Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3251–3255, April 1992
Neurobiology

Rat brain expresses an alternatively spliced form of the dihydropyridine-sensitive L-type calcium channel α2 subunit

(ion channel/primary structure/*in situ* hybridization/RNA splicing)

HYUNG-LAE KIM, HYUN KIM, PATRICIA LEE, RON G. KING, AND HEMIN CHIN*

Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

Communicated by Marshall Nirenberg, January 6, 1992

ABSTRACT **We have cloned and sequenced a cDNA (rB-α2) encoding an α2 subunit of the voltage-sensitive L-type calcium channel (dihydropyridine receptor) of rat brain. The cDNA (3823 base pairs) encodes a protein of 1091 amino acids with a M_r of 123,822. The deduced amino acid sequence of rB-α2 cDNA is highly similar (95% amino acid identity) to that of rabbit skeletal muscle α2 subunit. The rB-α2 protein is distinct from the previously cloned skeletal muscle α2-subunit protein [Ellis, S. B., Williams, M. E., Ways, N. R., Brenner, R., Sharp, A. H., Leung, A. T., Campbell, K. P., McKenna, E., Koch, W., Hui, A., Schwartz, A. & Harpold, M. M. (1988) *Science* 241, 1661–1664] because it contains an insertion of 7 amino acid residues and a deletion of a 19-amino acid segment between putative transmembrane domains 1 and 2. We show that the rB-α2 and skeletal muscle α2-subunit transcripts are the variants produced by alternative splicing of the primary transcript and that they are differentially expressed in brain and skeletal muscle, respectively.**

The slowly inactivating voltage-sensitive L-type Ca^{2+} channel activated by strong depolarization is sensitive to organic Ca^{2+} channel blockers such as 1,4-dihydropyridines (DHP), phenylalkylamines, and benzothiazepines. The biochemical and molecular properties of the DHP-sensitive L-type Ca^{2+} channel have been well characterized from skeletal muscle and heart. Purified skeletal muscle Ca^{2+} channel is an oligomeric protein composed of two high-molecular polypeptide subunits (α1 and α2) and three smaller subunits (β, γ, and δ) (reviewed in refs. 1 and 2). Molecular cloning studies have elucidated the primary structure of the DHP-sensitive Ca^{2+} channel α1 subunit from both skeletal muscle (3) and cardiac muscle (4). The cloned skeletal and cardiac muscle α1 subunits express functionally active, DHP-sensitive Ca^{2+} channels when transfected into fibroblasts or microinjected into myotubes (5, 6). Thus, the α1 subunit appears to have the structural features required for Ca^{2+} channel function and contains the binding sites for Ca^{2+} antagonists. Subsequent cloning of cDNAs encoding the α2, β, and γ subunits of rabbit skeletal muscle (7–10) has allowed electrophysiologic characterization of coexpressed cloned Ca^{2+} channel subunits and also has demonstrated specific functional roles for these subunits to modulate Ca^{2+} channel function. While the α1 subunit mRNA alone produced Ca^{2+} currents in *Xenopus* oocytes, coinjection of mRNAs for skeletal muscle α2 and β subunits together with that for the α1 subunit produced very high levels of expression (11–13).

Although not as well characterized as their cardiac and skeletal muscle counterparts, brain DHP-sensitive Ca^{2+} channels are widely distributed throughout the central nervous system and have a similar molecular structure consisting of α1, α2, β, and δ subunits (14). By using the skeletal muscle α1-subunit cDNA as a probe, a number of cDNA clones with sequence homology to the skeletal muscle α1 subunit have recently been isolated from neuronal tissues (15–18). On the other hand, brain cDNA clones that are homologous to other subunits of the DHP-sensitive Ca^{2+} channel have not been isolated. Our goal is to elucidate the molecular structure and functional roles of each neuronal Ca^{2+} channel subunit. In the present study, we have cloned a cDNA encoding an isoform of the α2 subunit of a DHP-sensitive Ca^{2+} channel isolated from rat brain.† Our findings indicate that there are multiple variants of the α2 subunit generated by alternative splicing of the primary transcript, and that these variants are differentially expressed in rat brain and skeletal muscle.

MATERIALS AND METHODS

Isolation of Rat Brain Ca^{2+} Channel α2-Subunit (rB-α2) cDNAs, Subcloning, and DNA Sequencing Analysis. Approximately 6×10^5 recombinant plaques from a rat hippocampus cDNA library in Lambda Zap II (a gift from J. Boulter of the Salk Institute, La Jolla, CA) were screened with the oligonucleotide probes whose sequences were derived from rabbit skeletal muscle DHP-sensitive Ca^{2+} channel α2-subunit cDNA (7). Two probes were prepared by using an automatic DNA synthesizer (Applied Biosystems model 380B): probe R1 corresponded to nucleotide residues 1391–1439, and probe R2 corresponded to nucleotide residues 1482–1528 (7). Plaques were transferred to nitrocellulose filters; the filters were prehybridized and hybridized at 42°C in a solution containing 2× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7.2), 20% formamide, 0.1% SDS, Denhardt's solution (0.2% polyvinylpyrrolidone/0.2% bovine serum albumin/0.2% Ficoll), 20 mM sodium pyrophosphate, and 100 μg of denatured salmon sperm DNA per ml.

The oligonucleotide probes were labeled by T4 polynucleotide kinase and hybridized for 18 hr to DNA adsorbed to filters at 1×10^6 cpm per ml of hybridization solution. Filters were washed with 2× SSC containing 0.1% SDS for 20 min at room temperature four times, with 0.2× SSC containing 0.1% SDS for 5 min at 42°C and finally with 6× SSC containing 0.1% SDS for 30 min at 55°C. Autoradiography was carried out for 40 hr at −80°C with Kodak X-Omat AR film. Two plaques that were positive for both of the probes were selected and purified, and the plasmids were excised *in vivo* from the plaques to obtain rB-α2C11 and rB-α2C51 plasmid clones according to the manufacturer's instruction

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Abbreviations: DHP, 1,4-dihydropyridine; rB-α2, rat brain DHP-sensitive calcium channel α2 subunit; RT, reverse transcription.

*To whom reprint requests should be addressed at: Laboratory of Molecular Biology, Building 36, 3D-02, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M86621).

(Stratagene). Both strands of these plasmids were sequenced. Oligonucleotide primers were synthesized according to prior sequence information. Sequencing was performed by using the dideoxynucleotide chain-termination method (19) with Sequenase (United States Biochemical).

The screening procedure was repeated to obtain the rB-α2C52 clone from a rat cerebellum Lambda Zap cDNA library (a gift from Jim Boulter) as shown in Fig. 1 with another set of two 42-mer oligonucleotide probes (N1: 5′-CAT GAT TGG CCA TCA GAA GAA ATC CAC CAT CAT CTA GAA TGA-3′ and N2: 5′-CAC ACG GAT CCC TGA TTG AAG TTT TGG TAA AAT TTT CTA TCC-3′) that corresponded to the 3′ end of clone rB-α2C51.

RNA Hybridization. Total RNA was isolated from young adult rat tissues by using the guanidinium isothiocyanate/CsCl method (20). Poly(A)$^+$ RNA was purified by using the Poly(A) Quick mRNA isolation kit (Stratagene). Approximately 20 μg of total RNA from adult rat brain or 5 μg of poly(A)$^+$ RNA from other rat tissues was fractionated by formaldehyde/agarose gel electrophoresis, transferred to a supported nitrocellulose paper (BRL), and fixed by baking at 80°C for 2 hr. The RNA was hybridized with a nick-translated 1.2-kilobase (kb) *Xba* I fragment from the rB-α2C51 cDNA probe at 37°C for 20 hr in 6× SSC containing 20% formamide, 0.5% SDS, 5× Denhardt's solution, and 100 μg of yeast tRNA and 100 μg of denatured salmon sperm DNA per ml. Filters were washed with 1× SSC containing 0.1% SDS four times for 30 min each at room temperature and with 0.2× SSC containing 0.5% SDS for 45 min at 50°C.

Reverse Transcription (RT) and PCR. The first-strand cDNA was synthesized by using Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase (BRL). Approximately 1 μg of total RNA from brain and skeletal muscle was incubated at 37°C for 60 min in 20 μl (final reaction volume) containing 10 units of cloned Mo-MuLV reverse transcriptase, 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, bovine serum albumin (0.1 mg/ml), 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, and 25 pmol of downstream 3′ primers. After incubation, 10 μl of the first-strand reaction mix was amplified in a 50-μl reaction mix that included 25 pmol of upstream primer and 25 pmol of downstream primer for 30 cycles (1 min at 94°C, 2 min at 55°C, and 2 min at 72°C). The PCR products were separated on a 3% agarose gel, and the DNA was transferred to a nitrocellulose membrane. The PCR primers used were: 5′ upstream primer, 5′-TGA CAC CAC GTT TTA CAC TGT GCC-3′; and 3′ downstream primer, 5′-CTC CAC TTT CTC CGT CTA TCA T-3′.

To examine differential expression in brain and skeletal muscle, the blot was probed with ^{32}P-labeled oligonucleotide probes specific either for the longer skeletal muscle form (probe S, 5′-GTC GGT ATA CCC ACA CCA ATA GGC-3′) or common to both forms (probe C, 5′-ATC GAG AAA ATC CAG TGT GAC CGG-3′) (see Fig. 5*A*).

***In Situ* Hybridization.** *In situ* hybridization was performed essentially as described (21). Briefly, frozen sections (12 μm thick) were cut, thaw-mounted on gelatin-coated slides, fixed, and dehydrated immediately before hybridization. The plasmids containing a 3823-base-pair (bp) rB-α2 cDNA in opposite orientations were linearized with *Hin*dIII. Antisense and sense riboprobes (730 bp in length, including the vector sequence) were transcribed and labeled with T7 and T3 phage RNA polymerases using a Riboprobe system (Promega) in the presence of uridine 5′-(α-[^{35}S]thio)triphosphate (1000–1500 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq). The sections were incubated overnight at 54°C in hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.7% Ficoll, 0.7% polyvinylpyrrolidone, 0.7% bovine serum albumin, 0.15 mg of yeast tRNA per ml, 0.33 mg of denatured salmon sperm DNA per ml, 20 mM dithiothreitol, and the ^{35}S-labeled RNA probe (1 × 10^6 cpm per 75 μl of hybridization solution); washed in 2× SSC; treated with RNase A (20 μg/ml; Boehringer Mannheim) for 30 min at 25°C; and washed sequentially for 60 min in 2× SSC at 50°C, for 50 min in 0.2× SSC at 55°C, and for 50 min in 0.1× SSC at 60°C. The slides were dried and exposed to film (β-max Hyperfilm; Amersham) for 4 days.

RESULTS AND DISCUSSION

Primary Structure of DHP-Sensitive Ca^{2+} Channel α2 Subunit Encoded by rB-α2 cDNA. A Lambda Zap II rat hippocampus cDNA library was screened with two oligonucleotide probes, R1 and R2, whose sequences were derived from rabbit skeletal muscle α2-subunit cDNA (7). The two overlapping λ clones, identified as rB-α2C11 and rB-α2C51 in Fig. 1, were obtained by characterizing plaques positive to both probes. Both strands of the cDNA inserts derived from clone rB-α2C11 (2.6 kb) and clone rB-α2C51 (1.5 kb) were sequenced completely. The amino acid sequence deduced from the overlapping rB-α2C11 and rB-α2C51 cDNAs is very similar to that of skeletal muscle Ca^{2+} channel α2-subunit cDNA, residues 16–681 (7). Such an observation provided evidence that the rB-α2C11 and rB-α2C51 cDNAs encoded a Ca^{2+} channel α2 subunit expressed in rat brain. Oligonucleotide probes whose sequences had been derived from the 3′ end of the rB-α2C51 clone were then used to isolate a clone that contained a cDNA insert extending to the 3′ end of the coding region. The sequence of the resulting rB-α2C52 clone contained an in-frame stop codon but did not extend to the poly(A)$^+$ tail. Together these overlapping clones comprise a contiguous cDNA sequence, designated rB-α2 cDNA, containing an open-reading frame of 3273 nucleotides flanked by a 164-nucleotide segment of the 5′ untranslated sequence and a 386-nucleotide 3′ untranslated sequence.

The deduced amino acid sequence of the rat brain DHP-sensitive Ca^{2+} channel α2-subunit cDNA (rB-α2 cDNA), shown in Fig. 2, is about 95% identical to that of the rabbit skeletal muscle counterpart. However, there are the following differences. The rB-α2 cDNA is shorter by 26 amino acid residues when compared with rabbit skeletal muscle α2 subunit. There is a deletion of a 19-amino acid segment corresponding to skeletal muscle residue positions 507–528 and an insertion of 7-amino acid residues located after residue Thr-617. There are also deletions of 1 or 2 amino acids, shown in Fig. 2.

Furthermore, the primary structure of rB-α2 cDNA suggests that a single α2-subunit gene encodes the α2- and δ-subunit proteins of rat brain DHP-sensitive Ca^{2+} channel. Skeletal muscle α2-subunit cDNA encodes a precursor mol-

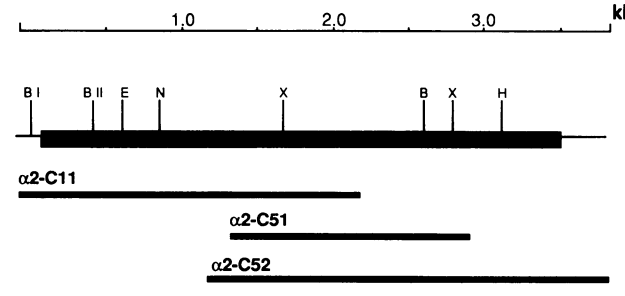

FIG. 1. Partial restriction enzyme map and cloning strategy for rB-α2 cDNA. A linear composite map of the full-length cDNA is shown. The protein-coding sequence contained within the rB-α2 cDNA is indicated by the solid box, and the 5′ and 3′ untranslated sequences are shown as thin-line extensions. The different clones analyzed are shown as thick solid lines below the map. Relevant restriction endonuclease sites are: B I, *Bgl* I; B II, *Bgl* II; E, *Eco*RI; X, *Xba* I; B, *Bam*HI; and H, *Hin*dIII.

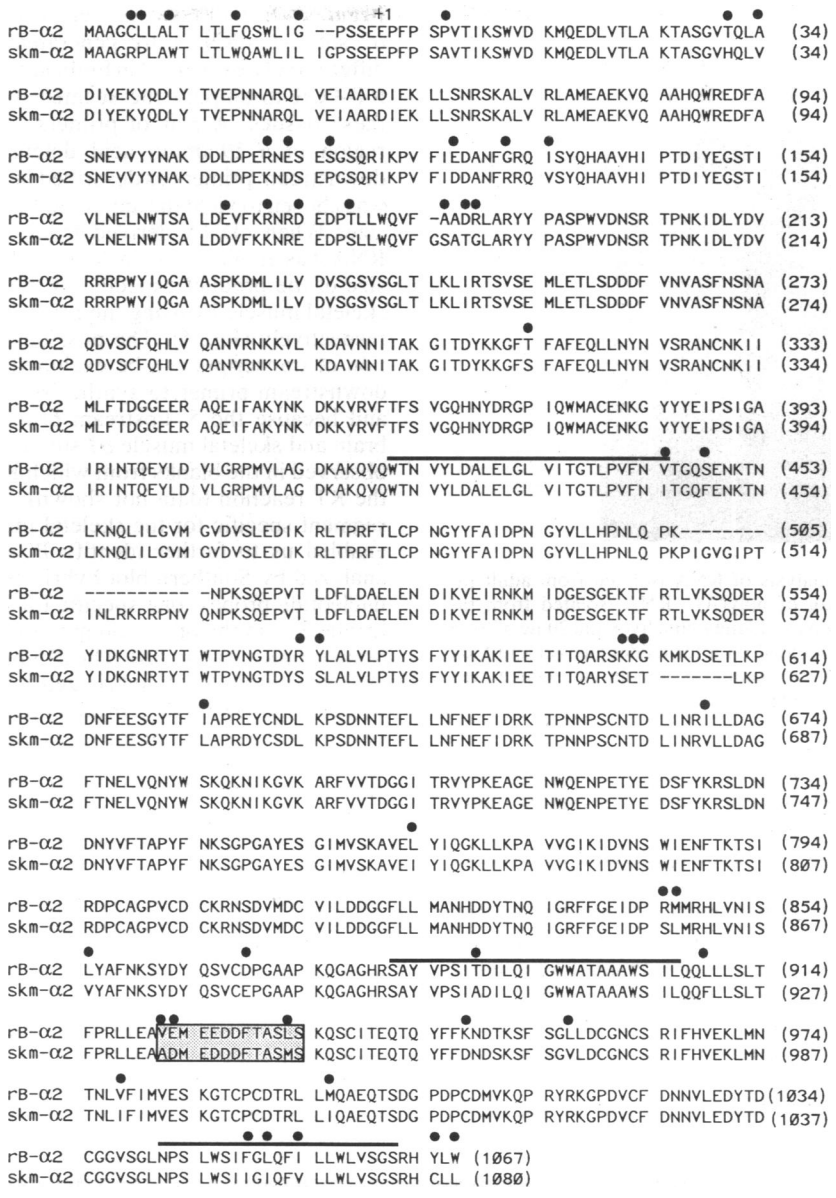

FIG. 2. The primary structure of the Ca^{2+} channel encoded by rB-α2 mRNA compared to rabbit skeletal muscle Ca^{2+} channel α2-subunit cDNA. The deduced amino acid sequence of the two Ca^{2+} channel α2-subunit cDNAs: rB-α2 cDNA and rabbit skeletal muscle α2 subunit cDNA—is shown in the one-letter amino acid code. rB-α2 cDNA (upper line of each pair) is aligned with the skeletal muscle (skm-α2) sequence (bottom line of each pair) reported (7). Gaps are indicated by spaces. The deduced amino acid residues of rB-α2 cDNA are numbered beginning with the N-terminal amino acid after cleavage of the putative signal peptide sequence. The putative transmembrane segments are indicated by solid lines. The solid circles indicate the amino acids whose sequences are different between brain and skeletal muscle. The sequences in the box indicate the N-terminal amino acids of the δ subunit of the DHP-sensitive Ca^{2+} channel.

ecule that is posttranslationally modified to produce mature α2 and δ polypeptides (7, 22, 23). The amino-terminal amino acid sequence of purified δ polypeptide of rabbit skeletal muscle occurs at residues 935–947 within the deduced amino acid sequence of skeletal muscle α2-subunit cDNA (shown as the shaded box in Fig. 2), indicating that cleavage of the δ polypeptide from the precursor occurs between Ala-934 and Ala-935 (22, 23). The corresponding sequence in rB-α2 cDNA occurs as Ala-925 and Val-926. Except for the three amino acids that exhibit conservative substitution (valine for alanine, glutamic acid for aspartic acid, and leucine for methionine), the N-terminal amino acid sequence of the putative δ polypeptide from rB-α2 cDNA is identical to that of skeletal muscle (22, 23). This highly conserved amino acid sequence between the α2-subunit cDNAs from brain and skeletal muscle indicates that these cDNAs are produced from a single gene.

Expression of α2-Subunit mRNA in Various Rat Tissues. Tissue-specific expression of the rat brain α2-subunit mRNA is shown in Fig. 3. An mRNA transcript of $\approx$8000 nucleotides that hybridized with the rB-α2 cDNA probe was most abundant in the brain and skeletal muscle. Lower levels of the mRNA species hybridizing with the α2 cDNA probe were present in heart and lung. No hybridization was observed with poly(A)$^+$ RNAs isolated from kidney or liver. The weakly hybridizing mRNA species of 3800 nucleotides, corresponding to the size of the cloned cDNA in skeletal muscle, may be the mature α2-subunit transcript present there.

To assess the overall distribution of rB-α2 mRNA throughout the body, we conducted *in situ* hybridization with a sagittal section of a whole 21-day-old rat fetus (Fig. 4). Label was most concentrated in the brain, especially in the hypothalamus, cerebral cortex, olfactory bulb, pons, and superior colliculus. Relatively high levels were present in skeletal

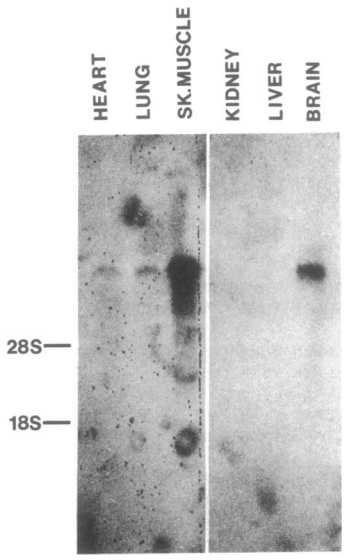

FIG. 3. Northern blot analysis of RNA isolated from adult rat tissues. Approximately 5 μg of poly(A)$^+$ RNA isolated from rat heart, lung, skeletal (SK.) muscle, kidney, and liver and 20 μg of total RNA from brain were fractionated on a formaldehyde gel and then transferred to a supported nitrocellulose membrane, which was hybridized with a nick-translated probe derived from rB-α2C51 clone (*Xba* I fragment, 1.2 kb).

muscle, especially in the tongue. Moderate densities were apparent in the heart and intestine. Label was almost undetectable in the liver and lung. The general pattern of α2-subunit mRNA distribution is in good agreement with that of Northern blot analysis, except for lung. Although the reason for this discrepancy is not clear, it may be due to the fact that the lung is not functional at this embryonic stage since the presence of significant amounts of Ca^{2+}-channel α2-subunit protein was first detected in adult lung with specific monoclonal antibodies (24). Thus, it would be of interest to examine developmental expression of α2-subunit mRNA in rat tissues.

rB-α2 cDNA Represents a Shorter Isoform of the DHP-Sensitive Ca^{2+}-Channel α2 Subunit Preferentially Expressed in Brain. To further test whether the rB-α2 and skeletal muscle α2 subunit mRNAs are generated from a single gene but differentially expressed in brain and skeletal muscle, we used RT–PCR to amplify the segment of mRNAs isolated from these tissues. A pair of primers, which flanked the rB-α2 region of a 19-amino acid deletion (5′ upstream and 3′ downstream primers as described in *Materials and Methods*), was chosen to yield the amplified PCR products with differing size. When cDNA derived from brain or skeletal muscle RNA was amplified, a single major band was observed at the size of the product expected of the α2 subunit of brain and skeletal muscle by using the primer pair, 198 bp and 255 bp, respectively (Fig. 5). The possibility of selective amplification of transcripts can be excluded because we used the 3′ downstream primer to synthesize the first strand of cDNA and because the 5′ upstream primer was identical in both brain and skeletal muscle α2 subunits. No PCR product was observed in the blanks from which RNA had been omitted in the RT reaction (data not shown). The presence of a DNA segment specific for the skeletal muscle α2-subunit isoform enabled us to further identify PCR products, which were analyzed by Southern blot hybridization with the two oligonucleotide probes, one specific for the skeletal muscle form (probe S) and the other common to both skeletal muscle and brain (probe C) (Fig. 5*B*). The 255-bp amplified product from skeletal muscle RNA hybridized with both probes S and C, whereas the 198-bp PCR product from the brain RNA sample only hybridized with probe C. These results indicate that the two RT–PCR products reflect the α2-subunit transcript present in brain and skeletal muscle. The absence of both the smaller 198-bp product in the skeletal muscle RNA sample and the larger 255-bp product in the brain RNA sample is an important indication that the cloned skeletal muscle α2 subunit cDNA and brain α2 subunit cDNA are produced by alternative splicing of the primary transcript.

Highly conserved cDNA sequences of brain and skeletal muscle α2 subunits, the identical size of mRNA transcripts, a similar tissue distribution pattern of mRNA, and differential expression of isoforms in brain and skeletal muscle are all consistent with the idea that the brain and skeletal muscle DHP-sensitive Ca^{2+} channel α2-subunit isoforms are the spliced variants of the same α2-subunit gene. After completing the work described here, we then mapped the α2-subunit

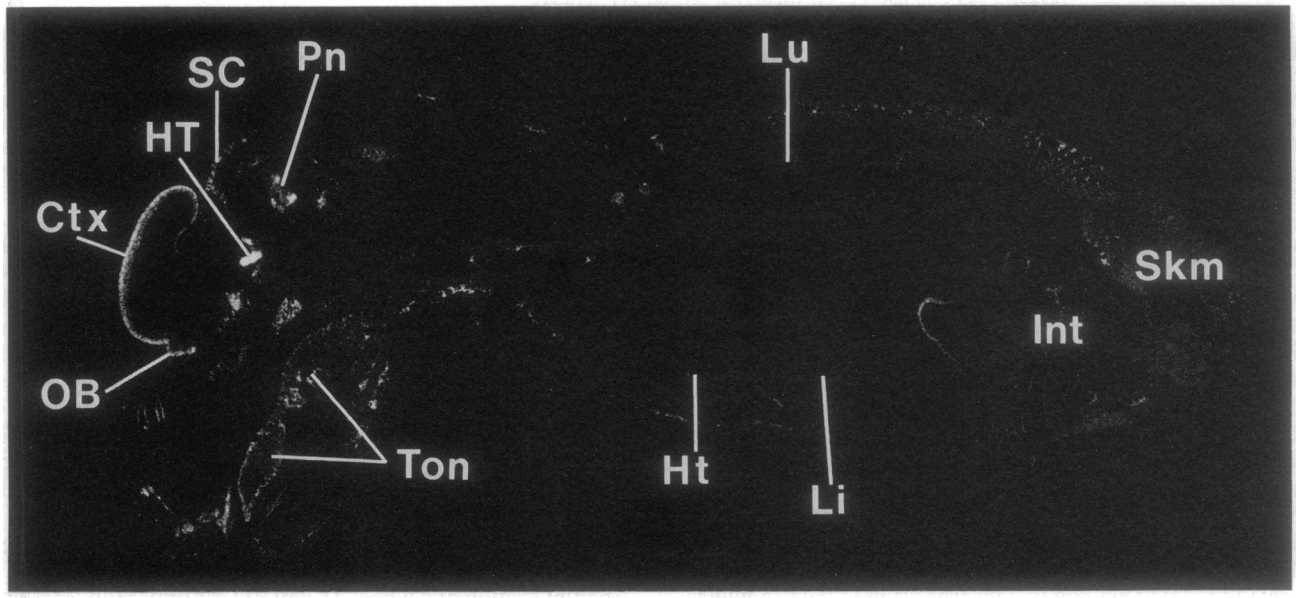

FIG. 4. Localization of rB-α2 mRNA in a 21-day-old rat embryo by *in situ* hybridization. Negative film image of *in situ* hybridization of sagittal section. Ctx, cerebral cortex; HT, hypothalamus; Ht, heart; Int, intestine; Li, liver; Lu, lung; OB, olfactory bulb; Pn, pons; SC, superior colliculus; Skm, skeletal muscle; Ton, tongue.

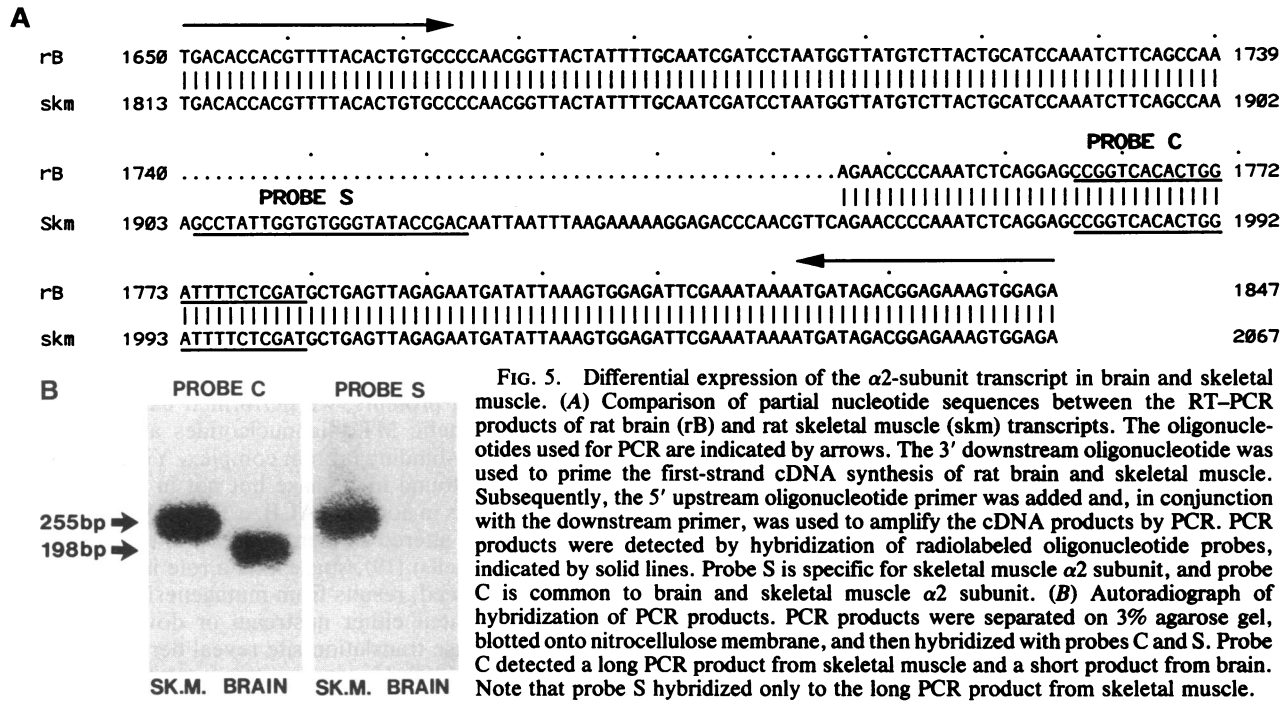

FIG. 5. Differential expression of the α2-subunit transcript in brain and skeletal muscle. (*A*) Comparison of partial nucleotide sequences between the RT–PCR products of rat brain (rB) and rat skeletal muscle (skm) transcripts. The oligonucleotides used for PCR are indicated by arrows. The 3′ downstream oligonucleotide was used to prime the first-strand cDNA synthesis of rat brain and skeletal muscle. Subsequently, the 5′ upstream oligonucleotide primer was added and, in conjunction with the downstream primer, was used to amplify the cDNA products by PCR. PCR products were detected by hybridization of radiolabeled oligonucleotide probes, indicated by solid lines. Probe S is specific for skeletal muscle α2 subunit, and probe C is common to brain and skeletal muscle α2 subunit. (*B*) Autoradiograph of PCR products. PCR products were separated on 3% agarose gel, blotted onto nitrocellulose membrane, and then hybridized with probes C and S. Probe C detected a long PCR product from skeletal muscle and a short product from brain. Note that probe S hybridized only to the long PCR product from skeletal muscle.

gene, using the 1.2-kb *Xba* I fragment of rB-α2 cDNA as probe, at a single locus on the mouse chromosome 5 (H.C., C. A. Kozak, B. Mock, and H.-L.K., unpublished data).

Multiple protein isoforms are commonly generated by an alternative splicing mechanism from a single gene (25). The structural differences found in the splicing variants often result in altered physiologic functions of the proteins they encode as well, as is the case for the voltage-sensitive K^+ channels (26). Physiologic implication of the differences between brain and skeletal muscle forms of the α2 subunit is not yet known. It will be of interest to compare the electrophysiologic properties of the Ca^{2+} channels reconstituted with the brain α2-subunit isoform with those of the channels reconstituted with the skeletal muscle α2-subunit isoform.

We thank Mrs. Devera Schoenberg for editorial assistance.

1. Campbell, K. P., Leung, A. T. & Sharp, A. H. (1988) *Trends Neurosci.* **11**, 425–430.
2. Catterall, W. A., Seagar, M. J. & Takahashi, M. (1988) *J. Biol. Chem.* **263**, 3535–3538.
3. Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1987) *Nature (London)* **328**, 313–318.
4. Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S. & Numa, S. (1989) *Nature (London)* **340**, 230–233.
5. Tanabe, T., Beam, K. G., Powell, J. A. & Numa, S. (1988) *Nature (London)* **336**, 134–139.
6. Perez-Reyes, E., Kim, H. S., Lacerda, A. E., Horne, W., Wei, X., Rampe, D., Campbell, K. P., Brown, A. M. & Birnbaumer, L. (1989) *Nature (London)* **340**, 233–236.
7. Ellis, S. B., Williams, M. E., Ways, N. R., Brenner, R., Sharp, A. H., Leung, A. T., Campbell, K. P., McKenna, E., Koch, W., Hui, A., Schwartz, A. & Harpold, M. M. (1988) *Science* **241**, 1661–1664.
8. Ruth, P., Roehrkasten, A., Biel, M., Bosse, E., Regulla, S., Meyer, H. E., Flockerzi, V. & Hoffman, F. (1989) *Science* **245**, 1115–1118.
9. Jay, S. D., Ellis, S. B., McCue, A. F., Williams, M. E., Vedvivk, T. S., Harpold, M. M. & Campbell, K. P. (1990) *Science* **248**, 490–491.
10. Bosse, E., Regulla, S., Biel, M., Ruth, H. E., Flockerzi, V. & Hofmann, F. (1990) *FEBS Lett.* **267**, 153–156.
11. Varadi, G., Lory, P., Schultz, D., Varadi, M. & Schwartz, A. (1991) *Nature (London)* **352**, 159–162.
12. Lacerda, A. E., Kim, H. S., Ruth, P., Perez-Reyes, E., Flockerzi, V., Hofmann, F., Birnbaumer, L. & Brown, A. M. (1991) *Nature (London)* **352**, 527–530.
13. Singer, D., Biel, M., Lotan, I., Flockerzi, V., Hofmann, F. & Dascal, N. (1991) *Science* **253**, 1553–1557.
14. Ahlijanian, M. K., Westenbroek, R. E. & Catterall, W. A. (1990) *Neuron* **4**, 819–832.
15. Hui, A., Ellinor, P. T., Krizanoa, O., Wang, J.-J., Diebold, R. J. & Schwartz, A. (1991) *Neuron* **7**, 35–44.
16. Perez-Reyes, E., Wei, X., Castellano, A. & Birnbaumer, L. (1990) *J. Biol. Chem.* **265**, 20430–20436.
17. Snutch, T. P., Tomlinson, W. J., Leonard, J. P. & Gilbert, M. M. (1991) *Neuron* **7**, 45–57.
18. Mori, Y., Friedrich, T., Kim, M.-S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofman, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T. & Numa, S. (1991) *Nature (London)* **350**, 398–402.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
20. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
21. Simmons, D. M., Arriza, J. F. & Swanson, L. W. (1989) *J. Histotechnol.* **12**, 169–181.
22. De Jong, K. S., Warner, C. & Catterall, A. (1990) *J. Biol. Chem.* **265**, 14738–14741.
23. Jay, S. D., Sharp, A. H., Kahl, S. D., Vedvick, T. S., Harpold, M. M. & Campbell, K. P. (1991) *J. Biol. Chem.* **266**, 3287–3293.
24. Morton, M. E. & Froehner, S. C. (1989) *Neuron* **2**, 1499–1506.
25. Breitburt, R. E., Andreadis, A. & Nadal-Ginard, B. (1987) *Annu. Rev. Biochem.* **56**, 467–495.
26. Jan, L. Y. & Jan, Y. N. (1989) *Cell* **56**, 13–25.