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Isolation and Functional Characterization of the 5′-Upstream Region of Mouse P/Q-Type Ca^{2+} Channel α_{1A} Subunit Gene

Eiki Takahashi,*,†,[1] Yoshiyuki Murata,* Tohru Oki,* Norimasa Miyamoto,* Yasuo Mori,‡,§ Naoyuki Takada,§ Hiroshi Wanifuchi,§ Nobuko Wanifuchi,§ Ken-ichi Yagami,† Tetsuhiro Niidome,* Isao Tanaka,* and Koichi Katayama*,†

*Tsukuba Research Laboratories, Eisai Co. Ltd., 5-1-3, Tokodai, Tsukuba, Ibaraki, 300-2635, Japan; †Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki, 305-8575, Japan; ‡Department of Information Physiology, National Institute for Physiological Science, Okazaki, Aichi, 444-8585, Japan; and §Institute of Molecular Pharmacology and Biophysics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0828

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The ω-agatoxin-IVA-sensitive P/Q-type Ca^{2+} channel is predominantly expressed in the nervous system. To dissect the molecular mechanisms underlying the neuron-specific expression of the P/Q-type channel, we have isolated and characterized the 5′-upstream region of the mouse α_{1A} subunit gene. A transcription start site appeared to exist at −269 bp upstream from the start codon as found by 5′ RACE analysis. The proximal promoter of the α_{1A} subunit gene lacks a typical TATA box, but contains several transcription factor binding sequences, including two Sp1 sites. When linked to a placental alkaline phosphatase (PLAP) reporter gene to examine the promoter activity, the 6.3-kb (−6,273 to +269) 5′-upstream region, but not a smaller 3.0-kb construct (−3,021 to +269), was able to drive the reporter gene in neuron-like PC12 cells. In contrast, neither of these constructs enhanced the PLAP expression in fibroblast NIH3T3 cells. The sequence between 6.3 and 3.0 kb of the 5′-upstream region did not show promoter activity in either of the cell lines, but enhanced TK promoter activity in PC12 cells, though not in NIH3T3 cells. These results suggest that neuron-specific elements of the α_{1A} subunit gene are likely to be located in the distal upstream regions (−6,273 to −3,021) of the 5′-upstream sequence.

High voltage-sensitive Ca^{2+} channels regulate Ca^{2+} entry into cells and thereby contribute to various physiological functions including membrane excitability, gene expression, neurotransmitter release and synaptic plasticity in the nervous system (1). Several types of Ca^{2+} channels (L, N, P, Q and R) have been identified based on their pharmacological and physiological properties (2, 3, 4, 5). These Ca^{2+} channels have been reported to be composed of at least three subunits, α_1, α_2-δ, and β subunits (6). The α_1 subunit is a pore-forming component, functions as the voltage-sensor, and is capable of generating Ca^{2+} channel activity in heterogeneous expression systems (7). Molecular cloning studies have revealed that α_{1S}/α_{1C}/α_{1D}, α_{1A}, α_{1B} and α_{1E} genes encode the α_1 subunits of the dihydropyridine (DHP)-sensitive L-type channels, ω-agatoxin-IVA (ω-Aga-IVA)-sensitive P/Q-type channel, ω-conotoxin-GVIA (ω-CgTx-GVIA)-sensitive N-type channel and DHP/ω-Aga-IVA/ω-CgTx-GVIA-insensitive R-type channel, respectively (7, 8, 9, 10).

A non-L-type subfamily, unlike the L-type subfamily, is predominantly expressed in the nervous system (8, 9, 10). In addition, distinct expression patterns of α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E} subunit genes in the nervous tissues have been demonstrated (11). Considering that the nervous tissues are composed of highly heterogeneous cell populations with distinct functional features, it is important to understand the molecular mechanisms that regulate the temporal and spatial expression patterns of α_1 subunit genes. Recent studies have provided information about the transcriptional mechanisms for the rat L-type α_{1D} (12) and human N-type α_{1B} (13) subunit genes, but little is known about the P/Q-type α_{1A} subunit gene. Because of the specific expression patterns, it is of interest to examine the molecular mechanisms of P/Q-type α_{1A} subunit gene expression and to compare them with those involved in L-type α_{1D} and N-type α_{1B} subunit gene expression. Here we describe the isolation of an 8.4-kb genomic

[1] To whom correspondence should be addressed at Tsukuba Research Laboratories, Eisai Co. Ltd., 5-1-3, Tokodai, Tsukuba, Ibaraki, 300-2635, Japan. Fax: 81-298-47-2037. E-mail: e2-takahashi@eisai.co.jp.

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clone containing the 5′-upstream region of mouse α_{1A} subunit gene and the functional characterization of a 6.3-kb upstream promoter region in terms of its transcriptional control of the P/Q-type Ca^{2+} channel α_{1A} subunit gene.

MATERIALS AND METHODS

Genomic library screening and identification of α_{1A} subunit genomic clones. Approximately 9×10^5 recombinant phages from a TT2 cell genomic DNA library (14) were screened with two rabbit α_{1A} subunit cDNA probes, 601 bp *Hin*dIII-*Not*I fragment and 222 bp *Sal*I-*Hinc*II fragment from the plasmid pSPCBI-2 (9). A 13.5-kb clone TT2-5 was isolated, subcloned and sequenced. A bacteriophage library, CBA genomic DNA library, was constructed by cloning CBA mouse liver genomic DNA into the ZAP expression vector (Stratagene). Approximately 2.4×10^4 recombinant phages from the CBA genomic DNA library were screened with a probe, 760 bp *Bam*HI-*Apa*I fragment from TT2-5, and a 8.4-kb clone CBA-104 was isolated. A 8,397 bp *Bgl*II-*Eco*RI fragment from CBA-104 was subcloned into the *Bam*HI-*Eco*RI site of pBluescript II KS- (Stratagene) to yield pBS104. The nucleotide sequence of the 8,397 bp *Bgl*II-*Eco*RI fragment from CBA-104 will appear in the EMBL and GenBank databases under the Accession Number AB025352.

Poly(A)$^+$ RNA preparation and RACE analysis. The 5′-end of mouse α_{1A} subunit cDNA was isolated by means of a 5′ RACE experiment using a 5′ RACE System for Rapid Amplification of cDNA Ends kit (Gibco BRL). The 5′ RACE procedure was carried out according to the manufacturer's instructions. Briefly, an oligonucleotide, GSP3 (5′-CGGCTGCTGTTGCTGCGGCGA-3′, Fig. 1) was used as a primer to synthesize first-strand cDNA from CBA mouse brain poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated with a FastTrack 2.0 kit (Invitrogen) according to the manual. After the hydrolysis of RNA with RNaseH, cDNA was purified with a GlassMax DNA isolation spin cartridge (Gibco BRL) and subjected to oligo-dC tailing reaction. Polymerase chain reaction (PCR) of dC-tailed cDNA was carried out with a 5′ RACE abridged anchor primer (5′-GGCCACGCGTCGACTAGTACGGGGGGGGGG-3′) and α_{1A} subunit gene-specific primer GSP3 under the following conditions; 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 7 min. An aliquot of this reaction mixture was used as a template for a second PCR reaction using an abridged universal amplification primer, AUAP (5′-GGCCACGCGTCGACTAGTAC-3′) and the nested primer GSP5 (5′-TGCTCAGGCTGGCCGGGCTGT-3′, Fig. 1). The reaction product was subcloned into pT7Blue(R)T-Vector (Novagen) and sequenced with a Dye Terminator Cycle Sequence kit (Applied Biosystems) using U19 and T7 primers with an ABI Prism 377 DNA sequencer (Applied Biosystems). The computer analysis of potential transcription factor binding sites was carried out using GENETYX software (Soft Development, Tokyo).

Reverse transcription (RT)-PCR. First strand cDNA was synthesized with 1 μg of poly(A)$^+$ RNA by using a SuperScript preamplification system (Life Technologies Inc.). Following the first strand cDNA synthesis, PCR reactions were carried out for 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 7 min. The primers used are as follows: forward 5′-GAGATGATGGCCATTTGGCCCAAC-3′ (+5,484 to +5,508, Genbank Accession Number, U76716); reverse 5′-TCAGAGATGGTACTGAGGTCA-3′ (+6,041 to +6,062).

Plasmid constructions. Plasmids for transient expression assay to identify transcriptional regulatory element(s) in the mouse Ca^{2+} channel α_{1A} subunit gene were constructed by utilizing the placental alkaline phosphatase (PLAP) reporter gene vector, pUG-BGH-PLAP, which was essentially described previously (15). To construct a pBS104H vector, a *Hin*dIII linker was inserted into the *Not*I site of the plasmid pBS104. The pBS104HH vector was made by inserting a *Hin*dIII linker into the translational start site at +269 relative to the transcriptional site in pBS104H, using polymerase reaction (PCR)-based mutagenesis. The plasmid pα_{1A}6.3-PLAP was constructed by inserting the *Hin*dIII-*Hin*dIII fragment of pBS104HH into the *Hin*dIII site of pUG-BGH-PLAP. The plasmid pα_{1A}3.0-PLAP was constructed by inserting the *Eco*RV-*Eco*RV fragment of pα_{1A}6.3-PLAP into the *Eco*RV site of pBluescript II KS-. p6-3BS104 was made by removing the *Eco*RV-*Eco*RV fragment from pBS104. To construct pTK-PLAPI, which contains the thymidine kinase (TK) promoter driving a PLAP reporter gene, a *Xho*I linker was inserted into the *Xba*I site of pTK-PLAP (15). The plasmid p6-3α_{1A}TK-PLAP was generated by inserting the *Spe*I-*Xho*I fragment of p6-3BS104 into the *Spe*I-*Xho*I site of pTK-PLAPI. The plasmid p6-3α_{1A}PLAP was generated by removing the *Hin*dIII-*Xho*I fragment from p6-3α_{1A}TK-PLAP.

Cell culture, transient transfection and PLAP assay. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5% horse serum. NIH3T3 cells were grown in DMEM with 10% FCS. All culture media were supplemented with 100 units/ml of penicillin and 100 μg/ml streptomycin To examine the activity for expression of the mouse α_{1A} subunit gene, we carried out transient expression assay using undifferentiated PC12 cells and NIH3T3 cells. The cells were plated at a density of 1×10^5 cells in a collagen-coated 6-well dish. Twenty-four hours after the seeding, the culture was washed extensively to remove non-adherent cells and the medium was replaced. On the second day, transfection was carried out with FuGENE 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany) according to the manual. One microgram of the plasmid containing the mouse α_{1A} subunit gene promoter region fused to the PLAP reporter gene and 1 μg of the pSV-$\beta\beta$-galactosidase control vector (Promega, Madison, WI) were co-transfected into the cells. Twenty-four hours after the transfection, the medium was replaced and culture was continued for an additional 24 h. The culture supernatant was drawn from each sample and the PLAP activity was determined as described previously (15). Data represent the means and standard deviations from three independent experiments.

RESULTS

Cloning and Nucleotide Sequence of the 5′-Upstream Region of Mouse a_{1A} Subunit Gene

A 13.5-kb clone TT2-5 was isolated from a TT2 cell genomic DNA library which was established from an F1 embryo between C57BL/6 and CBA mice (14) by screening with two rabbit α_{1A} subunit cDNA probes. Southern blot and sequence analyses revealed that the 13.5-kb TT2-5 clone was identical to the CBA, but not the C57BL/6, mouse genome (data not shown). An 8.4-kb clone CBA-104 was then isolated from the CBA mouse genomic DNA library with a probe from TT2-5. Two overlapping clones, TT2-5 and CBA-104, taken together, contained 6.3kb of the 5′-upstream region, the first exon and 13.0 kb of the first intervening sequence. The 8,397 bp *Bgl*II-*Eco*RI fragment from the clone CBA-104 was subcloned, and sequenced, and a part of the sequence is shown in Fig. 1.

Mapping of the 5′ End of Mouse a_{1A} Subunit cDNA

The region of the mouse α_{1A} subunit gene in which transcription was likely to be initiated was determined

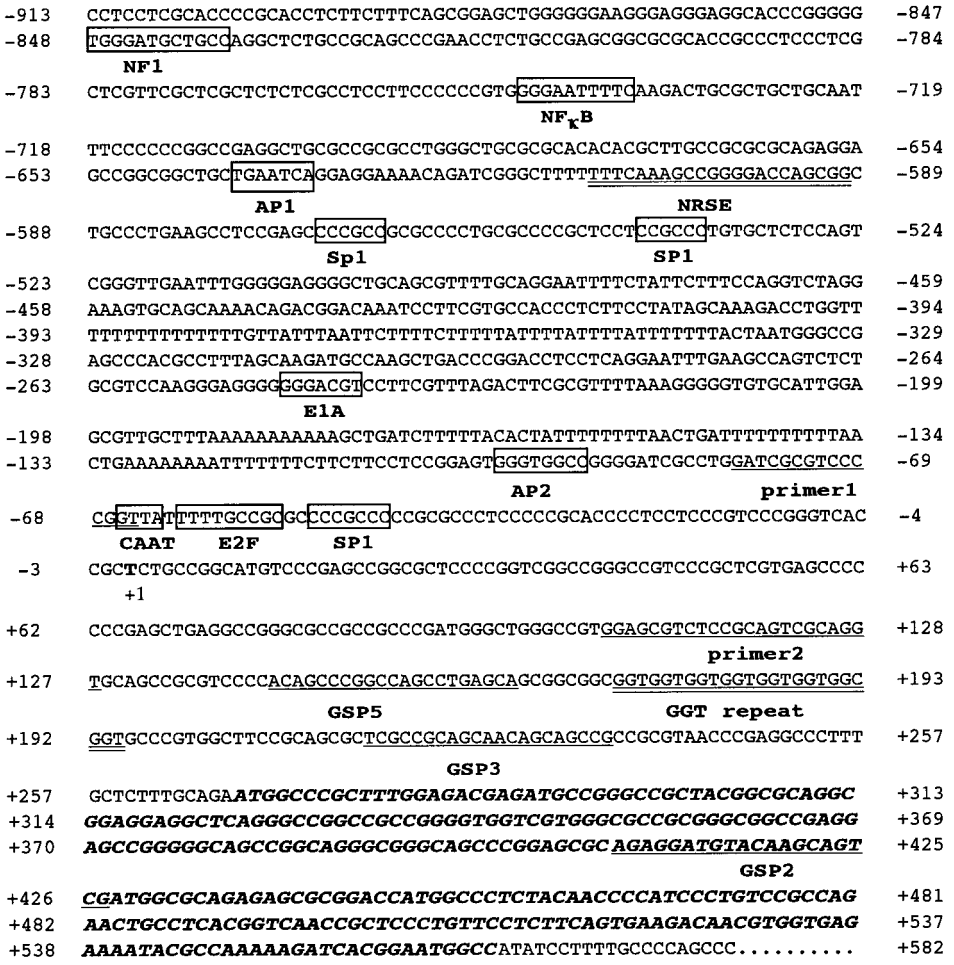

FIG. 1. Nucleotide sequence of the 5′-upstream region of the mouse P/Q-type Ca^{2+} channel α_{1A} subunit gene. The first nucleotide of the 5′ RACE product is indicated as bold T and arbitrarily designated as +1. Putative transcription factor binding sites are boxed. The sequence of the first exon is indicated by italic letters in bold. The primers used for 5′ RACE and RT-RCR analyses are underlined. The GGT repeat and NRSE-like site are double-underlined.

by comparing the sequence of the 5′ RACE product with that of the corresponding genomic DNA. The comparison indicated that the transcription start site was at −269 bp upstream from the translation start site, arbitrarily indicated as +1 (Fig. 1). We also performed reverse-transcription PCR (RT-PCR) experiments to confirm this transcription start site. The predicted 333 bp fragment was amplified with primer 2 (Fig. 1) and GSP2 (Fig. 1), but the predicted 516 bp fragment was not amplified with primer 1 (Fig. 1) and GSP2 (data not shown). These results suggest that the transcription start site is located between primer 1 and primer 2. The 8,397 bp sequence from clone CBA-104 contains 6,272 bp of the 5′-upstream region, 566 bp of the first exon (269 bp of the 5′-untranslated region and 297 bp of coding region) and 1,559 bp of the first intron. The sequence within the putative promoter region of 500 bp upstream from the first exon lacked a typical TATA box. But the region proximal to the transcription start site contained two Sp1 sites (−50 to −43 and −49 to −42), which are likely to be important for the basal transcriptional machinery in TATA-less promoters (16). A CAAT box-like sequence (−66 to −63) was present in the opposite strand. In addition, the following binding sites were located: E2F (−61 to −53), AP-2 (−99 to −92), E1A (−247 to −241), AP1 (−631 to −625), NF-κB (−747 to −738) and NF1 (−848 to −837).

mRNA Expression of Ca^{2+} Channel α_{1A} Subunit in Cultured Cell Lines

To examine the endogenous expression of α_{1A} subunit gene, RT-PCR analysis was performed for neuron-like PC12 cells, which are reported to possess the P/Q-type Ca^{2+} channel (17), and fibroblast NIH3T3 cells. The PCR of mRNA, without reverse transcription, was performed as a negative control (data not shown). RT-PCR yielded an amplified product corresponding to the predicted size from PC12 cells but not from NIH3T3

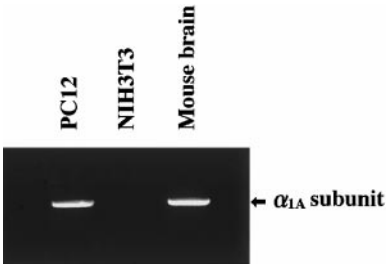

FIG. 2. RT-PCR analysis of the endogenous P/Q-type Ca^{2+} channel a_{1A} subunit gene. The first strand cDNA was reverse-transcribed using poly(A)$^+$ RNA from each cell or tissue and amplified with a specific a_{1A} subunit gene primer. The PCR products were electrophoresed on a 1.5% agarose gel.

cells (Fig. 2). Sequencing of these PCR products confirmed the α_{1A} cDNA sequence (data not shown).

Activity of α_{1A} Subunit Promoter in Cultured Cell Lines

To address whether the 6.3-kb of 5′-upstream region of the mouse α_{1A} subunit gene includes the regulatory sequences utilized in PC12 cells or NIH3T3 cells, we made fusion gene constructs, pα_{1A}6.3-PLAP (−6,273 to +269) and pα_{1A}3.0-PLAP (−3,021 to +269) (Fig. 3). The promoterless construct pUG-BGH-PLAP (basic-PLAP) served as a background control and the pTK-PLAP construct as a positive control. PLAP activity was measured after transient transfection. Figure 4 shows that the pα_{1A}6.3-PLAP construct, but not the pα_{1A}3.0-PLAP construct, could efficiently drive PLAP expression in PC12 cells. In NIH3T3 cells, the two fusion gene constructs have no promoter activity. To examine whether the sequence between 6.3 and 3.0 kb of the 5′-upsream region (−6,273 to −3,021) contributes to PC12-specific expression and lacks promoter activity, we conducted a transient expression assay using p6-3α_{1A}TK-PLAP and p6-3α_{1A}PLAP (Fig. 3). Figure 5 shows that the activity of the TK promoter was enhanced by the presence of the sequence between 6.3 and 3.0kb of the 5′-upstream region (−6,273 to −3,021) in PC12 cells, but not in NIH3T3 cells. The promoterless p6-3α_{1A}PLAP construct did not show promoter activity in either of the cell lines.

DISCUSSION

We have cloned and molecularly analyzed the 6.3-kb 5′-upstream region of the mouse P/Q-type Ca^{2+} channel α_{1A} subunit gene. The predicted promoter region has sequences for two putative Sp1 sites (−50 to −43 and −49 to −44) and a CAAT box-like sequence (−66 to −63), but lacks a typical TATA box. The rat α_{1D} and human α_{1B} subunit genes also lack a typical TATA box (12, 13). Thus, TATA-less promoters may be a common feature of the Ca^{2+} channel α_1 subunit genes. Another characteristic sequence of at −611 to −590, found (Fig. 1) by computer-assisted analysis, is homologous to the neuronal-restrictive silencer element (NRSE) identified in neuron-specific genes such as SCG10 gene (18). A transcription factor, NRSE-binding factor, binds to the 21-bp NRSE site in the 5′-upstream region of neuron-specific genes to repress selectively the transcription of these genes in non-neuronal cells (19). The NRSE-like sequence found in the α_{1A} subunit gene was composed of 22 bp, not 21 bp, but 16 bp of its 5′ sequence showed 69% sequence identity and 5 bp of its 3′sequence showed 80% identity to the corresponding sequences from SCG10 gene. To assess whether the foregoing putative 22-bp silencer site can negatively

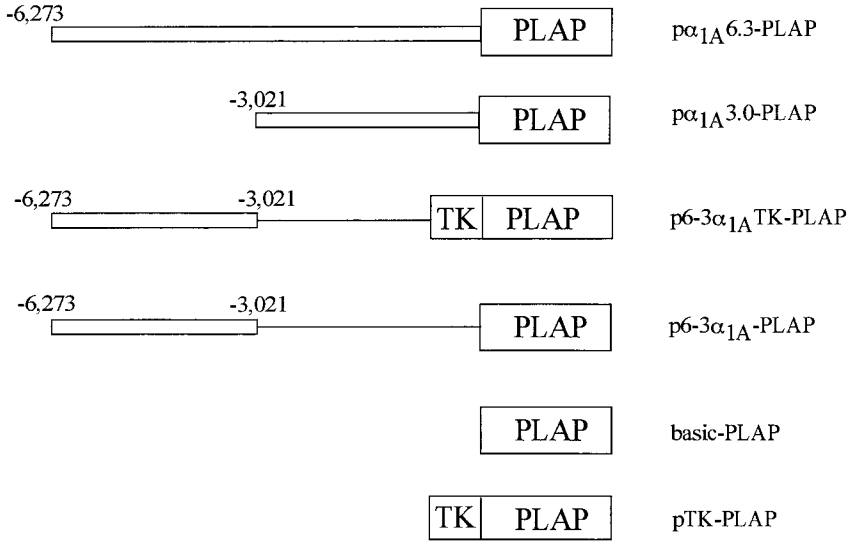

FIG. 3. Plasmid constructions used in transient transfection assay. Construction is described in Materials and Methods.

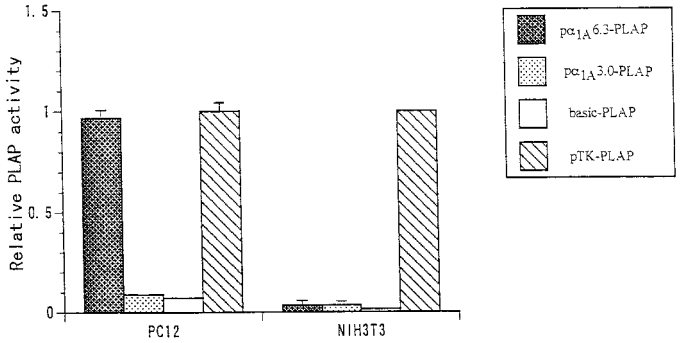

FIG. 4. Promoter activity of the 5′-upstream region of the mouse P/Q-type Ca^{2+} channel α_{1A} subunit gene. PLAP activity of different deletion constructs is shown relative to that of the vector pTK-PLAP. All values are normalized to the pSV-β-galactosidase vector, which was co-transfected into cells. Results represent the mean relative activities ± standard deviations of three independent experiments.

regulate a different promoter irrespective of sequence, as would be expected if it functions analogously to the silencer present in SCG10 gene, we placed the *Sma*I-*Sma*I fragment (−854 to −11), which contains the putative 22-bp silencer site, in front of the TK promoter driving a luciferase reporter gene. When introduced into NIH3T3 cells, the *Sma*I-*Sma*I fragment (−854 to −11) did not inhibit transcriptional activity of the TK promoter (data not shown). This result suggests that the 22-bp NRSE-like site is not functional and the *Sma*I-*Sma*I fragment (−854 to −11) may not contain other negative *cis*-regulatory elements to confer neuron-specific expression of genes.

The transcription of the rat α_{1D} subunit gene is reported to be regulated by a positive *cis*-regulatory element for neuron-specific expression that consists of an $(ATG)_7$ trinucleotide repeat in the coding region of the first exon (12). Mouse α_{1A} subunit gene also contains the $(GGT)_7$ trinucleotide repeat with a GGC interruption before the last GGT in the 5′-untranslated region (Fig. 1). However, it is unlikely that the $(GGT)_7$ trinucleotide repeat of the α_{1A} subunit gene is a functional element for transcription, because pα_{1A}3.0-PLAP could not efficiently drive PLAP expression in PC12 cells. The activity of the human α_{1B} subunit gene proximal promoter was apparently expressed in neuronal and non-neuronal cells (13), but such an expression pattern was not detected in the case of the mouse α_{1A} subunit gene. In addition, deletion analysis of the human α_{1B} subunit gene revealed the existence of a negative *cis*-regulatory element for neuron-specific expression in the distal upstream region (−3,992 to −1,788), while the mouse α_{1A} subunit gene contained a positive *cis*-regulatory element in the distal upstream region (−6,272 to −3,019). These different expression mechanisms for the α_1 subunit genes are likely to contribute to the diversity in expression patterns of Ca^{2+} channels in the nervous system.

To dissect the mechanisms for the expression of α_{1A} subunit gene, an analogy can be drawn with the case of the Kv3.1 potassium channel gene (20). When fused to the chloramphenicol acetyltransferase reporter gene, 5.3 kb of the 5′-upstream region of the Kv3.1 potassium channel gene was active in driving the reporter gene in PC12 cells, but not in NIH3T3 cells. Within this region, a cyclic AMP (cAMP) response element (CRE) motif was demonstrated to be responsible for the cell-specific expression. Our computer-assisted analysis indicated that the distal upstream region of the α_{1A} subunit gene contains two putative CRE motifs (−4,023 to −4,019 and −3,330 to −3,326). The CRE motif, known to play a crucial role in the expression of multiple neuronal proteins, has been shown to be present in several genes, such as the dopamine β-hydroxylase gene (21). In the 6.3-kb 5′-upstream region of the α_{1A} subunit gene, the CRE motif was identified only in this region (−6,272 to −3,019). It is likely that the CRE motif of

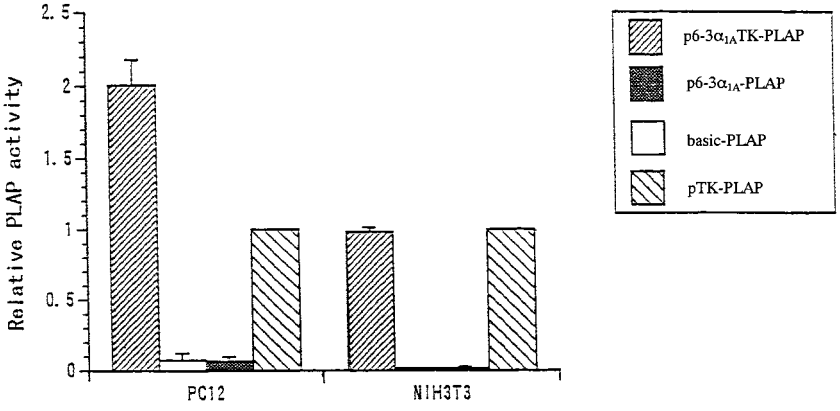

FIG. 5. Element analysis for the cell-specific expression of the P/Q-type Ca^{2+} channel α_{1A} subunit gene. PLAP activities of different deletion constructs are expressed relative to the vector pTK-PLAP. All values are normalized to the pSV-β-galactosidase vector, which was co-transfected into the cells. Results represent the mean relative activities ± standard deviations of three independent experiments.

the α_{1A} subunit gene also contributes to specific expression of the α_{1A} subunit gene in the nervous system.

In summary, we have identified a region in the 5′-upstream part of the mouse P/Q-type α_{1A} subunit gene ($-6{,}272$ to $-3{,}019$) that contains a positively *cis*-acting regulatory element likely to be responsible for the neuron-specific expression of the α_{1A} subunit gene. Further studies, including deletion analysis and production of transgenic mice, should help to clarify in detail the mechanism of expression of the mouse P/Q-type α_{1A} subunit gene.

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