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Transcript Scanning Reveals Novel and Extensive Splice Variations in Human L-type Voltage-gated Calcium Channel, \( \text{Ca}_{\text{v}1.2} \) \( \alpha_1 \) Subunit*§

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The L-type (\( \text{Ca}_{\text{v}1.2} \)) voltage-gated calcium channels play critical roles in membrane excitability, gene expression, and muscle contraction. The generation of splice variants by the alternative splicing of the pore-forming \( \text{Ca}_{\text{v}1.2} \) \( \alpha_1 \)-subunit (\( \alpha_1.2 \)) may thereby provide potent means to enrich functional diversity. To date, however, no comprehensive scan of \( \alpha_1.2 \) splice variation has been performed, particularly in the human context. Here we have undertaken such a screen, exploiting recently developed “transcript scanning” methods to probe the human gene. The degree of variation turns out to be surprisingly large; 19 of the 55 exons comprising the human \( \alpha_1.2 \) gene were subjected to alternative splicing. Two of these are previously unrecognized exons and two others were not known to be spliced. Comparisons of fetal and adult heart and brain uncovered a large IVS3-S4 variability resulting from combinatorial utilization of exons 31–33. Electrophysiological characterization of such IVS3-S4 variation revealed unmistakable shifts in the voltage dependence of activation, according to an interesting correlation between increased IVS3-S4 linker length and activation at more depolarized potentials. Steady-state inactivation profiles remained unaltered. This systematic portrait of splice variation furnishes a reference library for comprehending combinatorial arrangements of \( \text{Ca}_{\text{v}1.2} \) splice exons, especially as they impact development, physiology, and disease.

Rapid influx of \( \text{Ca}^{2+} \) through the \( \text{Ca}_{\text{v}1.2} \) channels initiates physiological responses like gene expression, neurotransmitter release, cardiac or smooth muscle contraction, and regulation of \( \text{Ca}^{2+} \)-dependent ion channels (1–4). In these capacities, the functional profile of \( \text{Ca}_{\text{v}1.2} \) calcium channels can be customized by combinatorial assembly of the \( \text{Ca}_{\text{v}1.2} \) \( \alpha_1 \) with several different auxiliary \( \beta \)- and \( \alpha_6 \)-subunits (5). Even greater flexibility in functional tuning could arise from alternative splicing of the \( \alpha_1 \)-subunit genes (6); splicing of the human \( \alpha_1.2 \) subunit gene is known to generate variants with tissue-specific biases and with distinct pharmacological properties (7). At present, 15 of 53 known exons (Fig. 1) of the human \( \alpha_1.2 \) gene have been reported to be subjected to alternative splicing (8). However, the full set of possible splice loci and variations and their distributions in heart and brain could far exceed this initial view.

Recently, genome-wide analyses suggest that as high as 74% of human genes are alternatively spliced (9). Alternative splicing of pre-mRNA has been implicated in development, physiology, and pathophysiology, and the inclusion or exclusion of exons can be regulated in a tissue-specific or temporal manner (10, 11). Splice variations of human \( \alpha_1.2 \) subunit confer on the channel isoforms altered properties such as sensitivity to blockade by antagonists, regulation by protein kinase, current density, and activation and inactivation characteristics (12–14). However, these studies have reported generally on the impact of alternative splicing on \( \text{Ca}_{\text{v}1.2} \) channel functions by characterizing limited numbers of cDNA clones isolated from a single library.

We decided to employ the transcript scanning method (15) to search systemically for the full suite of alternatively spliced exons of the human \( \alpha_1.2 \) subunit. Four human cDNA libraries (fetal and adult brain and heart cDNA libraries) were investigated to determine tissue or developmental regulation of the utilization of splice exons. In this study, we have discovered novel splice exons and have uncovered extensive splice variations especially in the IVS3 and IVS3-S4 linker region. To illustrate functional diversity generated by alternative splicing of the \( \alpha_1.2 \) subunit, we performed electrophysiological recordings of the IVS3-S4 splice variants for the following reasons. (i) The IVS3-S4 region is subjected to extensive splicing. (ii) The addition of just two amino acids in the same linker region in the \( \text{Ca}_{\text{v}2.1} \) and \( \text{Ca}_{\text{v}2.2} \) channels showed altered biophysical and pharmacological properties (16, 17). (iii) The equivalent region was suggested to form part of the voltage-sensor paddle in the potassium channel, KvAP (18, 19). Our results showed that the \( \text{Ca}_{\text{v}1.2} \) splice variations at the IVS3-S4 region generated a range of current-voltage relationships and voltage-dependent activation characteristics that correlated with the linker length.

**Experimental Procedures**

Transcript Scanning by PCR—The transcript scanning method has been described in detail by Mittman et al. (15) and Soong et al. (20) for the systematic identification of loci for alternative splicing of the following voltage-gated calcium channel genes: \( \text{CACNA1I} \), \( \text{CACNA1G} \), and \( \text{CACNA1A} \). Here we briefly describe the method. Pairs of primers

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were used to amplify 20 amplicons covering overlapping regions of the entire human Cav1.2 α1-subunit. The size of the PCR products ranged from 360 to 670 bp, spanning between 1 and 5 exons. We used 2–5 lots of fetal and adult brain and fetal and adult human cDNA libraries (Quick-clone cDNA, catalog numbers 7129-1, 7187-1, 7168-1, and 7121-1; and Marathon-Ready cDNA, catalog numbers 7402-1, 7400-1, and 7404-1, Clontech; fetal heart cDNA, catalog number D8864-01, Gene Pool; Invitrogen) for the transcript scanning reactions. Details of the method are found in the Supplemental Material or in Soong et al. (20).

**Determination of Distribution of IVS3–54 Splice Variations by Nested PCR—** Flanking primers were employed for PCR across exons 28–37 (primers 23 and 28; supplemental Table S1) or across exons 30–35 (primers 25 and 26; supplemental Table S1) of the human Cav1.2 α1-subunit from fetal and adult brain and heart cDNA libraries. The amplicons were subcloned into pGEM-T Easy vector and transformed into DH10B Escherichia coli cells. About 280 colonies from each group were picked as templates in a second nested PCR by using exon 31-specific forward primer (5′-CAGCCATGAGCATTGCATT-3′) or exon 32-specific forward primer (5′-TGGTATGATATTCAACTC-3′) and a common reverse primer (5′-CTTACACAGGCATGGACG-3′). cDNA fragments of the five splice combinations (+31, -32, and +33); (-31, +32, and +33); -31, +32, and -33; (-31, +32-6 nt, and +33), and (-31, +32-6 nt, and -33) were subcloned into pGEM-T Easy vector and used as controls for the colony PCR screening experiments. The different splice combinations were differentiated based on their distinct migration patterns in 4–5% agarose gels. To verify the accuracy of the gel analysis, plasmids extracted from representative colonies were sent for DNA sequencing.

**Construction of Full-length Human Cav1.2 Splice Variants—** The parental full-length human Cav1.2 α1-subunit (α1C77WT) in pBluescript vector was kindly provided by Dr. R. D. Zuhlke (Swiss Agency for Therapeutic Products, Switzerland). The exon combination of α1C77WT is as follows: 1–20, 22–30, 32–44, and 46–50 (21, 22). PCR fragments containing variation A (-31, 32-6 nt, and -33), variation B (-31, 32-6 nt, and +33), and variation C (-31, +32, and -33) were amplified from an adult heart cDNA library using primers 23 and 28 (supplemental Table S1), whereas variation D (-31, +32, and +33) was amplified from a fetal heart cDNA library. The four splice variations (supplemental Table S1), whereas variation D (-31, +32, and +33) were used to amplify 20 amplicons covering overlapping regions of the entire Cav1.2 α1-subunit. The size of the PCR products ranged from 360 to 670 bp, spanning between 1 and 5 exons. We used 2–5 lots of fetal and adult brain and fetal and adult human cDNA libraries (Quick-clone cDNA, catalog numbers 7129-1, 7187-1, 7168-1, and 7121-1; and Marathon-Ready cDNA, catalog numbers 7402-1, 7400-1, and 7404-1, Clontech; fetal heart cDNA, catalog number D8864-01, Gene Pool; Invitrogen) for the transcript scanning reactions. Details of the method are found in the Supplemental Material or in Soong et al. (20).

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**Diversity of α1,2 Subunit**

**A. Backbone structure of Cav1,2 α1-subunit**

**B. Transcript-scanning reactions**

**C.**
reported elsewhere (Fig. 1). The method (Fig. 1B) required PCR amplifications across 1–5 exons producing products of 360–670 bp in size. Careful analyses by DNA sequencing of the PCR products and examination of all exon-exon junctions revealed all possible splicing loci of the human α₁₂β subunit in a given tissue. Fig. 1C is an example in which amplicon 30–35, subcloned into the pGEM-T Easy vector and screened by colony PCR, demonstrates splice variations at the IVS3-S4 region when analyzed by electrophoresis in 5% agarose gel. DNA sequencing confirmed the combinatorial identities of 4 of the possible 12 splice combinations. The entire 12 IVS3-S4 splicing permutations are generated by the inclusion or exclusion of exons 32, 32-6 nt, and 33 (six combinations), exons 31 and 33 (four combinations), and combinations of either (+31, +32, and +33) or (+31, +32-6 nt, and +33). We found all the IVS3-S4 splice variations by screening human fetal and adult heart and brain cDNA libraries (Table I).

Novel and Extensive Splice Variations Revealed by Transcript Scanning—Fig. 2 illustrates the distribution of splice sites across the entire length of the human Ca,1.2 message. Four pairs of exons are by and large spliced in a mutually exclusive manner: 1/1a, 8/8a, 21/22, and 31/32. Six exons including 9, 9*, 10*, 33, 45, and 45* are alternatively spliced, whereas seven other exons are spliced at the alternate junctions namely 3, 7, 15, 17, 32, 41, and 45* (Table I, also see supplemental Figs. A and B for the detailed splicing patterns). Exons 32 and 45* are unique as they could be spliced either as alternate exons or at alternate donor sites. Junctional splicing at the acceptor site of exon 17 could either produce an insertion of a 9-nucleotide (9-nt) sequence (...ACTGGCCAG...) without disrupting the reading frame or an insertion of 12 nt (...CTGACTGGCCAG...) giving rise to an in-frame TGA stop codon immediately 3’ of exon 16. The 12-nt insertion might produce a two-domain hemichannel as exon 16 resides on the II-III loop of α₁₂β subunit.

Exons 9* and 10* are novel and have not been described for the human α₁₂β subunit transcript in the literature, whereas exons 3 and 9 have not been reported to be subjected to alternative splicing. Exon 9* is a 75-nt insert between exons 9 and 10, whereas exon 10* adds 120 nt to the mRNA between exons 10 and 11 in the I-II loop of the channel (GenBank™ accession numbersAY562395 for 9* andAY562396 for 10*). It is notable that 10* was not annotated as a potential exon in the database but was detected by transcript scanning in 1 of 8 clones of amplicons 8–11 that were sequenced. Essentially, the canonical splice sites and a pyrimidine tract flanking 10* confirm it as an exon. Predictably, a number of splicing events would produce nonfunctional channels as follows: insertion of 4 nt at the 5’ end of exon 3; deletion of 73 nt in 15; deletion of both 8 and 8a or 8, 8a, and 9; deletion of both 21 and 22; and the deletion or inclusion of both 31 and 32 (Table I and supplemental Figs. A and B). The 4-nt insertion into the 5’ end of exon 3 shifts the reading frame of the α₁₂β transcript. This would give rise to a premature stop with the production of only the N terminus of the α₁₂β subunit, and it would be interesting to determine whether this short cytoplasmic fragment may play any dominant-negative regulatory role. What is also intriguing was the large number of splice variations detected in the IVS3-S4 region where we identified all 12 combinations derived from random inclusion or exclusion of 31/32, or 32-6 nt, and 33.

The transcript scanning method was unable to detect splicing of the first or last exon as the method requires flanking exons for the design of PCR primers. However, exon 1a has been reported in human, rabbit, and rat (12, 26, 27). By using 1a-specific primers (primers 1 and 2, see supplemental Table S1), we were able to detect exon 1a expression in the human

Table I

| Locus | Splice variation profile | Known human splice variants | Ref | Human Whole Brain | Human Adult Heart | Human Fetal Heart | Human Fetal Brain |
|-------|-------------------------|-----------------------------|-----|-------------------|-------------------|------------------|------------------|
| 1     | 1a                      | +                           | 12  | -                 | -                 | +                | -                |
| 2     | 1b                      | -                           | 28  | +                 | +                 | +                | +                |
| 3     | 2a                      | -                           | 33  | +                 | +                 | -                | +                |
| 4     | 3a                      | -                           | 32  | +                 | +                 | +                | +                |
| 5     | 4a                      | -                           | 11  | +                 | +                 | +                | +                |
| 6     | 5a                      | -                           | 10  | +                 | +                 | +                | +                |
| 7     | 6a                      | -                           | 9   | +                 | +                 | +                | +                |
| 8     | 7a                      | -                           | 8   | +                 | +                 | +                | +                |
| 9     | 8a                      | -                           | 7   | +                 | +                 | +                | +                |
| 10    | 9a                      | -                           | 6   | +                 | +                 | +                | +                |
| 11    | 10a                     | -                           | 5   | +                 | +                 | +                | +                |
| 12    | 11a                     | -                           | 4   | +                 | +                 | +                | +                |

For each locus, the splice variations are displayed by the numbering of the exons in the boxes, and alternative splicing of exons is shown to have occurred when the numbering of the exons is not in numerical sequence. Splicing at alternate junctional acceptor or donor sites is indicated by a hatched box for insertion and an open box for deletion. Extensive IVS3-S4 splice variations have been found at locus 10. Splice variants in human α₁₂β subunits detected in this study and reported in the literature are indicated by ‘*’. 
Diversity of α₁,1.2 Subunit

FIG. 2. Diagrammatic representation of exons of the human α₁,1.2 subunit, and the loci of various splice variations are indicated. There are 19 exons subjected to splicing: 8 are alternate exons indicated by black boxes (9, 9*, 10*, 33, 45, and 45*); 8 are mutually exclusive exons indicated by hatched boxes (1, 1a, 8, 8a, 15, 17, 32, 41, 31, and 32); and 7 exons are spliced at the junctions indicated by open boxes (3, 7, 15, 17, 32, 41, and 45*). Exons 32 and 45* can be spliced out entirely as an exon or at the donor site. Two exons, 9* and 10*, are novel exons as described in this report; the total number of exons in α₁,1.2 subunit is now 55.

A.

Exon 1a - 2

Exon 30 - 35

B.

FIG. 3. Tissue expression of exon 1a and exon 45. A, PCR across 30–35 amplicon (lower panel) indicated the presence of Ca₁,1.2 channels in human aorta and fetal and adult heart or brain. Exon 1a-specific primers reveal exon 1a containing Ca₁,1.2 channels (upper panel) present in human heart (lane 1) and human fetal heart (lane 2) but undetectable in human aorta (catalog number 7425-1, Marathon-Ready™ cDNA) (lane 3), human adult whole brain (lane 4), or human fetal brain (lane 5). Lane 6 is the water control. B, exon 45 was detected in Ca₁,1.2 channels from human fibroblast cell line, Mrč5 (lane 2), but not in human fetal brain (lane 1), human adult heart (lane 3), or human fetal heart (lane 4). Instead, exon 45* was detected in all the tissues other than in the human fibroblast cell line, Mrč5.

heart but not in the aorta or brain (Fig. 3A). In all our scanning, we have been unsuccessful in detecting exon 45 in human heart or brain, but we found that exon 45 was expressed at a high level in the human fibroblast cell line Mrč5 (Fig. 3B). This observation agreed with the report of exon 45 detected in human fetal skin fibroblast cell line CRL 1475 (28). Instead, we detected exon 45* in the human heart and brain cDNAs but not in fibroblast. We were also unable to detect the splice profile of the hippocampal cDNA clone h54 engineered into α₁C,kb (29), which included the deletion of 17 nt from 3' end of exon 40 (40A), exon 40B, and the addition of 132 nt at the 5' end and 30 nt at the 3' end of exon 43 (43A). However, such a cDNA clone might have been constructed from incompletely spliced nuclear pre-mRNA (13).

Transcript scanning has revealed 40 splice variations in the human α₁,1.2 subunit transcript, and of these we discovered 16 novel variations (Table I and supplemental Figs. A and B). Two of these 16 novel splice variations have been reported in other species. Exon 9* was found in rabbit lung, mouse brain, and a rat smooth muscle cell line A7R5 (30–32), whereas the 9-nt insertion at exon 17 was reported in rat brain (33). Inspection of the CACNAIC gene (NT_009759, gi:29805200) published in the GenBank™ data base demonstrated the use of the conventional GU-AG (34) donor and acceptor splice sites in almost all of the splice exons (supplemental Figs. A and B). One exception is the splicing between the invariant exons 2 and 3, which do not follow the GU-GU or AU-AC rule (34), but employed the AU-GA splice donor and acceptor sites. The other three exceptions are the insertion of 4 nt into exon 3 which employed the unconventional AU-UU splice sites, whereas the insertion of either 9 or 12 nt into exon 17 used the unconventional GU-GU splice sites. Altogether, we have identified 12 loci, 19 splice exons, and 40 splice variations in the human α₁,1.2 subunit. This report therefore provides a comprehensive examination of all splice loci and splice combinations to highlight the rich repertoire of genetic variations in the human Ca₁,1.2 channel.

In-depth Analysis of Predominant Splice Combinations in IVS3-S4—Transcript scanning provided a quick glimpse of the possible splicing events for most of the exons in the α₁,1.2 message. Because of the numerous PCRs performed on four cDNA libraries as templates, we could only screen a limited number of bacterial colonies (8–32 colonies) and sequenced a small number of PCR products showing different sizes (2–4 clones). It is conceivable that we might not detect low abundant splice exons expressed in different tissues or developmental stages. The data in Table I show the splice exons we detected in two types of tissues but do not define any proportions of spatial or temporal distributions. To generate such profiles, a more vigorous investigation of the distribution of splice variations was conducted for the IVS3 and IVS3-S4 linker region that are encoded by exons 31, 32, and 33 (33, 35). We decided to investigate the functional diversity in this region because inclusion or exclusion of a dipeptide in the same region in Ca₂,1.2 and Ca₉,2.2 channels produced altered biophysical and pharmacological characteristics (16, 36). Two colony libraries containing either PCR products of amplicon 30–35 (~500 bp) or amplicon 28–37 (~1240 bp) were generated and assessed to exclude the possibility of preferential PCR of one splice combination over another. Here more than 280 bacterial colonies of each recom-
Diversity of α1,2 Subunit

Parameters of current-voltage relationships of αC77WT and splice variants

The I-V curves were fitted by

\[ I = \frac{g_{\text{max}}(V - E_{\text{rev}})(1 + \exp(V - E_{\text{rev}})/\kappa)}{\kappa} \]

where \( g_{\text{max}} \) is maximum conductance; \( E_{\text{rev}} \) is reversal potential; \( V \) is voltage at 50% of \( I_{\text{rev}} \) activation; \( k \) is slope factor; and \( n \) is number of tested cells.

### Table II

| Splice variants | \( V_{\text{rev}} \) (mV) | \( h_L \) | \( n \) |
|-----------------|--------------------------|-------|-----|
| αC77WT          | 48.0 ± 1.13              | -9.3 ± 1.28 | -5.99 ± 0.32 | 8   |
| αC77-A          | 46.3 ± 0.72              | -18.8 ± 0.97 | -5.80 ± 0.28 | 11  |
| αC77-B          | 46.7 ± 0.64              | -14.2 ± 0.60 | -6.39 ± 0.25 | 16  |
| αC77-C          | 47.1 ± 0.58              | -16.1 ± 0.86 | -6.31 ± 0.26 | 16  |
| αC77-D          | 45.7 ± 0.30              | -11.8 ± 0.94 | -7.28 ± 0.34 | 9   |

\( p < 0.05 \) compared with the αC77WT channel (one-way ANOVA and Tukey test).

**FIG. 4. Distribution of IVS3-S4 splice variations in fetal and adult heart and brain.** A, comparison of the amino acid sequences of the IVS3-S4 splice variations of WT and variations A–D. B, the bar chart displays the predominant splice combination, WT (~60%), in fetal heart and fetal and adult brain. Adult heart has two almost equal populations of WT (39%) and C (31%) splice variations. Others include the seven rare splice variations listed in Table I. The standard error of the distribution of each variation in a tissue is shown. \( N_{\text{clone}} \) is the number of clones screened in each cDNA library. Fetal brain, \( N_{\text{clone}} = 285 \); fetal heart, \( N_{\text{clone}} = 234 \); adult heart, \( N_{\text{clone}} = 552 \). The \( z \) test was used to determine the statistical significance between the altered expression of variation \( D \) in brain and variations WT, A, and C in heart as indicated by ** (\( p < 0.01 \)).

Biconat cDNA library containing the IVS3-S4 region were screened for the usage of exons 31, 32 (32-6 nt), and 33. Both libraries provided corroborating results on the distribution of the IVS3-S4 splice variants in the cDNAs examined. Of the 12 splice combinations, at least 5 were predicted to form functional channels (Fig. 4A and Table I). Detailed analysis of the colony PCR screens indicated that the predominant splice combination for the fetal heart and the fetal and adult brain at the IVS3-S4 region was WT (~31, +32, and +33). This splice combination represented between 59 and 68% of the clones screened (Fig. 4B). There were, however, two co-dominant isoforms in the adult heart that included 39% of WT (~31, +32, and +33) and 31% of variation C (~31, +32, and +33). Whereas on the other extreme, variation A (~31, +32-6 nt, and ~33) was detected at low levels representing between 1% in adult brain to 7% in adult heart. Variation B (~31, +32-6 nt, and +33) was expressed at a more even level between 11 and 16% in all the cDNA libraries. The 3-fold increase in variation C (~31, +32, ~33) of adult (31%) over fetal (10%) heart could have arisen by developmental regulation of exon 33 (Fig. 4B and supplemental Table S2). Most interestingly, there is a 10-fold lower expression of variation C in adult brain when compared with adult heart pointing to a tissue or physiological difference in expression (supplemental Table S2). A puzzling observation is that ~2–5% of the clones screened either include or exclude the pair of mutually exclusive exons 31/32 that will likely generate nonfunctional channels (Table I and supplemental Fig. B). The strict rule of mutual exclusivity in the inclusion or exclusion of either 31 or 32 did not apply to the α1,2 subunit transcript. Nonetheless, the combination +31 and -32 was represented between 8 and 16%, whereas the combination ~31 and +32 ranged from 82 to 88% of the clones examined.

**Alternative Splicing Generated Electrophysiological Differences in Ca,1,2 Variants**—The reference Ca,1,2 channel, αC77WT, that contained the predominant IVS3-S4 splice variation is designated the wild type (WT) clone (21, 22). Whole-cell \( I_{\text{rev}} \) was measured to compare the current-voltage (I-V) relationships between the WT and the four splice variants αC77-A, -B, -C, and -D (Table II). The \( V_{\text{rev}} \) for the αC77WT was ~9.3 ± 1.28 mV (\( n = 8 \)), and three splice variants (αC77-A, -B, and -C) containing exon 32 and a shorter IVS3-S4 linker region demonstrated a shift in the I-V curve into the hyperpolarized direction. Approximate fits of the I-V relationships hinted at differences in the voltage dependence of activation, with the half-point of voltage activation for single Boltzmann terms (\( V_{\text{rev}} \)) yielding significant differences. The I-V curve of variant αC77A shifted ~9.5 mV (‐18.8 ± 0.97 mV, \( n = 11 \)), αC77-B shifted ~4.9 mV (‐14.2 ± 0.60 mV, \( n = 16 \)), and αC77-C shifted ~6.8 mV (‐16.1 ± 0.86 mV, \( n = 16 \)) to the left (Fig. 5). Nonetheless, the difference between the \( V_{\text{rev}} \) for the WT and αC77WT was not statistically significant. The reversal potentials for all the channel splice variants were similar.

To scrutinize further the differences in activation properties between variants, we conducted tail-current analyses, yielding G-V curves as shown in Fig. 6. The channels were activated by a voltage family of 20-ms test pulses (~40 to 120 mV, \( \Delta V = 10 \) mV), and tail currents were measured after repolarization to ~50 mV for 10 ms (Fig. 6A). The \( V_{\text{act}} \) of the splice variants (Table III) were left-shifted in the hyperpolarization direction as compared with the αC77WT (10.4 ± 2.0 mV, \( n = 11 \)) by ~11.2 mV for αC77A (~0.8 ± 1.0 mV, \( n = 7 \), \( p < 0.01 \)), by ~5.3 mV for αC77B (~5.1 ± 1.4, \( n = 10 \), \( p < 0.05 \)), and by ~10.4 mV for αC77C (~0.2 ± 1.2, \( n = 9 \), \( p < 0.01 \)). There was no statistical difference for the \( V_{\text{act}} \) when compared with the αC77-D variant that contained equal IVS3-S4 linker lengths, even though 12 of 28 amino acids coding for exons 31/32 are different. Compared with I-V relationships, the tail-current analysis provides a more quantitative and rigorous assessment of activation properties. In fact, fits to these data indicated that the activation process was better described by a dual Boltzmann characterization, which only becomes obvious with the tail-current analysis. Half-activation as determined by the dual Boltzmann fits (\( V_{\text{act}} \)) confirmed the rank order of effects of splice variation on the voltage dependence of activation.

There appeared to be a correlation between the IVS3-S4 linker length (33) of the variants and the amount of hyperpolarizing shift in the voltage dependence of activation when compared with the WT (Fig. 6C). This is evident in the large shift in the \( V_{\text{act}} \) by ~10 to ~11 mV for a deletion of 11–13 aa compared with a smaller shift of 5 mV for a 2-aa deletion in the
linker. Similar results are also demonstrated for the shift in I-V relationships. Absolute differences between $V_{1/2}$ (determined by qualitative I-V curve analysis) and $V_{1/2,act}$ (determined from tail G-V analysis) are not surprising, given the limited and qualitative view of activation afforded by the I-V curve data. However, we cannot exclude the possibility that the amino acid sequence in the linker may play a role in altering the biophysical properties of the splice variants when compared with the WT channels.

The steady-state inactivation protocol involves eliciting a test pulse at 10 mV for 30 ms that preceded a family of depolarizing prepulses held for 15 s and then followed by another test pulse evoked at +10 mV for 104 ms. The voltage dependence of steady-state inactivation of all the splice variants when assessed in comparison to the $\alpha_{1C77}^{WT}$ was found to be similar (Fig. 7). The differences in their $V_{1/2, inact}$ values were of no statistical significance as determined by the one-way ANOVA and Tukey tests (Table IV).

The electrophysiological characterizations of the $\alpha_{1C77}^{WT}$ and variants revealed the range of phenotypic variations of the genetic isoforms derived from alternative splicing at a single locus. Although at least 5% of the 12 variants form functional channels, most of the other variants are likely nonfunctional. These variants mainly have the pair of mutually exclusive exons 31/32 included or excluded or all three exons 31/32/33 deleted. Such splicing events will possibly disrupt the topology of the $\alpha_{1C77}$ channels at the C terminus from IVS3 onward, as the proteins are still produced in-frame without encountering any premature stop codon until the end of the C terminus.

DISCUSSION

Systematic Identification by Transcript Scanning Revealed Extensive Splice Variations of $\alpha_{1.2}$ Subunit—We have exploited a transcript scanning method (15, 20) to obtain a comprehensive screen of alternative splicing in the human $\alpha_{1C77}^{WT}$ gene. In the process, we have discovered novel exons, and we obtained evidence of remarkably extensive heterogeneity encompassing a total of 40 splice variants. In particular, 18 of the 55 exons in the $\alpha_{1.2}$ gene were subjected to splicing, either by the inclusion or exclusion of the entire exon or at alternate splice junctions. The exception is exon 1/1a that has been reported to be derived by the activation of alternate promoters (37, 38). Overall, the theoretical number of $\alpha_{1.2}$ splice variations produced by random splice decisions at each locus would be staggering (219 combinations for the $\alpha_{1C77}^{WT}$ subunit). This could support enormous diversity in channel functional properties, for which our systematic compendium of splice variation (Fig. 2, Table I, and supplemental Figs. A and B) serves as a simplifying reference library.

Potential Functional Consequences of Various Splice Variants

Alternative Splicing at N Terminus of $\alpha_{1.2}$ Subunit—In the N terminus, exon 1/1a has been reported to be derived by the activation of alternate promoters, and we confirmed that the expression of exon 1a is cardiac muscle-specific (37, 38). Protein kinase C was shown to mediate the attenuation of the inhibitory effect of exon 1a via the phosphorylation of a channel site or auxiliary protein (39). On the other hand, the role of the entire N-terminal fragment produced by the 4-nt insertion at the 5′ end of exon 3 requires further investigations. One possibility is that it may interfere with G$\beta$ binding to the N terminus or alleviate the inhibition of channel function (40, 41).

Splice Variations in Domain I and Novel Alternatively Spliced Exons in I-II Loop—In domain I, the junctional splicing

FIG. 5. Current-voltage relationships of WT and splice variants. A, exemplar test pulses of the WT and splice variants stepped from -90 to -50, -30, -10, 0, or 30 mV. B, a family of whole-cell currents recorded from human $\alpha_{1C77}^{WT}$ calcium channel or its splice variants with rat $\beta_{2A}$ and $\alpha_{1.3}$ subunits in HEK293 cells. Ensemble of whole-cell I-V relationships were obtained by fitting the Boltzmann function. The whole-cell I-V relationships were obtained by holding the cell at -90 mV before stepping to various potentials from -50 to 50 mV ($\Delta V = 10$ mV) over 900 ms. $V_{1/2}$ values and the numbers of the recorded cells for each splice variant are presented in Table II.
at exon 7 would result in the deletion of 4 aa in the IS5-S6 P-loop region, and how this may affect Ca\(^{2+}\) permeation is unknown. The exon 8/8a form the IS6 segment, and they affect the sensitivity of Ca\(_{1.2}\) channels to the dihydropyridine block (7). In the I-II loop, we only detected exclusion of exon 9 together with 8 or 8a possibly giving rise to nonfunctional channels as IS6 (exon 8/8a), and the \(-\alpha\) interacting domain (exon 9) (42, 43) will be missing; besides, the protein will be translated out-of-frame with a premature stop. On the other hand, exons 9* and 10* have not been reported in human Ca\(_{1.2}\) subunit. It has been hypothesized that 9* of the rat Ca\(_{1.2}\) subunit may be a target for phosphorylation (31).

Possible Generation of Hemichannels from Alternative Splicing at Domain II and II-III Loop—There is also the possibility that hemichannels, produced in Cav1.2 subunit by the deletion of 73 nt from exon 15 (domain II, P-loop) or the insertion of a 12-nt at the 5'-end of exon 17 (II-III loop), may act in a dominant-negative fashion to dilute out the \(\alpha\) subunit interaction (44).

![Figure 6](image6.png)

**FIG. 6.** Different human Ca\(_{1.2}\) calcium channel splice variants confer distinctive activation behavior to the L-type channels. A, exemplar tail currents evoked by repolarizations to \(-50\) mV after depolarizing test pulses at \(-40, 0, 40,\) or \(90\) mV. B, ensemble of activation properties of \(\alpha\)C77WT and splice variants obtained from traditional tail-activation protocol. The channels were activated by a variable-voltage family of 20-ms test pulses, from \(-40\) to \(120\) mV, and tail currents were recorded on repolarization to \(-50\) mV. Normalized \(G_{pmax}/G_{max}\) versus \(V\) curves were generated from a dual Boltzmann function. \(\overline{V}_{1/2}\) values and the numbers of the recorded cells for each splice variant are presented in Table III. C, correlation of IVS3-S4 linker length to hyperpolarized shift in I-V curve and voltage-dependent activation of variants. The IVS3-S4 linker is estimated to be 24 aa long consisting of part of exon 31 or 32, the entire exon 33, and part of exon 34 (33). The IVS3-S4 linkers are either of the same length (variant D) or shorter by 13 (variant A), 2 (variant B), or 11 aa (variant C).

![Figure 7](image7.png)

**FIG. 7.** Steady-state inactivation properties of Ca\(_{1.2}\) calcium channels are insensitive to splice variation at IVS3-S4 region. A, exemplar current traces after 15-s conditioning depolarizing pulses evoked at \(-120, -90, -60, -40,\) or \(-20\) mV. B, ensemble of steady-state inactivation properties obtained from traditional steady-state inactivation protocol. The channels were evoked by the steady-state protocol in which a 30-ms normalizing pulse to 0 mV is followed by a variable-voltage family, from \(-120\) to \(20\) mV, of 15-s conditioning pulses and a 104-ms test pulse to 0 mV. Plots of the steady-state inactivation, \(h(15\text{s})\), as a function of voltage of conditioning pulse were obtained from normalized data points obtained from peak values of prepulse and test pulse currents (\(h(15\text{s})/I_{test}/I_{prepulse}\)). The smooth curves were generated from a single Boltzmann function. \(V_{1/2}\) inact values, and the numbers of the cells recorded for each splice variant are presented in Table IV.

**TABLE III**

| Splice variants | \(V_{1/2}\) act (mV) | \(n\) |
|----------------|---------------------|-----|
| \(\alpha\)C77WT   | 10.4 ± 2.0          | 11  |
| \(\alpha\)C77-A    | \(-0.8 ± 1.0^a\)    | 7   |
| \(\alpha\)C77-B    | 5.1 ± 1.4^a         | 10  |
| \(\alpha\)C77-C    | \(-0.02 ± 1.2^a\)   | 9   |
| \(\alpha\)C77-D    | 11.4 ± 2.3          | 6   |

\(^a\) \(p < 0.01\) when compared with the \(\alpha\)C77WT channel (unpaired \(t\) test).

\(^b\) \(p < 0.05\).
5′ end of exon 41 adds 19 aa to the pre-IQ3 region of the channel, and how this may modulate the tethering of the calmodulin to the C terminus of Ca_{1.2} and impact on Ca^{2+}-dependent inactivation would have to be determined (52). The deletion of 187 nt from exon 45* produces a truncated C terminus that removes the serine cAMP-dependent protein kinase A site in exon 48 (S1928) (53, 54). To determine the physiological consequence of the junctional deletion at this low expressing splice exon would require further investigations. However, exon 45* has been implicated to be important for oxygen sensing (55, 56). Most interestingly, the role of exon 45, expressed specifically in fibroblast, has not been characterized.

**TABLE IV**

| Splice variants | k | n |
|-----------------|---|---|
| a1C77WT         | -39.14 ± 2.43 | -8.0 ± 0.69 | 6 |
| a1C77-A         | -40.73 ± 2.27 | -8.2 ± 0.69 | 6 |
| a1C77-B         | -42.25 ± 1.34 | -9.8 ± 0.45 | 8 |
| a1C77-C         | -39.46 ± 2.77 | -9.2 ± 0.79 | 8 |
| a1C77-D         | -39.98 ± 1.17 | -9.9 ± 0.40 | 7 |

Electrophysiological and Pharmacological Properties of Domains III or IV Splice Variations—In the IIIIS2 segment, the exons 21/22 imparted differences in the voltage dependence of the dihydropyridine block of the Ca_{1.2} variants (21), although we have demonstrated that 31/32 (IVS3) did not produce different biophysical properties of the Ca_{1.2} channels. Contrary to a reported higher representation of exon 31 in normal ventricular myocardium (45), we found a >82% representation of exon 32 in whole heart, which is in agreement with the expression in rat heart (46). The deletion of 6 nt or 2 aa (-VN-) from exon 32 that resulted in a 5-mV hyperpolarizing shift of the I-V curve is reminiscent of the effect of a 2-aa insertion on the channels (16, 36, 47). However, the mechanistic generation of the 2-aa inclusion or exclusion is different as the -NP- and -ET-dipeptides are encoded on a 6-nt exon in the α_{1.2} and α_{2.2} subunit genes, respectively, whereas the deletion of the 6-nt in α_{1.2} is via the use of alternate donor splice site (supplemental Fig. B). A number of splice variants have been reported that display altered biophysical, biochemical, or pharmacological properties (6, 8, 16).

**Activation Potential and IVS3-S4 Linker Length**—Here we have shown an extensive splice variation in the IVS3-S4 region that produced an apparent correlation of voltage-dependent activation to IVS3-S4 linker length, providing a basis for future in-depth investigations into structure-function relationships in channel activation. However, in the Shaker potassium channels, the amount of shift in the voltage activation curves was not strictly proportional to the S3-S4 linker length but instead showed periodicity in half-activation voltages (48). Our study, on the other hand, is based on native splice variants that produced correlative and depolarizing half-activation potential shifts to increasing linker lengths. A possible physiological correlate, at least in the heart, relates to the channel behaviors of the IVS3-S4 splice variants influencing either the shape or the duration of ventricular action potentials (49). Transgenic mouse models could be employed to evaluate the biological consequences of the shifts in half-activation potentials arising from the splice variations of the IVS3-S4 in specific tissues. Our results are consistent with the depolarizing shift in activation potential with longer IVS3-S4 linker lengths observed in the P/Q-type α_{2.1} and N-type α_{2.2} calcium channel splice variants (16, 17) but different from the hyperpolarizing shift seen in mutated linker lengths shorter than 7 aa in the Shaker potassium channel (50).

**Alternative Splicing at C Terminus of α_{1.2} Subunit**—In the C terminus, the EF-a and EF-b exons of Ca_{2.1} or Ca_{2.2} confer different Ca^{2+}-dependent regulation, cell-specific localization, or current density (11, 51). The α_{1.2} subunit does not contain any mutually exclusive EF-a or EF-b exon. We also did not find EF-a or EF-b in other L-type α_{1.1}, α_{1.3}, or α_{1.4} subunits (data not shown). Nonetheless, the insertion of the 57 nt at the

**Potential for Customization of Splicing Profiles in Relation to Physiology**

Alternative splicing has been shown to play crucial roles in sex determination, development, physiology, and disease (57–59). The number of splice variations can be as many as thousands in the Discarr gene (60). The actual number of splice variations is possibly much lower than the theoretical permutations because the splicing event is probably not random but linked (61). It is therefore conceivable, with a nonrandom utilization of splice exons, to describe a predominant genetic combination consisting of invariant backbone and splice exons in different tissues and under various cellular conditions.

Here we showed that exon 9* was expressed in adult heart and not brain, but the transcript scanning method could not provide information on the combinatorial profile of other splice exons. Similarly, we demonstrated developmental regulation of exon 33 in the heart, but we were unable to determine whether utilization of other splice exons was changed in tandem. Even though the transcript scanning method has uncovered novel and new splice variations, it may still not be able to detect rare splice variants or splice variants expressed in a cell-specific manner or under certain developmental or physiological states.

It is puzzling why a cell requires an enormous number of channel splice combinations exhibiting equally diverse phenotypic variations. This situation makes it extremely difficult to determine the biological role of a single splice exon. Nonetheless, the biological role of a splice exon may be examined in transgenic mouse models by the specific ablation of a single exon in the channel gene.

What would be equally intriguing is to understand the potential for customization of splice combinations in response to internal or external cues or pathological conditions and to determine whether the alteration in splice exon usage is an adaptation or a consequence of change. To better understand splicing profiles, a more vigorous and quantitative method is needed to assess tissue and temporal regulation of splice exon expression. Probing for splice combinations of full-length libraries of the α_{1.2} subunit constructed from different tissues obtained under various conditions will allow tracking of the utilization of exons in isolation or in combination with other splice exons.

The comprehensive elucidation of the splice variations of the human α_{1.2} subunit in this report represents a significant step toward understanding the complexity of Ca_{1.2} calcium channels. It will provide a framework to investigate the role of alternative splicing of α_{1.2} in generating Ca_{1.2} channel functional variations to fine-tune membrane excitability, excitation-transcription coupling and smooth or cardiac muscle contraction.

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