

# Multiple transcripts of sodium channel *SCN8A* (Na<sub>v</sub>1.6) with alternative 5′ and 3′ untranslated regions and initial characterization of the *SCN8A* promoter

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Received 16 July 2004; accepted 3 September 2004

Available online 7 December 2004

## Abstract

To identify the transcriptional start sites of the neuronal channel *SCN8A*, we carried out 5′RACE (rapid amplification of cDNA ends) with RNA from human and mouse brain. We recovered four mutually exclusive 5′ untranslated exons (exon 1a to exon 1d) that map to a 1.8-kb region of genomic DNA located ~70 kb upstream of the first coding exon. The same 5′ untranslated exons are expressed in central, peripheral and sympathetic nervous system and in embryonic and adult brain. A 4.8-kb genomic fragment containing these 5′ exons demonstrated promoter activity in transfected MN-1 cells. In transgenic mice, transcription of the 4.8-kb promoter was restricted to brain and spinal cord. The 4.8-kb promoter contains eight consensus Sp1-binding sites and two Inr sites. A potential NRSE/RE-1 site is located nearby. Two active polyadenylation sites identified by 3′RACE are conserved in human, mouse, and chicken *SCN8A*. Sequence comparison of human and mouse *SCN8A* identified 12 conserved noncoding elements whose effect on transcription was tested in transfected cells.

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**Keywords:** Promoter (genetics); Transcription initiation site; Sodium channels (genetics); 5′ Untranslated regions; 3′ Untranslated regions; Conserved sequence; Polyadenylation; *SCN8A*; Sp1 transcription factor; RE1-silencing transcription factor

## Introduction

Voltage-gated sodium channels are responsible for the rising phase of the action potential during neuronal firing. The transcriptional regulation of the sodium channels genes is poorly understood and the work on promoter characterization is in an early stage. Four closely related sodium channel genes have a broad expression pattern throughout the central nervous system (CNS) and peripheral nervous system: *SCN1A*, *SCN2A*, *SCN3A*, and *SCN8A*. Mutations in *SCN1A* and *SCN2A* are responsible for two inherited

epilepsy syndromes, GEFS+ and BFNIS [1–3]. De novo mutations of *SCN1A* have been identified in patients with severe myoclonic epilepsy of infancy [4]. Mutations of *SCN8A* in the mouse result in movement disorders ranging in severity from mild ataxia and tremor to severe dystonia and paralysis [5–7]. A modifier gene that influences *Scn8a* related disease was recently isolated [7–9].

*SCN8A* encodes the 260-kDa sodium channel  $\alpha$ -subunit Na<sub>v</sub>1.6, which contains the pore-forming and voltage-sensitive domains of the channel [10]. Na<sub>v</sub>1.6 is localized in axonic, dendritic, presynaptic, and postsynaptic membranes and is the major sodium channel in mature nodes of Ranvier [11,12]. Particularly high levels of expression have been observed in cerebellum, cochlear nucleus, olfactory bulb, and dorsal root ganglia.

Transcripts of *Scn8a* can be detected in mouse brain as early as embryonic day (E)<sup>1</sup> 12.5. Transcript abundance increases around the time of birth and reaches adult levels

**Abbreviations:** As1, alternatively spliced internal untranslated exon 1; BAC, bacterial artificial chromosome; CPE, cytoplasmic polyadenylation element; cpm, counts per minute; E, embryonic day; NCS1, noncoding conserved sequence 1; P, postnatal day; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

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by postnatal day (P) 21 [13]. Modulation of sodium channel expression has been described in diabetic neuropathy [14] and inflammatory demyelination [15]. In addition to *SCN8A*, neurons contain several other voltage-gated sodium channels and the proportions of different channels vary in different parts of the nervous system [16].

The promoters of *SCN2A*, *SCN3A*, and the cardiac channel *SCN5A* were recently identified [10,17–19]. To initiate the analysis of transcriptional regulation of *SCN8A*, we have identified the gene promoter and characterized the expression of the endogenous promoter and derived constructs. We have also identified alternative 5′ and 3′ untranslated regions (UTRs) that may influence transcription or translation of *SCN8A* transcripts.

## Results

### Four conserved 5′-untranslated exons of *SCN8A*

To identify the transcription start sites for *Scn8a*, 5′-rapid amplification of cDNA ends (5′-RACE) was carried out on

poly(A)<sup>+</sup> RNA from mouse brain, spinal cord, and dissected brain regions that included cortex, cerebellum, retina, striatum, hippocampus, olfactory bulb, brainstem, and retina. The reverse primer was located in the first coding exon of *Scn8a*. Sequencing of more than 150 5′-RACE products from 11 tissues identified 42 different types of clones, with each type defined by its unique length or by recovery from independent experiments. Each “of the 42” clones contained one of four alternative 5′-untranslated exons, designated exon 1a to exon 1d (Fig. 1A) (GenBank accession nos. AY510077, AY510079, AY510081, and AY510082). Each exon terminates in a consensus splice donor site and is directly spliced to the first coding exon (exon 1). Ninety percent of 5′-RACE clones contained either exon 1a or exon 1b (Fig. 1A), including clones from all of the brain regions listed above. Approximately 10% of the 5′-RACE clones contained the sequence of a 149-bp internal noncoding exon [alternatively spliced internal untranslated exon 1 (Asi1)] between the 5′ exon and the first coding exon 1 (GenBank accession nos. AY510078 and AY510080). There was no preferential association of Asi1 with exons 1a–1d (data not shown). Asi1 is flanked by consensus splice donor and splice

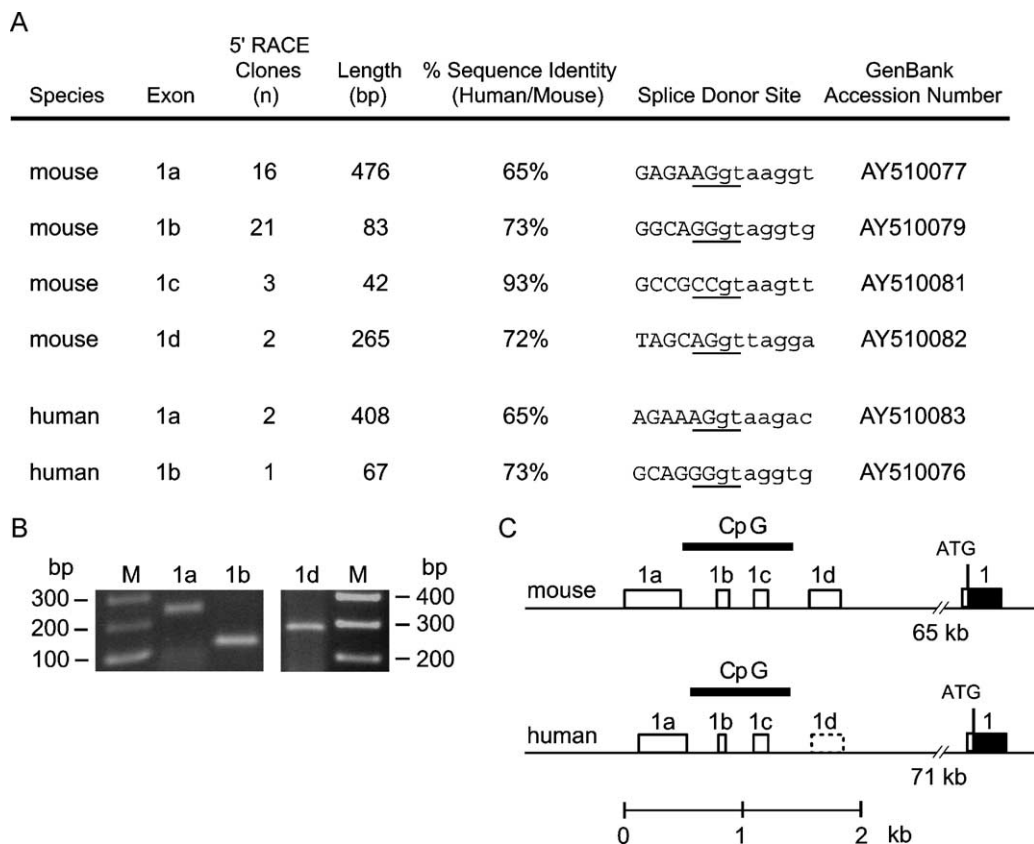


Fig. 1. Identification of four untranslated exons encoding the 5′UTRs of *SCN8A* transcripts. (A) Each independent 5′RACE clone was amplified from poly(A)<sup>+</sup> RNA in a different experiment or recognized by its unique insert length. Exon size was determined by the longest 5′RACE clone. The 3′-end of each exon terminates in a consensus splice donor site. Human clones were amplified from cerebellar poly(A)<sup>+</sup> RNA. (B) RT-PCR amplification of mouse brain total RNA with forward primer in the untranslated exon and reverse primer in the first coding exon (exon 1). The predicted products were obtained from exon 1a (238 bp) using primers 16 and 11 (Supplemental Table 1), exon 1b (171 bp) using primers 18 and 6, and exon 1d (280 bp) using primers 19 and 6. M, molecular weight marker. (C) Evolutionary conservation of promoter organization in mouse and human *SCN8A*. The promoters of mouse and human *SCN8A* are located 65 and 71 kb upstream of the first coding exon, respectively. Dotted box, human genomic sequence with 72% identity to mouse exon 1d; transcription of this exon has not been confirmed.

acceptor sites in genomic DNA. Because the first coding exon of *Scn8a* contains an in-frame stop codon upstream of the initiator methionine codon [20], the choice of 5' exon and the inclusion of *Asi1* do not affect the protein sequence.

To determine whether the 5' noncoding exons are evolutionarily conserved, we analyzed 5'-RACE products from human cerebellar RNA. The human RACE products contained sequences corresponding to mouse exon 1a and exon 1b, with 67 and 72% sequence identity, respectively (Fig. 1A) (GenBank accession nos. AY510076 and AY510083). As in the mouse, the human exons terminate in consensus splice donor sites and are spliced directly to the first coding exon (Fig. 1A). The human orthologue of exon 1c is present in a reported *SCN8A* cDNA clone (GenBank accession no. NM014191). Mouse exon 1d is represented in the predicted position in human genomic DNA, with 72% identity to mouse exon 1d, but human exon 1d has not been detected in transcripts. Sequences corresponding to *Asi1* were not recovered in human 5'-RACE products and were not detected in the finished sequence of the human bacterial artificial chromosome (BAC) clone that contains the other 5' exons.

#### Transcription of the 5'-untranslated exons

Expression of the 5' exons was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) of mouse brain cDNA using a forward primer in each noncoding exon with a reverse primer in the first coding exon. RT-PCR products were gel-purified and sequenced. The predicted products were obtained for exon 1a, exon 1b, exon 1d, and *Asi1* (Fig. 1B). Exon 1c could not be analyzed by RT-PCR, because of the high GC content of 92%. None of the RT-PCR products contained more than one of the 5' exons 1a–1d. This is consistent with the absence of splice acceptor sites in the genomic sequence upstream of these exons.

To search for additional untranslated exons upstream of exon 1a, we carried out 5'-RACE on human and mouse brain RNA using reverse primers in exon 1a. The recovered 5'-RACE products contained contiguous genomic sequence only. The longest products recovered were 405 bp in human and 475 bp in mouse. Transcription of these sequences was confirmed by RT-PCR using forward primers close to the 5' ends of the longest human and mouse products (data not shown).

To determine whether transcription is independently initiated at exon 1b, rather than read-through from exon 1a, we carried out a ribonuclease protection assay. The 579-bp riboprobe contained the 3'-end of exon 1a, the 5'-end of exon 1b, and the intervening 308-bp intron (Fig. 2). RNA from mouse brain protected 126 bp from exon 1a and 78 bp from exon 1b (Fig. 2), but did not protect the full-length 512-bp fragment that would be protected by a read-through transcript (not shown). This is consistent with our failure to amplify the 308-bp intron by RT-PCR using a forward

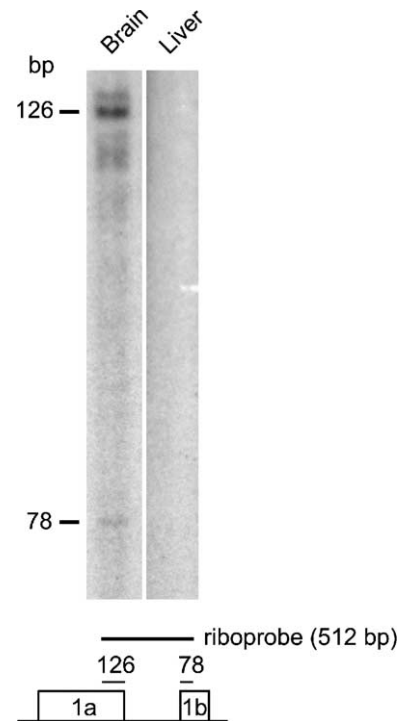


Fig. 2. Independent transcription initiation of exon 1a and exon 1b. An RNase protection assay was carried out with a 512-bp riboprobe containing portions of exon 1a and exon 1b and the intervening 308-bp intron. Fragments of 126 and 78 bp corresponding to the included portions of exon 1a and exon 1b were protected by total RNA from mouse brain. We did not detect larger fragments that would have been protected by read-through from exon 1a through the intron (not shown). RNA from mouse liver did not protect the riboprobe, consistent with the lack of expression of *Scn8a* in liver.

primer in exon 1a and a reverse primer in exon 1b (not shown). The data indicate that exon 1a and exon 1b are mutually exclusive first exons that are independently transcribed from distinct transcription start sites.

#### Genomic clustering of the 5'-untranslated exons

Sequence alignment of the 5'-RACE products with mouse and human genomic sequence using BLAST2 software demonstrated that the four untranslated exons are clustered in a 1.8-kb region located 65 kb upstream of the first coding exon in the mouse and 71 kb upstream in human (Fig. 1C). The order and arrangement of the four 5' exons is conserved in the two species (Fig. 1C). The promoter region also contains a CpG island, an incomplete LINE-1 element, and an incomplete SINE element from the MIR family. *Asi1* is located 37 kb upstream of the first coding exon in mouse but not in human.

#### Promoter fragments containing the 5'-untranslated exons are active in cultured cells

A 4.8-kb genomic fragment containing exons 1a, 1b, 1c, and 1d, with 1.5 kb of upstream sequence and 1.5 kb of

downstream sequence, was cloned into the pGL3-Basic expression vector upstream of a 154-bp fragment containing the endogenous splice acceptor site from the first coding exon and the firefly luciferase cDNA (Fig. 3C). Deleted subclones were prepared using the indicated restriction sites. Constructs were transiently transfected into the mouse motor neuron/neuroblastoma hybrid cell line MN-1 [21]. The expression of endogenous *Scn8a* in MN-1 cells (Fig. 3A) indicated that the requisite transcription factors are

present in these cells. To control for transfection efficiency, firefly luciferase activity from the *Scn8a* construct was normalized to the activity of Renilla luciferase produced by the cotransfected plasmid pRL-SV40.

The activity of the full-length 4.8-kb fragment (construct 1, Fig. 3C) was 100× higher than the promoterless vector. Deletion of exon 1a (construct 2) or exon 1d (construct 4) did not significantly reduce expression. Full activity was retained by constructs 5 and 6, which contain exon 1b and

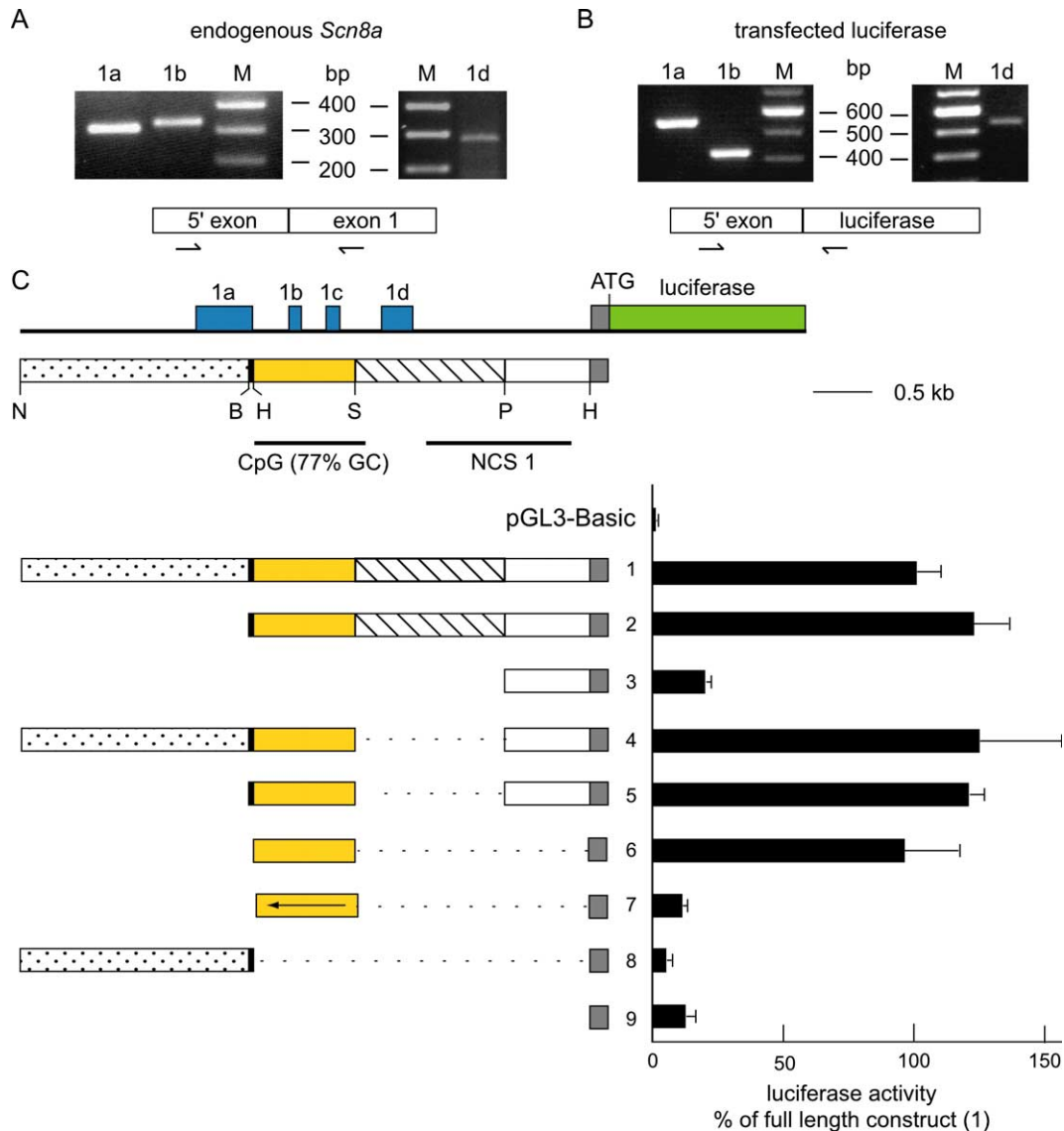


Fig. 3. The *Scn8a* promoter is active in MN-1 cells. (A) Expression of endogenous *Scn8a* in MN-1 cells. RT-PCR of total RNA was carried out with a forward primer in each 5'noncoding exon and a reverse primer in the first coding exon. The predicted products for exon 1a (307 bp), exon 1b (325 bp), and exon 1d (280 bp) are shown. Exon 1a was amplified with primers 16 and 22 (Supplemental Table 1); exon 1b was amplified with primers 18 and 22; exon 1d was amplified with primers 19 and 22. These primers are more efficient than the primers used in Fig. 1B to amplify the same transcripts. M, molecular weight marker. (B) MN-1 cells transfected with the full-length promoter (construct 1) produce full-length, correctly spliced luciferase transcripts. RT-PCR was carried out with a forward primer in exon 1a, 1b, or 1d and reverse primer in the luciferase cDNA. Predicted products for transcription from exon 1a (553 bp) using primers 16 and 22 (Supplemental Table 1), exon 1b (421 bp) using primers 18 and 22, and exon 1d (532 bp) using primers 19 and 22 are shown. The identity of RT-PCR products was confirmed by sequencing. (C) Expression of a series of promoter constructs. Firefly luciferase activity was normalized against cotransfected Renilla luciferase to control for transfection efficiency. Normalized luciferase activity is presented as the percentage of the activity of the full-length 4.8-kb construct. The data presented are means  $\pm$  SD for at least three independent experiments for each construct. For each experiment, the average of triplicate transfections was calculated. N, *Nhe*I; B, *Bgl*II; P, *Pme*I; S, *Sac*II; H, *Hind*III. Arrow, reverse orientation.

exon 1c with the associated CpG island containing several Sp1 sites. Reversing the orientation of the 0.85-kb promoter fragment in construct 6 resulted in loss of activity (construct 7). Constructs 3 and 9 lacking all of the 5' exons were inactive, as was construct 8, which contains only exon 1a with 21 bp of the downstream intron. These data indicate that the 0.85-kb fragment containing exons 1b and 1c and the CpG island is sufficient for promoter activity in MN-1 cells.

To determine which transcription start sites are used in the transfected cells, we carried out RT-PCR with forward primers in exon 1a, exon 1b, and exon 1d and a reverse primer in the luciferase cDNA. We obtained products corresponding to transcript initiation from all three 5' exons (Fig. 3B). All products were spliced directly to the splice acceptor site from the first coding exon of *Scn8a*. The 4.8-kb promoter fragment (GenBank accession no. AY510075) thus recapitulates the features of in vivo brain transcription in transfected MN-1 cells.

#### Tissue-specific expression of a 4.8-kb *Scn8a* promoter in transgenic mice

To evaluate in vivo promoter activity, construct 1 (Fig. 4A) was microinjected into fertilized mouse eggs. We analyzed transcripts from two independent lines of mice, Tg17 and Tg52, to control for potential effects of chromosomal insertion site. The predicted 2-kb luciferase transcripts were detected by Northern blot in brain RNA from the two transgenic lines but not in nontransgenic brain (Fig. 4C, arrow). The endogenous *Scn8a* transcripts were present in all three samples (Fig. 4C). RT-PCR products containing exon 1a, exon 1b, exon 1d, and luciferase coding sequence were recovered from adult brain and spinal cord (Figs. 4B and 4D). The expression of exon 1c could not be tested by RT-PCR due to its high GC content. Transgene transcripts were not detected in nonneuronal tissues (Fig. 4D). The ubiquitously expressed control transcript *Scnm1* was amplified from all samples (Fig.

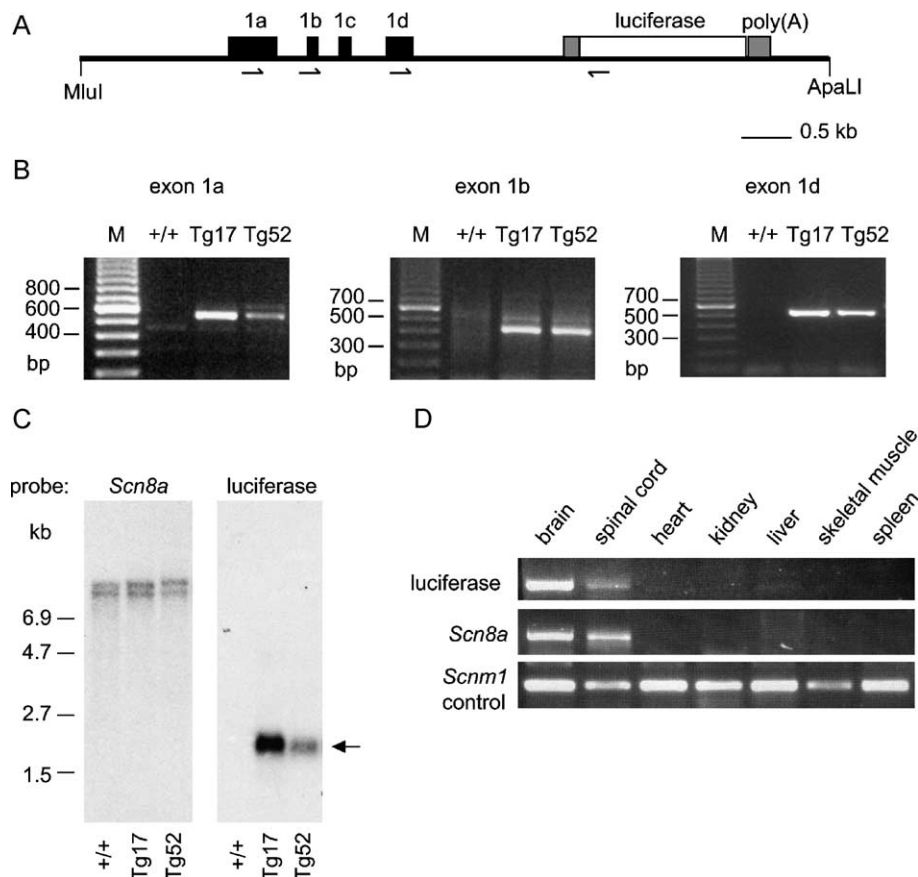


Fig. 4. Expression of a 4.8-kb *Scn8a* promoter in transgenic mice. (A) Structure of the 7.4-kb transgene with the 4.8-kb mouse *Scn8a* promoter upstream of the luciferase coding sequence. (B) Transgene expression in brain. RT-PCR was carried out on total brain RNA from transgenic lines Tg17 and Tg52. +/+, nontransgenic control. The forward primers in exons 1a, 1b, and 1d are 16, 18, and 19 (Supplemental Table 1), respectively, and the reverse primer in the luciferase gene is 22. The primer positions are shown in (A). The predicted products of 553, 423, and 532 bp were obtained and confirmed by sequencing. M, molecular weight marker. (C) A Northern blot with 2  $\mu$ g of poly(A)<sup>+</sup> RNA from brain was hybridized first with a 2.3-kb *Scn8a* coding fragment ( $6 \times 10^9$  cpm/ $\mu$ g) and then with a 1.0-kb luciferase coding fragment ( $4 \times 10^9$  cpm/ $\mu$ g). Film exposure was for 2 h (*Scn8a* probe) or 6 h (luciferase probe). (D) Tissue distribution of transgene expression in line Tg17. RT-PCR was carried out with forward primer 16 (Supplemental Table 1) in exon 1a and reverse primer 22 in the luciferase gene. Transcripts of endogenous *Scn8a* were amplified with forward primer 33 in exon 1 and reverse primer 34 in exon 6. The ubiquitously expressed control *Scnm1* was amplified as previously described using primers 35 and 36 [10].

4D). The data demonstrate that the 4.8-kb promoter fragment directs tissue-specific expression of the luciferase transgene in the same tissues that express the endogenous *Scn8a* gene.

#### *Promoter usage in peripheral and sympathetic nervous systems*

To search for additional transcription start sites, RNA was prepared from mouse dorsal root ganglia (DRG) and superior cervical ganglia (SCG). 5'-RACE was carried out with a reverse primer in the first coding exon of *Scn8a*. Sequencing the 5'-RACE products identified 16 unique clones from DRG and 13 unique clones from SCG. The 29 products contained the same 5' exons that were identified in the CNS (Table 1). The results indicate that the transcription start sites used in the peripheral and sympathetic nervous systems are the same as those identified in brain.

#### *Developmental expression of the *Scn8a* promoter*

Brain RNA was isolated at E14.5, P0.5, P7.5, and P20. RT-PCR was carried with forward primers in exons 1a, 1b, or 1d and a reverse primer in the first coding exon. Products containing exons 1a, 1b, and 1d were obtained from all samples (Table 1). Thus, the three noncoding exons are transcribed throughout development.

#### *Two alternative polyadenylation sites in *Scn8a* transcripts*

Human and mouse genomic sequence contain two conserved consensus polyadenylation signals (AATAAA) downstream of the translation termination codon in exon 24. Poly(A) 1 is located 2.5 kb downstream of the stop

codon and poly(A) 2 is located 5 kb downstream of the stop codon. To determine whether both poly(A) signals are active, we carried out 3'-RACE experiments on mouse brain RNA using nested forward primers located upstream of each site (Fig. 5A). Both sets of primers generated 3'-RACE products containing poly(A) tracts that begin 18–29 bp downstream of the polyadenylation signal and are not present in genomic DNA. Both polyadenylation signals are conserved in the chicken *Scn8a* gene, at positions 2.5 and 4.4 kb downstream of the stop codon (Fig. 5C). Sequence comparison between the three species demonstrates that the conservation of the 3' UTR is highest near the polyadenylation sites (Fig. 5B). Two cytoplasmic polyadenylation elements (CPEs) are located ~50 and ~150 bp upstream of the first polyadenylation signal. These two CPEs and the surrounding sequence are conserved in human, mouse, and chicken.

Previous studies detected two size classes of *Scn8a* transcripts on Northern blots of human and mouse brain RNA, with lengths of ~9 and ~12 kb [5,6,22,23]. We hybridized a Northern blot with one probe from the coding sequence of *Scn8a* and one probe specific for the long 3'-UTR. The 3'-UTR probe hybridized only with the 12-kb transcript (Fig. 5B). The coding sequence probe detected both transcripts. This result supports the conclusion that shorter *Scn8a* transcripts terminate at the first polyadenylation site, whereas the longer transcripts utilize the second polyadenylation site.

#### *Identification and analysis of evolutionarily conserved noncoding sequences*

To identify potential transcriptional regulatory elements, we compared human and mouse genomic DNA sequence spanning the 138-kb interval between the closest upstream gene (*SLC4A8*) and the second coding exon of *SCN8A*, using VISTA (VISualization Tool for Alignments) software (<http://www-gsd.lbl.gov/vista/>) [21]. We identified a 1.2-kb element, noncoding conserved sequence 1 (NCS1), with 78% sequence identity that is located immediately downstream of exon 1d (Fig. 6 and Supplemental Fig. 1A). Mouse NCS1 matched only the orthologous rat and human sequences in a BLAST search of the GenBank database. There were no matches to expressed sequence tags or other genomic sequence. NCS1 does not contain repetitive elements or simple sequence detected by Repeat Masker (<http://repeatmasker.genome.washington.edu>) or by hybridization of genomic DNA with an NCS1 probe (Supplemental Fig. 1B). Neither exons nor long open reading frames were identified in NCS1 by Grail, Genefinder, Genemark, Fex, Hexon, or Fgene (<http://www.hgmp.mrc.ac.uk/NIX/>). Alignment of human and mouse NCS1 did not detect internal repeats (Supplemental Fig. 1C). NCS1 thus appears to be a unique, evolutionarily conserved noncoding element. However, deletion of NCS1 did not affect promoter activity in transfected MN-1 cells (e.g., construct 6, Fig. 4C).

Table 1  
Broad distribution of 5'-UTR expression

RNA	Exon 1a	Exon 1b	Exon 1c	Exon 1d
<b>Region</b>				
Adult brain	+	+	+	+
Cerebellum	+	+	nd	+
Retina	+	+	+	+
Cortex	+	+	nd	+
Striatum	+	+	nd	+
Spinal cord	+	+	nd	+
Olfactory bulb	+	+	nd	+
Hippocampus	+	+	nd	+
Brainstem	+	+	nd	+
Dorsal root ganglia	+	+	+	+
Superior cervical ganglia	+	+	+	+
<b>Development</b>				
E14.5 head	+	+	nd	+
P0.5 head	+	+	nd	nd
P7.5 brain	+	+	nd	+
P20 brain	+	+	nd	+

Exons 1a, 1b, and 1d were detected by 5'-RACE and/or RT-PCR, Exons 1c was detected by 5'-RACE. nd, not done.

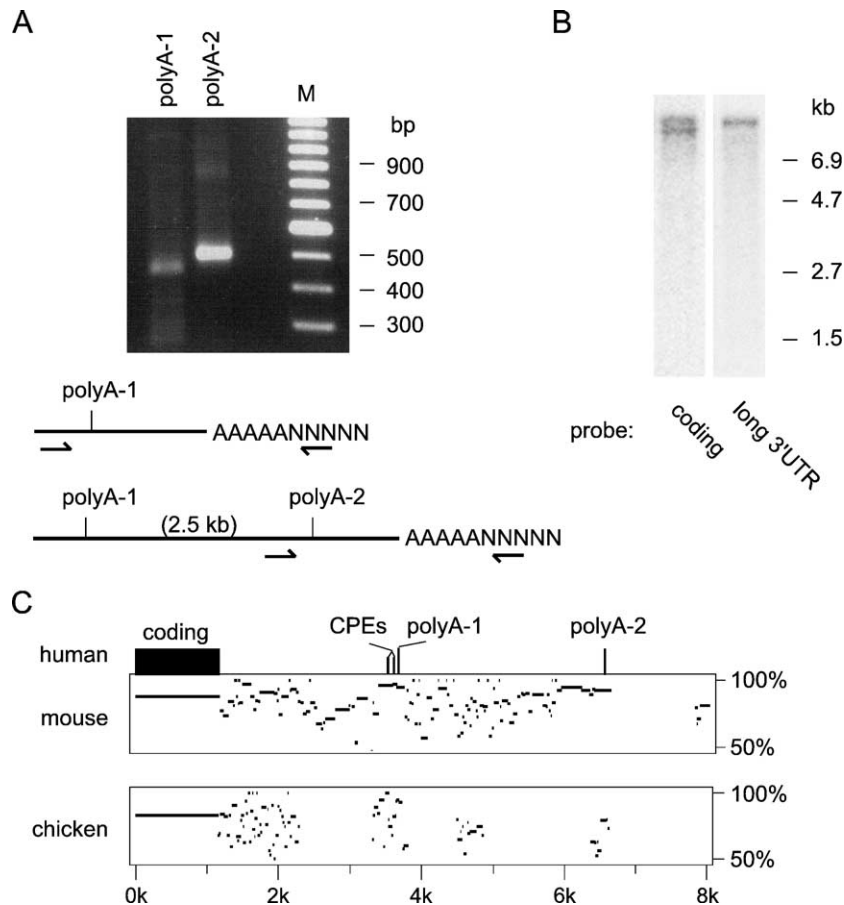


Fig. 5. *Scn8a* contains two alternative sites of polyadenylation. (A) 3'RACE products from mouse brain RNA were obtained by nested PCR using forward primers upstream of each polyadenylation signal with a reverse primer complementary to the oligonucleotide tag on the oligo(dT) primer. (B) Northern blot of mouse brain poly(A)<sup>+</sup> RNA. The 2.3-kb *Scn8a* probe containing exons 2–13 hybridizes with two classes of *Scn8a* transcripts, approximately 9 and 12 kb in length. The 639-bp probe specific to the long 3'UTR hybridizes exclusively with the 12-kb transcripts. (C) Conservation of 3'UTR sequence in human, mouse, and chicken *Scn8a*. The percentage identity to human exon 24 is indicated in the PipMaker plot.

Eleven other conserved noncoding sequences, between 100 and 300 bp in length with  $\geq -75\%$  sequence identity, were identified in this 138-kb interval (Supplemental Fig. 2A). Each of these fragments was amplified from mouse genomic DNA and cloned upstream of the minimal SV40 promoter in the pGL3-Promoter vector (Promega). Constructs were transfected into cultured cells and luciferase activity was measured as described above. None of the fragments had significant positive or negative effects on luciferase expression in MN-1 cells (Supplemental Fig. 2B) or the nonneuronal cell line NIH-3T3 (not shown).

We also compared the large intron of *SCN8A*, located between the 5' noncoding exons and the first coding exon, with the corresponding introns of *SCN1A*, *SCN2A*, and *SCN3A* (Supplemental Fig. 3). These four paralogous genes share pan-neuronal expression domains, although *SCN8A* diverged from the others more than 300 million years ago [24]. Sequence comparison using BLAST2 and PIPmaker software did not identify any paralogous conserved elements meeting the criteria of  $\geq 75\%$  sequence identity spanning  $\geq 100$  bp.

## Discussion

*The four 5' noncoding exons of SCN8A are conserved in human and mouse*

The features of the promoter region and 5' and 3'-UTRs of *Scn8a* are summarized in Fig. 6. Extensive 5'RACE analysis identified four mutually exclusive 5'exons that are clustered within 1.8 kb of genomic DNA (Fig. 6A). Exons 1a and 1b are the major transcription start sites in RNA samples from a variety of developmental stages, brain regions, and neuronal tissues although the high GC content of exon 1c (92%) may have resulted in underrepresentation in our analysis. Exons 1a, 1b, 1c, and 1d are transcribed from independent start sites. Their orientation and spacing are conserved in human and mouse and the percentage sequence identities are within the average range of  $70 \pm 13\%$  for mammalian 5'-UTRs [25,26], except for the GC-rich exon 1c, which exhibits 93% sequence identity. The internal noncoding exon Asi1 is present in 10% of mouse transcripts but is not conserved in human.

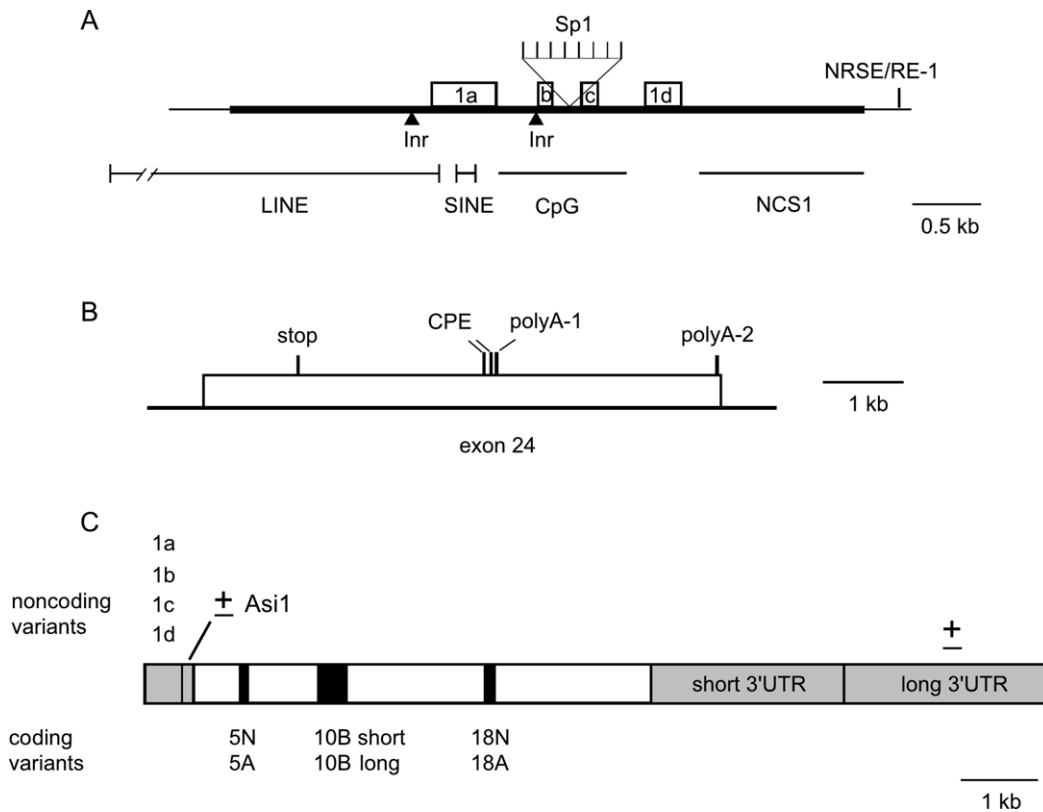


Fig. 6. Promoter and multiple transcripts of *Scn8a*. (A) Organization of the promoter region. The 4.8-kb active promoter is represented by the heavy line. The positions of four untranslated exons, 1a–1d, a CpG island, eight consensus Sp1-binding sites, a 133-bp partial SINE element, and a 3.3-kb partial LINE element are shown. A potential NRSE/RE-1 site is located 200 bp downstream of the 4.8-kb promoter. Initiation consensus sites (Inr) are located upstream of exons 1a and 1b (triangles). (B) Organization of the 3'UTR. Two polyadenylation signals are located 2.5 and 5 kb downstream of the stop codon in exon 24. The first polyadenylation signal is preceded by two consensus CPEs. (C) Combinatorial generation of multiple *Scn8a* transcripts. Sixteen transcripts differing in noncoding regions (gray), and a total of 128 transcripts differing in coding and noncoding sequences, can be generated from alternative exons.

The *SCN8A* promoter lacks a TATA box, but contains a CpG island that is rich in Sp1 sites (Fig. 6A). Two copies of the initiator sequence YYANWYY [27] are located near the noncoding exons (Fig. 6A, triangles). The promoter also contains an L1 LINE element and a MIR class SINE element that inserted prior to the divergence of human and mouse lineages (Fig. 6A).

MN-1 cells were selected for promoter analysis because of their neuronal origin and expression of the endogenous *Scn8a* gene. The smallest promoter fragment with full activity in MN-1 cells was an 850-bp fragment containing exon 1b and exon 1c. Constructs that did not contain this fragment were inactive (Fig. 3C, constructs 3, 8, and 9), suggesting that it contains essential regulatory elements. The 850-bp fragment includes most of the large CpG island and the eight consensus Sp1-binding sites, which may contribute to its strong promoter activity. Although exons 1a and 1d are expressed in MN-1 cells (Fig. 3A), deletion of exon 1a (construct 2) and exon 1d (construct 4) did not reduce expression (Fig. 3C). This may be a consequence of the overexpression of constructs in transfected cells, which may permit compensatory transcription from other 5' exons in the construct.

The 4.8-kb *Scn8a* promoter fragment was tested in transgenic mice to confirm that the promoter fragment is active *in vivo*. Expression of exons 1a, 1b, and 1d was detected in brain and spinal cord, but not in nonneuronal tissues. Exon 1c was not tested because it cannot be assayed by RT-PCR, due to the high GC content. The 4.8-kb fragment thus appears to contain the minimal elements necessary for tissue-specific regulation of *Scn8a* expression. The active expression of genomic fragments containing the 5'-noncoding exons and the widespread expression of these exons in *Scn8a* transcripts support the conclusion that we have identified the major promoter region of *SCN8A*.

Like *SCN8A*, the *BDNF* gene contains a cluster of alternative 5'-UTRs, but unlike *SCN8A*, each *BDNF* 5' exon has a distinct expression pattern within the nervous system [28]. The same 5'-UTRs of *SCN8A* are expressed throughout the central, peripheral, and sympathetic nervous systems and during prenatal and postnatal development (Table 1). Relative expression of these exons was not quantified, but we found no evidence of spatial or temporal specificity of transcript initiation at the alternative start sites. Rather, the promoter appears to function as a single unit that is either active or inactive.

### *Alternative polyadenylation signals are conserved in human, mouse, and chicken*

Two alternative polyadenylation signals are located 2.5 and 5 kb downstream of the stop codon of mouse and human *Scn8a* (Fig. 6B) and there are corresponding signals 2.5 and 4.4 kb downstream of the stop codon in chicken *Scn8a*. Polyadenylation at these two sites generates two size classes of *Scn8a* transcripts that differ by 2.5 kb. The overall sequence identity of the 3'-UTRs from mouse and human is 74%, within the average range of  $71 \pm 12\%$  [25]. The 0.3-kb region directly upstream of each polyadenylation signal exhibits 90% identity in human and mouse. These sequences may influence the choice of polyadenylation site or affect the stability of the mRNA.

Functional CPEs must be located immediately proximal of the polyadenylation site [29]. There are two consensus CPE sequences located 50 and 150 bp upstream of the first polyadenylation site of *Scn8a*, a position similar to the CPEs that influence polyadenylation and dendritic transport of the  $\alpha$ CaMKII mRNA [30,31]. The evolutionarily conserved short and long transcripts could thus be directed to different subcellular compartments or be subject to different translational regulation. Although the Na<sub>v</sub>1.6 protein has been detected in dendrites [12], localization of the mRNA has not been reported.

### *Evolutionary conservation as a predictor of transcriptional regulatory sequences*

Several recent studies used evolutionary conservation to identify noncoding sequences that regulate transcription of associated genes [32–34]. We tested the enhancer activity of 12 highly conserved noncoding sequences: NCS1, a 1.2-kb fragment with 78% sequence identity located downstream of exons 1a–1d, and 11 other fragments between 0.1 and 0.3 kb in length with sequence identity between 75 and 84% (Supplemental Figs. 1A and 2A). None of these elements influenced expression in transfected MN-1 or NIH-3T3 cells. Since integration into chromatin structure is required for the activity of some types of regulatory elements, these conserved sequences are candidates for further analysis in stably transfected cells or transgenic mice.

### *A potential NRSE/RE-1 site near the SCN8A promoter*

One transcription factor with an established role in sodium channel regulation is NRSF/REST, which binds to the NRSE/RE-1 sequence and represses expression in nonneuronal cells by a chromatin modification mechanism [35–37]. The best characterized NRSE/RE-1 is located 1 kb upstream of the distal promoter of rat *Scn2a*. A 21-bp consensus NRSE sequence was defined by experimental analysis of 24 naturally occurring sites, testing both transcriptional activity and protein binding [38]. We searched for matches to this consensus in the 138-kb

interval between the upstream *SLC4A8* gene and the second coding exon of human *SCN8A*. The closest match, TTCAG CACCA CAGCA AGAAA C, is located 2 kb downstream of exon 1d and contains six mismatches to the consensus (Fig. 6A). The position of this potential NRSE is conserved in mouse and rat, with 17/21 and 18/21 bp sequence identity to the human element, respectively. This element is not required for restriction of *Scn8a* expression to the nervous system, since the 4.8kb promoter fragment that exhibited tissue specificity in transgenic mice did not contain the NRSE (Fig. 6A). Nonetheless, the location of this site near the active promoter and its conservation in human and rodent genes warrant further analysis.

As one test of the likelihood that this is an active NRSE, we calculated the weighted matrix score for the 24 reported REST elements [38], using the MATCH program and the TRANSFAC database (<http://www.gene-regulation.com/>). The matrix scores ranged from 0.88 to 1.0 for the 20 active elements and from 0.84 and 0.87 for the 4 inactive elements. The matrix values for the NRSE sites from human, mouse, and rat *SCN8A* were 0.83, 0.75, and 0.76, respectively, which are lower than the values for the experimentally inactive sites.

### *Diversity of Scn8a transcripts*

Two major full-length *SCN8A* transcripts of approximately 9 and 12 kb have been detected on Northern blots of brain RNA from human and mouse [5,6,22,23]. The open reading frame of *SCN8A* is only 6 kb in length. The experiments reported here demonstrate that the full-length transcripts include short 5'-UTRs (0.2 to 0.6 kb in length) and either a short 3'-UTR of 2.5 kb or a long 3'-UTR of 5 kb. We demonstrated that the variation in the 3'-UTR is responsible for the two size classes and only the 12-kb transcripts contain the long 3'-UTR. The presence of conserved CPEs near the terminus of the short 3'-UTR suggests that the two classes of transcripts could differ in subcellular localization or translational regulation.

Additional diversity of *SCN8A* transcripts is produced by alternative splicing of coding exons. There are two pairs of alternative coding exons, 5A/5N and 18A/18N [13,39]. Exon 18N has an in-frame stop codon and is transcribed only in the CNS during fetal development and at a very low level in nonneuronal tissues in the adult [13]. Two coding variants differing by 11 amino acids are produced by the alternative splice donor sites of exon 10 [20]. In this paper, we have described molecular heterogeneity of the 5'-UTR and 3'-UTR of *SCN8A*. This variation may influence translation initiation or efficiency, binding of regulatory proteins, mRNA stability, or subcellular location of different classes of *Scn8a* transcripts [40–44]. Sixteen transcripts differing in the 5'-UTR and/or 3'-UTR can be derived from the noncoding elements identified here and if the coding variants are included, 128 transcripts of *SCN8A* can be generated (Fig. 6C). The functional consequences of this newly recognized molecular heterogeneity will be a subject for future analysis.

## Materials and methods

### RNA isolation

Total RNA was extracted from tissues and cells using the TRIzol reagent (Life Technologies). Poly(A)<sup>+</sup> RNA was prepared using the PolyAtract mRNA Isolation System IV (Promega). Total RNA from human brain was purchased from Ambion, Inc. Chicken brain poly(A)<sup>+</sup> RNA was purchased from BD Biosciences Clontech. Elutes were quantified by OD<sub>260/280</sub> readings and quality was assessed on 1.2% agarose gels.

### 5'-RACE

5'-RACE was carried out using the GeneRacer Kit (Invitrogen) with 150 ng of poly(A)<sup>+</sup> RNA and the 5'-RACE System (Invitrogen) with 125–200 ng of poly(A)<sup>+</sup> RNA or 1–4 µg of total RNA. The 5'-RACE System was used with normal and GC-rich protocols. The reverse primers 1–5 and 46 (Supplemental Table 1) for reverse transcription were complementary to the first coding exon of *SCN8A* (exon 1) or, in a few experiments, to exon 1a. The reverse primers for PCR and nested PCR were primers 6–10 and 11–15 (Supplemental Table 1). PCR amplification was initiated by 2 min of denaturing at 94°C followed by 33 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C with a final extension step of 6 min at 72°C. In some experiments, discrete products were visualized by ethidium bromide staining of agarose gels, purified using the QIAquick Gel Extraction Kit (Qiagen), and sequenced at the University of Michigan Sequencing Core (<http://seqcore.brcf.med.umich.edu/>). In other cases, mixed PCR products were cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). Inserts with unique lengths were identified by restriction digestion and electrophoresis and sequenced from vector primers.

### 3'-RACE

3'-RACE was carried out on 240 ng of mouse brain poly(A)<sup>+</sup> RNA using the GeneRacer Kit (Invitrogen). RACE products were amplified using reverse primers 38–39 (PCR) and 40–41 (nested PCR) (Supplemental Table 1) and visualized on agarose gels. PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced as above.

### RT-PCR

First-strand cDNA was synthesized in 20-µl reactions containing as template 200 ng of mouse brain poly(A)<sup>+</sup> RNA, 200 ng of human brain poly(A)<sup>+</sup> RNA, or 2 µg of total RNA from MN-1 cells, with 50 units of Superscript II Reverse Transcriptase (Life Technologies) and 50 ng random hexamers or 0.5 µg oligo(dT) primer. The first-

strand cDNA (1 µl) was amplified in a 25-µl reaction volume using reverse primers 6, 8, 11, 22, or 46 and forward primers 16–21 or 37 (Supplemental Table 1) as indicated in the figure legends. Amplification was carried out for 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, and terminated with 6 min at 72°C.

### Ribonuclease protection assay

Ribonuclease protection was carried out using 30 µg of total RNA from mouse brain or liver with the RPA III kit (Ambion). The probe was transcribed from a gel-purified PCR fragment amplified from mouse BAC RP23-319B16 using primers 23 and 24 (Supplemental Table 1) and cloned into the pGEM-T Easy vector (Promega). The [ $\alpha$ -<sup>32</sup>P]CTP-labeled antisense riboprobe was generated by T7 *in vitro* transcription using the MaxiScript kit (Ambion). After gel purification of the labeled riboprobe, RNA was hybridized overnight at 42°C with 1 × 10<sup>5</sup> counts per minute (cpm) of riboprobe. Digestion with a mixture RNase A and RNase T1 was performed at 37°C for 30 min. The digested products were separated by electrophoresis on a 5% acrylamide 8 M urea denaturing gel and visualized by autoradiography after drying of the gel.

### Southern blot

The Southern blot was prepared as previously described [45]. A 411-bp probe was amplified by PCR from C57BL/6J genomic DNA using primers 25 and 26 in NCS1 (Supplemental Table 1). The probe was gel-purified and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Megaprime DNA Labeling System (Amersham).

### Northern blot

The Northern blot was prepared as previously described using 2 µg of mouse brain poly(A)<sup>+</sup> RNA [22]. A 2320-bp *Scn8a* cDNA probe (exons 2 to 13) was amplified by RT-PCR from mouse brain cDNA using primers 42 and 43 (Supplemental Table 1). A 1023-bp luciferase cDNA probe sequence was digested from the pGL3-Basic vector using restriction enzymes *Ava*I and *Nar*I. A 639-bp probe corresponding to the unique part of the long 3'-UTR was amplified by PCR from C57BL/6J genomic DNA using primers 44 and 45 (Supplemental Table 1). Probes were gel-purified and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\alpha$ -<sup>32</sup>P]dATP using the Megaprime DNA Labeling System (Amersham).

### Luciferase constructs

The “full-length” 4.8-kb construct was prepared in three steps. A 154-bp genomic “splice acceptor” extending upstream from the first ATG codon of *Scn8a* was amplified from mouse BAC clone RP23-319B16 using primers 27 and 28 (Supplemental Table 1) and cloned into the pGL3-Basic

luciferase reporter vector (Promega) directly upstream of the luciferase translation start site. A 159-bp DNA fragment containing the 3'-end of the conserved element NCS1 was amplified from the BAC clone using primers 29 and 30 (Supplemental Table 1), gel-purified, and cloned upstream of the splice acceptor fragment. A 4.6-kb *NheI* fragment containing the four untranslated exons was isolated from the mouse BAC, gel-purified, and cloned directly upstream of the other fragments. Deletion constructs were generated using the restriction enzymes shown in Fig. 3C. The identity of each construct was verified by restriction digestion and sequencing. The pGL3-Control vector (Promega) containing the SV40 promoter/early enhancer was used as a positive control in the luciferase experiments.

#### Cell culture and transfection

MN-1 is a hybrid cell line derived by fusion of mouse embryonic motor neurons from strain C57BL/6J with the neuroblastoma cell line N18NEO [46–48]. N18NEO is a subline of N18TG2 cells from mouse strain A/J [49]. MN-1 and NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies) at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells were plated at 4 × 10<sup>5</sup> cells per well in 6-well plates 24 h before transfection. The medium was changed to Dulbecco's modified Eagle's medium with 10% fetal bovine serum 1 h before transfection. Cells were transfected with 0.001 to 1 µg test plasmid and 10 ng of a plasmid encoding Renilla luciferase, pRL-SV40 (Promega), using the Fugene 6 transfection reagent (Roche) in the ratio 3 µl Fugene to 1 µg DNA. Cell lysates were collected 40–48 h posttransfection. The activities of firefly luciferase and Renilla luciferase were assayed using the Dual-Luciferase Reporter Assay System (Promega). Luminescence was detected using a Monolight 301D luminometer (PharMingen) for 10 s with a 2-s delay as per instructions. Triplicate transfections were carried out for each construct in each experiment. The reported values are means ± standard deviations of data from three or more independent experiments.

#### Transgenic mice

A 7.4-kb *ApaI/MluI* fragment containing the 4.8-kb sodium channel promoter and the luciferase cDNA was isolated from the 9.7-kb full-length luciferase construct, gel-purified, and microinjected into fertilized (C57BL/6 × SJL) F<sub>2</sub> eggs at the University of Michigan Transgenic Animal Core (<http://www.med.umich.edu/tamc/>). Founders were identified by PCR amplification of the transgene using primers 31 and 32 (Supplemental Table 1) under the following PCR conditions: 94°C for 2 min, followed by 32 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 6 min. Founders were bred to C57BL/6J mice to generate transgenic

offspring. Two independent transgenic lines, Tg17 and Tg52, were characterized. Transgene transcripts were detected by RT-PCR amplification of 4 µg of total RNA using the forward primers 16, 18, or 19 and reverse primer 22 (Supplemental Table 1). Expression of endogenous *Scn8a* and *Scnm1* was detected by RT-PCR with primers 33–34 and 35–36, respectively (Supplemental Table 1).

#### Human, mouse, and chicken DNA sequence comparison

Finished genomic sequence for the 5'-end of *SCN8A* was obtained from human chromosome 12 BAC clone RP11-285E4 (GenBank accession no. AC025097) and mouse chromosome 15 BAC clone RP23-319B16 from strain C57BL/6J (GenBank accession no. AC104833), which was sequenced at our request by the TRANS-NIH BAC sequencing program. Genomic sequence for the 3'-end of *SCN8A* was obtained from human BAC clone RP11-932I11 (GenBank accession no. AC068987) and mouse BAC clone RP23-274F23 (GenBank accession no. AC104834). The genomic sequence of chicken exon 24 was acquired from contig 229.3 of the *Gallus gallus* whole-genome shotgun sequence assembly (GenBank accession no. AADN01044394). Sequences were compared using the VISTA program [21] (<http://www-gsd.lbl.gov/vista/>), which uses a nongapped alignment algorithm with adjustable resolution. The conservation calculation was carried out with the finished sequence for the mouse BAC as the reference sequence and a sliding window of 100 bp. Human and mouse 5'-UTR and 3'-UTR sequences were compared using the BLAST2 sequences program (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>). PIPmaker [50] (<http://bio.cse.psu.edu/pipmaker/>) was used with default settings to generate the dot-matrix comparison of human and mouse NCS1 and the 3'-UTR sequence conservation report. The CpG islands were identified using the EMBOSS CpGReport program (<http://www.ebi.ac.uk/emboss/cpgplot/>). We calculated the matrix scores for the NRSE/RE-1 sequences using the MATCH program and the TRANSFAC database (<http://www.gene-regulation.com/>).

#### Enhancer activity of conserved sequence elements

Sequence comparison was carried out on mouse and human genomic sequence spanning the 138-kb interval from the closest upstream gene (*SLC4A8*) to the second coding exon of *SCN8A* using the VISTA program. Eleven evolutionarily conserved sequences meeting the criterion of =75% sequence identity for ≥100 bp were selected for evaluation of enhancer function. Each element was amplified by PCR from mouse genomic DNA, using the primer pairs E2–E12 (available from the authors upon request). Amplified fragments were cloned upstream of the SV40 promoter in the vector pGL3-Promoter (Promega). Clones were sequenced before transient transfection into MN-1 and NIH-3T3 cells.

## Acknowledgments

This work was supported by NIH Research Grants NS34509 (M.H.M.) and NS44047 (A.P.L.). V.L.D. acknowledges support from the Neuroscience Training Program of the University of Michigan (T32 MH14279) and the Scottish Rite Schizophrenia Foundation. The superior cervical ganglia sample was provided by Leslie K. Sprunger. We thank Jason C. Dugas and Ben A. Barres for critically reading the manuscript.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ygeno.2004.09.002](https://doi.org/10.1016/j.ygeno.2004.09.002).

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