THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 38, Issue of September 19, pp. 24008–24015, 1997
Printed in U.S.A.
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Alternative Splicing of the Sodium Channel *SCN8A* Predicts a Truncated Two-domain Protein in Fetal Brain and Non-neuronal Cells*

(Received for publication, April 24, 1997, and in revised form, July 1, 1997)

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The voltage-gated sodium channel α subunit SCN8A is one of the most abundant sodium channels in neurons from brain and spinal cord. We have identified two alternatively spliced exons, 18N and 18A, that encode transmembrane segments S3 and S4 in domain III. Exon 18N is expressed in fetal brain and non-neuronal tissues. Transcripts with exon 18N have a conserved in-frame stop codon that predicts the synthesis of a truncated, two-domain protein similar to the fetal form of the muscle calcium channel. The proportion of transcripts containing exon 18N is highest in mouse fetal brain between E12.5 and P1.5; at later ages transcripts containing exon 18A predominate. This developmental program is recapitulated in P19 cells during retinoic acid-induced neuronal differentiation. Non-neuronal tissues contain a low level of *SCN8A* transcripts containing exon 18N. *SCN8A* thus provides a new model of differentiation specific splicing. Genomic analysis of *SCN8A* from human, mouse, and fish demonstrated a conserved structure in which exon 18N is located 300–500 bp upstream of exon 18A. Duplication of exon 18 thus preceded the divergence of fish and mammals. The genomic organization, developmental regulation, and coding content of exons 18N and 18A closely resemble the previously described alternate exons 5N and 5A of the neuronal sodium channel genes. Our proposal that the evolutionary origin of exons 18N and 18A was by duplication of exons 5N and 5A is consistent with other evidence that the four-domain cation channels arose by two rounds of duplication from a single-domain ancestral channel.

Voltage-gated sodium channels are responsible for the rapid membrane depolarization associated with action potentials in nerves and muscles. Ten members of the sodium channel α subunit gene family have been mapped in the human and mouse genomes (1, 2), and several more related cDNAs have been identified. The coding sequences of these family members are unusually highly conserved, but the genes exhibit different patterns of expression in neurons, glia, skeletal muscle, and cardiac muscle. The neuronal α subunit proteins also differ with regard to their site of membrane localization in cell bodies or processes. The 260-kDa α subunit protein contains four transmembrane domains (I–IV), each with six membrane spanning segments (S1 to S6). The transmembrane domains are linked by cytoplasmic loops and form the walls of a sodium-selective pore that is lined by four pore-forming segments. Accessory β subunits modulate channel characteristics but are not required for channel function in transfected cells (3).

The *SCN8A* gene is expressed in neurons of brain and spinal cord (1, 4) and is most closely related to the neuronal channels *SCN1A* (type 1), *SCN2A* (type 2), and *SCN3A* (type 3), with approximately 79% amino acid sequence identity. Mutations in the mouse gene, *Scn8a*, are responsible for neurological dysfunction in the mutant "motor endplate disease" (*med*) (1). Complete loss of *Scn8a* results in progressive paralysis of the hind limbs, muscle atrophy, and juvenile lethality, due to failure of evoked neurotransmitter release at the neuromuscular junction (5). One missense mutation within the S4/S5 cytoplasmic loop of domain III and two splicing defects have been identified in spontaneous mutant alleles at this locus (6, 7).

Mammalian sodium channel α subunit genes are known to undergo two types of alternative splicing. The alternative exons 5N and 5A encode part of segment S3 and all of S4 of domain I in the neuronal α subunits *SCN2A* (8, 9) *SCN3A* (9), *SCN8A*[1] and Schwann cell channel Na_s (10). There are either one or two differences in the 30 encoded amino acids, and in all of these genes residue 7 is Asp in 5A and Asn or Ser in 5N. The functional consequence of this substitution is not known. Transcripts containing exon 5N predominate in fetal and neonatal brain, whereas in adult brain the predominant transcript contains exon 5A. Alternate splicing of sodium channels also occurs in the cytoplasmic loop between the first two transmembrane domains. Variation in length of this loop has been observed in cDNAs from *SCN1A, SCN3A* (11), and *SCN8A* (1).[2]

The complete intron/exon structure of the *SCN8A* gene was recently determined in our laboratory.[1] The borders of most of the 28 exons correspond to those described for the cardiac channel gene *SCN5A* (12) and the muscle channel gene *SCN4A* (13, 14). To facilitate comparison, we have numbered the exons of *SCN8A* in accordance with those of *SCN4A*. We have found that *SCN8A* contains two alternative copies of exon 18 that differ in their tissue-specific and developmental patterns of expression.

MATERIALS AND METHODS

RT-PCR[3]—Total RNA was prepared from dissected tissues using the Trizol reagent (Life Technologies, Inc.). First strand cDNA synthesis reactions (20 μl total volume) contained template RNA (10 μg of brain RNA or 5 μg of RNA from P19 cells and non-neuronal tissue), 200 units

* This work was supported in part by National Institutes of Health Research Grant NS34509 (to M. H. M.) and the NeuroScience Network of the Canadian National Centers of Excellence (to M. W. McB.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by the President's Fellowship in Organogenesis, University of Michigan, and National Institutes of Health Training Program in Genetics Grant T32 GM 07544.

[1] J. Galt *et al.*, manuscript in preparation.

[2] N. W. Plummer and M. H. Meisler, unpublished observations.

[3] The abbreviations used are: RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); RA, retinoic acid.

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TABLE I
Sequence of primers used for PCR
The first species listed is the source of the sequence; the others are also amplified. m, mouse; h, human; f, fish.

Primer	Sequence	Species	Location	Reference
A	^{3004}AAATGGACAGCCTATGGCTTC	m, h	F exon 17	Burgess *et al.* (1)
B	^{3367}TCACCTCGTCGATTTCGAACCG	m	R exon 19	Burgess *et al.* (1)
C	TGTTGACATCTTCAATTTCAAATCGG	h	R exon 19	Footnote 1
D	AGGTTCCATTAAGTTTGTCTGGC	m, h, f	F exon 18N	Genbank U97672
E	^{3141}AAGGGACTTTATGGCACCTAGTTCC	m, h	R exon 18A	Burgess *et al.* (1)
F	TTTAATGGGCCCTAGATCGGAGTAGC	f	R exon 18A	Genbank D37977
G	TTGTTAGAGGGGCAAGGGTAAG	f	F intron 18N/18A	Genbank U97673
H	CAATGTTTCTCCATGTCGCTCTTGGC	f	R intron 18N/18A	Genbank U97673
I	AAATCCCATGTTGGCAGAGCTGAG	m, h	F intron 17/18N	Genbank U97672
J	TCCCAGAAGCTCCCATTAAATTAAG	m, h	R exon 18N	Genbank U97672
K	AAAGGGCGAGGGTAAGGCTCTTG	m, h	F exon 18N	Genbank U97672

of Superscript II reverse transcriptase (Life Technologies, Inc.), and a mixture of random hexamer and poly(dT) primers. The first strand cDNA product (1 μl) was used as template in 50-μl PCR reactions with final concentrations of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatin, 3 mM $MgCl_2$, 400 μM dNTP, 0.3 μM primer A, and 0.3 μM primer B. A single round of amplification (36 cycles) was initiated by denaturation for 2 min at 95 °C followed by 30 s each at 65, 72, and 95 °C. Primer sequences are given in Table I.

cDNA Libraries—*SCN8A* cDNA fragments were isolated from the human adult brain library BR1 (15) by PCR amplification using primers A plus C. The mouse neonatal brain library (Stratagene 936309) and human fetal brain library (Stratagene) were amplified with primer B, and the vector primer M13 F. PCR conditions were as described above.

Genomic Sequence—The exons of *SCN8A* have been numbered to correspond with those of *SCN4A* (13, 14). Exons 17, 18, and 19 of *SCN8A* correspond to nucleotides 2902–3075, 3076–3198, and 3199–3483 of the published mouse brain cDNA sequence (Ref. 1 and GenBank U26707). Genomic sequence of exons 18A and 18N and the intervening intron was obtained from human, mouse, and fish as described below and deposited in GenBank with accession numbers U97671, U97672, and U97673, respectively.

P1 clone 2207 containing a portion of the mouse *Scn8a* gene (1) was amplified using primers A plus E. The 6-kilobase pair PCR product was cloned in the vector pGEM-5Z (Promega) that had been modified as described (16) and propagated in *Escherichia coli* strain DH5. The insert was sequenced using primers E, I, J, and K. The overlapping P1 clone 2206 (1) was digested with *Eco*RI, and a 500-bp fragment containing exons 5N and 5A was subcloned and sequenced using vector primers.

P1 clones containing human *SCN8A*[1] were isolated by Genome Systems (St. Louis) from a human library (17). P1 DNA was digested with *Eco*RI and subcloned in the vector pGEM-11Z (Promega). Ampicillin-resistant colonies were arrayed in microtiter dishes, replicated onto nylon filters (Hybond N, Amersham Corp.), and hybridized with a 1.3-kilobase pair RT-PCR product containing nucleotides 3123–4447 of the mouse *Scn8a* cDNA (1). Subclones containing exon 18A were identified by hybridization with the oligonucleotide (5′GGTGCCATAAAGTCCCTTAGGACC) and amplified with primers E plus I. The 587-bp PCR product was sequenced with primers D, E, and I to obtain the sequence of exon 18N and the adjacent downstream intron. The same subclone was amplified with primer J plus the vector primer M13 F to obtain the sequence of the intron upstream of exon 18N.

Genomic DNA from the pufferfish *Fugu rubripes*[4] was amplified with primers D plus F. The 750-bp PCR product was sequenced with primer D to provide the 3′ end of exon 18N and the adjacent downstream intron. The remaining portion of exon 18N and the upstream adjacent intron was obtained by inverse PCR. Genomic DNA (1 μg) in a total volume of 200 μl was digested with *Nsi*I, heat treated at 65 °C to inactivate the enzyme, and ligated with T4 DNA ligase (Life Technologies, Inc.). Circularized DNA fragments were ethanol precipitated and resuspended in 10 μl of distilled water. Aliquots (2 μl) were amplified with primers G and H by denaturation for 2 min at 95 °C followed by 35 cycles at 95 °C (30 s), 63 °C (30 s), and 72 °C (3 min). The 1-kilobase pair inverse PCR product was sequenced with primer H.

DNA Sequencing—PCR products were excised from ethidium bromide-stained gels and purified with QIAEX beads (Qiagen) according to the manufacturer's instructions. Purified DNA was either sequenced manually by ^{33}P-labeled dideoxynucleotide incorporation (Thermo Sequenase kit, Amersham Corp.) and polyacrylamide gel electrophoresis or sequenced by incorporation of fluorescently labeled dideoxynucleotides with analysis on an ABI373A automated sequencer in the University of Michigan Core Facility (R. Lyons, Director). Sequence alignments were performed with MacVector version 4.5 software (Eastman Kodak).

P19 Cell Culture—P19 cells were cultured essentially as described (18). Briefly, to initiate differentiation, cells were plated into bacterial grade Petri dishes at 10^5 cells/ml in medium containing either 1% dimethyl sulfoxide or 3×10^{-7} M retinoic acid. After 2 and 4 days the medium was changed on the aggregates of cells and the cultures expanded. On day 5 the cell aggregates were plated onto tissue culture grade plastic and medium was replenished every 2 days. Cells were harvested at 2-day intervals during the course of the differentiation regime, and RNA was isolated in Trizol reagent as described above.

RESULTS

Three Scn8a Transcripts in Mouse Brain—Alternative splicing of exon 18 was first indicated by analysis of cDNA fragments amplified from brain cDNA libraries. Using a forward primer from the vector and a reverse primer from exon 19, products that were spliced directly from exon 17 to exon 19 were obtained from a mouse neonatal brain library and a human fetal brain library. To extend the analysis to primary cDNA, total RNA from mouse brain was amplified with a forward primer from exon 17 and a reverse primer from exon 19 (Fig. 1, *top*). Three products of 364, 309, and 241 bp were obtained (Fig. 2). Sequencing of the gel-purified cDNA fragments demonstrated identity in the regions transcribed from exons 17 and 19 but differences in the internal sequences (Fig. 1). The sequence of the 364-bp product contains exons 17, 18A, and 19 and corresponds to the full-length *Scn8a* cDNA transcript from adult mouse and rat brain (1, 4). The 241-bp fragment is spliced directly from exon 17 to exon 19, and is designated Δ18. This transcript maintains an open reading frame but lacks sequences encoding the S3 and S4 segments of domain III. In the 309-bp product, exons 17 and 19 are separated by sequence apparently derived from an alternate exon, designated 18N. This transcript contains an in-frame stop codon that would generate a truncated protein of 1034 amino acids, 60% of the full-length channel protein (1). The predicted structure of this novel, two-domain form of *Scn8a* is indicated in Fig. 3.

Exon 18N Is Transcribed in Human Brain cDNA—A human adult brain cDNA library (15) was amplified with primers A plus C from exons 17 and 19. Three products corresponding in size to those in neonatal mouse brain were obtained. Comparison of the sequences of the human and mouse cDNA fragments (Fig. 1) revealed 90% nucleotide sequence identity (61/68 bp) within exon 18N. The position of the in-frame stop codon is conserved in human and mouse, indicating that it was present prior to the divergence of the two species.

Exon 18A from human and mouse *Scn8a* is 123 bp in length.

[4] UK MRC Human Genome Mapping Project Resource Center, http://www.hgmp.mrc.ac.uk.

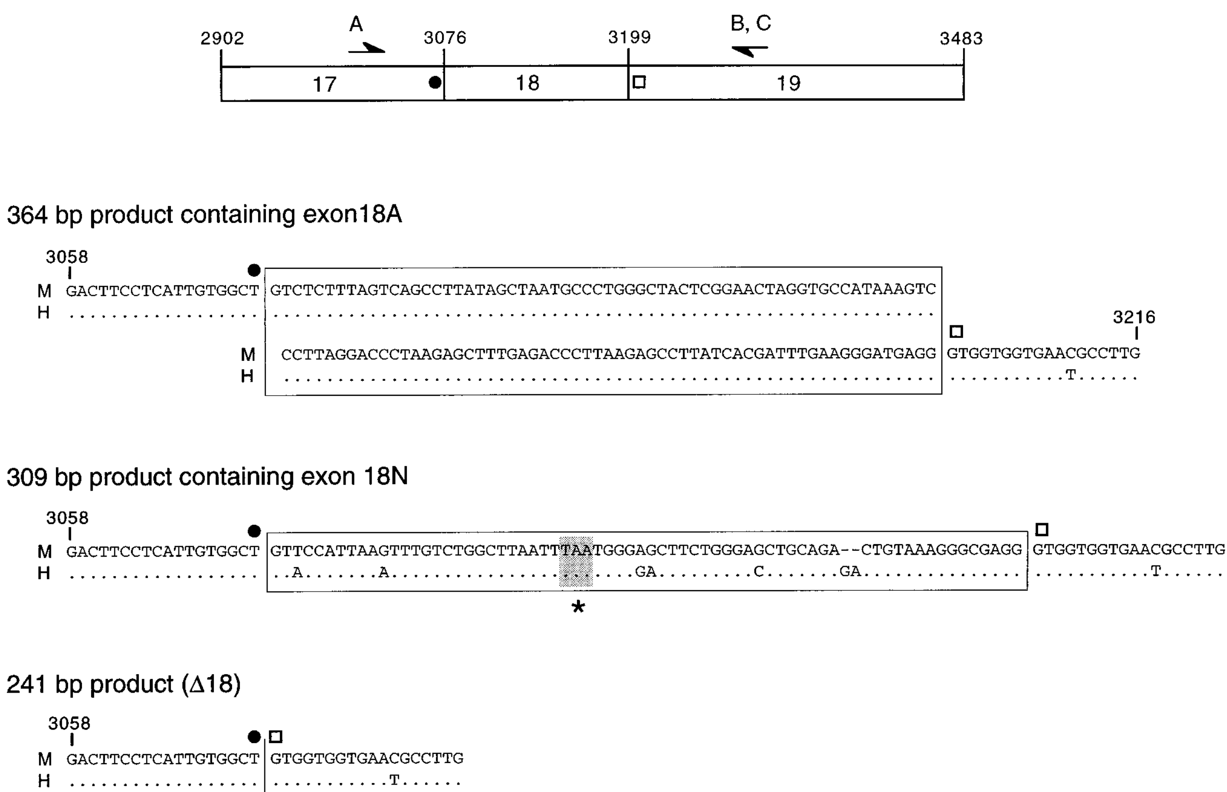

FIG. 1. **Three *SCN8A* transcripts in mouse and human brain.** cDNA fragments spanning nucleotides 3004–3367 from exons 17–19 were amplified by RT-PCR from mouse brain RNA using primers A and B. Gel electrophoresis of the products is shown in Fig. 2. Partial sequences of the three products is presented here, beginning with nucleotide 3058. The corresponding human sequences (see text) are shown with *dots* representing identity to the mouse sequence. *M*, mouse; *H*, human; ●, 3′ end of exon 17; □, 5′ end of exon 19.

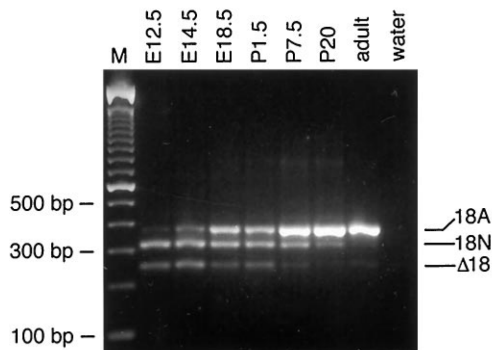

FIG. 2. **Alternative splicing of *Scn8a* in developing mouse brain.** First-strand cDNA from total brain RNA was amplified with primers A and B (Fig. 1). RNA from E12.5 and E14.5 was prepared from dissected head tissue, the other samples are from brain. PCR products (10 μl) were electrophoresed through 2% agarose and visualized by ethidium bromide fluorescence. Sequences of the three products are given in Fig. 1. *M*, 100-bp ladder (Life Technologies, Inc.).

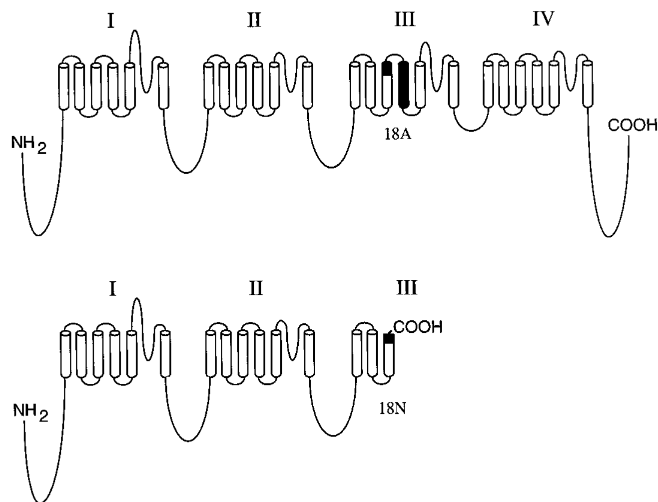

FIG. 3. **Proteins predicted by *SCN8A* transcripts containing exon 18A or 18N.** The indicated structure is based on the model of Catterall (47). The four transmembrane domains are designated I to IV. The filled segments represent the portions of domain III encoded by exons 18A (*top*) and 18N (*bottom*).

Human exon 18N contains 70 bp, and mouse exon 18N contains 68 bp. Thus, in addition to the in-frame stop codon, transcripts containing exon 18N have an altered reading frame downstream of 18N.

Developmental Pattern of Alternative Splicing of Exon 18N and 18A—The pattern of *Scn8a* splicing changes during development (Fig. 2). At embryonic day 12.5 (E12.5), the predominant transcripts are the 18N and Δ18 forms. On E14.5, all three transcripts are present at comparable levels. At E18.5 and later, the 18A form predominates. Expression of Δ18 and 18N decreases after birth, while there is a substantial increase in expression of the 18A form. The significant increase in total *Scn8a* expression after birth that is seen in Fig. 2 can also be detected using RNase protection assays, Northern blotting, and *in situ* hybridization.[2,5]

Evolutionary Conservation of Genomic Organization of Exons 18A and 18N—To determine the organization of exons 18N and 18A, the intervening intron was amplified by PCR of genomic DNA from mouse, human, and the fish *Fugu rubripes*. Using a forward primer from exon 18N and a reverse primer from exon 18A, PCR products ≤700 bp in length were obtained

[5] K. Schaller and J. Caldwell, personal communication.

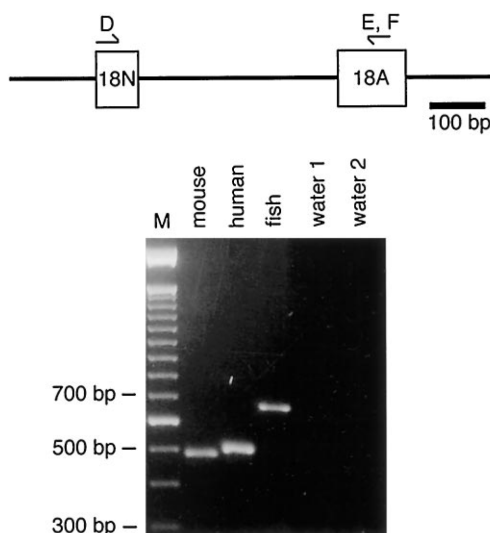

FIG. 4. **Genomic organization of exons 18N and 18A in three vertebrates.** Genomic DNA was amplified with primers D plus E (human and mouse) or D plus F (fish). After denaturation for 2 min at 95 °C, PCR was carried out for 32 cycles at 95 °C (30 s), 62 °C (30 s), and 72 °C (60 s). Products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorescence. Controls lacking template contained primers D plus E (water 1) or D plus F (water 2). *M*, 100-bp size marker ladder (Life Technologies, Inc.).

from the three genomic DNA samples (Fig. 4). This result demonstrates that exon 18N is located upstream of exon 18A and that the two exons are separated by a short intron. The complete intron was isolated and sequenced using P1 clones as templates for amplification of the human and mouse genes and inverse PCR of genomic DNA to isolate the fish gene. The sequence of the intron as well as exon 18N and flanking sequence from three species are aligned in Fig. 5.

Compared with human and mouse, exon 18N from the fish contains a one-nucleotide deletion and an in-frame stop codon located 5 bp upstream of the stop codon in the mammalian genes (Fig. 5). Thus exon 18N of the fish gene also predicts a truncated protein with the secondary structure represented in Fig. 3. The amino acid sequence encoded by fish exon 18N is identical to human and mouse at 5 of the 7 amino acid residues (Fig. 8*A*; discussed below). The nucleotide sequence similarity in exon 18N decreases downstream of the stop codon, as expected for noncoding sequence.

The length of the intron between exon 18N and exon 18A is 375 bp in human, 353 bp in mouse, and 501 bp in fish. There is an unusually high sequence identity of 70% between the human and mouse introns (counting insertions and deletions as single changes). Three-way sequence comparison identified one highly conserved segment of 24 bp designated conserved element 1 (CE1) adjacent to a eukaryotic branchpoint consensus site (Fig. 5). This potential splice regulatory element is identical in human and mouse and differs by only three nucleotide substitutions in the fish gene. CE1 is located immediately upstream of the splice acceptor site for exon 18A in the fish gene and is separated from the acceptor site in the human and mouse genes by an expanded poly(T) tract (Fig. 5).

Exon 18N Is Widely Expressed in Non-neuronal Tissues—All tissues examined contain sufficient *Scn8a* transcripts for detection by RT-PCR. A distinctive pattern of splicing was observed in neuronal and non-neuronal tissues. In brain and spinal cord, the major transcript contains exon 18A, as expected for tissues in which voltage-gated sodium channels are functional. In marked contrast, the Δ18 and 18N forms predominate in all of the non-neuronal tissues tested (Fig. 6). The 309-bp product from sciatic nerve RNA, which is largely derived from Schwann cells, was sequenced to confirm that it contains exon 18N. The low level of exon 18A containing transcript in the sciatic nerve sample could be derived from Schwann cells or from RNA in the axons of motor neurons, which are known to express SCN8A. The data demonstrate tissue-specific regulation of the alternative splicing of exons 18N and 18A.

Developmental Switch of Exons 18N and 18A in P19 Cells—Treatment of the mouse embryonic carcinoma cell line P19 with retinoic acid induces differentiation into a mixed population of cells that include neurons, glia, and fibroblast-like cells (19, 20). P19-derived neurons become electrically excitable (21), form functional synapses, and express many neuron-specific genes (22). Treatment with dimethyl sulfoxide (Me_2SO) induces an alternate pathway of differentiation and generates cells with features of cardiac muscle, skeletal muscle, and epithelium (20, 23). We examined the expression of *Scn8a* during treatment with retinoic acid and Me_2SO. Uninduced P19 cells, and cells treated with Me_2SO, express *Scn8a* transcripts of the 18N and Δ18 types that are characteristic of non-neuronal tissues (Fig. 7). After treatment for 8 days with retinoic acid (RA), transcripts containing exon 18A are detected. The faint band that migrates between 18A and 18N is a heteroduplex that can be regenerated from gel-purified fragments 18A and Δ18 by mixing and reannealing (data not shown).

The time course of induction of exon 18A was reproducible. Post-mitotic neurons appear in these cultures relatively synchronously at day 5, and their membranes become electrically active a few days later (24). P19 cells thus recapitulate the tissue-specific splicing observed *in vivo* and provide an experimental system for biochemical analysis of the alternative splicing of *Scn8a*.

DISCUSSION

Potential Function of the Truncated Two-domain Sodium Channel Encoded by Exon 18N—The observed evolutionary conservation in human, mouse, and fish strongly suggests that alternative splicing of exons 18N and 18A has a functional role that has been maintained by selection. The precise developmental and tissue-specific regulation of the alternative splicing of 18N and 18A, and its recapitulation during the induction of neuronal differentiation in retinoic acid-treated P19 cells, are also suggestive of function. In the absence of selection, the accumulation of mutations in sequences required for regulated splicing would be expected to eliminate the expression of 18N. In fact, transcripts containing exon 18N were detected in both human and mouse tissues.

Two mechanisms for selective pressure to express exon 18N may be considered as follows: the truncated protein itself may have some function in non-neuronal tissues, or the splicing of 18N may be a "fail-safe" mechanism that produces a nonfunctional truncated protein to prevent the synthesis of full-length, active channel protein. Two observations indicate that the truncated protein is unlikely to function as a sodium channel. An expression construct producing a protein containing domains I and II of the channel *Scn2a* did not produce sodium currents when tested in the *Xenopus* oocyte system (25). In addition, undifferentiated P19 cells that express exon 18N do not exhibit sodium currents (26–28). Alternative functions for the truncated protein might be derived from its binding sites for β subunits (3) or other membrane-associated proteins such as ankyrin and neurofascin (29, 30). Expression of the truncated SCN8A protein in oocytes or mammalian cells could be used in the future to test for these activities.

The fail-safe hypothesis assumes that the promoter of *SCN8A* is not completely neuron-specific, resulting in a low

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                                                          H ttagttaaaagtgagagatagaaggatgtatttaaaagatagagatagaactcacttaagcga
                                                          M ag...gatggt..gnctt.ca.g.tggaag..atc.tctg.c..gg...gaggga.t.tg.g

H gttctcatggagccacagttcttcttgggaactgcaagtcagccatggta---cttattgaacgttttcattgttgttaatgtcgtgacgcatcagattctcaaacaaatg
M .ccagagccatgt..aggatgc.......gaga.-....--..-....ggtg.....c.ct.cg..tc..t-........a....t...t.a.......----..

H gtttttgtcaaccaggagtaaaagagcttgtccctcttggaaagccaatggtgaatgagctgaggagggggcagct----tgtgtggagtatgtgtatttatctgtattc
M ....c............-...g............g.c.....t..c...t..gca.............-.....gaggg......a.g..................
F                                                                                    acatact.g.aca.g....ct.......

            18N
H ttttccatag|GTACCATTAAATTTGTCTGGCTTAATTTAA-TGGGGACTTCTG------------------GGACCTGCAGAGACTGTAAAGGGCGAGG|gtaaggctc
M ..........|..T......G................-....AG.....------------------...G......--..............|.......a.
F ...ct.t...|..T.....-G.......T...GA...TG....ATT..A..CCTCAGCCAAGAGCGACAT...GAAA..TT--G.TAG.G....A...|.........
                                     *    *

H ttgtcgtctgttcaggctcctctccttccagtggaacttcctcactctctccctctcctgtcagcctctccccttttccaacttgtgtgtatgttctaccgccttctcgat
M .....c..c..................ac..g..c..t..ctc.c.cat.c.ct.a.c.cct.c..t.t.-----...c..c....g.....g.......c.ttc
F .caa-.......gg.tt.tt.taatcag.

H tttct-----cttccctgttgtttttcttctgcc--tctcaaccacctttccttccaggtcgtttgagtcaagttgaaataggcagctggtgttacccacttcctctac-
M c.c..tctct......t.gtgg.......c...tc....................................g.....t.aa.ta.gc.g..gg.ggta.ccac

                                                      -------  CE1
H --------ctggggggcccaat---ctgtgtctgcagtgctaggggctttattctaacactctggaacctctgtttctttcttttttttttttttttttttttttttt
M ttccgctg.......gt.ccccgc....taaat...........g.................................t.c....c....c..............--
F                                   c....ctagtga.tatt..cac.......g.t.........t..g----------------------------------

                                                     18A
H ttttttttttggtnaccttttttgttttttgttttctcttctgtttctgtgtag|GTCTCTTTAGTCAG
M ----------------------------------...........t.......|..............
F ----------------------------------..tc.......t.......|..G...A.......
```

FIG. 5. **Conservation of exon 18N and adjacent introns in human, mouse, and fish.** Complete sequences have been deposited at GenBank with accession numbers U97671 (human), U97672 (mouse), and U97673 (fish). The small portion of the fish intron with sequence similarity to the mammalian genes is shown. The conserved element CE1 is labeled; the 7 bp within CE1 that match the eukaryotic branchpoint consensus are indicated with the *dotted line*. *, stop codon.

M brain spinal cord sciatic nerve liver kidney lung stomach spleen thymus testis water

500 bp — 300 bp — 100 bp — 18A 18N Δ18

FIG. 6. **Alternative splicing of exon 18 in neuronal and non-neuronal tissues.** First strand cDNA from adult mouse tissues was amplified with primers A and B. Aliquots of the PCR reactions (5 μl for brain and spinal cord, 15 μl for other tissues) were electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorescence. *M*, 100-bp size marker ladder (Life Technologies, Inc.).

level of leaky transcription in other tissues. If the *Scn8a* channel has a significant open probability at the resting potential of non-neuronal cells, then expression of small numbers of channel molecules could have deleterious effects on sodium homeostasis or membrane potential. The production of active channels would be significantly reduced in non-neuronal cells if incorporation of exon 18A requires a neuronal specific splicing factor, and exon 18N is substituted in the absence of the tissue-specific factor.

Further work will be required to test these hypotheses and to characterize the role of the exon 18N product. Specific anti-SCN8A antibodies would be useful to evaluate the stability and subcellular distribution of the truncated protein. However, because of the high degree of amino acid sequence conservation among the sodium channel α subunits, it is difficult to generate specific antisera, and we and others have been unsuccessful in generating specific anti-SCN8A sera. Overexpression of the truncated protein in transfected cells, or deletion of exon 18N by targeted mutation in the mouse, could be used in the future to evaluate the function of this novel gene product.

The expression pattern for the Δ18 transcript lacking exon 18 is similar to that of 18N, although its abundance in brain is lower. Inefficient splicing of exon N could be responsible for generation of Δ18. Although the reading frame downstream of exon 18 is preserved in the Δ18 transcript, the encoded protein would lack the entire transmembrane segment 4 with its voltage sensor function. In addition, the downstream transmembrane segments are likely to be inserted into the cell membrane in reverse orientation. The product of the Δ18 transcript is therefore not likely to be a functional channel. The non-channel or fail-safe functions discussed above are both applicable to the Δ18 protein.

Any proposed role for 18N or Δ18 must take into account the phenotype of mice with null mutations of *Scn8a*. These mice appear to develop normally and do not exhibit abnormal brain histology. They do not demonstrate obvious dysfunction prior to P10, the time of initiation of progressive paralysis and muscle atrophy (1, 6). The death of homozygous null mice at 3 weeks of age is presumably due to lack of functional SCN8A channels containing exon 18A. However, it is possible that biochemical abnormalities associated with absence of the truncated protein during fetal development have been missed.

A Two-domain, Truncated Product of a Voltage-gated Cal-

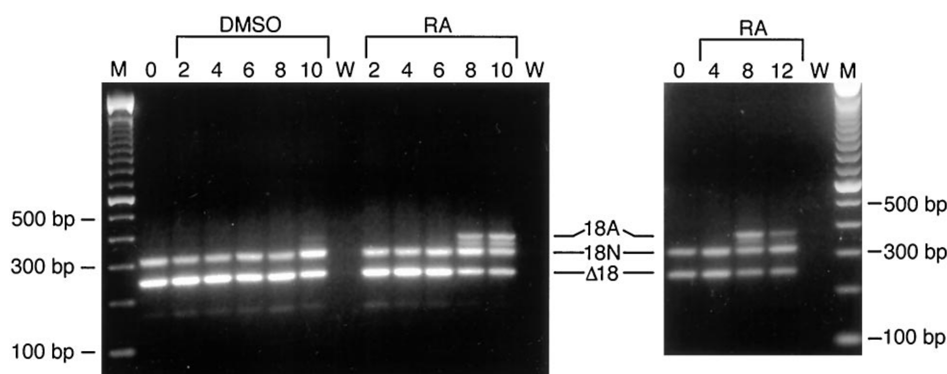

FIG. 7. **Regulated splicing of exon 18 in differentiating P19 cells.** First strand cDNA from P19 cells treated with Me_2SO (*DMSO*) or retinoic acid (*RA*) was analyzed by RT-PCR. Two independent RA experiments are shown. PCR products (10 μl) were electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorescence. The length of treatment with Me_2SO or RA is indicated in days. *M*, 100-bp ladder (Life Technologies, Inc.); *0*, untreated cells; *W*, water control.

cium Channel Gene—A truncated, two-domain isoform of the muscle voltage-gated calcium channel gene that is expressed during fetal development has been identified (31). The two-domain calcium channel isoform is predominant in newborn muscle, and the standard four-domain protein is predominant in adult muscle. Splicing from S2 of domain II into S2 of domain IV results in a two-domain protein with a normal domain I and a chimeric second domain. Western blot analysis demonstrated that the two-domain protein is translated and localized to the membrane (31). Although the two-domain form of SCN8A is produced by different splicing mechanism, the similarity in structure and developmental regulation of the predicted proteins suggests that they may serve a similar function.

Mutations of the neuronal calcium channel α subunit *CACNL1A4* are associated with inherited episodic ataxia, EA2 (32). Two of the mutations detected in human patients predict the synthesis of a two-domain protein containing domain I, domain II, and a small portion of domain III, very much like the predicted product of *SCN8A* exon 18N. The dominant inheritance of EA2 in these patients suggests that the truncated protein can interfere with normal channel activity.

Alternate Exons with In-frame Stop Codons—There are precedents for generation of two transcripts with different termination codons from a single gene. An alternative exon 3 in the fibroblast growth factor receptor 1 gene introduces an in-frame stop codon upstream of the transmembrane domain, generating a secreted protein (33, 34). An alternative exon in the epidermal growth factor receptor also generates a secreted isoform lacking the transmembrane domain (35). In this case, the truncating exon functions as a final exon and provides both in-frame stop codon and polyadenylation signal. In contrast, exon 18N of *SCN8A* is followed by a functional splice donor site that is spliced normally to exon 19. Transcripts of the mouse interleukin 4 receptor containing an in-frame stop codon have also been observed (36), and a stop codon is introduced by exon skipping in the serine/threonine kinases *CLK2* and *CLK3* (37).

Evolutionary Origin of Exons 18A and 18N—The internal homology between the transmembrane domains of the four-domain sodium and calcium channels and their similarity to the single-domain potassium channel suggested that the sodium and calcium channels evolved by two sequential gene duplications (38, 39). The greater degree of sequence identity between domain pairs I–III and II–IV in the four-domain channels indicates that the two-domain intermediate persisted for an interval sufficiently long to permit divergence of domains I and II. Since the four-domain calcium and sodium channels are found in both arthropods and vertebrates, the duplication from two-domain to four-domain channel must have occurred more than 500 million years ago.

The sequence similarity within the short coding region of exon 18N and the first portion of exon 18A (Fig. 8*A*) is consistent with their origin by exon duplication. The amino acid sequences encoded by exons 5N and 5A in domain I are identical at 28/30 residues, indicating that they are also the product of an exon duplication event (8–10). For both exon pairs, 18N/18A and 5N/5A, the intervening intron is relatively small; the upstream exon is expressed earlier in development than the downstream exon, and the encoded amino acids comprise transmembrane segments S3 and S4 of their respective domains I and III. These similarities are consistent with a common origin for the two exon pairs. The data can be accounted for by a model in which the duplication event that generated exons 5N and 5A occurred in the two-domain ancestral gene, and the subsequent duplication of the two-domain intermediate to the modern four-domain channel also produced exons 18N and 18A from exons 5N and 5A (Fig. 9).

Additional support for the origin of exons 5N and 5A early in the evolution of this gene family is provided by the presence of a corresponding exon pair in the *Drosophila* voltage-gated calcium channel α subunit gene (40). The alternative exons IS4a and IS4b encode the transmembrane segments S3 and S4 of domain I. The predicted amino acid sequence of the *Drosophila* exons contains a region with sequence identity of 11/17 residues with mammalian *SCN8A* exons 5N and 5A (Fig. 8*B*). The data are consistent with duplication of exon 5 at the two-domain stage prior to the divergence of sodium and calcium channels (Fig. 9).

Exons 5N and 5A were first recognized from the divergent sequences present in cDNA clones for *SCN1A* and *SCN2A*. Similarly, we identified the alternative splicing of exon 18 by analysis of cDNA sequences. Divergence in the sequences encoding exon 18 have not been reported to date for cDNAs from the neuronal channels most closely related to *SCN8A*, namely *SCN1A, SCN2A,* and *SCN3A*, nor for the more distantly related muscle and cardiac genes, *SCN4A* and *SCN5A*. In view of the apparent ancient origin of both pairs of exons, it would be of interest to look for duplication of these exons in genomic DNA clones for other members of the voltage-gated channel gene family.

The Conserved Intron Element CE1—The mechanism regulating the alternative splicing of 18N and 18A is not known, but it is likely to involve splicing factors with affinity for sequence elements in the adjacent introns. For example, a protein complex has been shown to mediate inclusion of a neuron-specific exon of c-*src* by binding to a 33-bp conserved intron sequence,

A

```
18A  human  V S L V S L I A N A L
     mouse  . . . . . . . . . . .
     fish   . . I . . . V . . . .
18N  human  . P . - N . S G L I *
     mouse  . P . - - . S G L I *
     fish   . P . . C . S *
```

B

```
5N    ---YITEFVNLGNVSALRTFRVLRALKTISVIP
5A    ---.V....D.....................
IS4a  AMTIFAEANIDVDLRM..S.....P..LV.R..
IS4b  FMTQYPQIGPEVDLRT..AI....P..LV.G..
```

FIG. 8. **Amino acid sequences encoded by duplicated exons.** *A,* exons 18A and 18N from human, mouse, and fish. The first 11 residues, of the total of 41, are shown. *B,* comparison of exons 5N and 5A from mouse *Scn8a* with exons IS4a and IS4b from the *Drosophila* voltage-gated calcium channel *Dmca1A*. The *Dmca1A* sequence is from Peixoto *et al.* (40). *Dots* represent amino acid identity. Mouse exons 5N and 5A were sequenced as described under "Materials and Methods."

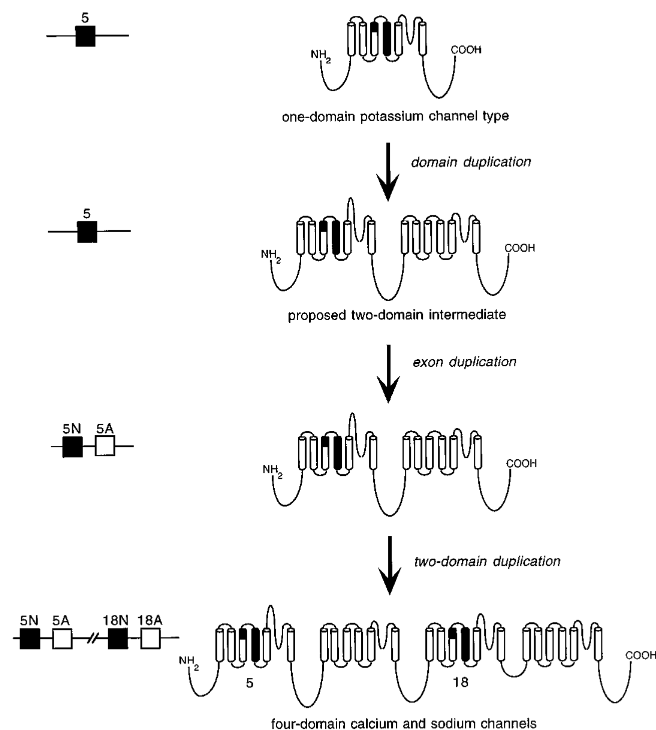

FIG. 9. **Proposed model for the evolutionary origin of exon 18N.** The evidence in support of the two-domain intermediate in the evolution of the four-domain voltage-gated ion channels has been reviewed (38, 39).

the downstream control sequence (41). Like *Scn8a*, expression of the neuron-specific c-Src protein is induced by retinoic acid in P19 cells (42). Although *SCN8A* does not contain a site related to the downstream control sequence of c-*src*, the 24-bp element CE1 is conserved in *SCN8A* from human, mouse, and fish (Fig. 5). The first 7 bp of CE1 match the eukaryotic consensus branchpoint (43), and the location of CE1 indicates that this is the functional branchpoint. The sequence of CE1 is not related to the recently defined binding sites for the polypyrimidine tract binding protein (44) or the neuron-specific RNA binding protein Nova-1 (45).

Sequences adjacent to the branchpoint are thought to be favorable sites for regulation of splicing. The *Drosophila* sodium channel gene *para* contains seven alternatively spliced exons, and 14 evolutionarily conserved intron elements are located adjacent to these exons (46). Two of the conserved intron elements of *para* contain a consensus branchpoint. Although a BLAST search of the public sequence data bases did not identify sequences related to CE1, its evolutionary conservation and intriguing location make it a worthwhile candidate for functional analysis.

Alternative Splicing of Scn8a in P19 Cells—In the course of normal brain development, expression of exon 18A replaces that of 18N during the neonatal period. This pattern appears to be recapitulated in P19 cells, a model of neuronal differentiation (22). Untreated P19 cells contain Δ18 and 18N transcripts. A variety of neuron-specific genes are expressed 8–10 days after initiation of retinoic acid of P19 cells, including neuron-specific enolase *N*-methyl D-aspartate (NMDA) receptors, and the glutamic acid decarboxylases GAD1 and GAD2 that catalyze neurotransmitter synthesis. To this list we can now add the mature form of *SCN8A*. The availability of a cultured cell line in which alternative splicing of *SCN8A* is regulated will facilitate identification of the factors involved in this developmental switch.

Acknowledgments—We thank our laboratory colleagues for generous contributions of reagents: Daniel Burgess for the sequence of a human Δ18 cDNA clone, Wonhee Jang for first strand cDNA from non-neuronal tissues, and David Kohrman for total RNA from fetal brain. Leslie Sprunger and David Kohrman provided valuable technical advice. We thank Alan Goldin, Lori Isom, David Kohrman, and Leslie Sprunger for critical reading of the manuscript.

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