Tissue-specific expression of two human $Ca_v1.2$ isoforms under the control of distinct 5′ flanking regulatory elements

Li Pang[a], Gideon Koren[b], Zhiguo Wang[a], Stanley Nattel[a,c,*]

[a] *Department of Medicine, Montreal Heart Institute, and University of Montreal, 5000 Belanger St. East, Montreal, QC, Canada H1T 1C8*
[b] *Division of Cardiology, Brigham and Women's Hospital, Boston, MA 02215, USA*
[c] *Department of Pharmacology, McGill University, Montreal, QC, Canada H3G 1Y6*

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Abstract Transcriptional regulation may be important for L-type Ca^{2+} channel α_{1C} subunit ($Ca_v1.2$) gene expression. In this study, we found two human $Ca_v1.2$ isoforms, one strongly and selectively expressed in heart and the other with apparently ubiquitous expression. The promoter for the cardiac isoform has an 'initiator' sequence, and is active in neonatal cardiomyocytes but not in cardiac fibroblasts, H9C2 cells, human aorta-vascular smooth muscle and HEK293 cells. The promoter for the ubiquitously expressed isoform is of the 'housekeeping' type and is active in all cell types examined. These data indicate specific expression patterns of two human $Ca_v1.2$ isoforms under the control of distinct 5′ flanking regulatory sequences.

Key words: Exon; Heart failure; Regulatory element

1. Introduction

L-type Ca^{2+} channel α_{1C} subunit ($Ca_v1.2$), encoded by CACNA1C [1], is widely expressed and plays vital roles in electrophysiology and excitation–contraction coupling. There is important transcriptional regulation of cardiac $Ca_v1.2$. Changed $Ca_v1.2$ expression is associated with cardiac arrhythmias and congestive heart failure [2–4]. Alterations in Ca^{2+} channel properties in developing rat hearts result from transcriptionally regulated expression changes [5]. β-Adrenergic stimulation increases $Ca_v1.2$ mRNA expression in neonatal myocytes, followed by increased L-type Ca^{2+} current [6]. Protein kinase A regulates $Ca_v1.2$ expression in rat myocytes by altering transcription rate [7]. The 5′ flanking region of rat CACNA1C shows several potential transcription factor (TF) binding sites, including a putative cAMP response element, which could mediate β-adrenergic effects [8]. Recently, alternatively spliced first exons have been described in human CACNA1C [9–11], with one isoform mainly expressed in the heart and the other driven by a different promoter mainly expressed in vascular muscle cells [10,11]. In this study, we cloned these exons and their 5′ flanking regions and studied tissue-specific expression.

2. Materials and methods

2.1. RNA and genomic DNA isolation

Total RNA was extracted from normal and failing human left ventricle (LV), right ventricle, atrium and septum, human aorta vascular smooth muscle cells (HA-VSMC) and HEK293 cells with TRIzol™ (Invitrogen). Human tissues were obtained from the Réseau de tissus pour études biologiques tissue bank under procedures approved by the Human Research Ethics Committee of the Montreal Heart Institute. Additional human RNA samples were purchased from Clontech. Human genomic DNA was isolated from whole blood with Promega Wizard Genomic DNA Purification kits.

2.2. Rapid amplification of cDNA ends (RACE)

The transcription start sites of human CACNA1C transcripts were determined with Ambion's RNA ligase-mediated 5′RACE kit. Gene-specific primers (GSPs) for 5′RACE were designed based on the human CACNA1C exon 2 (GenBank accession number Z26257): GSP1: GTGGAGCTGACTGTGGAGATGGT; GSP2: CTGCCGCATTGGCATTCATGTTG. Polymerase chain reaction (PCR) products were subcloned into PCR2.1 (Invitrogen) and sequenced.

2.3. Quantification of CACNA1C transcripts

TaqMan quantitative assay of CACNA1C transcripts was performed with real-time two-step reverse transcription PCR (GeneAmp 5700, PE Biosystems), involving an initial reverse transcription with random primers. Primers for real-time PCR crossed the junctions of exon 1 (each alternative isoform) and exon 2: isoform A, forward primer (FW): TGCCAACACGGAGGTCAAG, reverse primer (REV): TTGGAACCTCCAGGAGAGATGT; isoform B, FW: GGTCCATGGTCAATGAGAATACG, REV: GGCTCCCATAGTTGGAACCTT. Absolute quantification was obtained with the use of a plasmid containing Exon 1A-2 and Exon 1B-2 sequences as a standard. Human GAPDH control reagents (Applied Biosystems) were used as internal controls. Products of amplification were also verified by gel electrophoresis and dissociation curve analysis with SYBR green.

2.4. PCR amplification of putative promoter regions and construction of promoter-luciferase fusion plasmids

PCR was performed with human genomic DNA as a template, using Advantage and Advantage-GC genomic polymerase mixes (Clontech). PCR primers were designed based on GenBank sequence AC005342. Luciferase-containing constructs were purchased from Promega. The PGL3 promoter contains the SV40 promoter element (but not the enhancer) upstream of the luciferase gene and PGL3-Basic includes only the luciferase gene with an upstream multiple cloning site for introducing putative promoter fragments. PCR prod-

*Corresponding author. Fax: (1)-514-376 1355.
E-mail address: nattel@icm.umontreal.ca (S. Nattel).

Abbreviations: $Ca_v1.2$, L-type Ca^{2+} channel α_{1C} subunit; CACNA1C, L-type Ca^{2+} channel α_{1C} subunit gene; TF, transcription factor; LV, left ventricle; HA-VSMC, human aorta vascular smooth muscle cells; 5′RACE, 5′ rapid amplification of cDNA ends; GSPs, gene-specific primers; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; FW, forward primer; REV, reverse primer

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ucts were subcloned into PCR2.1 first, and then cloned into the polylinker region of PGL3-Basic with appropriate restriction enzymes.

2.5. DNA sequencing and analysis

The integrity and orientation of all constructs were confirmed by restriction endonuclease analysis and DNA sequencing. Results were analyzed with BLAST and Clone Manager V6.0 (Sci-Ed). TF binding sites were analyzed with MatInspector V2.2.

2.6. Cell culture

HA-VSMC were grown in modified F-12K medium (American Type Culture Collection) containing 10% fetal bovine serum (FBS; Invitrogen), 10 mM HEPES, 10 mM TES, 0.05 mg/ml L-ascorbic acid, 0.01 mg/ml insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite and 0.03 mg/ml endothelial cell growth supplement. Cells in passage 22–24 were used for transfection. HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. H9C2 cells were grown in DMEM containing 10% FBS.

2.7. Neonatal rat cardiomyocyte culture

One- to 2-day-old rats were decapitated, hearts aseptically removed, dissected, minced, and exposed overnight to 0.05% trypsin (4°C). The next day, cells were dissociated with collagenase (Worthington), centrifuged (500×*g*, 4°C), and pre-plated in culture flasks (60 min, 37°C). Subsequently, non-attached cells (myocytes) were plated on 24-well plates in DMEM/F-12 medium (Invitrogen) containing 10% FBS, 0.1 mM L-ascorbic acid, 0.1 mM bromodeoxyuridine (Sigma), insulin–transferrin–sodium selenite (Invitrogen) and 10^{-9} M triiodothyronine. Over 90% of cells in culture were observed to beat.

2.8. Neonatal fibroblast culture

Neonatal rat cardiac fibroblasts were prepared as for cardiomyocytes, with adherent cells obtained during the pre-plating procedure cultured in the same medium but without bromodeoxyuridine. Cells were maintained to confluence and then plated in 24-well plates for transfection.

2.9. Transfection and luciferase assay

Cells were transfected with 1 μg PGL3-target DNA (firefly luciferase vector) and 0.1 μg PRL-TK (TK-driven *Renilla* luciferase expression vector, a control for transfection efficiency, Promega) with Lipofectamine-2000 (Invitrogen). Following transfection (48–72 h), cells were washed with phosphate-buffered saline and lysed with 150 μl passive lysis buffer (Promega). Luciferase activity was measured with a dual luciferase reporter assay kit (Promega) on a Lumat LB9507 luminometer.

2.10. Statistics

Each experiment was performed in triplicate. Reported values represent the mean of three to five separate experiments. Results are mean ± S.E.M., and statistical comparison was by Student's *t*-test.

3. Results

3.1. 5′RACE results

Two isoforms were defined by 5′RACE (Fig. 1). Isoform A (524 bp) contains a 3′ 57-bp sequence identical with CACNA1C exon 2 (Z26257). The following 467-bp region, identical to part of the human 12p13.3 P1-derived artificial chromosome RP5-1096D14 sequence (AC005342), will be termed exon 1A. Isoform B (660 bp) was obtained based on two products (647 and 660 bp), identical except for 13 bp at the 5′ end. The 3′ 57-bp region is identical to CACNA1C exon 2 (Z26257) and the following 420-bp region is identical to CACNA1C exon 1 (Z26256). The 603-bp 5′ sequence is identical to the corresponding sequence of AC005342 and will be termed exon 1B. The first two exon–intron junctions meet the definition of 'donor site' sequences: AG-gt. The 5′RACE products correspond to sequences in human chromosome 12 working draft sequence segment NT_024408, containing the CACNA1C gene.

3.2. Tissue and regional distribution

Isoform A was strongly expressed in heart (Fig. 2A). For non-cardiac tissues, only uterus and adrenal gland showed significant concentrations of isoform A transcripts (Fig. 2A). Isoform B was expressed across a broad range of tissues and cell types (Fig. 2B). In all non-cardiac tissues, isoform B/A transcript concentration ratios exceeded 1, with ratios of transcript B to A at least 8:1 (Fig. 2C).

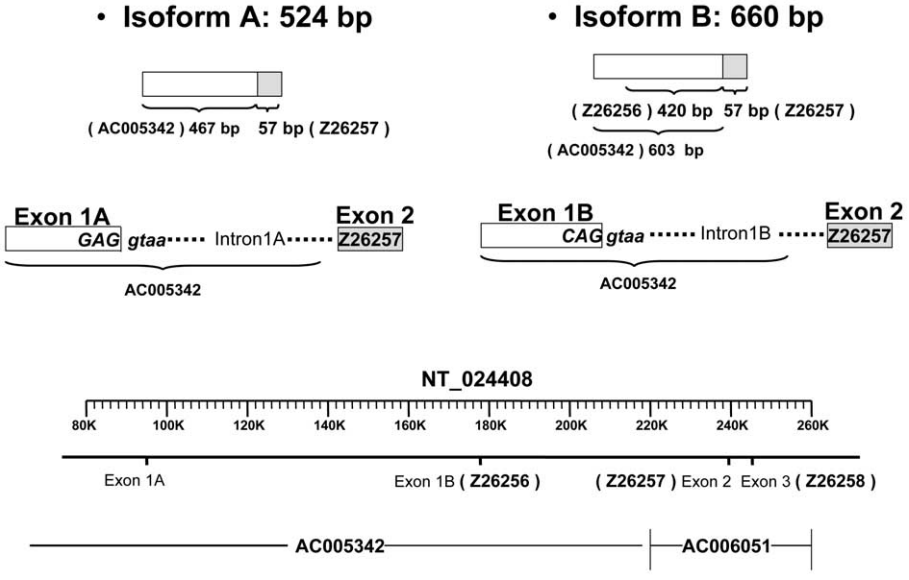

Fig. 1. 5′RACE results and proposed gene structure. Two 5′RACE products and nucleotide homology with reported GenBank sequences. The two junctions of first exon–intron boundaries meet the definition of 'donor site' sequences. The location and sizes of exons and introns are according to GenBank: NT_024408.

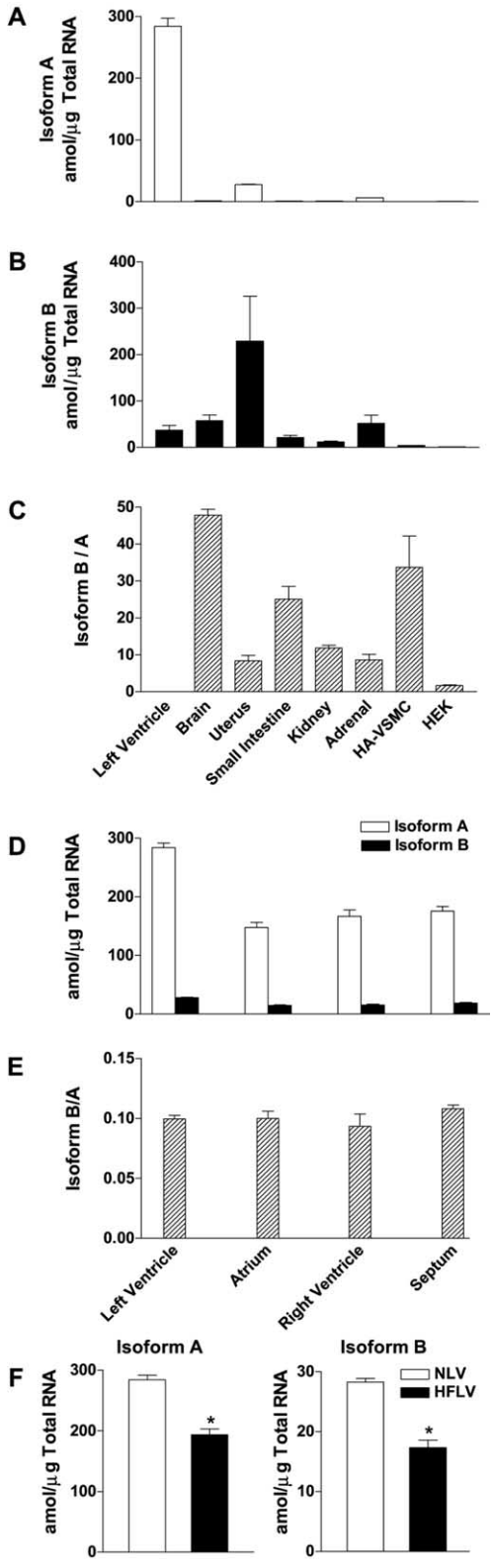

Fig. 2. Isoform distribution with tissue and disease. A: Isoform A expression in different tissues and cell types. B: Isoform B expression in different tissues and cell types. C: Isoform B/A expression ratios. D: Absolute isoform expression in different regions of normal hearts ($n=4$). E: Isoform B/A ratios in different cardiac regions. F: Isoform expression in congestive heart failure. NLV, normal left ventricle ($n=6$); HFLV, failing LV ($n=7$).

Isoform A transcript concentrations were highest in LV free wall (Fig. 2D). Isoform B/A transcript concentration ratios were consistent at ~0.1 across regions (Fig. 2E). Transcript levels for both isoforms were reduced in failing LV (Fig. 2F), without change in relative expression.

3.3. 5′ upstream regions

Putative *cis*-acting regulatory elements in the sequences immediately upstream of exon 1A and exon 1B were analyzed. The putative CACNA1C promoter A had no apparent TATA element and was not GC-rich, but there was an 'initiator' (5′-Py-Py-A-N-A/T-Py-Py, Py = C/T) sequence [12]. Unlike GC-rich promoters, such promoters are usually not constitutively active [12]. The putative CACNA1C promoter B contained features of 'housekeeping-type promoters': absence of TATA or CAAT boxes, presence of several GC-rich Sp1 consensus elements.

3.4. Promoter activity

CACNA1C promoter-luciferase fusion plasmids were transiently transfected into neonatal cardiomyocytes, cardiac fibroblasts, H9C2, HA-VSMC, and HEK293 cells. The activities of promoter A constructs in cardiomyocytes were five- to nine-fold of PGL-basic, with substantial activity in the −436/+80 region and detectable further activity in the region between −1393 and −1062 (Fig. 3B). Promoter A constructs failed to show activity in any non-cardiomyocyte cell types (Fig. 3C–F). Promoter B had substantial activity in all cell types (Fig. 4B–F). The inclusion of nucleotides −1727 to −613 reduced promoter activity, suggesting negative regulatory elements.

Cardiac-specific activity of promoter A was maintained in the shortest (−436/+80) construct studied (Fig. 3). We compared potential TFs in this region between promoter A and the corresponding rat promoter (which drives expression in vascular tissue [8]) and found that promoter A possesses unique Nkx2.5 (−223) and E box (+30) elements. We examined the effects of their mutation (Nkx2.5: from AACTTGA to AGCTCAG; E box: from CACTGG to GGATTG) on activity of the PGL3-(−436/+80) construct in neonatal rat cardiomyocytes and HA-VSMC. Wild-type PGL3-(−436/+80) showed luciferase activity 8.85 ± 1.36-fold that of PGL3-Basic in atrial cells and 7.17 ± 0.52-fold in ventricular cells, versus 1.13 ± 0.08-fold in HA-VSMC (7.8- and 6.3-fold cardiac selectivity for atrium and ventricle versus HA-VSMC respectively). Mutation of the Nkx2.5 sequence resulted in 5.09 ± 0.21- and 4.35 ± 0.40-fold activity in atrial and ventricular myocytes, compared to 0.84 ± 0.15 in HA-VSMC (6.0- and 5.2-fold cardiac selectivity in atrium and ventricle). Mutation of the E box resulted in 4.90 ± 0.17-fold activity in atrial and 3.00 ± 0.52 in ventricular myocytes, versus 1.01 ± 0.07 in HA-VSMC (4.8- and 3.0-fold cardiac selectivity).

4. Discussion

We have identified alternative CACNA1C exon 1 isoforms in the human genome, cloned their respective 5′ upstream regions and shown differences in potential regulatory elements that correspond to differences in tissue-specific expression. The relative expression of these isoforms is consistent across regions in the human heart and is unaffected by congestive

heart failure, although the expression of both is down-regulated in the failing heart.

Recently, a longer N-terminal $Ca_v1.2$ isoform differentially regulated by protein kinase C has been described in human heart [9]. This isoform is encoded by exon 1A, which we found to show cardiac-selective expression, emphasizing its importance. Along with previous observations [10,11], our results support the arguments of Blumenthal et al. [9] for the potential importance of the long N-terminal variant in the human heart.

The N-terminal-encoding $Ca_v1.2$ cDNAs of rabbit heart [13] and rat aorta [14] share 80% and 69% residue identity with human isoform A. The rat CACNA1C promoter [8] has 51% residue identity with human promoter A (−1494 to +80). The rat promoter is active in pulmonary artery vascular smooth muscle cells [8], whereas promoter A is inactive in HA-VSMC. Our mutation studies suggested that some of the differences in cardiac selectivity between the rat and human promoters may be due to specific Nkx2.5 and E box sequences present only in the human promoter. A recent paper reported that a c-Ets-like motif (CAGGATGC) is essential for rat CACNA1C promoter activity in vascular smooth muscle cells [15]. In the corresponding region (−622) of human promoter A, the sequence differs slightly from the rat Ets-like motif, but deletion of this region in promoter A did not alter cardiac specificity (Fig. 3B). Ca^{2+} channel α_{1C} subunit cDNAs from rabbit vascular smooth muscle [16] and rat brain [17] share 80% and 75% residue identity with human isoform B, consistent with our findings regarding isoform B expression patterns.

H9C2 is a clonal cardiac cell line derived from embryonic rat heart [18] and expresses L-type Ca^{2+} currents with cardiac characteristics [19]. We were surprised that human CACNA1C promoter A had no promoter activity in H9C2 cells. However, H9C2 cells also express skeletal muscle L-type Ca^{2+} channels [20], consistent with a phenotype that is not cardiac-specific.

The rat and cat *ncx1* genes [21,22] encoding Na^+,Ca^{2+}-exchanger isoform 1 also have alternative promoters which control tissue distribution. These genes are similar to CACNA1C: the alternative first exons spread out over a large genomic region, a cardiac-specific promoter regulates the heart-specific isoform expression, transcription begins at an 'initiator' sequence and GATA box, E box and AP1 binding sites are present in the proximal promoter region. For both, the ubiquitously expressed isoform is strongly expressed in brain and is controlled by a housekeeping-type promoter.

There is growing evidence for $Ca_v1.2$ subunit diversity arising from alternative splicing of a primary transcript [10,11,23]. In this study, we show the existence of N-terminal variability in human $Ca_v1.2$ subunits and tissue-selective control by alternative promoters. This work provides a basis for further studies of factors controlling local $Ca_v1.2$ expression under

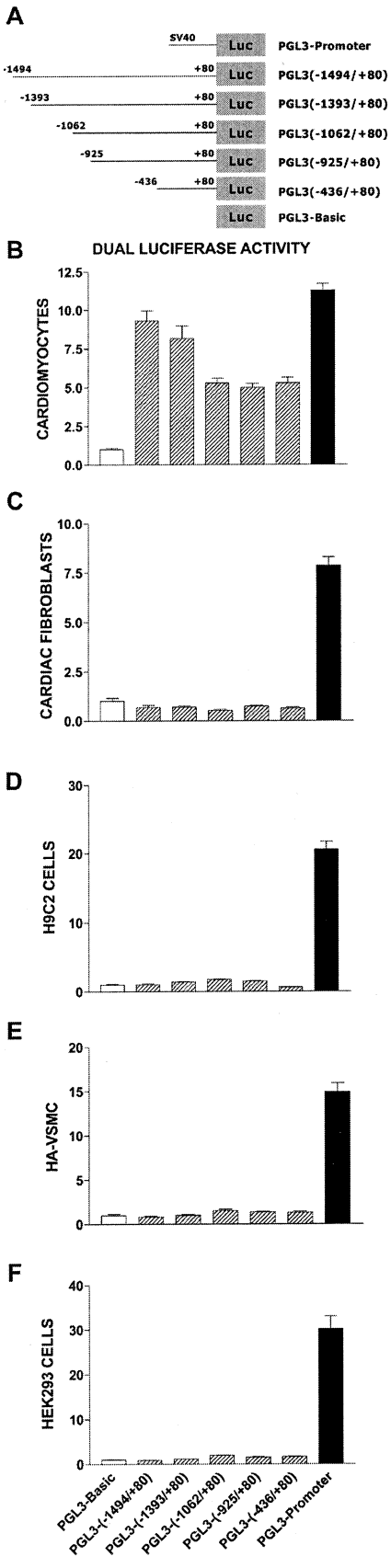

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Fig. 3. Promoter A activity in various cell types. Open bars, negative control (PGL3-basic); solid bars, positive control (SV40 promoter); hatched bars, promoter A-luciferase fusion plasmid constructs. Nucleotides of fusion plasmids are numbered with respect to the transcription start site (+1) identified by 5′RACE that is 55 nucleotides upstream of the transcription start site identified in [10].

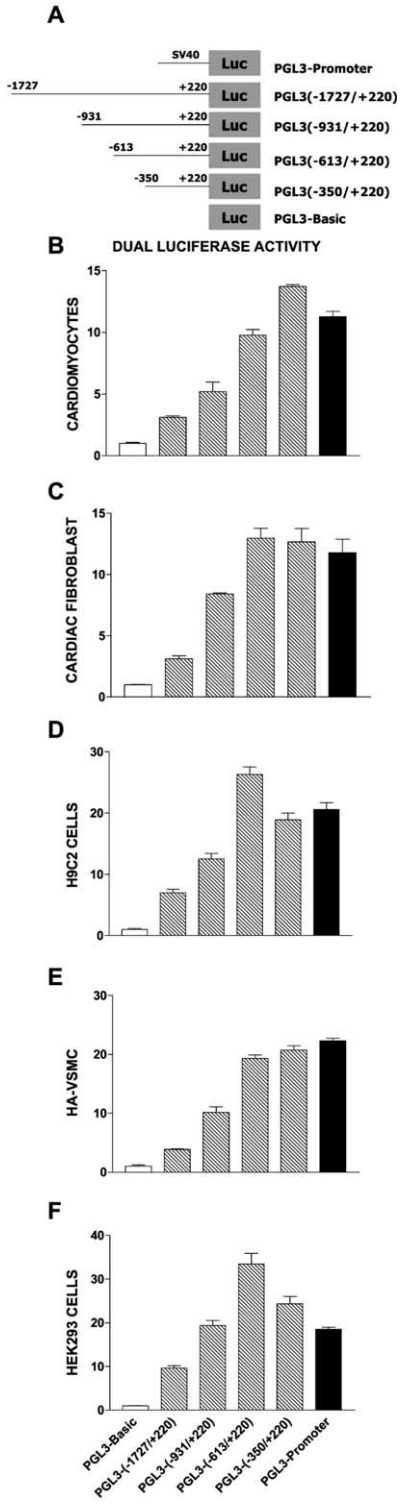

Fig. 4. Promoter B activity in various cell types. Open bars, negative control (PGL3-basic); solid bars, positive control (SV40 promoter); hatched bars, promoter B-luciferase fusion plasmid constructs. Nucleotides of fusion plasmids are numbered with respect to the 3′ transcription start site (+1) identified by 5′RACE.

physiological and pathological conditions. Alternatively spliced C-terminal exons have also been reported to show tissue-specific expression [23]. Further studies of the role of various alternatively spliced $Ca_v1.2$ isoforms and potential interactions between them would be interesting.

We found $Ca_v1.2$ mRNA to be down-regulated by heart failure. Many investigators have studied L-type Ca^{2+} current and corresponding $Ca_v1.2$ changes in experimental and clinical heart failure (for review, see [24]). The results have been variable, with the majority of studies showing a decrease and a significant number of studies showing no change. The variability is likely due to differences in the degree and mechanism of heart failure, limited sample sizes and (in clinical studies) the role of concomitant drug therapy, patient factors such as age, sex and ethnic differences, etc. It is interesting that despite significant down-regulation of CACNA1C expression, the isoform A/B ratio remained unchanged in failing hearts in the present study, suggesting the factors that drive strong cardiac-selective expression of isoform A and weaker cardiac expression of isoform B are not significantly altered by the important metabolic, structural, signaling and regulatory alterations in congestive heart failure.

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