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SHORT REPORT

Exclusion of the SCN2B gene as candidate for CMT4B

Alessandra Bolino[1], Marco Seri[2], Francesco Caroli[2], James Eubanks[3], Jayashree Srinivasan[3], Paola Mandich[4], Angelo Schenone[5], Aldo Quattrone[6,7], Giovanni Romeo[1,8], William A Catterall[3] and Marcella Devoto[1,9]

[1]*Laboratorio di Genetica Molecolare, Facoltà di Medicina, Università di Genova*
[2]*Istituto G Gaslini, Genova, Italy*
[3]*Department of Pharmacology, University of Washington, Seattle, WA, USA*
[4]*IBIG, Facoltà di Medicina, Università di Genova*
[5]*Dipartimento di Scienze Neurologiche e della Riabilitazione, Università di Genova*
[6]*Istituto di Neurologia, Facoltà di Medicina, Università di Reggio Calabria, Catanzaro*
[7]*Istituto di Medicina Sperimentale e Biotecnologia, CNR, Cosenza, Italy*
[8]*International Agency for Research on Cancer, Lyon, France*
[9]*Laboratory of Statistical Genetics, Rockefeller University, New York, NY, USA*

Charcot-Marie-Tooth disease type 4B (CMT4B) is a demyelinating autosomal recessive motor and sensory neuropathy characterised by focally folded myelin sheaths in the peripheral nerve. The *CMT4B* gene has been localised by homozygosity mapping and haplotype sharing in the 11q23 region. A cDNA encoding for the β2 subunit of the human brain sodium channel, *SCN2B*, has been recently assigned to the same chromosomal interval by FISH. The *SCN2B* gene has been considered a good candidate for CMT4B on the basis of protein homology, chromosomal localisation, and putative biological function of the coded product. In this paper, we report the genomic structure of the *SCN2B* gene consisting of 4 exons and 3 introns spanning a region of approximately 12 Kb. In addition, a search for mutations in patients affected with CMT4B as well as a refined physical localisation excludes *SCN2B* as the *CMT4B* gene.

Keywords: peripheral neuropathy; sodium channel; genomic structure; physical mapping

Introduction

Among the heterogeneous group of the autosomal recessive demyelinating hereditary motor and sensory neuropathies (HMSN) three pathological forms are now recognised, one with classical onion bulbs, one with basal lamina onion bulbs or Charcot-Marie-Tooth (CMT) type 4A,[1] and a third characterised by focally folded myelin sheaths or Charcot-Marie-Tooth type 4B (CMT4B).[2]

We previously reported a large inbred pedigree with recurrence of demyelinating motor and sensory neuropathy with focally folded myelin sheaths, CMT4B, inherited as an autosomal recessive trait.[3] Taking advantage of the particular inbred structure of the family, we used homozygosity mapping and haplotype sharing to localise, through a genome-wide search, the

Correspondence: Drs Marcella Devoto and Alessandra Bolino, Laboratorio di Genetica Molecolare, Facoltà di Medicina, Università di Genova, Istituto G. Gaslini, Largo Gaslini 5, 16148 Genova - Italy. Tel: +39 10 5636400; Fax: +39 10 3779797; E-mail: genseq@tin.it
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CMT4B gene on chromosome 11q23 in a 5 cM interval between D11S1332 and D11S917.[4]

Recently, a cDNA encoding the β2 subunit of the human brain sodium channel, *SCN2B*, was isolated and mapped by FISH on chromosome 11q23.[5] The voltage-gated sodium channels are membrane proteins responsible for the initiation and propagation of action potentials in most excitable cells. In the rat brain, these channels are a heterotrimeric complex composed of a pore-forming α subunit and two smaller auxiliary subunits, β1 and β2.[6,7] Human β2 is a 186 amino acid glycoprotein with a short intracellular COOH-terminal domain, a single transmembrane segment, and a large extracellular domain containing a predicted immunoglobulin (Ig) V-type fold with a disulfide bond and four N-linked glycosylation sites. The human protein has 89% amino acid sequence identity and 93% similarity to the rat β2 and, accordingly, many of the structural properties are conserved.[5,7]

Based on homology between the rat β2 and the cell adhesion molecule contactin, it has been postulated that β2 might interact with proteins of the extracellular matrix during the myelination process of the central and the peripheral nervous systems as suggested by the presence of β2 in rat dorsal root ganglia.[5] Correspondingly, the human β2 has the highest similarity to another cell adhesion molecule, the myelin protein zero (p0), a major component of the peripheral myelin which contains an extracellular Ig loop between two cysteine residues and N-linked glycosylation sites. Mutations in myelin p0 cause an autosomal dominant form of demyelinating CMT diseases, CMT type 1B.[8]

On the basis of the protein homologies, chromosomal localisation, and the postulated role during the myelination process, human *SCN2B* has been considered a potential candidate for CMT4B.

In the present study, we first characterised the genomic structure of the *SCN2B* gene and then demonstrated that *SCN2B* is not the *CMT4B* gene. A search for mutations in the coding region of *SCN2B* in CMT4B patients yielded negative results. In addition, a refined physical localisation showed that *SCN2B* maps approximately 20 cM distal to the critical region for the disease.

Materials and Methods

DNA Extraction

DNA was extracted from peripheral leukocytes of the CMT4B patients using standard methods.[9] DNA extraction from positive YAC clones was performed as described elsewhere.[10]

Genomic Structure of the SCN2B Gene and Mutation Analysis

PCR reactions on the *SCN2B* gene were performed according to standard protocols[9] using 500 ng of genomic DNA, 200 μM of each dNTP, 30 pmol of each primer, 1 or 1.5 mM of [Mg2 +], and 1.25 U of *Taq* polymerase, in a final volume of 50 μl. The same PCR conditions with 1 mM of [Mg2 +] were also used to amplify the four exons of the *SCN2B* gene in the CMT4B patients. The following couples of primers designed on the intron-exon boundaries were used (Figure 1 and Table 1): UTR-5' and EX1R for exon 1, with an annealing temperature of 55°C; EX2F and IR for exon 2, using an annealing temperature of 61°C; IF and IBR at annealing temperature of 59°C for exon 3; and finally, IBF2 and EBR3 for the last exon using an annealing temperature of 59°C.

Molecular cloning was carried out using the pCR II vector kit from Invitrogen following the manufacturer's instructions. Finally, cycle sequencing was performed using a PCR-dye primer and/or terminator protocol on an Applied Biosystem 373A Sequencer.

Physical Mapping

Physical mapping of the *SCN2B* gene was performed using the GenBridge 4 Radiation Hybrid panel[11] (Research Genetics, Huntsville, Alabama, USA) as well as the CEPH mega-YAC library (provided by the YAC screening Centre DIBIT, Milan, Italy).

Results and Discussion

Since only the 648 bp open reading frame (ORF) of the human *SCN2B* cDNA was available, we first characterised the *SCN2B* genomic structure. To this end, we designed two pairs of primers which were expected to amplify two overlapping fragments of an average size of 600 bp from human genomic DNA. This hypothesis was based on the homology to the highly conserved rat gene where two introns were identified in the coding sequence of the mature β2 protein.[7]

Using the first pair of primers, EAFN and EAR (Figure 1 and Table 1), located in the 5' portion of the cDNA, we were not able to obtain a single amplification product, probably because of the homology between this region of the gene and molecules belonging to the Ig superfamily. On the other hand, primers EBF and EBR (Figure 1 and Table 1) from the 3' portion of the coding sequence amplified a fragment of approximately 1.5 Kb which was cloned in the pCR II vector (Invitrogen Corporation, Carlsbad, California, CA, USA). The recombinant clones were entirely sequenced and an intron of 977 bp was identified.

In order to obtain a single amplification product containing the 5' portion of the gene using *SCN2B*

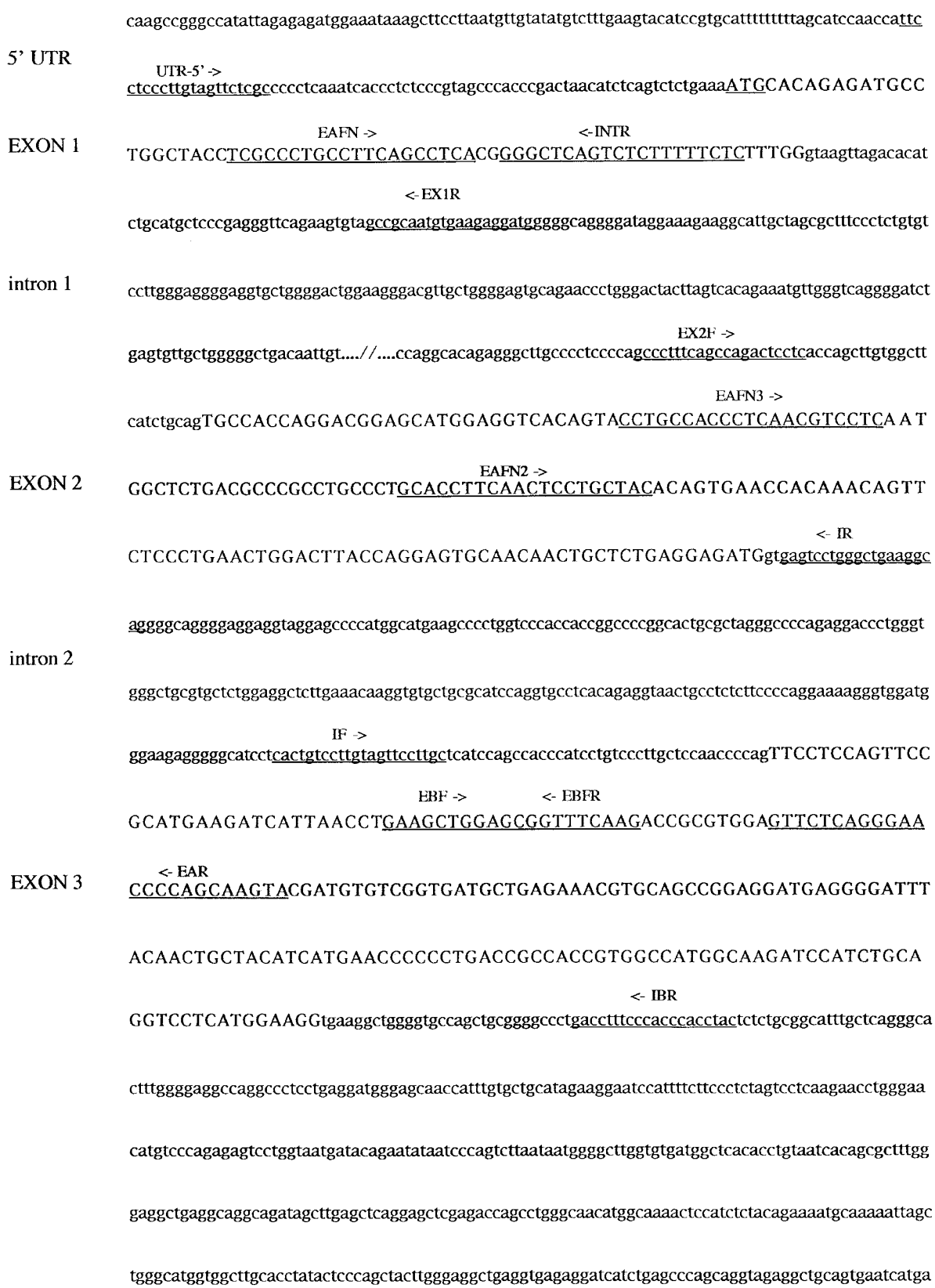

Figure 1 *Genomic sequence of the SCN2B gene which was submitted to GenBank under accession nos AF049496 and AF049497. Primers used in PCR amplification are underlined. The sequences of these primers are shown in detail in Table 1. The complete coding sequence of the SCN2B gene with the corresponding protein sequence was also submitted to GenBank under accession no. AF049498.*

ttgtgccactgcactccagcttgggcaacagagcaagaccctgtttcaaatgacaacaacagcaataataatgatggtgatgatggcagctttcattt

intron 3 attgggcacctctatgtctcagtcatagggctaagctctgtttatgcattatctcatcctcatgaaaaccttgagggaggtactattattaagcccat

tttacatgttagaaaactgaggttagagaggataagttacttaaggtcacgcaattcgtaagttgtacaattaggactcaaatttgggtctgtttgatc

ttaaagttcatgctcttaaatacttcatcacactagctcctctgggggctttgggtgtcctgggatttggaggtggcggcgcttcagggtcagtcactg

IBF2 ->
gattgcttttcggctttgctaaagtcctgtctgtggaggagagtaaagtcaggatggttccacgcatgccacgggtagtggggtgatgggggaggccc

ctttcccagccctggtgctcagtggctctgcttcctttccagAGCCCCCTGAGCGGGACTCCACGGTGGCCGTGATTGT

GGGTGCCTCCGTCGGGGGCTTCCTGGCTGTGGTCATCTTGGTGCTGATGGTGGTCAAGTGTGTGA

EXON 4

GGAGAAAAAAAGAGCAGAAGCTGAGCACAGATGACCTGAAGACCGAGGAGGAGGGCAAG

<- EBR
ACGGACGGTGAAGGCAACCCGGATGATGGCGCCAAGTAGtgggtggccggccctgcagcctcccgtgtcccgtc

<- EBR3
tcctcccctctccgccctgtacagtgaccctgcctgctcgctcttggtgtgcttcccgtgacctaggaccccagggcccacctggggcctcctgaac

3' UTR

ccccgacttcgtatctcccaccctgcaccaagagtgacccactctcttccatccgagaaacctgccatgctctgggacttgtgggccctggggagag

gagagaaagggctcccacctgccagtccctgggggggaggcaggaggcacatgttgagggtcccc

Figure 1 *continued*

Table 1 Primers used for PCR amplification of the *SCN2B* gene

Primer	*Sequence*	*Strand*	*Position*
UTR-5′	TTCCTCCCTTGTAGTTCTCGC	FORWARD	5′-UTR
EAFN	TCGCCCTGCCTTCAGCCTCA	FORWARD	EXON 1
INTR	GAGAAAAAGAGACTGAGCCC	REVERSE	EXON 1
EX 1R	CATCCTCTTCACATTGCGGC	REVERSE	INTRON 1
EX 2F	GCCCTTTCAGCCAGACTCCTC	FORWARD	INTRON 1
EAFN3	CCTGCCACCCTCAACGTCCTC	FORWARD	EXON 2
EAFN2	GCACCTTCAACTCCTGCTAC	FORWARD	EXON 2
IR	CTGCCTTCAGCCCAGGACTC	REVERSE	INTRON 2
IF	CACTGTCCTTGTAGTTCCTTGC	FORWARD	INTRON 2
EBF	GAAGCTGGAGCGGTTTCAAG	FORWARD	EXON 3
EBFR	CTTGAAACCGCTCCAGCTTC	REVERSE	EXON 3
EAR	TAXTTGCTGGGGTTCCCTGAGAAC	REVERSE	EXON 3
IBR	GTAGGTGGGTGGGAAAGGTC	REVERSE	INTRON 3
IBF2	GGTTCCACGCATGCCACGGG	FORWARD	INTRON 3
EBR	TGGCGCCATCATCCGGGTTGC	REVERSE	EXON 4
EBR3	AGCAGGCAGGGTCACTGTAC	REVERSE	3′-UTR

specific primer sequences, we extended the sequence of the cDNA to its untranslated regions. We performed a BLAST search of sequences similar to the human *SCN2B* coding sequence on the Expressed Sequence Tag human cDNA database (http://www.ncbi.nih.gov/dbEST/index.html. We retrieved EST clones with high homology to *SCN2B* and belonging to the same contig (THC 168161, TIGR database) and we partially sequenced two of them (IMAGE Consortium Clones ID = 25456 and ID = c–0cd03) which were expected to contain the untranslated regions of *SCN2B*. The sequence of 190 nt from the 5'-UTR and 236 nt from the 3'-UTR was determined and is shown in Figure 1.

Several forward primers were then designed from the 5'-UTR as well as the 5' end of the cDNA and used in combination with various reverse primers from the 3' end of the coding sequence. Only the pair of primers EAFN2 and EBFR (Figure 1 and Table 1) were able to amplify a product of approximately 450 bp which was cloned in pCR II. The recombinant clones were sequenced and an additional intron of 289 bp was found.

Because a PCR product had been obtained using primers UTR-5' with INTR and EAFN3 with IR (Figure 1 and Table 1), whereas no PCR product had been obtained using these primers in different combinations, we deduced that a large intron had to be present between primers INTR and EAFN3. In order to amplify this last intron, we screened by PCR the pools from a human CEPH Mega-YAC library using EAFN2/EBFR as primers and we found three positive YAC clones (665E10, 742F9, and 785E12). Clone 665E10 was used as a template in a long PCR reaction (Boehringer, Mannheim Corporation, Indianapolis, IN, USA) using primers EAFN and IR (Figure 1 and Table 1). A PCR product of more than 9 Kb was identified and cloned in pCR II. Only the intron–exon boundaries of an intron of approximately 9 Kb were sequenced as shown in Figure 1. This intron, located in the sequence coding for the cleaved signal peptide of SCN2B, was not identified in previous work on the rat gene.[7] Using primers designed on the intron–exon boundaries already established, we amplified the other two introns of 289 and 977 bp from the same YAC clone 665-E-10, thus confirming the results obtained starting from genomic DNA.

The established exon–intron structure of the *SCN2B* gene is schematically illustrated in Figure 2. Based on this gene structure, we searched for mutations in the *SCN2B* genes of our CMT4B patient sample consisting of five affected individuals from the inbred Italian pedigree used to map the *CMT4B* gene (1945, 273, 279, 287, and 1943 from the pedigree reported in reference 4), two affected sibs born from unaffected parents and one sporadic CMT4B case.[12] We amplified the 4 exons of the *SCN2B* gene using primers designed for the intron boundaries. Only the wild-type allele was found in all patients by analysis of the entire coding sequence of each gene.

At the same time, we performed a refined physical localisation of the *SCN2B* gene to confirm its previous location by FISH on chromosome 11q23. A search in the CEPH YAC contig map of the human genome[13] allowed us to assign the positive clones, retrieved through PCR screening of the CEPH YAC library, to two clusters of clones recognised by Généthon's markers D11S1340, D11S939, and D11S1356. These markers are located 20 cM distal to the critical interval for

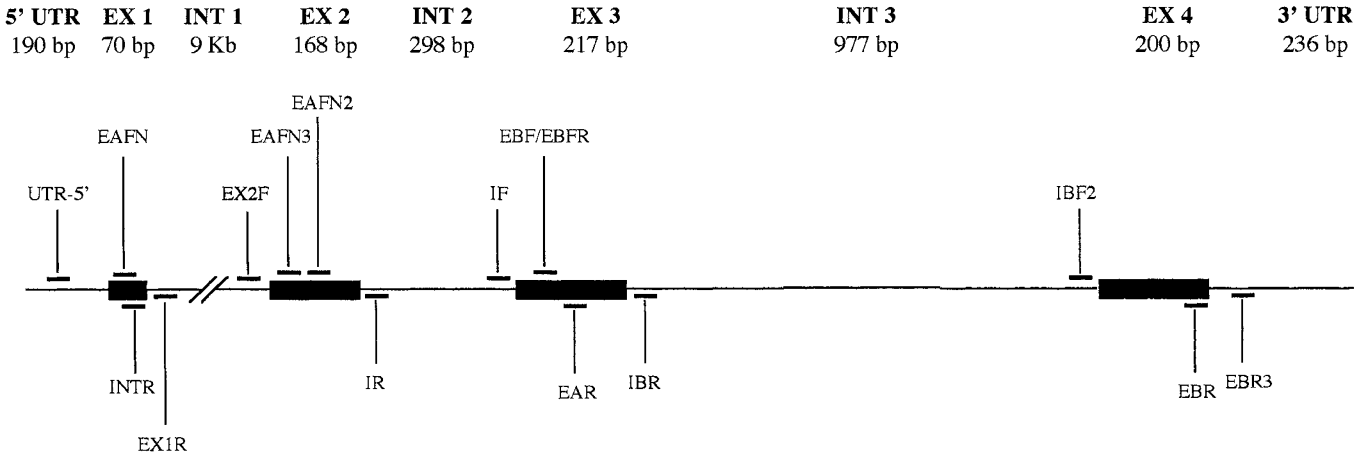

Figure 2 *The intron-exon organisation of the SCN2B gene is schematically reported. The size of each intron or exon is also indicated.*

CMT4B, which is delimited by markers D11S1332 and D11S917 located in 11q22.1, as determined from the same YAC contig map. To confirm these results which excluded the *SCN2B* gene from the region of interest, we screened by PCR a panel of radiation hybrids using EBF/EBR as primers (Figure 1 and Table 1). Thus, the *SCN2B* gene was mapped on chromosome 11q23 at 1.92 cR from the STS marker WI-7642 of the integrated map from the Whitehead Institute (http://www-genome.wi.mit.edu/). This STS is flanked by Généthon's markers D11S1340 proximally and D11S936 distally, which are located 20 cM distal to the lower limit of the CMT4B region. We therefore concluded that *SCN2B* is not the *CMT4B* gene. To our knowledge, no other disease has been mapped to the 11q23 region for which *SCN2B* might constitute a suitable candidate gene.

Acknowledgements

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