Expression of Diverse Na^+ Channel Messenger RNAs in Rat Myocardium

Evidence for a Cardiac-specific Na^+ Channel

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Abstract

This study examined the diversity of Na^+ channel gene expression in intact cardiac tissue and purified myocardial cells. The screening of neonatal rat myocardial cell cDNA libraries with a conserved rat brain Na^+ channel cDNA probe, resulted in the isolation and characterization of a putative rat cardiac Na^+ channel cDNA probe (pCSC-1). The deduced amino acid sequence of pCSC-1 displayed a striking degree of homology with the eel, rat brain-1, and rat brain-2 Na^+ channel, thereby identifying pCSC-1 as a related member of the family of Na^+ channel genes. Northern blot analysis revealed the expression of a 7-kb CSC-1 transcript in rat cardiac tissue and purified myocardial cells, but little or no detectable expression of CSC-1 in rat brain, skeletal muscle, denervated skeletal muscle, or liver. Using RNase protection and Northern blot hybridization with specific rat brain Na^+ channel gene probes, expression of the rat brain-1 Na^+ channel was observed in rat myocardium, but no detectable expression of the rat brain-2 gene was found. This study provides evidence for the expression of diverse Na^+ channel mRNAs in rat myocardium and presents the initial characterization of a new, related member of the family of Na^+ channel genes, which appears to be expressed in a cardiac-specific manner.

Introduction

Voltage-gated Na^+ channels are of major importance in the maintenance of organized cardiac conduction (1–4). Although the electrophysiological properties of cardiac Na^+ channels are similar to those of other excitable tissues (5), several studies have found important pharmacological differences among these channels (6–9). The activation of cardiac Na^+ channels by batrachotoxin displays positive cooperativity with the binding of agonists to the muscarinic receptor, thereby implying a possible regulatory interaction between these two membrane proteins (9), and treatment with isoproterenol can induce a Na^+ current in ventricular myocytes (10). Adult rat myocardium contains a class of Na^+ channels with a relatively low affinity for tetrodotoxin (tetrodotoxin insensitive), in addition to a class of tetrodotoxin-sensitive Na^+ channels similar to the brain Na^+ channels (6, 8). Whether these specialized functions of cardiac Na^+ channels are due to the presence of multiple, distinct cardiac Na^+ channel proteins is unknown (11, 12). Alternatively, this diversity of Na^+ channel activities might reflect a single Na^+ channel protein that is subject to post translational modification, regulation by another set of subunits or cofactors, or segregation into separate sarcolemmal membrane domains. Although the electrophysiology of cardiac Na^+ channels has been a subject of extensive study (13), relatively little information is currently available on the molecular characterization of individual Na^+ channels in the heart. The high degree of conservation of this transmembrane domain in the Na^+ channel genes of diverse species suggested the possible use of cDNA probes containing these conserved sequences to isolate and characterize related members of the family of Na^+ channel genes in rat myocardium.

Accordingly, the objective of this study was to examine the diversity and tissue specificity of rat myocardial Na^+ channel gene expression by: (*a*) the isolation and characterization of putative rat cardiac Na^+ channel cDNAs; and (*b*) the use of these cardiac cDNA probes and known rat brain Na^+ channel probes to characterize Na^+ channel mRNAs in intact cardiac tissue and purified myocardial cells. The results of this study provide evidence for the expression of diverse Na^+ channel mRNAs in rat myocardium and presents the initial characterization of a new, related member of the family of Na^+ channel genes, which appears to be expressed in a cardiac-specific manner.

Methods

Myocardial cell culture. Cultured neonatal rat myocardial cells were prepared as previously described (14–16). After being incubated in serum for 24 h, the cultures were washed and incubated in DMEM/medium 199 with 0.1 mM phenylephrine to induce cardiac mRNAs (17).

Isolation of RNA and construction of cDNA libraries. Total RNA was isolated from neonatal rat myocardial cells, adult rat heart, liver, and skeletal muscle by a guanidine-HCL procedure (18). The quality of the RNA was routinely assessed by size fractionation on formaldehyde-agarose gels, followed by ethidium bromide staining (19). Neonatal rat myocardial cell total RNA was poly A selected by oligo-dT cellulose chromatography (20), and used for construction of cDNA libraries in ZAP (Stratagene Corp., La Jolla, CA) by the method of Gubler and Hoffman (21). For these studies, three separate cDNA

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libraries were constructed: (*a*) a library containing cDNAs 1 kb and larger; (*b*) a library containing cDNAs 1–4 kb long; and (*c*) a library containing cDNAs 4 kb and larger.

Isolation of Na^+ channel cDNA clones. The cDNA libraries were plated at a density of ~ 8,000 plaque-forming units per 10-cm dish, and transferred to HAW filters (Millipore Corp., Milford, MA). The filters were denatured, neutralized, baked in vacuo at 80°C for 2 h, and hybridized with either restriction fragments or oligonucleotide probes, as previously described (22, 23). The restriction fragments were labeled with α-^{32}P-dCTP by either nick translation (24) or random hexamer labeling (25), and the oligonucleotide probes were end-labeled with α-^{32}P-ATP with polynucleotide kinase (19). The initial screenings of the cDNA libraries were performed with the cDNA insert of RB 211 (26) under conditions of moderate stringency (5× SSPE [20× SSPE = 3 M NaCl; 0.2 M NaH_2PO_4; 20 mM NaEDTA; pH 7.4], 5× Denhardt's, 0.1% SDS, 150 μg/ml denatured salmon sperm DNA; 5×10^5 cpm/ml probe; 50°C). Secondary screenings of these cDNA libraries with restriction fragments of pCSC-1 were performed under more stringent conditions (68°C). The filters were exposed overnight to XAR-5 film (Kodak) with Cronex enhancing screens (DuPont Co., Wilmington, DE) and positive clones were detected by autoradiography.

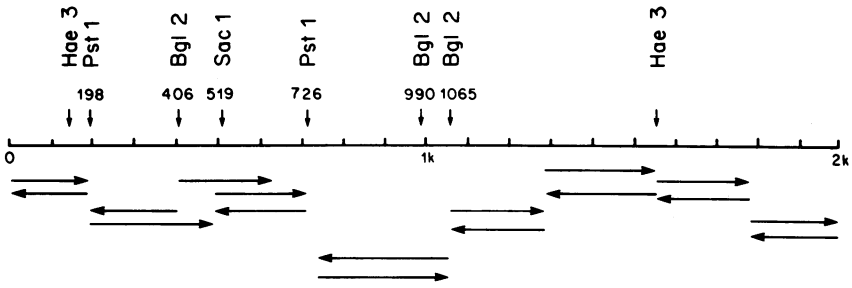
Analysis of cDNA clones by restriction mapping and DNA sequencing. Large-scale preparations of appropriate bacteriophage clones were obtained, as previously described (19), and the cDNA inserts were subcloned into PUC, M13, and SP6/T7 vectors. The positive clones were further identified by restriction mapping and by direct sequencing using a dideoxynucleotide termination technique with α-^{35}S-dATP (22, 23).

Hybridization studies. Total and poly A selected RNA were size fractionated on formaldehyde gels and transferred to nylon membranes by electroblotting. After prehybridization overnight, the filters were hybridized under stringent conditions (5× SSPE; 5× Denhardt's; 0.1% SDS, 50% formamide; 300 μg/ml denatured salmon sperm DNA; 500 μg denatured yeast tRNA; $2–8 \times 10^6$ cpm/ml probe; 60°C) with ^{32}P-labeled RNA probes derived from SP6/T7 vectors containing inserts of various Na^+ channel cDNAs. The filters were washed and autoradiography was performed with various durations of exposure (6–96 h). RNase protection studies were performed by a modification (26) of a previously described method (27).

Results and Discussion

Isolation and characterization of rat cardiac Na^+ channel cDNAs. To isolate cardiac Na^+ channel cDNA clones, size-selected neonatal rat myocardial cell cDNA libraries were screened with pRB211, a partial cDNA probe from the rat brain-2 Na^+ channel (26). pRB211 contains coding information for the S4, S5, and S6 transmembrane segments of the IV internal repeat of the brain Na^+ channel, a region that is highly conserved at the amino acid and nucleotide level between the rat brain-1/rat brain-2/eel Na^+ channel cDNAs (26, 28, 29). The cloning strategy assumed that these domains would be critical to Na^+ channel function and would therefore be present in related members of the family of Na^+ channel genes, such as cardiac Na^+ channel cDNAs.

As a result of multiple rounds of library screening (2×10^6 plaques), two positive clones were identified and subcloned into a PUC-derived plasmid vector (pCSC-1). Restriction mapping of the 2.0-kb cDNA insert of pCSC-1 was performed (Fig. 1), and the appropriate restriction fragments were sub-



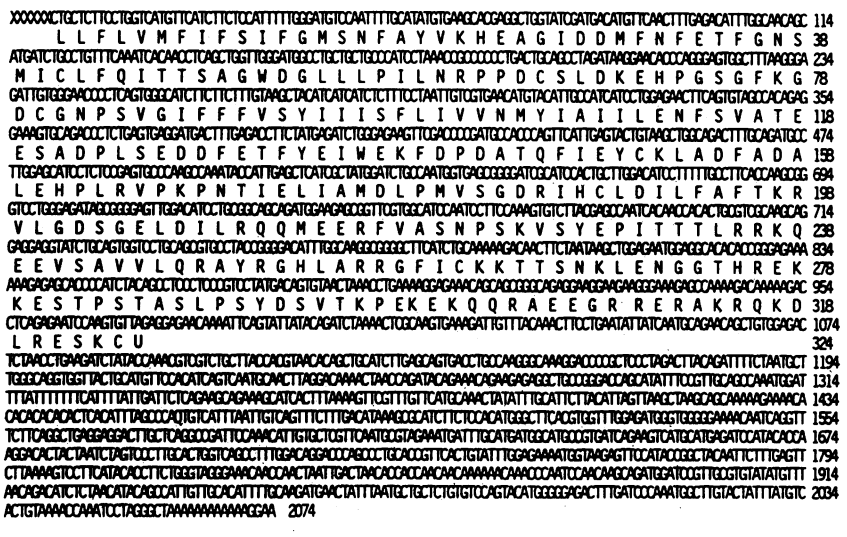


Figure 1. Restriction map, nucleotide sequence, and deduced amino acid sequence of pCSC-1. Size selected, neonatal rat myocardial cell cDNA libraries were constructed in λ ZAP (Stratagene Corp.) and were screened with a rat brain-2 cDNA probe that contained sequences encoding the conserved S4, S5, and S6 regions within the fourth homologous domain. As a result of screening 2×10^6 plaques, two positive clones (0.8 and 2.0 kb) were identified and were subcloned into M13 and the PUC based vector, pBS (Stratagene Corp.). Restriction mapping and sequencing identified the 0.8-kb clone as containing the 3′ region of the 2.0-kb cDNA. Detailed restriction mapping and sequencing of both strands of the 2.0-kb cDNA insert (pCSC-1) were performed using a combination of cDNA subclones and specific sequencing primers, as indicated. Translation of the nucleotide sequence revealed a single open reading frame that had > 80% homology to the deduced amino acid sequences of the rat brain-1 and rat brain-2 cDNAs. The numbers at the right margin of each line refer to the position of the last nucleotide (*top line*) or deduced amino acid residue (*bottom line*) in pCSC-1.

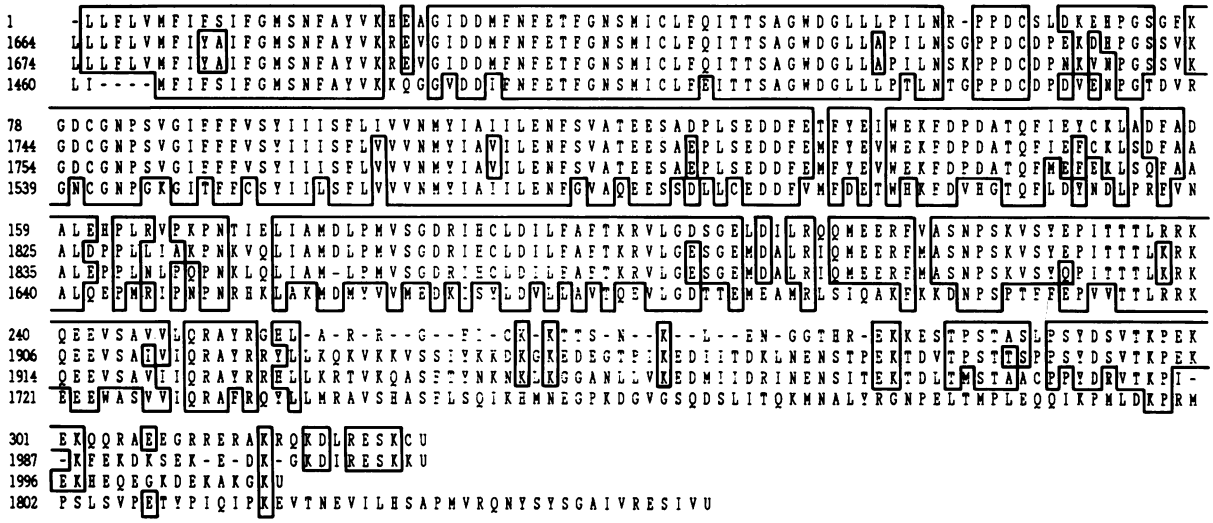


Figure 2. Comparison of the deduced amino acid sequence of CSC-1 with rat brain-1, rat brain-2, and eel Na^+ channel cDNAs. The deduced amino acid sequence of CSC-1 was aligned with the corresponding sequences of other known Na^+ channel cDNAs. The regions of identity of deduced amino acid sequences among these Na^+ channel cDNAs are enclosed in boxes, and areas of divergence are depicted as open spaces. The numbers at the left margin of each line refer to the position of the initial amino acid residue in CSC-1 (*first line*), rat brain-2 (*second line*), rat brain-1 (*third line*), and eel Na^+ channel (*last line*).

cloned into M13 single-stranded vectors for dideoxy nucleotide sequencing. The entire cDNA insert of pCSC-1 was sequenced in both directions, revealing a nucleotide sequence which was highly homologous (> 80%) with the reported nucleotide sequence of the rat brain Na^+ channel II gene (29). Translation of the nucleotide sequence of pCSC-1 identified a single, open reading frame (Fig. 1) with regions of extensive homology (ranging from 80 to 100%) with the deduced amino acid sequences of the eel and rat brain Na^+ channel genes (Fig. 2) (28, 29). As determined by alignment of the deduced amino acid sequences, pCSC-1 contained coding information for the S5 and S6 transmembrane segments of the IV internal repeat, the COOH terminus, and a portion of the 3′ untranslated region (Fig. 1). As expected, the highest degree of homology was found in the transmembrane domains, with evidence of areas of divergence in the COOH terminal portion of the protein (Fig. 2). The deduced amino acid sequence identifies pCSC-1 as a partial cDNA corresponding to a related member of the family of Na^+ channel genes. The 3′ untranslated regions of the eel, rat brain-1, rat brain-2, and CSC-1 cDNAs were highly divergent, thereby suggesting the utility of 3′ untranslated probes to specifically assess the expression of the corresponding individual Na^+ channel mRNAs.

Expression of rat brain and cardiac Na^+ channel mRNAs in various tissues. To initially examine the expression of known rat brain Na^+ channels in rat myocardium, RNA probes of the rat brain-1 and rat brain-2 Na^+ channel genes were used for RNase protection studies. As displayed in Fig. 3, hybridization of rat brain RNA with the rat brain-1 and rat brain-2 probes, resulted in the appearance of protected fragments that corresponded to the predicted size of the complementary regions of the respective RNA probes. Hybridization studies with cardiac RNA revealed protection with the rat brain-1 probe, but no detectable protection with the rat brain-2 probe. Accordingly, these studies indicated that the rat brain-1 Na^+ channel mRNA was expressed in rat myocardial tissue, whereas the rat brain-2 channel mRNA was not expressed at detectable levels.

To further characterize the various types of Na^+ channel mRNAs in rat myocardium, Northern blot hybridization studies were performed with rat cardiac poly A RNA and a panel of Na^+ channel gene probes: (*a*) a "common" Na^+ channel gene probe that contains sequences shared by all known Na^+ channel cDNAs (RB211); (*b*) a rat brain-1-specific probe (29); and (*c*) a CSC-1 specific probe derived from the 3′ untranslated region of pCSC-1. As displayed in Fig. 4, the common Na^+ channel probe (RB211) and the rat brain-1 probe hybridized to a 9-kb mRNA, the size of Na^+ channel mRNAs that have been described in other excitable tissues (27, 29, 30, 31, 32). However, hybridization of the same filter with the pCSC-1 specific probe resulted in the detection of a single 7-kb transcript. It should be noted that the detection of the 7-kb mRNA required an eightfold increase in duration of autoradiographic exposure (4–5 d) compared with the Northern blots obtained with either the RB211 or RB-1 probes (12–16 h), indicating a markedly lower level of abundance of the CSC-1-related transcript in rat myocardium. This lower level of abundance presumably accounted for the inability to detect the CSC-1 mRNA with the RB211 probe following shorter durations of autoradiographic exposure.

To examine the tissue-specific pattern of expression of the CSC-1 related Na^+ channel mRNA, Northern blots were performed with RNA derived from various excitable and nonexcitable tissues (Fig. 4). As expected, the rat brain-1 probe hybridized with a 9-kb transcript in cardiac and brain tissue, but did not hybridize with RNA derived from a nonexcitable tissue (liver). The CSC-1 probe hybridized exclusively with cardiac RNA, but did not provide a detectable signal with RNA derived from rat brain or rat liver. In addition, the CSC-1 probe did not hybridize to RNA derived from skeletal muscle or denervated skeletal muscle (Fig. 5). These results indicated

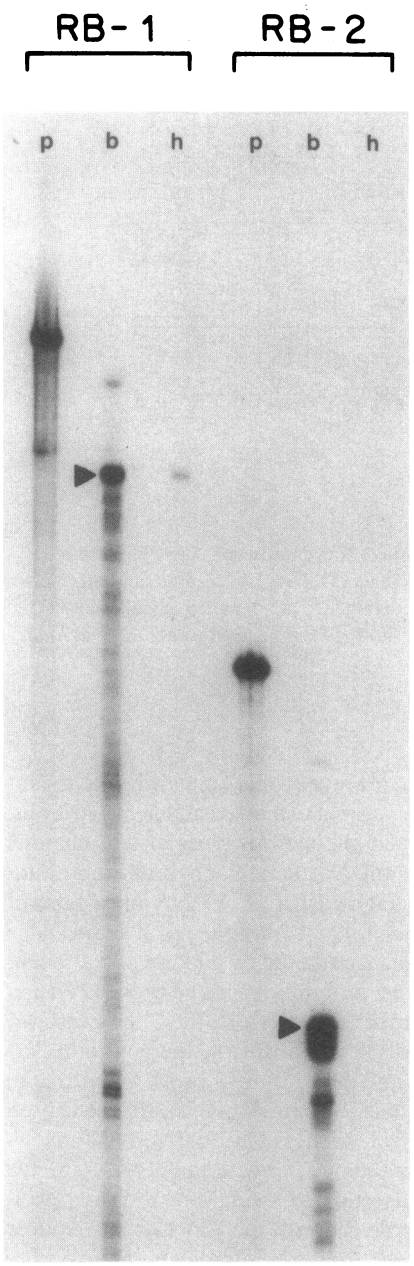


Figure 3. RNase protection analyses. Total RNA from rat brain and rat myocardium was isolated as described in Methods. RNA probes for the rat brain-1 (p7 Δ C) or the rat brain-2 (24 ET) were generated in SP6/T7 vectors and used for RNA protection analyses, as described in Methods. p, probe; b, brain; h, heart.

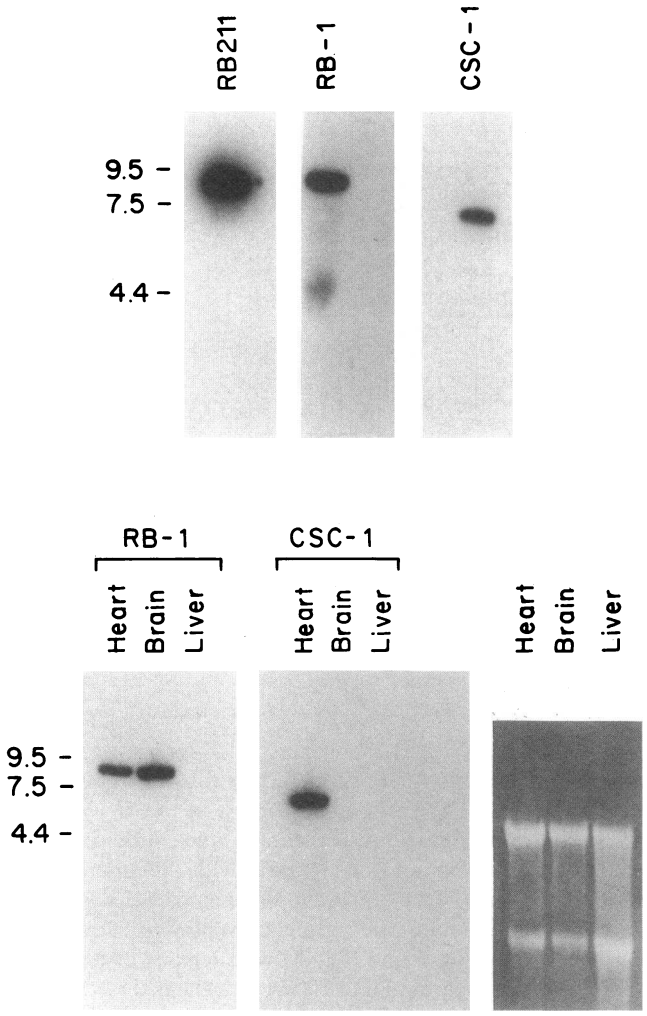


Figure 4. Assessment of Na^+ channel gene expression in rat myocardium by Northern blot hybridization analyses. Total RNA was isolated from various tissues and poly A selected, as described in Methods. (*Top*) 10 μg of cardiac poly A RNA was size fractionated on formaldehyde-agarose gels, transferred to nylon membranes by electroblotting, and hybridized with various probes. (*Top*) a Na^+ channel RNA probe that contained sequences conserved in all known Na^+ channel cDNAs (RB211; *left*); a specific rat brain-1 RNA probe (p7 Δ C; *middle*); or a CSC-1 specific RNA probe (*right*). The same filter was used for hybridizations with each individual probe and the size of the transcripts was determined by comparison to the migration of RNA standards (depicted at the left margin). (*Bottom*) RNA from rat heart, brain, and liver was size fractionated on formaldehyde-agarose gels, stained with ethidium bromide, transferred to nylon membranes by electroblotting, and hybridized to a specific rat brain-1 RNA probe (*left*) or a specific CSC-1 RNA probe (*middle*). For these studies, the same membrane was used for hybridizations with both probes and the size of the corresponding transcripts was determined by comparison to the migration of RNA standards. Ethidium bromide stain of the agarose gel before transfer (*right*).

that the CSC-1-related Na^+ channel appeared to be expressed in a cardiac-specific manner.

To exclude the possibility that the CSC-1 probe was hybridizing to RNA derived from the small amounts of neural tissue that are found in intact rat myocardium, Northern blot studies were performed with RNA derived from cultures of purified cardiac myocytes (> 99% myocytes, as assessed by indirect immunocytofluorescence with cardiac myosin light chain antisera [19]). As revealed in Fig. 5, the CSC-1 probe hybridized with equal intensity to both total rat heart RNA, and purified myocardial cell RNA, thereby indicating that the CSC-1 probe was indeed hybridizing to a rat cardiac mRNA.

In summary, this study provides evidence for the expression of diverse Na^+ channel mRNAs in rat myocardium. In addition, a new related member of the family of Na^+ channel genes (CSC-1) has been putatively identified in rat myocardial

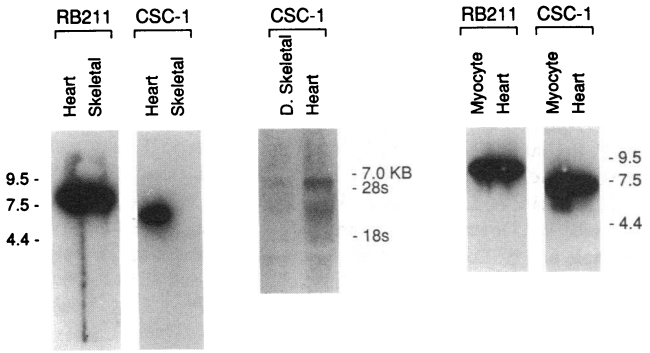


Figure 5. Pattern of expression of CSC-1 mRNA in skeletal muscle, denervated skeletal muscle, and purified myocardial cells. Experimental details are similar to Fig. 4. (*Left*) Hybridization of rat skeletal muscle RNA with the common RB211 RNA probe and with a CSC-1-specific probe. The size of transcripts was determined by comparison to the migration of RNA standards, depicted at the left margin. (*Middle*) Hybridization of total RNA derived from denervated skeletal muscle (*left lane*) and total rat cardiac RNA (*right lane*) with a CSC-1 specific RNA probe. Denervated skeletal muscle was prepared as previously described (29). (*Right*) Northern blot hybridizations with RB211 and a CSC-1 specific probe were performed with size fractionated RNA from adult rat heart and purified neonatal rat myocardial cells. The size of the transcripts was determined by comparison to the migration of RNA standards, depicted on the left margin.

cells. The cardiac-specific expression of this putative Na^+ channel mRNA invites speculation that the CSC-1 transcript might encode a Na^+ channel that displays cardiac specific pharmacological properties, such as tetrodotoxin insensitivity. However, any definitive assignment of the 7-kb CSC-1 transcript as a Na^+ channel mRNA and determination of its pharmacological properties awaits the expression of the full-length mRNA in surrogate systems.

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