Isolation and Characterization of the 5′-Upstream Region of the Human N-type Calcium Channel α1B Subunit Gene

CHROMOSOMAL LOCALIZATION AND PROMOTER ANALYSIS*

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电压敏感型钙离子通道 (VSCC)1 发现在细胞膜中许多可兴奋细胞能够调节钙离子进入，形成一个广泛的生理功能网络，比如细胞膜的可兴奋性，神经递质的释放，以及兴奋-收缩偶联等。这些功能是通过电压敏感型钙离子通道 (VSCC) 来调节的。

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was the 443-bp cDNA insert isolated from p60Z plasmid (12) corresponding to nucleotide residues 2803–3246 in GenBank™ accession no. M57682. Following hybridization the blot was rinsed in solution I (2 × SSC and 0.5% SDS) at room temperature for 15 min twice and washed in solution II (0.1 × SSC and 0.1% SDS) at 50°C for 40 min with a change of fresh solution. The blots were exposed to Biomax MR x-ray film (Eastman Kodak Co.) at –80°C for 3 days.

DNA Cloning and Sequencing—To isolate the promoter of the human α1b subunit gene, approximately 2 × 10⁷ recombinant phages from a human WI-38 lung fibroblast cell αFix genomic DNA library (Stratagene, La Jolla, CA) were screened with both the 230-bp α1b cDNA probe and a mixture of two oligonucleotide probes (hNAG1 and hNAG2). Oligonucleotide sequences are: hNAG1, 5′-CCA GCG GGT CTT CTA CAA GAC ATC GAT GCC GCA GGG CGC GG CAG-3′ (285–308, GenBank™ accession no. M94172); hNAG2, 5′-AGA GCC ACG GGC TGT CGG TGA AGC AGG TCT GCT GTC GGA GGA-5′ (328–371, GenBankTM7 accession no. M94172). Plaques were transferred to nitrocellulose filters, and the filters were prehybridized in 20% formamide, 5 × SSPE, 1 × Denhardt’s solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA for 4 h and hybridized overnight at 42°C with the 3²P-labeled probes at 1 × 10⁶ cpm/ml of hybridization solution. Filters were washed at room temperature in 2 × SSC, 0.2% SDS for 15 min three times and at 62°C in 0.1 × SSC, 0.1% SDS for 30 min twice. Autoradiography was carried out for 48 h at –80°C with Kodak X-OMAT AR film.

The genomic inserts isolated from positive plaques were subcloned into pGEM7Zf (+) plasmid (Promega, Madison, WI). Both strands of the genomic inserts were sequenced by the chain termination sequencing method (20). Sequence analysis and data base searches were performed with the GCG software package.

Primer Extension Analysis—A 21-mer antisense oligonucleotide primer (NAPE1, as indicated in Fig. 2), complementary to a portion of the first exon (56–76; GenBank™ accession no. M94172) of the human N-type calcium channel α1b gene, was end-labeled with 3²P by T4 polynucleotide kinase. The 3²P-labeled NAPE1 was annealed to 2 μg of human neuroblastoma SH-SYSY cell poly(A) RNA in 40 μm PIPES (pH 6.8), 1.25 mM EDTA (pH 8.0), 125 mM NaCl, and 75% formamide for 1 h at 42°C. Hybrids were ethanol-precipitated and extended by avian myeloblastosis virus (AMV) reverse transcriptase in a mixture containing 0.06 μg of actinomycin D. Extension products were analyzed on 8% polyacrylamide-urea sequencing gels.

Fluorescence in Situ Hybridization—The plasmid containing a 9.5-kb human α1b subunit genomic insert (pNAG Sac2-2) was labeled with biotin-DUTP by nick translation. The labeled probe was hybridized to formaldehyde-fixed metaphase chromosomes from phytohemagglutinin-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2 × SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceine conjugated avidin. The slides were counterstained with propidium iodide and analyzed.

Plasmid Construction—The human α1b gene-luciferase fusion plasmid was constructed by subcloning into the polylinker region of pG2L2 Basic vector (Promega) with the following restriction fragments from the human α1b genomic clone pNAG Sac2-2: a 4.0-kb BamHI/BssHI fragment (∼39921), a 1.7-kb XhoI/BssHI fragment (∼17881), and 0.1-kb NotI/BssHI fragment (∼1100). Another deletion constructs were generated by Discrete-Delete ExoIII/mung bean nuclease deletion kit (Epicentre Technologies, Madison, WI). All plasmids were sequenced to determine the deletion end points and to exclude the possibility of recombination in host Escherichia coli. The control plasmids pRSVL (a gift from Dr. Sung O Huh; Sloan-Kettering Institute, New York) and pCMVβ (Stratagene, La Jolla, CA) contain the Rous sarcoma virus (RSV) promoter fused to the luciferase gene and the cytomegalovirus (CMV) promoter fused to the β-galactosidase gene, respectively.

Cell Culture—Human neuroblastoma SH-SYSY and BE(2)-C cells, which were provided by Dr. June Biedler (Sloan-Kettering Institute, New York), were grown in 1:1 Eagle’s minimal essential medium and Dulbecco’s modified Eagle’s medium containing 10% FBS, 100 μg/ml streptomycin, and 100 μg/ml penicillin G. Human neuroblastoma N280Y and human glial cell line U251 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS and 5% horse serum. HeLa and HepG2 cells were grown in Eagle’s minimal essential medium with 10% FBS. All culture media were supplemented with penicillin G (100 units/ml) and streptomycin (100 μg/ml).

Fig. 1. Comparison of the tissue distribution of the calcium channel between human α1b and α1d subunit mRNAs. 3²P-Labeled α1b (A) and α1d (B) probes were hybridized to the human multiple tissue Northern blots as described under “Materials and Methods.” A 230-bp PCR product containing the N-terminal domain of the human α1b cDNA and a 443-bp fragment including the intracelular loop domain II of the rat α1d cDNA were used as hybridization probes. The tissues tested were heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). Sizes are indicated in kilobase pairs.

Transient Transfection and Luciferase Assay—Equimolar amounts of the human α1b gene-luciferase plasmids (3 μg) were used for the shortest deletion construct (−110L) and pCMVβ were cotransfected into subconfluent cells in 60-mm culture dishes using LipofectAMINE (Life Technologies, Inc.). Cells were harvested 24 h after transfection and lysed in 1 × cell culture lysis reagent, and activities were assayed using luciferase assay reagent (Promega). The light emitted was integrated over a 15-s interval on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) and expressed as light units.

β-Galactosidase was monitored by an assay kit in the same lysate (Promega). The luciferase activity of fusion constructs was normalized to β-galactosidase activity and expressed as a percentage of the RSV promoter activity of pRSVL.

Reverse Transcription (RT)-PCR Analysis—Total RNAs were prepared by a guanidinium thiocyanate-phenol extraction method (21). First strand cDNA was synthesized using 1 μg of total RNA, which had been treated with RNase-free DNase, by using SuperScript premplification system (Life Technologies, Inc.). Following the first strand cDNA synthesis, PCRs were done in a 50-μl reaction mixture containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 100 μM of each dNTP, 1.25 units of Taq polymerase (Perkin-Elmer), and 50 pmol each of primer pairs for α1b subunit gene and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used were as follows (the nucleotide residue number and accession nos. are in parentheses): CaCh SpI, 5′-ACG CCA TCA TCA TCG GCA TGC TGG-3′ (4838–4863, M29965); CaCh AsoI, 5′-CCT AGG ATG GAA GAA TCG CGT GT-3′ (5143–5165, M92965); GAPDH-S, 5′-AGA CAT TGT TGC TGT CAT CAA CAG CTA-3′ (108–129, M17701; GAPDH-AS, 5′-ATG AGC CCT TCG ATG CCA AAG-3′ (5258–5276, M17701). The PCR was performed for 30 cycles (1 min at 94°C, 2 min at 60°C and 2 min at 72°C). The final extension was carried out at 72°C for 5 min at the end of cycling. The amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

RESULTS

Differential Expression of α1b and α1d Transcripts—The distribution of α1b and α1d Ca²⁺ channel transcripts in human tissues was examined by Northern blot analysis. A single 10.5-kb transcript of the α1β gene was present in the brain, but there was no hybridized band detected in other human tissues examined (Fig. 1A). The size of the α1b mRNA present in human brain is similar to the 10-kb rat brain transcript (15). The α1d cDNA probe, which is derived from the least conserved intracelular linker of the II and III loop of the α1d subunit (10), strongly hybridized to the transcripts of two sizes, 9.5 and 8.5 kb, in the brain, placenta, lung, liver, kidney, and pancreas. An additional band of 6.5 kb was detected in the kidney and pancreas (Fig. 1B). No bands were detected in the heart and
skeletal muscle, indicating that the α1D probe did not cross hybridize to the L-type α1C and α1S subunit transcripts. The 9.5-, 8.5- and 6.5-kb sizes of the human α1D transcripts are similar to those present in rat brain (10, 22), but smaller than the 11-kb transcript found in pancreatic islets (23). Thus, expression of N-type Ca$^{2+}$ channel α1B subunit, unlike the DHP-sensitive L-type channel which shows a broad distribution pattern in various tissues, is limited to nervous tissues.

Cloning and Nucleotide Sequence of the 5'-Upstream Region of Human α1B Subunit Gene—Screening a human lung fibroblast genomic library yielded two overlapping clones which, taken together, contained 6.5 kb of the 5'-upstream region, the first exon, and 2.5 kb of the first intervening sequences. A major portion, approximately 4.7 kb, of the human α1B genomic clone was sequenced and is shown in Fig. 2. The 4670-nt sequence contains 3991 nt of the 5'-flanking region, 430 nt of the first exon (148 nt of 5'-untranslated region and 282 nt of coding sequence), and the 249-nt part of the first intron. The exon 1 sequence is identical to that of the human N-type Ca$^{2+}$ channel cDNA reported by Williams et al. (24).

Multiple GC boxes (GGGCGG), Sp1-binding sites (25), are found in the proximal 5'-flanking region between -388 and -75. Two of the GC boxes overlap with the NGFIA-binding site (GGCGGGCG) (26) located at nucleotides -118 to -110. Two potential AP1-binding sites (TGAGTCAG) (27) are located at -2572 and -2724. Interestingly, nine copies of a 39-bp direct repeat are found in the region between -2280 and -2530.

An inverted sequence of the core motif CCAGGAG (14) shared by several neuron-specific genes is found at -1719 to -1713. This consensus element or its inverted sequence occurs in the 5'-flanking region of the genes encoding the rat type II Na$^{+}$ channel (14), the rat peripherin (30), the mouse neurofilament (31), the rat SCG10 (32), rat GAP-43 (33), and the mouse synapsin II (34).
reverse transcriptase, and the extended products were separated by polyacrylamide-urea gel electrophoresis. A predominant band of 79 nt and a weaker band of 83 nt were detected on an autoradiogram (Fig. 3, lane 1). No extended products were observed when extension reaction was performed with E. coli tRNA templates which was used as a control for specificity of hybridization (Fig. 3, lane 2). From the size of the extended products and the location of the NAPE1 oligonucleotide primer, we were able to place major and minor initiation sites at 148 and 152 nt upstream of the translation start site, respectively.

Analysis of the sequence immediately upstream of the transcription initiation sites reveals that the $\alpha_{1B}$ subunit gene promoter contains a CCAAT box (−59 in antisense orientation) but lacks a typical TATA consensus motif. The sequences surrounding the major (GGTGAGGC) and minor transcription initiation sites (GTCGGGTG) are different from the initiator sequence (CTCANTCT) present in the promoter region of TATA-less genes, including the synapsin I gene (35) (the underlined nucleotide represents the transcription initiation site). In addition, this promoter is highly rich in G+C content and 72 CpG and 85 GpC dinucleotides are located in a region of 500 bp (positions −400 to +100).

Chromosomal Localization of $\alpha_{1B}$ Gene—Fluorescent in situ hybridization using the biotin-labeled probe resulted in specific labeling of the distal end of long arm of chromosome 9 (Fig. 4A). A second experiment was carried out in which a chromosome 9 centromere-associated satellite probe was cohybridized with the human $\alpha_{1B}$ genomic probe to confirm the identity of the specially labeled chromosome. This experiment showed the specific labeling of the centromeric heterochromatin and the distal long arm of chromosome 9 (Fig. 4B). Measurement of 10 specifically hybridized chromosome 9s demonstrated that the human Ca$^{2+}$ channel $\alpha_{1B}$ gene is located at a position which is 97% of the distance from the centromere to the telomere of chromosome arm 9q, an area that corresponds to band 9q34. A total of 80 metaphase cells were examined with 65 exhibiting specific signals.

Cell Type-specific Expression by 5'-Flanking Region of the $\alpha_{1B}$ Gene Promoter—To address whether the 5'-flanking sequence of the human $\alpha_{1B}$ subunit gene contains the regulatory sequences utilized in a cell type-specific manner we made a fusion gene construct −3992L, containing a 4.0-kb 5'-flanking sequence of the $\alpha_{1B}$ gene (−3992 to +86) linked to the promoterless luciferase reporter vector pGL2-Basic. This plasmid was transfected into a variety of neuronal and nonneuronal cell lines and assayed for luciferase activity. As controls, the plasmids pGL2-Basic and pRSVL were transfected into parallel cultures of each cell line. In all the cell lines tested, the pGL2-Basic plasmid was ineffective in driving expression of luciferase activity, while transfection of the pRSVL resulted in high levels of expression. The results of such an analysis are shown in Fig. 5A. In neuronal cells such as SH-5YSY, BE(2)-C, NS20Y, NG108-15, and PC12 cells, luciferase activities from the $\alpha_{1B}$ fusion gene construct −3992L, were approximately 40–60% of those from the RSV promoter. In contrast, reporter gene expression was very low, maximally 5% of RSV activity, in the glioma cell line U251 as well as in the nonneuronal HeLa and HepG2 cells. Interestingly, the luciferase gene was poorly expressed in one of the mouse neuroblastoma-rat glioma hybrid cell lines, 140-3 cells, consistent with our recent electrophysiological studies showing that 140-3 cells do not express any of high voltage-activated currents (36).

RT-PCR, which was carried out to detect the endogenous $\alpha_{1B}$ mRNA expression in the same cell lines used in transfection studies, yielded the amplified product corresponding to the predicted size of 355 bp in SH-5YSY, BE(2)-C, NS20Y, NG108-15, and PC12 cells but not in 140-3, U251, HeLa, and HepG2 cells (Fig. 5B). The level of endogenous $\alpha_{1B}$ gene expression in NS20Y, NG108-15, and PC12 cells, as judged by the intensity of the amplified bands on agarose gels, seemed to be higher than in SH-5YSY and BE(2)-C cells, suggesting that there is a good correlation between reporter gene expression from the $\alpha_{1B}$-luciferase fusion gene construct and endogenous $\alpha_{1B}$ gene expression. Taken together these results, we conclude that the 4.0-kb 5'-flanking sequence contains the cis-regulatory elements important for directing expression of the $\alpha_{1B}$ gene in

![Figure 3](image3.png)

**Figure 3.** Mapping of the transcription initiation site of the human N-type calcium channel $\alpha_{1B}$ gene. Primer extension using 2 μg of poly(A)$^+$ RNA of human neuroblastoma SH-5YSY cells (lane 1) or 2 μg of E. coli tRNA was carried out as described under "Materials and Methods." The arrowheads indicate two transcription initiation sites. On the left is shown a sequencing ladder which was used as a size marker.

![Figure 4](image4.png)

**Figure 4.** Fluorescence in situ hybridization of the human $\alpha_{1B}$ gene to human metaphase chromosome. The chromosome location of the human $\alpha_{1B}$ gene was determined by fluorescent in situ hybridization as described under "Materials and Methods." A, $\alpha_{1B}$ gene probe alone. B, $\alpha_{1B}$ gene probe was cohybridized with a 9-centromere-associated satellite probe. The arrows indicate the fluorescent signal of the $\alpha_{1B}$ subunit gene.
a neuron-specific manner.

The Distal Upstream Region of the \( \alpha_{1B} \) Gene Promoter for Neuron-specific Expression—To locate a cis-acting regulatory element for neuron-specific expression of the \( \alpha_{1B} \) gene, a series of \( \alpha_{1B} \)-luciferase fusion plasmids were constructed and transfected into NS20Y cells and HeLa cells. Progressive 5’ deletions between nucleotides 23992 and 2110 were made from the 23992L construct using either specific restriction enzymes or exonuclease III digestion protocols. As shown in Fig. 6A, a deletion from 23992 to 21788 resulted in an approximately 10-fold increase in luciferase activity in HeLa cells, but no change in NS20Y cells, indicating the presence of a repressor element between 23992 and 21788 that inhibits the reporter gene expression in HeLa cells. Further deletions of the region between 21788 and 21289 had little effect on luciferase activity in both cell lines (Fig. 6A). However, removal of the region from 21289 to 21057 resulted in a small but significant 2.0-fold increase only in HeLa cells, suggesting that this region may contain another weak repressor element. Extension of 5’ deletions to nucleotide 2110 gradually reduced the luciferase activity in NS20Y and HeLa cells (Fig. 6A).

**DISCUSSION**

In this study we report the cloning, chromosomal localization, and molecular analysis of the 5’-flanking region of the human N-type \( \text{Ca}^{2+} \) channel \( \alpha_{1B} \) subunit gene. A single 10.5-kb \( \alpha_{1B} \) mRNA transcript was detected only in the brain among the human tissues examined, whereas \( \text{L-type} \) \( \alpha_{1B} \) mRNAs were detected in a variety of tissues (Fig. 1). The \( \alpha_{1B} \) transcripts...
were generated from the single α1B gene, which was mapped to the distal end of the long arm of human chromosome 9 (Fig. 4), utilizing the major transcription start site located at 148 nt and the minor start site located at 152 nt 5′-upstream from the ATG translation start site (Fig. 3). The 4.0-kb 5′-flanking sequence of the α1B gene contained a promoter which was capable of directing expression of the α1B transcript in neuronal cells and repressing its expression in nonneuronal cells (Fig. 5). Deletion analysis of α1B subunit-luciferase fusion gene constructs indicated the presence of cis-acting regulatory elements located in the distant upstream region (−3992 to −1788) that may be critical for the neuron-specific expression of the α1B subunit gene (Fig. 6).

Over the past decade, considerable progress has been made in elucidating molecular mechanisms for the transcriptional activation of tissue- and cell type-specific expression of genes in nonneuronal cell types, such as erythrocytes, lymphocytes, and hepatocytes. More recently, the molecular bases for neuron-specific gene expression has also been examined (reviewed in Refs. 37 and 38). The transcription factor, termed neuronal-restrictive silencer element (NRSE)-binding factor (NRSF) (39–41) binds a 21-bp NRSE sequence present in the 5′-upstream region of neural-specific genes to selectively repress the transcription of these genes in nonneuronal cells.

The restricted expression of the N-type α1B gene in the central nervous system and wide distribution of L-type α1D transcripts provide us with an excellent opportunity to examine and compare molecular bases governing Ca2+ channel gene expression. Consistent with the broad mRNA expression within and outside of the central nervous system, we did not find any sequences with similarity to the NRSE sequence in the 5′-upstream region of the rat α1D gene. Furthermore, the transcription of the rat α1D gene is regulated by both cis-acting positive and negative elements in the 5′ promoter region and by an enhancer that consists of (ATG)3-trinucleotide repeats (19). Inspection of 5′-flanking sequence of the human α1B gene, however, revealed the nucleotide sequence (NRSE-α1B) homologous to the NRSE (nucleotide −810 to −799 as shown in Fig. 2). Although overall sequence identity to the 21-bp NRSE consensus sequence is 57%, the 5′ half of 10-bp NRSE-α1B fragment showed a 50% sequence identity to that of the NRSE. We have subcloned NRSE-α1B into the 5′ upstream of SV40 promoter linked to luciferase reporter gene to test whether or not an NRSE-α1B could function as a repressor element in nonneuronal cells. Luciferase activity assay showed that one or two copies of this putative motif did not affect the SV40 promoter activity, whereas one copy of the NRSE from SCG 10 gene was sufficient to repress its activity to 30% of the control in HeLa cells (data not shown). Since the promoter activity of 4.0-kb 5′ flanking region of the human α1B gene in various cell lines was in excellent agreement with RT-PCR analysis of the endogenous α1B mRNA expression (Fig. 5), we used the two of these lines, NS20Y and HeLa, to search for the cis-acting regulatory elements further 5′ upstream of the α1B gene. In vitro transient transfection of truncated α1B-luciferase fusion gene indicated that the region between −3992 and −1788 contains a repressor element(s) responsible for the neuron-specific expression of the N-type α1B subunit gene (Fig. 6). Since sequence analysis did not indicate the presence of any sequence with similarity to the NRSE, a repressor functional in the N-type α1B subunit gene may be distinct from the ones already identified. Further studies are required to establish whether this region contains a unique neuron-specific element that is capable of binding a NRSE.

Our results in the present study suggest that selective repression by cis-regulatory elements is responsible for neuron-specific expression of the human Ca2+ channel α1B subunit gene as is the case for the type II Na+ channel and other genes exclusively expressed in the nervous system. In addition to the NRSE that is the primary determinant for the tissue specificity, the core promoters are also important for conferring substantial neuronal specificity to several genes such as synapsin I, II, and myelin basic protein (34, 42, 43). In contrast, the activity of the human α1B subunit gene core promoter (−110L plasmid) was apparently similar in NS20Y and HeLa cells (Fig. 6A), indicating that the core promoter itself does not confer the neuron specificity to the α1B gene. However, we cannot rule out the possibility of concerted interactions between cell type-specific distal upstream repressor element(s) and the general minimal promoter.

In summary, we have presented an initial characterization of the human N-type Ca2+ channel α1B subunit gene and identified a region in the 5′-upstream of the gene (−3992 and −1788) that contains negatively acting cis-regulatory elements responsible for neuron-specific expression of the α1B gene. Further deletion analyses of the region between −3992 and −1788 and the studies of the DNA-protein interactions between transcription factors and the putative repressor elements should help to elucidate the molecular mechanisms of transcriptional regulation underlying spatiotemporal expression of VSCC α1 subunit genes in the nervous systems.

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