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Primary structure, chromosomal localization, and functional expression of a voltage-gated sodium channel from human brain

(ionic channels/human genome/tetrodotoxin/excitable membranes/chromosome 2)

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ABSTRACT **A cDNA library derived from human cerebral cortex was screened for the presence of sodium channel α subunit-specific clones. Ligation of three overlapping clones generated a full-length cDNA clone, HBA, that provided the complete nucleotide sequence coding for a protein of 2005 amino acids. The predicted structure suggests four homologous repeats and exhibits greatest homology and structural similarity to the rat brain sodium channel II. A second cDNA clone, HBB, that encodes a different subtype of sodium channel was isolated. Hybridization of DNA fragments from the 3′ untranslated region of HBA and PCR with primers derived from HBB with human–hamster somatic cell hybrids localized these clones to human chromosome 2. *In situ* hybridization to human metaphase chromosomes mapped the structural genes for both HBA and HBB sodium channels to chromosome 2q23–24.3. The sodium channel HBA gene product was expressed by transfection in CHO cells. Expressed HBA currents were voltage-dependent, sodium-selective, and tetrodotoxin-sensitive and, thus, exhibit the biophysical and pharmacological properties characteristic of sodium channels.**

Signaling in the brain is mediated by the activity of voltage-gated and ligand-gated ion channels. Sodium channels, archetypes of the multimember family of voltage-gated channels, are responsible for the rising phase of action potentials in electrically excitable cells (1) and have been extensively studied in a variety of cells, including human neurons (1, 2). Sodium channels vary in subunit composition and complexity (1, 3, 4). In rat brain, the channel complex consists of an α subunit of $M_r \approx 260{,}000$ and two nonidentical β subunits of $M_r \approx 39{,}000$ and 37,000 (3, 4). The α subunit is sufficient to form a functional channel, as demonstrated by heterologous expression of cDNAs (3–6); the role of the smaller subunits is not yet understood.

Sodium channels are the targets of a variety of clinically valuable drugs, such as local anesthetics, anticonvulsants, or antiarrhythmics, and of various toxins (1, 4). However, little is known about the molecular structure and specific pharmacology of human brain channels, goals hitherto hampered by the limited accessibility of the tissue to experimental manipulation. Availability of cDNA clones for these proteins and their expression in heterologous systems would provide powerful tools to investigate their fundamental structure and pharmacological properties and to assess efficacy of therapeutic intervention. Localization of sodium channel genes to human chromosomes is an important step in exploring their involvement in the etiology of heritable neuropsychiatric disorders that have been mapped and in developing molecular markers for clinical screening programs (7).

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We report here the complete amino acid sequence of the α subunit of a human brain sodium channel,¶ its localization to human chromosome 2q23–24.3, and its expression from cDNA in CHO cells. A preliminary account of this report was presented elsewhere (8).

MATERIALS AND METHODS

Isolation of cDNA Clones. Standard molecular biological techniques were carried out as described (9). A cDNA library in the vector λ ZAP II was constructed using mRNA from a section of human frontal cortex (10). A fraction of the cDNA library enriched in inserts >4 kilobases (kb) contained 8×10^6 individual recombinants. About 5×10^5 plaques were screened with a probe containing 1.2 kb of the 3′ coding region of human spinal cord sodium channel cDNA (D. H. W. and M. M., unpublished results). Hybridization was carried out in 30% (vol/vol) formamide/5× SSPE (750 mM NaCl/50 mM NaH_2PO_4/5 mM EDTA, pH 7.5)/0.1% SDS/salmon sperm DNA (200 μg/ml) for 24 hr at 42°C. Final washings were done in 0.1× SSPE/0.1% SDS at 55°C for 30 min. To obtain a clone containing the 5′ terminus, a 1-kb fragment from the 5′ end of the clone HB8 (Fig. 1) was used to screen 9×10^5 additional plaques. Recombinants in λ ZAP II were rescued with helper phage R408 (Stratagene) and used for further analysis. Sequencing of double-stranded DNA was carried out with T7 DNA polymerase (United States Biochemical) by the dideoxynucleotide chain-termination method (11).

Construction and Expression of a Full-Length cDNA Clone. A full-length cDNA clone in the expression vector pSVK3 (Pharmacia) (12) encompassing the three overlapping clones HB19, HB8, and HB6 (Fig. 1) was constructed. The *Pst* I–*Pst* I (positions −108 to 1396) fragment of HB19, the *Pst* I–*Nhe* I (positions 1396–5158) fragment of HB8, and the *Nhe* I–*Pst* I [position 5158 to the termination codon and ≈200 base pairs (bp) of the 3′ untranslated region (UTR) with a *Pst* I site derived from the multiple cloning site of the vector] fragment subcloned from HB6 were ligated into the *Pst* I site in pSVK3. This construct was used for expression studies.

CHO-K1 cells (ATCC CCL61) were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum. CHO cells (plated at ≈25% confluency in 35-mm dishes) were transfected with HBA cDNA in pSVK3 vector (5 μg) by calcium phosphate precipitation followed by a glycerol treatment (9). Cells were plated for electrophysiological recordings 48 hr later.

Chromosomal Localization. A human–hamster somatic cell hybrid mapping panel (13) and fluorescence *in situ* hybrid-

Abbreviations: nt, nucleotide(s); UTR, untranslated region.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M94055).

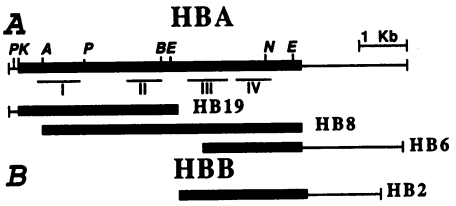

FIG. 1. Restriction map of human brain sodium channel clones. (*A*) HBA, a full-length clone resulting from ligation of three overlapping clones (HB19, HB8, and HB6). Diagram indicates their relative positions. Thick lines, coding region; thin lines, noncoding region. Thin bars denoted as I–IV represent the four homologous repeats. A, *Acc* I; B, *Bgl* II; E, *Eco*RI; K, *Kpn* I; N, *Nhe* I; P, *Pst* I. (*B*) Clone HB2 represents a different sodium channel subtype HBB, the position of which is indicated in the diagram.

ization (14) were used for chromosomal location (Table 1). DNA was isolated from each cell line, parental CHO cells, and human fibroblasts. HBA was localized by Southern blot analysis using a 1.6-kb *Eco*RI fragment from the 3′-UTR of clone HB6 (Fig. 1). Presence of a human-specific restriction fragment indicated that the cell line carried the human chromosome containing the HBA gene. HBB was localized using a 300-bp PCR-amplified fragment. Primers derived from the DNA sequence of the 3′-UTR of HB2 (Fig. 1) were 5′-CTTGTTTCCCATGTAAATAAAC-3′ (forward) and 5′-TTGTAGAATGAATAGCCTAC-3′ (reverse). PCR amplification revealed a human-specific amplification product but did not generate an amplification product using hamster DNA alone.

Fluorescence *in situ* hybridization was carried out using human metaphase chromosomes (14). To determine the chromosomal location of HBB, a 0.9-kb *Eco*RI–*Hin*dIII fragment from the 3′-UTR of clone HB2 was used to isolate a human cosmid clone from a library prepared in the vector pWE15. The location of HBA was determined using a mixture of clones HB8, HB19, and HB6 as a probe. Probes were labeled by random-hexamer-primed DNA synthesis in presence of biotinylated dUTP and dCTP, prehybridized with repetitive DNA to eliminate signals from repetitive sequences, and hybridized to human metaphase chromosomes (14). Hybridization was visualized using streptavidin-conjugated fluorescein isothiocyanate. Chromosomes were counterstained with propidium iodide to reveal a chromosomal banding pattern and images were collected using a Bio-Rad MRC600 confocal microscope. Positional assignments were made according to the apparent G-band location determined on >30 metaphases. Two positions of hybridization were seen on >90% of chromosomes.

Electrophysiological Recordings, Data Acquisition, and Analysis. Membrane currents from CHO cells were recorded in the whole-cell configuration 3–7 days after transfection (2, 15). Cells were bathed in standard saline {140 mM NaCl/3 mM KCl/2 mM $CaCl_2$/2 mM $MgCl_2$/5 mM glucose/5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes), pH 7.3}. Osmolarity was adjusted at 310 milliOsmolar with mannitol. The pipette solution was 140 mM KCl/2 mM $MgCl_2$/5 mM EGTA/5 mM glucose/5 mM Tes, pH 7.3. Voltage stimulation and data acquisition were performed with a microcomputer equipped with pCLAMP 5.5.1 software (Axon Instruments, Burlingame, CA). Capacitative currents were analog compensated and leakage currents were corrected using the P/6 protocol (16).

RESULTS AND DISCUSSION

Isolation and Characterization of cDNA Clones. Several overlapping cDNA clones encoding fragments of human brain sodium channel α subunit were isolated (Fig. 1*A*). Restriction mapping and partial sequence analysis established that three overlapping clones (HB6, HB8, and HB19) encompassed the entire coding region for a protein most closely related to rat brain sodium channel II. The human brain sequence is denoted as HBA. Of the six clones isolated, five were of the HBA type. Clone HB8 extends from nucleotide (nt) 446 in the coding region to nt 6005, only 10 bp before the end of the coding region. Notably, 5560 bp of coding region was obtained from a single clone, HB8, conclusively demonstrating that this sequence belongs to a single subtype of sodium channel. The remaining sequence on the 3′ end of this transcript was obtained from the overlapping clone, HB6. Clone HB6 starts at nt 3962 in the coding region, and after the termination codon at nt 6016, extends 2.25 kb in the 3′-UTR. The sequence succeeding the termination codon shows no resemblance to 3′-UTRs of other sodium channels. A poly(A) tail or the canonical AATAAA polyadenylylation signal (17) is missing at the 3′ end of the HB6 clone. Presumably, the 3′-UTR of this transcript is longer than the 2.25-kb stretch of clone HB6. Clone HB19 contains 145 bp of the 5′-UTR and extends 3366 bp into the coding region. The 5′-UTR carries four ATG triplets succeeded by in-frame termination codons. The 5′-UTR retained in the expression construct has three out-of-frame ATG triplets, the presence of which does not prevent its expression. The 5′-UTR shows 92% homology with the corresponding sequence from rat brain sodium channel IIA, whereas the 3′-UTRs are completely divergent (5). Lack of resemblance in the 3′-UTR suggests alternative splicing in this region and/or transcription from a different chromosomal gene.

Restriction enzyme digestion patterns and partial sequence analysis indicate that clone HB2 (Fig. 1*B*) codes for a different isoform of sodium channel. It consists of 2871 bp of coding sequence from the 3′ end succeeded by ≈800 bp of 3′-UTR. HB2 starts at residue 1039, numbered according to the alignment with rat brain sodium channel II, and encompasses homologous repeats III and IV. Homology with HBA is 90% and with rat brain sodium channels I, II, and III is 89%, 91%, and 89%, respectively.

Primary Sequence. The decoded amino acid sequence of the human brain sodium channel α subunit HBA is shown in Fig. 2. HBA codes for a protein of 2005 amino acids. At the nucleotide level, it shows 80%, 80%, and 77% homology with rat brain sodium channels I–III, respectively. At the amino acid level, sequence identity is 88%, 97%, and 88%, respectively. Therefore, this clone represents the human homolog of the brain-specific sodium channel subtype II. Identity is significantly lower with human (72%; ref. 19) or rat (73%; ref.

Table 1. Discordance

	Chromosome																						
Probe	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
HB6 (HBA)	3	0	2	2	19	2	2	4	2	2	2	3	2	2	2	2	3	2	2	2	2	2	1	2
HB2 (HBB)	3	0	8	6	22	9	4	7	4	4	6	6	4	8	5	5	3	9	11	3	10	5	4	2

Localization of human brain sodium channel clones HBA and HBB to human chromosome 2. For subtype HBA, Southern blot analysis with a probe from the 3′-UTR of clone HB6 was used. For subtype HBB, PCR with primers from the 3′-UTR of clone HB2 was used. A zero discordance value signifies a perfect match between presence of a chromosome in a given cell line and identification of the probe location to the given cell line and, therefore, assigns the probe to the corresponding chromosome.

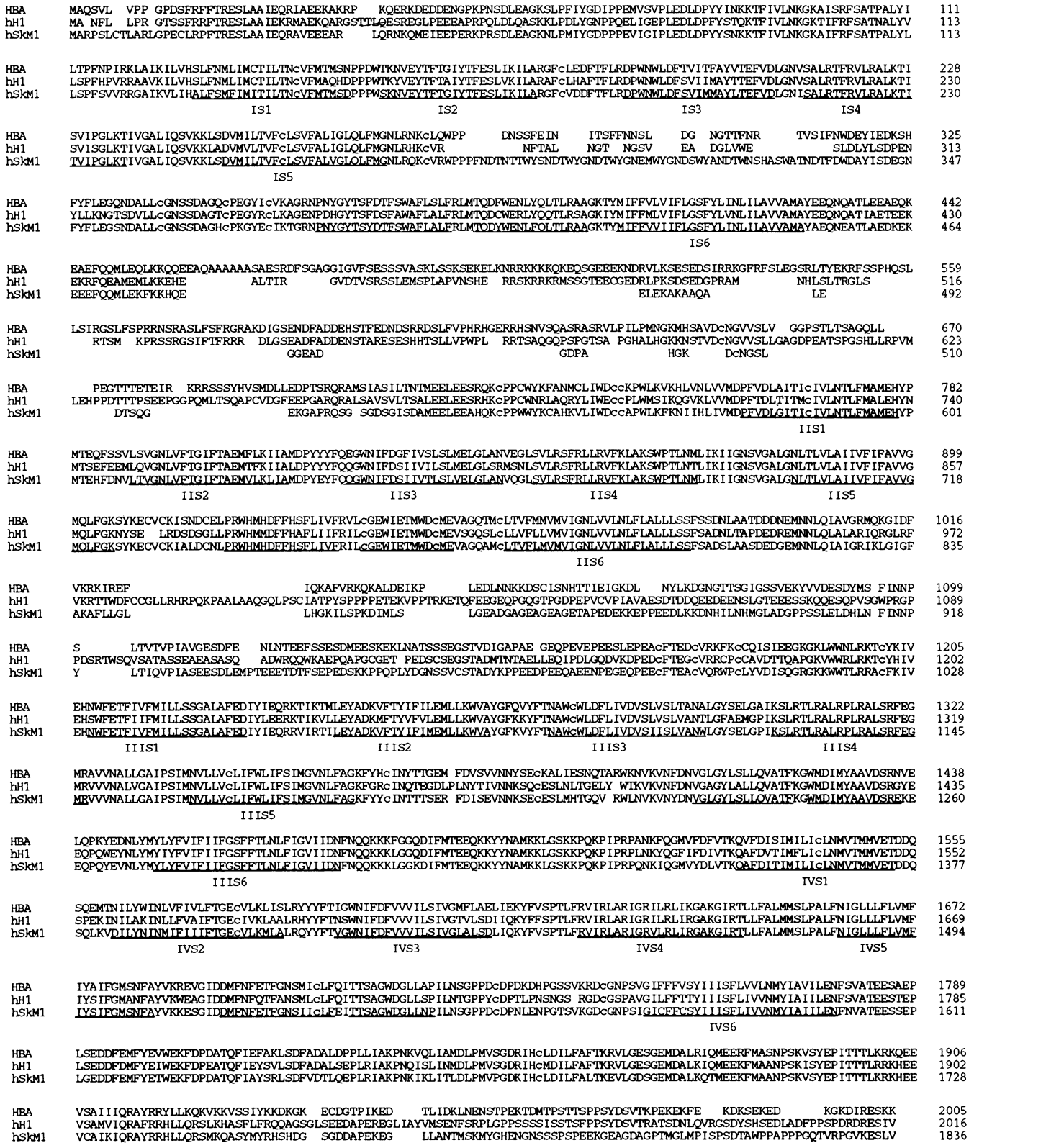

FIG. 2. Primary structure of the human brain sodium channel. Amino acid sequence of HBA, deduced from its cDNA, compared to sequences of hH1 (human heart, ref. 18) and hskM1 (human skeletal muscle, ref. 19). Numbers on the right correspond to amino acid positions. Within each of the four homologous repeats, postulated transmembrane segments are underlined. For HBA, potential glycosylation sites are at Asn-285, 291, 297, 303, 308, 340, 1368, 1382, and 1393; consensus sequences for phosphorylation by cAMP-dependent protein kinase are at positions 246–250, 550–554, 570–574, 607–611, 620–624, 682–686, 1048–1052, and 1926–1930; and consensus sequences for protein kinase C are at positions 13–16, 244–247, 494–497, 497–500, 531–534, 561–564, 568–571, 579–582, 606–609, 699–702, 1395–1398, 1506–1509, 1862–1865, 1899–1902, and 1991–1994.

20) skeletal muscle type I, human (66%; ref. 18) or rat (65%; refs. 21 and 22) cardiac muscle, *Electrophorus electricus* electroplax (60%; ref. 3), and *Drosophila* (47%; ref. 23) sodium channels. Evidently, evolutionary pressure com-

mands organ-specific rather than species-determined conservation.

Amino acid sequence alignments (24) and hydrophobicity plots reveal the presence of several canonical structural features predicted for other sodium channels (25, 26); a comparison of human brain sodium channel HBA with human cardiac muscle hH1 (18) and human skeletal muscle hskM1 (19) is illustrated in Fig. 2. For HBA, the four internal homology repeats (I–IV) extend from amino acids 129 to 427, 759 to 985, 1208 to 1475, and 1531 to 1778. Within each homologous repeat, there are eight potential transmembrane segments (underlined spans in Fig. 2), with S4 (Sd) exhibiting the 3-fold repeat of basic residues (25, 26).

The human brain and the cardiac or skeletal muscle channels differ in several amino acids, most of which occur in the loops connecting repeats I–II and II–III and do not change the assignment of potential glycosylation or phosphorylation sites postulated for other sodium channels (25, 26). Potential glycosylation sites (27) occur at nine asparagine residues assigned to extracellular loops C-terminal to the S5 segments of homologous repeats I and III. Consensus patterns for cAMP-dependent protein kinase phosphorylation (28) are identified in eight stretches. Further, 14 potential sequences for protein kinase C phosphorylation (29) are recognized. It is significant that all presumed phosphorylation sites are predicted to be in the cytoplasmic domain of the protein (25, 26). Acidic residues considered to contribute to the tetrodotoxin binding site are conserved and assigned to the segment denoted Sg (25) or SS2 (26) at positions 384, 387, 942, 945, 1426, and 1717 (30, 31). The HBA protein, thus, exhibits the structural features postulated for other sodium channels (3, 25, 26).

Mapping of Human Brain Sodium Channels to Human Chromosome 2. Southern blot analysis of a panel of 24 human–hamster cell hybrids assigned clone HBA to chromosome 2 (Table 1). A PCR with a different panel of 24 human–hamster cell hybrids mapped the structural gene of clone HBB to chromosome 2 (Table 1). *In situ* hybridization to metaphase chromosomes provided sublocalization of the structural genes for HBA and HBB to chromosome 2q23–24.3, illustrated in Fig. 3 *A* and *B*, respectively. Bright fluorescent spots emerge from the chromosome region corresponding to 2q23–24.3. It is plausible that different human genes encoding sodium channel proteins are arranged within this region (32) since the probe derived from clone HB8 encodes a segment of amino acids that is 95% identical with rat brain subtypes I, II, and III. Indeed, brain-specific sodium channels were localized to a broad region of chromosome 2q21–33 by screening a panel of somatic cell hybrids followed by *in situ* hybridization (32), and the brain sodium channel II was mapped to chromosome 2q22–23 by using a chromosomal microdissection–PCR (33). Therefore, our fluorescent *in situ* hybridization measurements provide a more accurate localization.

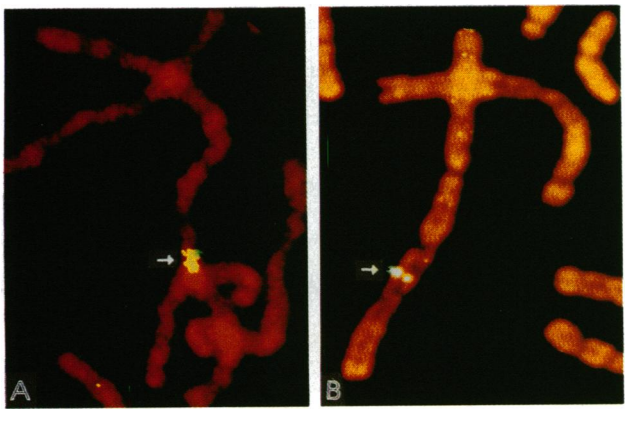

FIG. 3. Mapping of human brain sodium channel genes to human chromosomes by *in situ* suppression hybridization. Metaphase spreads of propidium iodide-counterstained chromosome 2 after hybridization with clone HBA (*A*) or clone HBB (*B*) probes. HBA probe was a mixture of cDNAs (HB19, HB8, and HB6; Fig. 1); HBB probe was a cosmid clone. cDNA clones HBA (*A*) and HBB (*B*) were biotinylated, hybridized, and detected with avidin-conjugated fluorescein isothiocyanate. Digitized images were obtained with a confocal laser scanning microscope (14). Specific fluorescence signals (spots) appear at 2q23–24 (indicated by arrow).

It is worth noting that in the mouse genome the three brain-specific sodium channel subtypes are clustered on chromosome 2 (34). In contrast, the skeletal muscle sodium channel SKM1, which exhibits 70% homology to clone HBA, was assigned to chromosome 17q23.1–25.3 (35). Hyperkalemic periodic paralysis (36) and paramyotonia congenita (37) were recently genetically linked to mutations in the skeletal muscle sodium channel gene. It is plausible that mutations will be uncovered in HBA as responsible for neuropsychiatric dysfunctions of unestablished etiology.

Transient Expression in Mammalian Cells. Electrophysiological properties of the sodium channel encoded by the HBA DNA sequence were characterized after transfection in CHO cells. A family of sodium currents in response to a series of 10-ms depolarizing voltage steps, from a holding potential of −100 mV, is illustrated in Fig. 4*A*. Currents increase to a peak

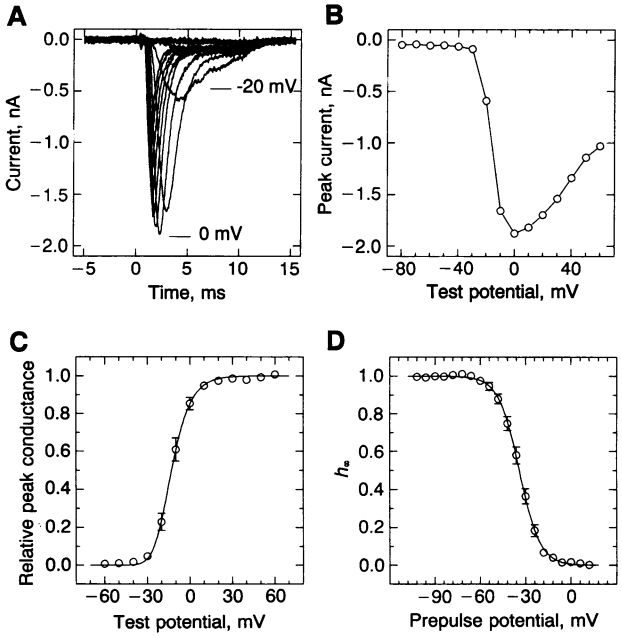

FIG. 4. HBA gene product is a voltage-gated sodium channel. Currents were recorded under voltage clamp from CHO cells transfected with HBA cDNA. (*A*) Family of sodium currents elicited by 10-ms voltage steps from a holding potential of −100 mV. Test potentials ranged from −80 mV to 60 mV in 10-mV increments. Currents were filtered at 3 kHz. (*B*) Peak currents plotted versus test potential for the records shown in *A*. (*C*) Peak conductance–voltage relationship calculated according to $g_{peak} = I_{peak}/(V - V_{Na^+})$, where V denotes the applied voltage and V_{Na^+} is the Nernst equilibrium potential. Solid line depicts the least-square fit to data points and is given by $g = \{1/[1 + \exp(V_{1/2} - V/a)]\}^3$, where $V_{1/2} = -24.08$ mV and $a = 8.3$ mV. Each point represents the mean ± SEM ($n = 18$). (*D*) Steady-state voltage dependence of inactivation determined using a series of 100-ms prepulses ranging from −102 mV to 12 mV followed by a 10-ms test pulse to 0 mV. Peak currents were normalized with respect to the maximum and plotted as a function of prepulse V. Solid line depicts the least-square fit to the data points given by $h_\infty = 1/[1 + \exp(V - V_{1/2}/a)]$, where $V_{1/2} = -34.07$ mV and $a = 6.85$ mV. Each point contains data from a minimum of 6 and a maximum of 17 experiments (mean ± SEM).

and then decline. Kinetics of early activation are sigmoidal and of subsequent decay approximately exponential. A comparison of the amplitude and kinetics of the currents recorded at −20 and 0 mV shows that both activation and current decay accelerate at progressively more depolarized potentials. The peak current–voltage (*I–V*) relationship is shown in Fig. 4*B*. Depolarization to voltages positive to −40 mV rapidly activates sodium currents. The peak current I_{peak} (1332 ± 487 pA) recorded in 18 cells was obtained at test potentials of 5.6 ± 8.6 mV (mean ± SD). In contrast, control CHO cells transfected with vector devoid of the HBA cDNA insert express an endogenous sodium channel with a I_{peak} of 201 ± 138 pA (n = 10) at a V of 18.8 ± 6.4 mV. The peak sodium conductance g_{peak} increased with depolarizing voltages to a maximum at a V of ≈20 mV, exhibiting a half-activation voltage at a V of −24 mV and an e-fold change in g_{peak}/8.3 mV (Fig. 4*C*). The Hodgkin–Huxley (38) inactivation parameter (h_∞) indicates that at a V of −34 mV half of the sodium currents were inactivated (Fig. 4*D*). Sodium currents were blocked by 100 nM tetrodotoxin.

Overall, the HBA gene product displays macroscopic sodium currents that resemble the transient time course, kinetics, selectivity, and tetrodotoxin-sensitivity characteristic of sodium channel currents recorded in a variety of cells (1) including the human cerebellar medulloblastoma cell line TE671 (2).

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