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Differential expression of two sodium channel subtypes in human brain*,**

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Two partial human brain sodium channel cDNA sequences (designated HBSC I and II) have been cloned and mapped to chromosome 2q23-2q24 by chromosome microdissection-PCR (CMPCR). The distribution of HBSC I and II mRNA in human brain was studied by means of a novel approach based on the ligase detection reaction. These studies demonstrate that HBSC I and II mRNA is heterogeneously distributed in brain, and that the relative ratio of the two forms can vary as much as 7-fold between different regions.

Sodium channel; cDNA cloning; Chromosomal mapping; Ligase detection reaction; Subtype distribution; Human brain

1. INTRODUCTION

The voltage-gated sodium channel is an important member of the family of selective ion channel proteins that contribute to the electrophysiological properties of excitable cells. In animal models, different properties of sodium channels can be distinguished both electrophysiogically and pharmacologically, e.g. tetrodotoxin-sensitive and -insensitive forms [1–3]. Although other factors may account for these differences, the recent cloning of multiple sodium channel genes from rat brain [4–6] and other soures [7–11] suggests that multiple sodium channel subtypes underlie these functional differences. We therefore initiated studies to determine whether multiple sodium channel subtypes are expressed in human brain. Previous work had led to the isolation of a partial sequence of a human brain sodium channel (HBSC) gene that was subsequently mapped to chromosome 2q22-2q23 by chromosome- microdissection PCR (CMPCR) [12]. That fragment provided the starting point for the work reported here, in which we describe the cloning and characterization of a cDNA sequence representing a second subtype of the human brain sodium channel and the relative distribution of subtype mRNAs in postmortem human brain regions.

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**Sequences described in this paper have been deposited in EMBL and GenBank, Acc. nos. X65361 and X65362.

Abbreviations: PCR, polymerase chain reaction; LDR, ligase detection reaction; CMPCR, chromosome microdissection PCR; LDM, ligase detection method; RT-PCR, reserve transcriptase PCR.

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2. MATERIALS AND METHODS

The sequence of the previously isolated HBSC II cDNA fragment was extended to the 3′ terminus by anchored PCR [13–15]. Poly(A)$^+$ RNA was isolated from adult postmortem human brain (frontal pole) using a FastTrack kit (InVitrogen, San Diego, CA) according to manufacturer's directions. 50 ng of mRNA was reverse-transcribed using 50 ng of a 57 nucleotide $(dT)_{17}$-R_i-R_o primer (5′AAGGATCCGTCGACATCGATAATACGACTCACTATAGGGATTTTTTTTTTTTTTTT3′). This 57 nucleotide sequence contains an oligo(17)dT linked to two sequences R_i (inner adapter primer, 5′GACATCGATAATACGAC3′) and R_o (outer adapter primer, 5′AAGGATCCGTCGACATC3′), providing a template for subsequent PCR primers. Reactions (41°C for 2 h) were performed in 20 μl buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM each of dNTP, 10 U of RNasin (Promega, Madison, WI) and 200 U of Moloney murine leukemia virus RNAse H$^-$ reverse transcriptase (SuperScript, Gibco BRL, Gaithersburg, MD). An aliquot of the resulting cDNA was used as template for second-strand cDNA synthesis and first round PCR amplification utilizing a sodium channel-specific primer, NaCh 6 (5′AACTCCATGATCTGCCTGTT3′), and the R_o primer. (For the origin of NaCh 6 and other sodium channel-specific primers mentioned below, see Fig. 1.) In order to increase the specificity during the amplification, 25 pmol each of nested primers, NaCh 11 (5′CCAAATTACAACCTCTGCT3′) and R_i, were used in second round PCR. Both rounds of PCR reactions were performed in 50 μl buffer containing 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 170 μg/ml BSA, 16.6 mM $(NH_4)_2SO_4$, 1.5 mM each of dNTP, 10% DMSO and 2.5 U of *Taq* polymerase (Perkin Elmer, Norwalk, CT). Thirty cycles of PCR (94°C for 1 min, 50°C for 2 min, and 72°C for 3 min) were carried out for both rounds of amplification. The products were isolated by agarose gel electrophoresis and cloned into plasmid pT_7T_3 using standard methods. DNA sequencing was performed according to [16].

To identify other HBSC subtypes, systematic PCR screening [17] was applied to a human fetal brain cDNA library containing 10^9 pfu/ml (λ-gt11, oligo-dT primed, average insert size 1.2 kb; cat. no. HL 1065b, ClonTech, Palo Alto, CA). The library was divided into 32 pools of clones. From each pool, an aliquot of phage lysate was boiled

for 5 min to release and denature the DNA. The DNAs were then used as templates in a PCR reaction using sodium channel-specific primers, NaCh 11 and NaCh 12 (5'GTCCTTCCTATACTAC-TAC3'). The PCR reactions were performed in 100 μl buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100, 20 mM each of dNTP and 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI). Thirty cycles of PCR (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) were carried out. The reaction products were analyzed by agarose gel electrophoresis. Those groups that gave positive PCR signals were amplified, subdivided and used for a second round of PCR selection. In this and subsequent rounds each positive pool was subdivided into 10 portions. A positive control with genomic DNA as template and a negative control without any template were run in parallel with each PCR assay. These procedures were reiterated until a single clone was isolated.

Chromosomal localization of HBSC I was carried out by CMPCR as described [12]. Briefly, Giemsa-banded metaphase chromosomes were dissected with glass needles using a micromanipulator. The microdissected chromosome fragments were then used as templates in PCR identification with gene-specific primers. To increase the specificity of the reactions, nested primers were used in the second round PCR amplification. The PCR product was analyzed by agarose gel electrophoresis after second-round amplification.

A ligase detection method (LDM) was developed to assess the relative distribution of HBSC I and II mRNA in postmortem human brain regions. A probe common to both subtypes, NaCh 24 (Fig. 4A), was 5' end-labeled with ^{32}P using T_4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD). For the LDM control experiment, 100 ng of cloned HBSC I DNA and /or 100 ng of cloned HBSC II DNA were incubated in the presence of 5 ng of labeled NaCh 24, 20 ng of unlabeled NaCh 24 and 25 ng of the subtype-specific primers NaCh 25 and/or NaCh 26 (Fig. 4A) in 50 μl of 20 mM Tris-HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 0.6 mM NAD, 0.1% Triton X-100 and 20 U of AMPLIGASE (Epicentre, Madison, WI), a heat-stable DNA ligase isolated from *Thermus aquaticus*. Reactions were carried out for 30 cycles of 94°C for 1 min followed by 65°C for 4 min. Aliquots were analyzed by autoradiography following gel electrophoresis. In subtype distribution studies, reverse transcriptase-PCR (RT-PCR) was used to increase the sensitivity of LDR reactions. Total RNA was isolated from frozen adult postmortem human brains with guanidium thiocyanate. First-strand cDNA was synthesized from 1 μg of total RNA with 200 U of reverse transcriptase (Superscript, RL) using the downstream primer, NaCh 27 (5'TCATCCTGGAGAACTTCAGT3'). Second-strand cDNA synthesis and PCR amplification were performed using primers NaCh 22 and NaCh 27. Buffer and PCR conditions were the same as described in the systematic PCR screening method. An aliquot of the amplified cDNAs was subjected to thirty cycles of LDR reactions as described above. Control experiments (data not shown) verified that prior amplification with PCR did not alter the ratio of LDR products.

3. RESULTS AND DISCUSSION

The human sodium channel sequence described previously was found to encode a peptide fragment homologous to the rat brain type II sequence beginning with amino acid 1,702, as numbered by Noda et al. [4]. The corresponding location of the nucleotide sequence is approximately 0.9 kb from the 3' end of the coding region of murine sodium channel cDNA. The human sequence was extended in the 3' direction by anchored PCR [13–15] yielding 0.9 kb products that were isolated and cloned into plasmid pT_7T_3. Four clones were subsequently sequenced, and three were found to be identical within the constraints of polymerase infidelity [18–20]. These three clones provided the basis for a consensus sequence extending the initial fragment to the 3' end of the coding region. Comparison of this extended sequence with the GenBank database revealed 77.3, 85.2 and 81.7% homology with genes encoding type I, II and III rat brain sodium channels, respectively. No significant homology with non-sodium channel sequences was found. On the basis of the relative homologies with rat brain subtypes, the human brain sodium channel represented by the 0.9 kb fragment has been designated HBSC type II. The alignment of the corresponding amino acid sequence with that for rat brain type II sodium channel is shown in Fig. 1. A fourth clone derived from anchored PCR was found to contain only 75% homology (data not shown) with HBSC II (or HBSC I described below). This sequence may represent a third human brain sodium channel subtype, but until a consensus sequence is available this assignment must remain tentative.

A second human brain sodium channel subtype was isolated using a modified systematic PCR screening

Table I

Regional distribution of human brain sodium channel subtype I and II mRNA

Brain region/ sample no.	Ratio HBSC I:HBSC II, replicates			Replicate mean ratio	Grand mean ± S.E.M.
	1	2	3		
FP 105	0.82	0.75	0.79	0.79	
FP 115	0.87	0.81	0.83	0.84	0.792* ± 0.015
FP 90-8	0.73	0.79	0.78	0.77	
BG 89-24	0.80	0.83	0.79	0.81	
BG 89-43	0.90	0.88	0.87	0.88	0.852* ± 0.012
BG 90-8	0.86	0.87	0.87	0.87	
CB 105	0.40	–	–	–	
CB 115	0.32	0.30	0.32	0.31	0.354* ± 0.018
CB 90-8	0.43	0.37	0.34	0.38	
MED 115	1.38	1.68	1.64	1.57	1.567* ± 0.094
SC 89-31	1.91	2.45	2.13	2.16	
SC 91-30	1.97	2.15	2.46	2.19	2.178* ± 0.095

Total RNA was prepared from 0.5 g samples of frozen postmortem human brain tissues using standard methods [15]. Five different regions were sampled, including cerebral cortex-frontal pole (FP), basal ganglia (BG), cerebellum (CB), medulla oblongata (MED), and spinal cord (SC). Arabic numbers following these designations in the table refer to different individual brains. Neuropathological examination of adjacent tissue was normal in each case. The age at death ranged from 44 to 94 years for these samples. Aliquots of RNA corresponding to 0.2 g tissue were treated to prepare cDNA and amplified by PCR with common sodium channel-specific primers as described in the text. The ratio of HBSC I:HBSC II was determined in triplicate for each sample using the ligase detection reaction. Replicates 1 and 2 report values for different aliquots of the same RNA preparation, whereas replicate 3 reports the value for a separate RNA extraction.

*Significantly different from all other grand means, $P < 0.007$

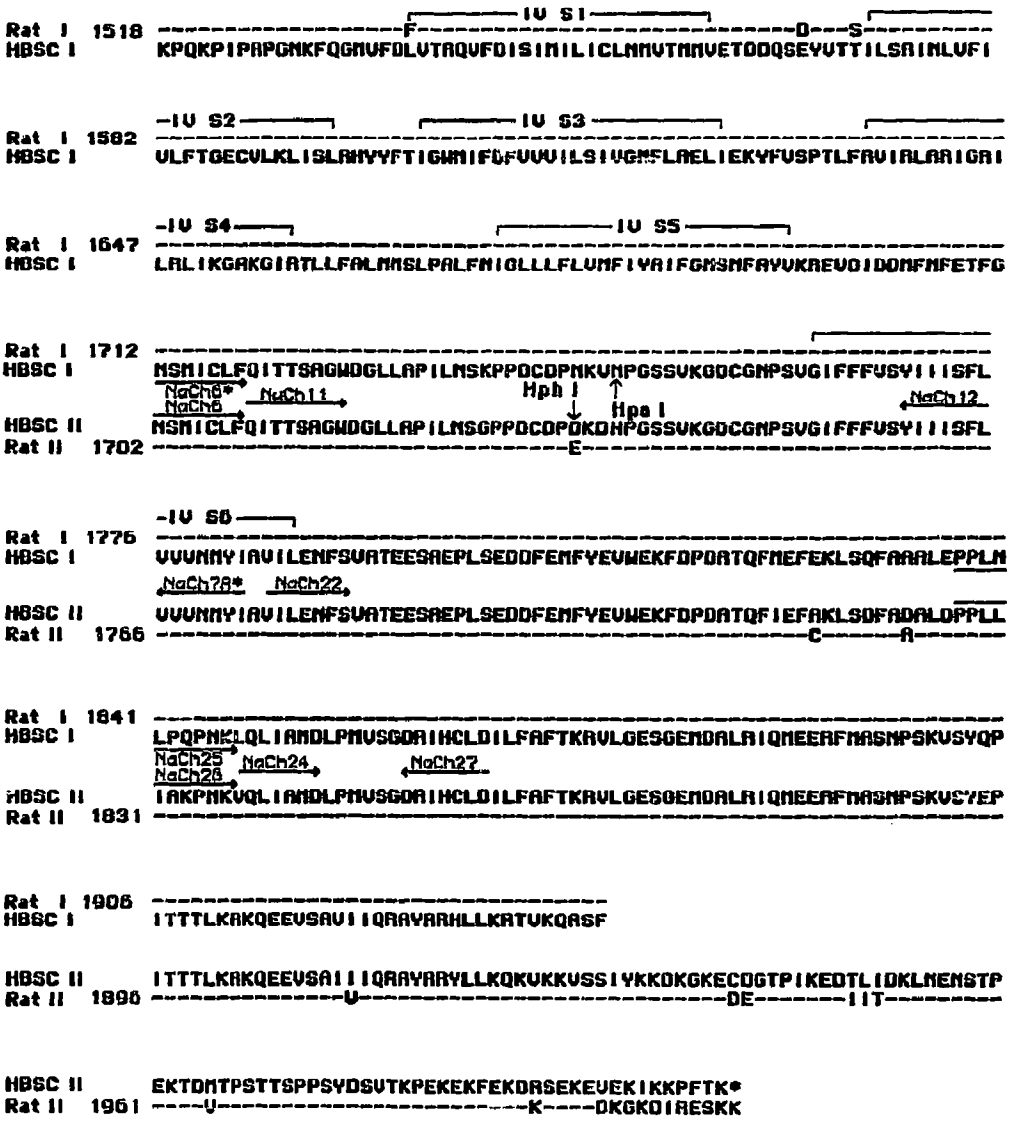

Fig. 1. Comparison of deduced amino acid sequences of human brain sodium channel fragments and rat brain sodium channels. Differences between the sequences are indicated by the letter code for the divergent residues. Dashes indicate identical residues. Numbers for residues and the putative transmembrane segments (IV S1–IV S6) are indicated according to published schemes [4]. The corresponding positions of primers referred to in the text are indicated by lines with arrowheads marking the 3′ end. The subtype-specific restriction enzyme cutting sites, *Hpa*I for HBSC I and *Hph*I for HBSC II, are also indicated. A stop codon found at the end of the cloned HBSC II sequence is labeled with an asterisk.

method [17] in which positive clones in a human fetal brain cDNA library were identified by PCR. Several positive clones containing inserts of 1–1.5 kb in size were isolated. The 1.3 kb insert from one of these, clone 8-8A, was characterized in detail. The sequence of clone 8-8A was examined against the GenBank database, revealing that it also exhibits significant homology only with previously cloned sodium channel genes. Among these, it shares 89.7, 84.9 and 84.5% homology with type I, II and III rat brain sodium channel sequences, respectively. Accordingly, the gene represented by clone 8-8A has been designated HBSC type I. Comparison of HBSC I and II sequences in the region of overlap reveals that they are identical over 87.5% of their length (Fig. 1). The deduced amino acid sequence of this HBSC I fragment spans the entire fourth repeated domain of the sodium channel structure, and in this region the homology with the rat type I sodium channel protein is strikingly high, there being only three amino acids at variance (Fig. 1). Not surprisingly, the characteristic sequence motif of positively-charged amino acids serving as a voltage sensor in transmembrane segment four [4] is also conserved in this human brain sodium channel cDNA.

Based on the sequence of HBSC I, NaCh 6* (5′AACAGCATGATCTGCCTATT3′) and NaCh 7A* (5′ATGTACATGTTCACCACAAC3′) were synthesized and used for CMPCR mapping of the gene. NaCh

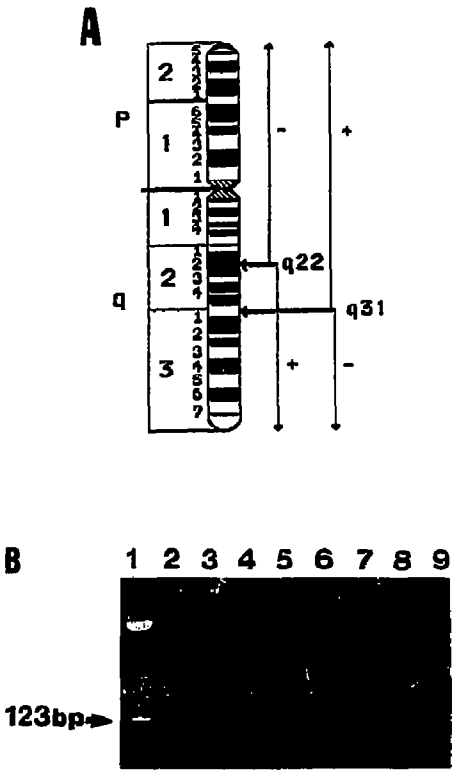

Fig. 2. Chromosomal localization of human brain sodium channel genes. (A) Diagram of Giemsa-banded chromosome 2. Microdissection sites are indicated by arrows. Chromosome fragments from the dissection sites to either short (p) or long (q) arm terminals were used as DNA templates for PCR. + and − signs indicate positive and negative CMPCR results. (B) Identification of subtype-specific CMPCR products. Lane 1, 123 bp molecular size marker. Lane 2–5, mapping analysis for HBSC I: lane 2, 172 bp HBSC I-specific CMPCR product obtained by using 2q22-qter fragment as template; lane 3, the 172 bp product can be digested by *Hpa*I; lane 4, the same product can not be digested by *Hph*I; lane 5, negative control in which no template was added in the reaction. Lane 6–9, mapping results for HBSC II: lane 6, 172 bp HBSC II-specific CMPCR product using 2q22-qter as template; lane 7, this product can not be cut by *Hpa*I; lane 8, the same 172 bp product is cut by *Hph*I, which is specific to HBSC II; lane 9, negative control.

11 and NaCh 12, which are common to both HBSC I and II, were used as nested primers for the second round of PCR amplification. Following second round amplification, susceptibility to two restriction enzymes, *Hpa*I and *Hph*I, was used to confirm the subtype identity of the CMPCR products. *Hpa*I and *Hph*I recognize sites located in the middle of the 172 bp NaCh 11/NaCh 12-amplified fragment and are specific to HBSC I and II, respectively (Fig. 1). Fig. 2 illustrates the CMPCR mapping result. A 172 bp HBSC I-specific PCR product was detected when microdissected chromosome fragments 2q22-qter and 2q31-pter were used as templates. This PCR product was not synthesized when fragments 2q22-pter and 2q31-qter were used. These results indicate that the HBSC I gene is located on chromosome 2q23-24, a locus identical or very close to HBSC II [12].

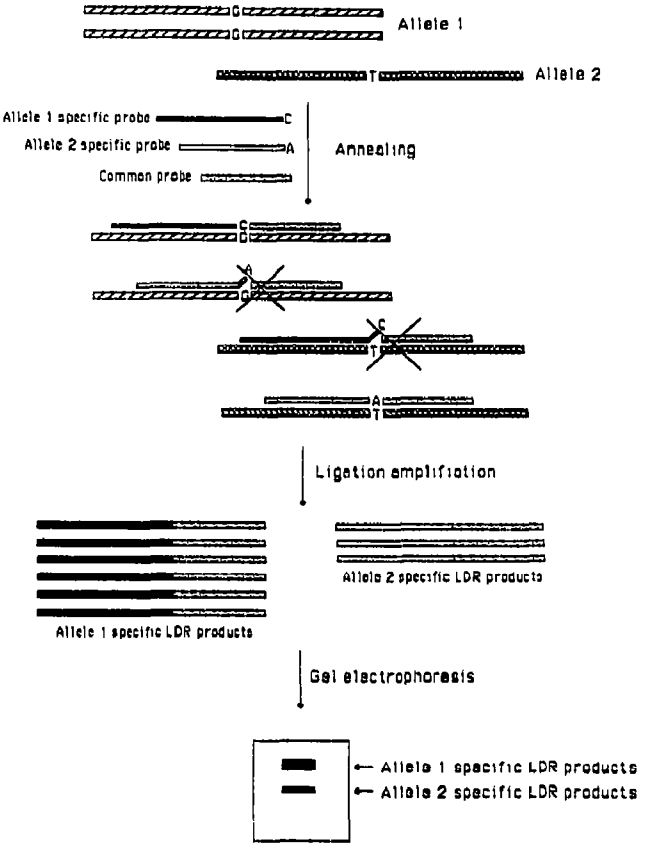

Fig. 3. Overview of the ligase detection method. Alleles 1 and 2 represent homologous DNA sequences nominally distinguished by a single nucleotide in the region to be targeted by the ligase enzyme. Shown here in a 2:1 ratio, these alleles provide the templates to which a set of oligonucleotide probes anneals so that the 3′ end of the upstream probe abuts the 5′ end of the downstream probe at the distinguishing nucleotide. When an allele-specific probe is hybridized to the 'correct' allele, the nucleotides at the juncture with the downstream probe are appropriately base-paired with the template, and the action of ligase links the two probes to form an allele-specific product. Note that even if the specific probe hybridizes to the 'wrong' allele (e.g., allele 1 probe with allele 2 and vice-versa), no product is formed since the nucleotide mismatch at the junction site precludes ligase-mediated linkage with the downstream probe. When a heat-stable ligase is used, the reaction can be carried out repetitively in a thermocycler, resulting in linear amplification of the products that accumulate in the ratio dictated by the relative amounts of the starting alleles. Gel electrophoresis separates the differently sized products and subsequent quantitation by autoradiography reflects the original 2:1 ratio of alleles 1 and 2.

The mRNAs encoding rat brain sodium channel subtypes are unequally distributed in the brain and are differentially regulated during developments [21–23]. We questioned whether the different sodium channel subtypes we have cloned are differentially distributed in human brain regions. Analysis of mRNA distribution has classically been achieved by means of Northern blot and/or in situ hybridization with subtype-specific probes. However, in view of the extensive sequence identity between HBSC I and HBSC II, these approaches may not offer the requisite selectivity. To circumvent this problem, the distribution of the human

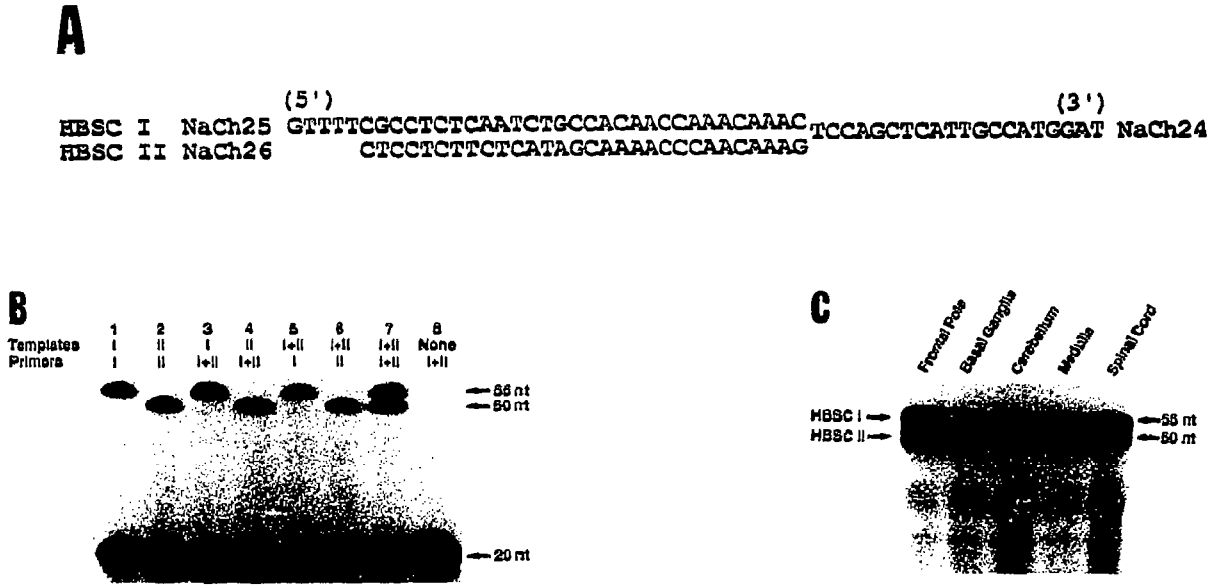

Fig. 4. Differential distribution of human brain sodium channel subtype mRNA. (A) Probes for LDM. Note that the 3' terminal nucleotide is different for NaCh 25 and 26. (B) Autoradiogram of LDM products from different combinations of templates and probes. (C) Representative autoradiogram of LDM results for HBSC I and II mRNA distribution in human brain. Differences in the ratio of subtype I and II LDM products in these brain regions, apparent upon visual inspection, were quantified by scanning densitometry (Table I). Prior to autoradiography, this gel was cut to eliminate the signal arising from labeled unligated NaCh 24 (20 nucleotides), which was present in excess.

brain sodium channel subtype mRNAs was assessed using a new approach based on the ligase detection reaction (LDR) and a thermostable DNA ligase [24–26] (Fig. 3).

The ligase detection method (LDM) applied here involves three probes. One probe, NaCh 24, was selected from the human sequence that is (i) common to both HBSC I and II, and (ii) adjacent at the 5' end to a different nucleotide in each of the two subtypes (Fig. 1 and 4A). Two additional probes were synthesized corresponding to HBSC I and II sequences; NaCh 25 (a 35mer) and NaCh 26 (a 30mer), respectively, both of which abut the 5' end of NaCh 24 (Fig. 1 and 4A). In the presence of appropriate template, the two oligonucleotides that are adjacent to each other can be linked covalently by DNA ligase if, and only if, the nucleotides at the junction site are correctly base-paired with those in template. The resulting signal is comprised of the appearance of a ligated oligonucleotide whose size is the sum of the two probes. Since the length of the subtype-specific probes is different, the expression of each subtype mRNA can be identified by the size of the LDM product. To ensure the specificity of the selected NaCh probes, the LDM reactions were tested in control experiments using cloned HBSC I and II DNA as templates in various combinations. The results presented in Fig. 4B demonstrate the excellent selectivity of the method. In the absence of either the appropriate probe or the appropriate template there is no detectable signal. Since probes for different targets can be designed with varying lengths, multiple sequences can be targeted in a single reaction vial and the results expressed as a ratio, obviating difficulties associated with intersample variability and the need to reference independent signals to some housekeeping mRNA such as β-actin. Another advantage of the method is the increased sensitivity that can be realized by repetitive cycling of the LDR reaction when a heat-stable form of the ligase enzyme is used, since the ligated product accumulates in a linear fashion.

To assess the relative distribution of the HBSC subtypes, total RNA was extracted [27] from five regions of different individual normal adult postmortem human brains (frontal pole, basal ganglia, cerebellum, medulla oblongata and spinal cord) and used in the LDM protocol. The final ligation products were separated on a denaturing polyacrylamide gel and the ratio of labeled 55 and 50 nucleotide bands (HBSC I:HBSC II) was measured by scanning densitometry of the corresponding autoradiograms (Fig. 4C). As indicated in Table I, the relative distribution of HBSC I and II mRNA in the five brain regions varies markedly, from a low value of 0.35 in cerebellum to a high of 2.18 in spinal cord (HBSC I:HBSC II). Where it was possible to sample multiple individual brains, it can be seen that the ratios in each region are quite consistent, suggesting that the values are characteristic and not an individual property. Relative to HBSC II, HBSC I is predominant in the more caudal regions of the central nervous system, reminiscent of the rostral-caudal gradients described for rat brain subtype I and II sodium channels [21].

The distribution of HBSC I and II mRNA suggests

that these human brain sodium channel genes are differentially regulated. However, the resolution of the CMPCR mapping procedure (about 10 million bp) is insufficient to distinguish whether this arises from independent transcriptional regulation of two separate genes in close proximity or from a single gene at the level of alternative splicing. Alternative splicing does not account for all forms of human sodium channels, since the gene encoding a human skeletal muscle sodium channel has previously been mapped to chromosome 17 [28]. The case for separate, yet closely linked, brain sodium channel genes has gained support from the recent work of Malo et al. [29] who reported that three separate brain α-subunit genes are clustered on the proximal segment of mouse chromosome 2. The authors suggested that gene duplication of a common ancestral sequence within a delimited chromosomal region may have provided a common mechanism for elaboration of mammalian brain sodium channel subtypes. On this basis, they also predicted that HBSC I and II genes would be found in close proximity on chromosome 2q, a prediction that is now borne out by the mapping data presented here.

The identification of human brain sodium channel subtypes and the finding that they exhibit similar distributional gradients as their homologous rat brain counterparts strengthens the hypothesis that very basic and important functional differences are conserved within these structures and/or their distribution. Additional studies are required to determine if these human brain sodium channel subtypes exhibit different intrinsic electrophysiological or pharmacological properties and if they are selectively targeted to different cellular domains.

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