced inhibition of promoter activity is mediated by sequences that control either basal levels or activated levels of transcription, we first tested the ability of the minimal promoter (−174/+105) to respond to $GABA_AR$ activation. Persistent exposure to GABA reduces minimal promoter activity to 52% ± 12% of control ($n = 3, P < 0.05$), which is similar to its effect on β1-P activity 47% ± 6% of control (n = 3) (Fig. 2*C*). Similarly, core promoter (−148/+105; T5.6) activity driven by a downstream Sv40-E is down-regulated by GABA (35% ± 6%, $n = 3, P < 0.01$). Sv40-E cannot mediate GABA regulation, because GABA does not alter Sv40-P activity in the presence of Sv40-E (Fig. 2*C*). The observation that the minimal promoter and the core promoter are both regulated by $GABA_AR$ activation suggests that down-regulation of β1-P activity may involve a change in the binding of general transcription factors.

Multiple Copies of the Inr Produce Promoter Activity That Is Autologously Regulated. We synthesized a 72-bp construct that contains three copies of the Inr and its flanking sequence [(−5/+19)$_3$, Fig. 4*A*] to test directly whether the Inr might constitute the core promoter and whether GABA could regulate its activity in a similar fashion to that of β1-P (Fig. 4*B*). To our surprise, whereas a single copy of the Inr (−5/+19) was without function, three copies produced activity that was equal to or greater than β1-P (Fig. 4*C*) and responded to chronic GABA treatment with reduced activity (Fig. 4*B*). In addition, (−5/+19)$_3$, like β1-P, exhibited negligible activity in transfected fibroblast cultures (data not shown). Point mutations at −1 and +2 [Mt(−1, +2)] within Inr were generated in the context of β1-P, reducing activity to the same extent as the internal deletion Δ−2/+18 (Fig. 4 *B* and *C*). The remaining activity, which is about 20% of β1-P, is not down-regulated by GABA, demonstrating that the Inr is critical for β1 promoter activity and GABA-induced down-regulation.

Sequence-Specific Binding to the Inr. DNase I footprinting analysis was used to determine whether neocortical nuclear extracts bind to the Inr of β1-P (Fig. 5). At least two distinct footprints can be seen. The first is marked by a hypersensitive band with protection immediately above and below. The second shows protection over a downstream region that contains the CGGGAAT sequence (Fig. 5*B*).

To identify nuclear protein binding sites, EMSA was performed on a 30-bp region of DNA containing Inr (−8 to +22). Two distinct complexes, C1 and C2, are formed on the addition of nuclear extracts (Fig. 6). Oligonucleotide competition confirms the importance of two sequences that were identified by footprinting. Substitution of ATTCC for GCAGG or TTCTT for GGTCC at −2 to +6 inhibits the ability of cold oligonucleotide to compete for the formation of C1 with little effect on competition for C2 (Fig. 6, lanes 4 and 5). In contrast, substitution of TTTATA for GGGAAT at +11 to +16 inhibits the ability of cold oligonucleotide to compete for both complexes, and produces a third complex, C3 (Fig. 6, lane 6). Interestingly, the abundance of C1 is significantly less in binding reactions that

contain nuclear extracts from GABA treated cultures (Fig. 7 *A* and *B*). Moreover, the decrease in the abundance of C1 is consistent with the decrease in β1 mRNA level (Fig. 7*C*), β1 protein, and $GABA_AR$, as measured by [^{3}H]flunitrazepam binding (Fig. 7*D*). Whereas fibroblast extracts bind to the Inr region, there is no change in C1 levels or promoter activity in response to GABA (data not shown).

Discussion

The expression of specific receptor subunits is critical for the functioning of chemical synapses and must be regulated with great fidelity to control neuronal phenotype. As a first step toward identifying the mechanisms and factors responsible for cell type-specific expression of $GABA_AR$ subunit genes and activity-dependent regulation of gene expression, we have isolated a tissue-specific, TATA-less minimal promoter for the human β_1 subunit of the $GABA_AR$. β1 promoter activity is subject to autologous down-regulation by persistent receptor activation. Transient transfection of primary monolayer cultures demonstrates that promoter activity is greatest in rat hippocampal cells, followed by rat neocortical, chicken brain, rat fibroblast, and rat muscle cells (Fig. 2), consistent with the distribution of β1 mRNA (28) and subunit protein (30) *in vivo*.

Functional assays in transfected neuronal cultures strongly suggest that the β1 promoter may be regulated by sequence-specific binding of transcription factors to a specific Inr and that binding is stabilized by upstream and downstream activating sequences. This conclusion is supported by several observations. First, an upstream sequence of 26 bp is sufficient to produce full activity from the core promoter, containing an Inr, and thus defines the minimal promoter (Fig. 3*A*). But removal of the 26-bp sequence in the context of β1-P does not decrease promoter activity, demonstrating that at least two mutually compensatory activating sequences are present in β1-P. Secondly, three concatenated copies of the Inr region produce full promoter activity (Fig. 4*C*) and additional Inr sequences in the β1 gene cannot compensate for the loss of the Inr in either the internal deletion $\Delta(-2/+18)$ or the mutagenized construct Mt(-1, $+2$). Finally, Inr $(-5$ to $+19)_3$ activity is autologously down-regulated by GABA, consistent with the reduction in activity seen for β1-P. These results demonstrate that the Inr at -2 plays an essential role in coupling activation of the core promoter to one or more upstream activating sequences and mediates GABA-induced down-regulation.

Competitive mobility shift analysis (Fig. 6), based on the results of DNase I footprinting (Fig. 5), was used to investigate whether GABA exposure alters the binding properties of nuclear proteins to sequences within the $-5/+19$ region. GABA (48 h) reduces the formation of C1 in nuclear extracts from neocortical cultures (Fig. 7). Results from oligonucleotide competition studies demonstrate that this reduction in binding occurs through the recognition of a 5-bp sequence that flanks the major start site of transcription (Fig. 1), indicating that GABA may produce an alteration in the binding of general transcription factors. In contrast, GABA treatment produces only a small change in the abundance of C2 (Fig. 7*B*).

General transcription factors acting at Inr elements are responsible for specifying the transcriptional start site in TATA-less promoters and certain TATA box-binding protein (TBP)-associated factors (TAFs) can distinguish between different promoters by recognizing variations in the sequence of the Inr (31). Sequence-specific binding of TAFs to an Inr in the absence of TBP can determine the responsiveness of the core promoter to activators (32). Moreover, the TFIID complex can bind to the Inr in the absence of any additional upstream sequence (33), and TAFs and TBP-related factors (TRFs) can mediate tissue-specific activity (34, 35). When taken together with these observations, the finding that three copies of the β1 Inr and its 10-bp 3′ flanking sequence produce full neural specific activity that is down-regulated by GABA, and that Mt(-1, $+2$) eliminates GABA-induced down-regulation and protein binding, suggests that transcriptional regulation by general factors may be an important mechanism for the control of gene expression in the nervous system.

We are unaware of any other finding indicating that persistent activation of a neurotransmitter receptor may regulate formation of the preinitiation complex on a receptor subunit gene. Regulation of the preinitiation complex can in turn control the number or kind of receptors present at the cell surface. Activity-dependent changes in the transcription of $GABA_AR$ subunit genes may underlie the alteration in $GABA_AR$ subunit composition that is believed to occur during neuronal differentiation and during the etiology of certain neurological disorders such as epilepsy.

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1. Roca, D. J., Rozenberg, I., Farrant, M. & Farb, D. H. (1990) *Mol. Pharmacol.* **37,** 37–43.
2. Montpied, P., Ginns, E. I., Martin, B. M., Roca, D., Farb, D. H. & Paul, S. M. (1991) *J. Biol. Chem.* **266,** 6011–6014.
3. Mehta, A. K. & Ticku, M. K. (1992) *Brain Res. Mol. Brain Res.* **16,** 29–36.
4. Lyons, H. R., Gibbs, T. T. & Farb, D. H. (2000) *J. Neurochem.* **74,** 1041–1048.
5. Leinekugel, X., Khalilov, I., McLean, H., Caillard, O., Gaiarsa, J. L., Ben-Ari, Y., Khazipov, R. (1999) *Adv. Neurol.* **79,** 189–201.
6. Poulter, M. O., Ohannesian, L., Larmet, Y. & Feltz, P. (1997) *J. Neurochem.* **68,** 631–639.
7. Tseng, Y. T., Wellman, S. E. & Ho, I. K. (1994) *J. Neurochem.* **63,** 301–309.
8. Swann, J. W., Pierson, M. G., Smith, K. L. & Lee, C. L. (1999) *Adv. Neurol.* **79,** 203–216.
9. Wilson, C. L., Maidment, N. T., Shomer, M. H., Behnke, E. J., Ackerson, L., Fried, I. & Engel, J. (1996) *Epilepsy Res.* **26,** 245–254.
10. Sutch, R. J., Davies, C. C. & Bowery, N. G. (1999) *Neurochem. Int.* **34,** 415–425.
11. Brooks-Kayal, A. R., Shumate, M. D., Jin, H., Rikhter, T. Y. & Coulter, D. A. *Nat. Med.* **10**, 1166–1172.
12. Russek, S. J. (1999) *Gene* **227,** 213–222.
13. Halliwell, R. F., Thomas, P., Patten, D., James, C. H., Martinez-Torres, A., Miledi, R. & Smart, T. G. (1999) *Eur. J. Neurosci.* **8,** 2897–2905.
14. Hill-Venning, C., Belelli, D., Peters, J. A. & Lambert, J. J. (1997) *Br. J. Pharmacol.* **120,** 749–756.
15. Sanna, E., Murgia, A., Casula, A. & Biggio, G. (1997) *Mol. Pharmacol.* **51,** 484–490.
16. Rabow, L. E., Russek, S. J. & Farb, D. H. (1995) *Synapse* **21,** 189–274.
17. Sigel E., Baur R., Trube G., Mohler H. & Malherbe P. (1990) *Neuron* **5,** 703–711.
18. McDonald, B. J., Amato, A., Connolly, C. N., Benke, D., Moss, S. J. & Smart, T. G. (1998) *Nat. Neurosci.* **1,** 23–28.
19. Triglia, T., Peterson, G. & Kemp, D. J. (1988) *Nucleic Acids Res.* **16,** 8186.
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1988) *Current Protocols in Molecular Biology* (Wiley, New York).
21. Edwards, J. B, Delort, J. & Mallet J. (1991) *Nucleic Acids Res.* **19,** 5227–5232.
22. Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G. & Cotman, C. W. (1993) *J. Neurosci.* **13,** 1676–1687.
23. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
24. Xia, Z., Dudek, H., Miranti, C. K. & Greenberg, M. E. (1996) *J. Neurosci.* **16,** 5425–5436.
25. Garrett, K. M., Saito, N., Duman, R. S., Abel, M. S., Ashton, R. A., Fujimori, S., Beer, B., Tallman, J. F., Vitek, M. P. & Blume, A. J. (1990) *Mol. Pharmacol.* **37,** 652–657.
26. Therrien, M. & Drouin, J. (1993) *Mol. Cell. Biol.* **13,** 2342–2353.
27. Kollmar, R. & Farnahm P. J. (1993) *Proc. Soc. Exp. Biol. Med.* **203,** 127–139.
28. Laurie, D. J., Wisden, W. & Seeburg, P. H. (1992) *J. Neurosci.* **12,** 4151–4172.
29. Kermekchiev, M., Pettersson, M., Matthias, P. & Schaffner, W. (1991) *Gene Expression* **1,** 71–80.
30. Li, M & De Blas, A. L. (1997) *J. Biol. Chem.* **272,** 16564–16569.
31. Verrijzer, C. P., Chen, J.-L., Yokomori, K. & Tjian R. (1995) *Cell* **81,** 1115–1125.
32. Chalkley, G. E. & Verrijzer, C. P. (1999) *EMBO J.* **17,** 4835–4845.
33. Pugh, B. F. & Tjian, R. (1991) *Genes Dev.* **11,** 1935–1945.
34. Chang, M. & Jaehning, J. A. (1997) *Nucleic Acids Res.* **25,** 4861–4865.
35. Hansen, S. K., Takada, S., Jacobson, R. H., Lis, J. T. & Tjian, R. (1997) *Cell* **91,** 71–83.