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Characterization of the human neurocan gene, *CSPG3*[1]

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Abstract

Neurocan is a chondroitin sulfate proteoglycan thought to be involved in the modulation of cell adhesion and migration. Its sequence has been determined previously in rat and mouse (Rauch et al., 1992. Cloning and primary structure of neurocan, a developmentally regulated, aggregating, chondroitin sulfate proteoglycan of the brain. J. Biol. Chem. 267, 19536–19547; Rauch et al., 1995. Structure and chromosomal location of the mouse neurocan gene. Genomics 28, 405–410). We describe here the complete coding sequence of the human neurocan mRNA, known as *CSPG3*, as well as mapping data, expression analysis, and genomic structure. A cDNA known as CP-1 was initially sequenced as part of a gene discovery project focused on characterizing chromosome 19-specific cDNAs. Sequence homology searches indicated close homology to the mouse and rat proteoglycan, neurocan (GenBank accession Nos X84727 and M97161). Northern analysis identified a brain-specific transcript of approx. 7.5 kb. A longer cDNA clone, GT-5, was obtained, fine-mapped to the physical map of chromosome 19 by hybridization to a chromosome-specific cosmid library, and sequenced. Full coding sequence of the mRNA indicates a 3963 bp open reading frame corresponding to a 1321 amino acid protein, similar to the protein length found in mouse and rat. The amino acid sequence of human neurocan shows 63% identity with both the mouse and rat sequences. Finally, genomic sequencing of a cosmid containing the complete neurocan gene was performed to determine the genomic structure of the gene, which spans approx. 41 kb, and is transcribed in the telomere to centromere orientation.

Keywords: Brain; Chromosomal mapping; Proteoglycan; Sequencing

1. Introduction

Neurocan is a brain- and nervous tissue-specific proteoglycan which plays an important role in modulating cell–cell and cell–matrix interactions during the development of nervous tissue. It belongs to a family of chondroitin sulfate proteoglycans whose primary structure includes an immunoglobulin domain followed by a series of tandem repeats at the N-terminus, and one or two epidermal growth factor-like domains, a lectin-like domain, and a regulatory protein-like sequence at the C-terminus. The internal portion of each proteoglycan has no homology with any of the other proteoglycans. Various members of this family, which also include aggrecan, versican, and brevican, are thought to play an important role in the inhibition of neural crest cell migration and may serve as a barrier in the migration of axons (Oakley and Tosney, 1991; Snow et al., 1990a,b; Pindzola et al., 1993). Neurocan has previously been shown to interact with specific molecules involved in cell adhesion and migration (Grumet et al., 1993, 1994; Milev et al., 1996), including three neuronally expressed cell adhesion molecules (N-CAM, Ng–CAM, and TAG-1/axonin-1) as well as an extracellular matrix protein (tenascin). All of these molecules colocalize with

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[1] The nucleotide sequence reported in this paper has been submitted to the GenBank Database under the name *CSPG3* and accession number AF026547.

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Abbreviations: cDNA, DNA complementary to RNA; CIAP, calf intestinal alkaline phosphatase; dbEST, database of expressed sequence tags; EGF, epidermal growth factor; FISH, fluorescent in situ hybridization; Ig, immunoglobulin; LLNL, Lawrence Livermore National Laboratory; mRNA, messenger ribonucleic acid; N-CAM, neural cell adhesion molecule; Ng–CAM, neuron–glia cell adhesion molecule; nt, nucleotide(s); *UTR*, untranslated region(s).

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neurocan (Grumet et al., 1993, 1994; Milev et al., 1996). In addition, neurocan has been shown to bind directly to neurons (Grumet et al., 1993) and to inhibit neurite growth (Friedlander et al., 1994).

Neurocan is under tight developmental regulation with respect to immunocytochemical localization, molecular size, and concentration in the cell. Immunocytochemical studies have shown that neurocan is expressed predominantly in the prospective white matter and is absent from the external granule cell layer in a 7 day postnatal rat cerebellum (Rauch et al., 1991; Grumet et al., 1993). However, in an adult brain, expression is highest in the molecular layer and completely absent in the white matter. The molecular weight also appears to be developmentally regulated. In the fetus, the predominant species is a 245 kDa (after removal of chondroitin sulfate chains) protein representing the full coding sequence of neurocan. At 7 days postnatal, a mixture of two forms is present, with molecular weights of 150 kDa and 245 kDa. The major species present in the adult brain is the 150 kDa form, which appears to be the result of in vivo proteolytic processing of the larger form (Rauch et al., 1992; Meyer-Puttlitz et al., 1995). This smaller form represents the C-terminal portion of the complete protein. Finally, studies in rat show that concentration of the neurocan protein decreases during development, from 20% of the total chondroitin sulfate proteoglycan protein at 7 days postnatal to 6% in adult tissue (Rauch et al., 1991).

This paper describes the coding sequence, chromosomal localization, expression analysis by Northern blot, and genomic structure of the human neurocan gene, *CSPG3*. This protein shows significant homology at the N-terminal and C-terminal ends, as well as a limited homology within the central region, with both mouse and rat neurocan protein. Northern analysis indicates a transcript of approx. 7.5 kb with brain-specific expression. The mRNA encodes a protein containing 1321 amino acids, as well as 2.3 kb of 3′ untranslated region. We have mapped *CSPG3* to chromosome 19p12-13.1, in a region previously shown to be syntenic with mouse chromosome 8.

2. Materials and methods

2.1. Isolation of cDNAs

The 1.4 kb CP-1 cDNA was isolated from a normalized infant brain cDNA library (Soares et al., 1994) as part of a chromosome 19-specific cDNA selection experiment. In an attempt to find a full-length cDNA, the GeneTrapper Kit (Life Technologies, Gaithersberg, MD) was used according to the manufacturer's recommended protocol and custom primers designed from the CP-1 sequence. From this experiment, a 5.0 kb cDNA referred to as GT-5 was isolated from a non-normalized adult brain cDNA library (Life Technologies No. 10418-010) which partially overlapped the original 1.4 kb CP-1 cDNA. Further GeneTrapper experiments yielded no larger clones. Screening of 10^6 clones from a random-primed adult brain cDNA library yielded no clones giving additional 5′ sequence. Finally, 5′-RACE (Clontech, Palo Alto, CA) experiments were attempted using primers RACE1 (5′-CTTTGGGACCAC-GCAGCAT-3′) and RACE2 (5′-CCAAGGGG-GACCAAAACAG-3′) on whole brain cDNA (Marathon-ready cDNA, Clontech) following the manufacturer's recommended protocol. No clones giving additional sequence were obtained using any of these methods.

2.2. Northern blot analysis

Northern blot analysis was performed using three blots: Human Multiple Tissue I (Clontech No. 7760-1: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas); Human Brain I (Clontech No. 7758-1: amygdala, caudate nucleus, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus, thalamus); and Human Fetal I (Clontech No. 7761-1: brain, heart, kidney, liver, lung). Approximately 2 μg of polyA$^+$ RNA was loaded onto each lane. Identical probes were made by PCR using ^{32}P-dCTP and vector primers T7 and Sp6 to amplify the GT-5 cDNA insert containing exons 7–15 and the 3′ UTR of *CSPG3*. Hybridization was performed as described above. Filters were exposed to PhosphorImager cassettes (Molecular Dynamics, Sunnyvale, CA) for 5 days and image analysis was performed using the Storm 860 (Molecular Dynamics).

2.3. Mapping to chromosome 19

A chromosome 19-specific cosmid library was prepared from flow-sorted hamster–human hybrid DNA at Lawrence Livermore National Laboratory (LLNL) as part of the National Gene Library Project as described previously (de Jong et al., 1989). Clones from this library have been randomly arrayed and spotted onto nylon filters using the Hewlett-Packard ORCA (Copeland and Lennon, 1994). To prepare the probe, the GT-5 cDNA insert was released from the vector by digestion with *Not*I and *Sal*I. The insert was random-labeled with ^{32}P-dCTP (Prime-It II, Stratagene), purified with a G-50 spin column (Worthington Biochemical, La Jolla, CA), and blocked with human Cot-I DNA (Life Technologies, Lakewood, NJ). Hybridization was overnight at 65°C, with the composition of the hybridization solution as follows: 0.6 M NaCl; 10 mM EDTA; 50 mM Tris (pH 7.4); 10% dextran sulfate; 2% SDS; and 0.1% sodium pyrophosphate. Washes were also performed at

65°C as follows: one rinse in 2 × SSC, 1% SDS followed by two 30 min washes in 0.2 × SSC, 1% SDS. Filters were exposed to PhophorImager cassettes (Molecular Dynamics) and analyzed with the Storm 860 (Molecular Dynamics). 25 cosmid clones were positive for the GT-5 probe. *Eco*RI fingerprinting data (Carrano et al., 1989) indicated that 22 of the 25 positive clones overlap in a contiguous stretch of genomic sequence spanning 948 kb (Ashworth et al., 1995). Multiple clones in this contig have been independently mapped to the region of 19p12-13.1 by fluorescent in situ hybridization (FISH).

2.4. DNA sequencing and analysis

The GT-5 cDNA clone was sequenced using a yeast Ty1-based transposon system (Perkin Elmer – Applied Biosystems Division, Foster City, CA) and the PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer – Applied Biosystems Division) with custom primers SD118 (5′-CATTGGATGCTGAGAATTCG-3′) and SD119 (5′-GCCGTCTATCCTGCTTGC-3′). Samples were run on a 377 (Perkin Elmer – Applied Biosystems Division) and sequence was analyzed using the Sequencher software package (Gene Codes Corporation, Ann Arbor, MI).

2.5. Establishment of transcript orientation

Two identical *Eco*RI southern blots were prepared from nine of the cosmids positive in the initial screening. Separate probes were made by PCR using custom oligos designed from the 5′ and 3′ ends of the GT-5 cDNA and hybridized as described above. The resulting positive fragments were compared with the *Eco*RI map of the cosmids to determine the orientation of the transcript.

2.6. Determination of exon/intron boundaries

Two subclone libraries were made from one cosmid (33494) which was predicted to contain *CSPG3* in its entirety. Cosmid DNA was isolated with a QIAGEN-500 (QIAGEN GmbH, Germany) maxi-prep and cut by *Sau*3A partial digestion. The digest was separated by electrophoresis on a 1% agarose gel and the region corresponding to 0.5–3 kb was isolated and gel-purified (EluQuik, Schleicher & Schuell, Keene, NH). Ten nanograms of the resulting DNA was ligated into *Bam*HI-cut M13 vector treated with CIP (Boehringer Mannheim, Indianapolis, IN) and transfected into XL-2 Blue competent cells (Stratagene). A plasmid library was constructed from a different aliquot of the same cosmid DNA. DNA was digested in separate reactions with *Bam*HI, *Sau*3AI, *Bgl*II, and *Bcl*I, run on a 1% agarose gel, and the region corresponding to 1.0–3.5 kb was size-selected. DNA was gel-purified (EluQuik, Schleicher & Schuell) and cloned into *Bam*HI-digested pBluescript KS$^-$ and transformed into DH10B cells (Life Technologies). Clones from both libraries were amplified by PCR using vector primers to check for inserts >500 bp. DNA was isolated from all clones with suitable inserts (QIAprep-8 M13 or QIAwell-8 plasmid kit, QIAGEN) and sequenced as described above. In addition, some sequences from a nebulized M13 library from adjacent cosmid 14150 were analyzed for exon sequence. Assembly was performed with the Sequencher software package (Gene Codes Corporation). cDNA sequence from mouse and human was compared with the assembled genomic sequence to predict exon/intron boundaries. In addition, the mouse cDNA sequence was used to identify the remaining 1.3 kb of open reading frame from human genomic sequence.

3. Results

3.1. Sequence analysis and exon/intron structure

The nucleotide sequence of the 5.0 kb GT-5 clone was determined using the dye-terminator kit from ABI. This sequence, in addition to the genomic sequencing described above, yielded 6310 bp corresponding to the entire coding sequence and 3′ UTR of human neurocan (GenBank accession No. AF026547). The open reading frame consists of 3963 bp corresponding to a 1321 amino acid protein.

CSPG3 spans at least 41 kb of genomic sequence as determined by the *Eco*RI map of the region, and consists of 14 exons which include the entire coding region and the 3′ UTR (Table 1). The mouse gene contains an additional exon in the 5′ untranslated region which has not been identified to date in humans. In order to avoid confusion, we have maintained the same numbering for each exon as in the mouse. Therefore our numbering starts at exon 2. We were unable to find a cDNA clone containing exons 1–6 after performing additional GeneTrapper experiments, screening of a random-primed brain cDNA library, 5′-RACE experiments, and scanning of an additional 12 kb of upstream genomic sequence.

Neurocan contains the characteristic N-terminal and C-terminal domains which share homology with other known proteoglycans, as well as a central, non-homologous region (Fig. 1). The N-terminal region is made up of an immunoglobulin domain, represented by exon 3 (amino acids 25–158), and a series of hyaluronic acid-binding tandem repeats in exons 4–6 (amino acids 159–357). This region shares 92% protein identity with both mouse and rat neurocan, and at least 50% amino acid identity with various other proteoglycan family members from different species, including monkey, mouse, rat, rabbit, dog, cow, pig, and chicken. Exons 7

Table 1
Intron/exon junctions of the human neurocan gene, compared with the mouse gene

Exon	Domain[a]	Splice acceptor	Exon size (bp)		Splice donor	Intron size (kb)
			Human	Mouse		
1	5′ UTR	N/A	N/D	95	N/D	N/D
2	SP	gatccag GATGGGG	74	80	GAAGAGG gtgagtt	1.9
3	IG	aacccag GCACACA	402	369	GTGACAG gtcantt	1.4
4	TR	cccacag GTGTTGT	175	175	CTGTTCG gtgaggg	0.1
5	TR	ttgtcag GTATCCT	128	128	CGTGGGG gtaagtc	0.5
6	TR	cccgcag GCGAGGT	294	294	TTCCGAG gtgcgtg	1.3
7	NH	cccacag CTCATCA	588	597	GGAGCTG gtgagtt	0.2
8	NH	gttgcag GTTCTGC	1359	1209	GAGGAGG gtgagta	>6.2
9	EGF	cctgcag TGCACTC	117	117	GAGATTG gtgagta	1.1
10	EGF	ctgccag ACATTGA	114	114	GAGAAAG gtgagtt	>3.1
11	LEC	ctcccag ACACCGA	159	159	ATTAATA gtagggg	>0.8
12	LEC	cctgcag GCTTTGG	83	83	CGGGCTG gtgagtg	>1.9
13	LEC	cctgcag CAATTTG	145	145	GGCACAG gtatgct	>3.3
14	CR	gtcccag TGCTCTG	183	183	ACCAAAC gtaagta	0.9
15	3′ UTR	cctgcag CCAGACG	2346	N/A		

Exon sequences are in capital letters; introns are in lower case.
[a]SP, signal peptide; IG, immunoglobulin-like; TR, tandem repeats; NH, non-homologous; EGF, epidermal growth factor-like; LEC, lectin-like; CR, complement regulatory-like; UTR, untranslated.

and 8 (amino acids 358–1007) represent the central, non-homologous portion of the gene, with no significant homologies found in the database with the exception of 57% (exon 7) and 29% (exon 8) amino acid identity with both the mouse and rat neurocan proteins. The C-terminal region is made up of an epidermal growth factor-like domain represented by exons 9 and 10 (amino acids 1008–1083), a lectin-like domain in exons 11–13 (amino acids 1084–1211), and a complement regulatory-like domain in exon 14 (amino acids 1212–1265). As can be expected, this entire region shows significant levels of homology to the proteoglycan family members, including 92% protein identity with the other neurocans and approx. 72% amino acid similarity with proteoglycans from other species.

3.2. Expression analysis

The results of the Northern analysis suggest that the *CSPG3* transcript is brain-specific (Fig. 2), although other nervous tissues were not tested. The 7.5 kb transcript is observed in both adult and fetal tissues, and in all brain regions tested. These results are similar to those determined previously in mouse and rat which estimate the mRNA size at approx. 7.5 kb, with expression confined to brain and nervous tissues. Scanning of dbEST using the *CSPG3* sequence reveals multiple cDNAs which are highly likely to be derived from this transcript. These cDNA clones originate from a variety of normalized and non-normalized brain tissues, including fetal (eight clones), infant (four clones), and adult brain (three clones) and a multiple-sclerosis brain (one clone). As in mouse, no clones originating from a tissue other than brain were found to have sequences derived from the neurocan transcript.

3.3. Mapping of CSPG3

CSPG3 maps to a well-characterized region of human chromosome 19 (Fig. 3). An *Eco*RI digest map, which includes the cosmids positive for the neurocan gene by hybridization, spans nearly 1 Mb of 19p12-13.1 (Ashworth et al., 1995). This map allowed us to localize neurocan to specific *Eco*RI fragments and therefore to determine its transcriptional orientation, with the 5′ end telomeric. Transcriptional orientation is from telomere to centromere; *CSPG3* is flanked on the telomeric side by *MEF2B* and on the centromeric side by a cluster of zinc finger genes. Additional transcripts which have been mapped to this region include *COMP*, *UBA52*, *ELL*, several trapped exons, and anonymous ESTs derived from IMAGE clones.

4. Discussion

In this study we have sequenced and characterized the human neurocan gene, *CSPG3*, and mapped it to chromosome 19p12-13.1. Study of its primary structure reveals a similarity to other known proteoglycans, most significantly with mouse and rat neurocan. All known members of the proteoglycan family display a high degree of homology with each other at the N-terminal and C-terminal regions of the protein. Functionally, it has been shown that the N-terminal region, and specifically the tandem repeats, is involved in the mediation

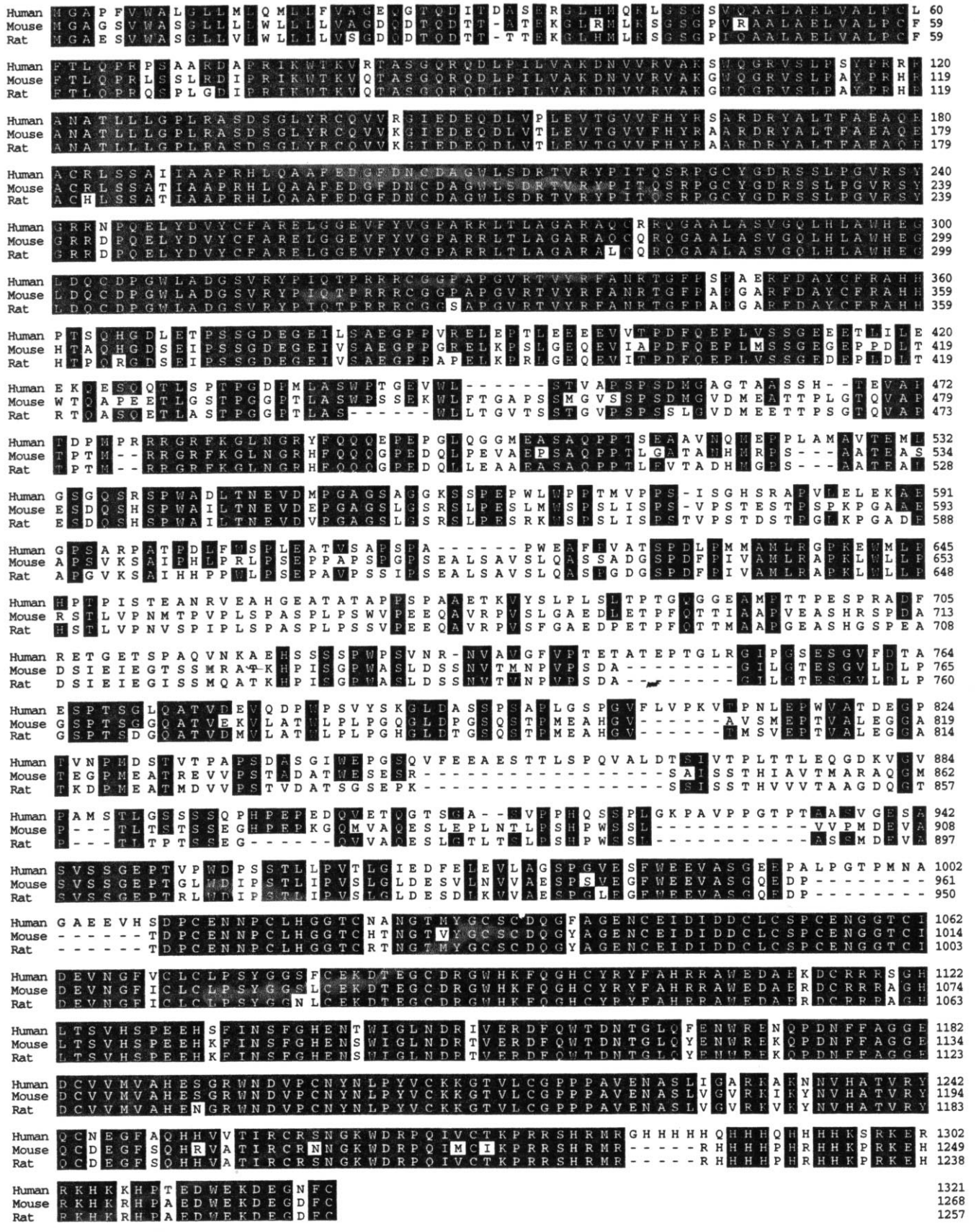

Fig. 1. Comparison of amino acid residues from human *CSPG3*, mouse neurocan, and rat neurocan. Dark shading indicates an exact match to the human protein sequence.

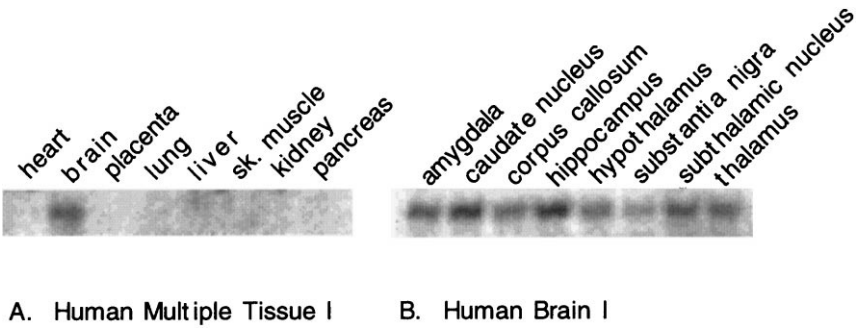

Fig. 2. Northern analysis reveals an approx. 7.5 kb transcript present only in brain tissues. The transcript is present in every brain tissue tested.

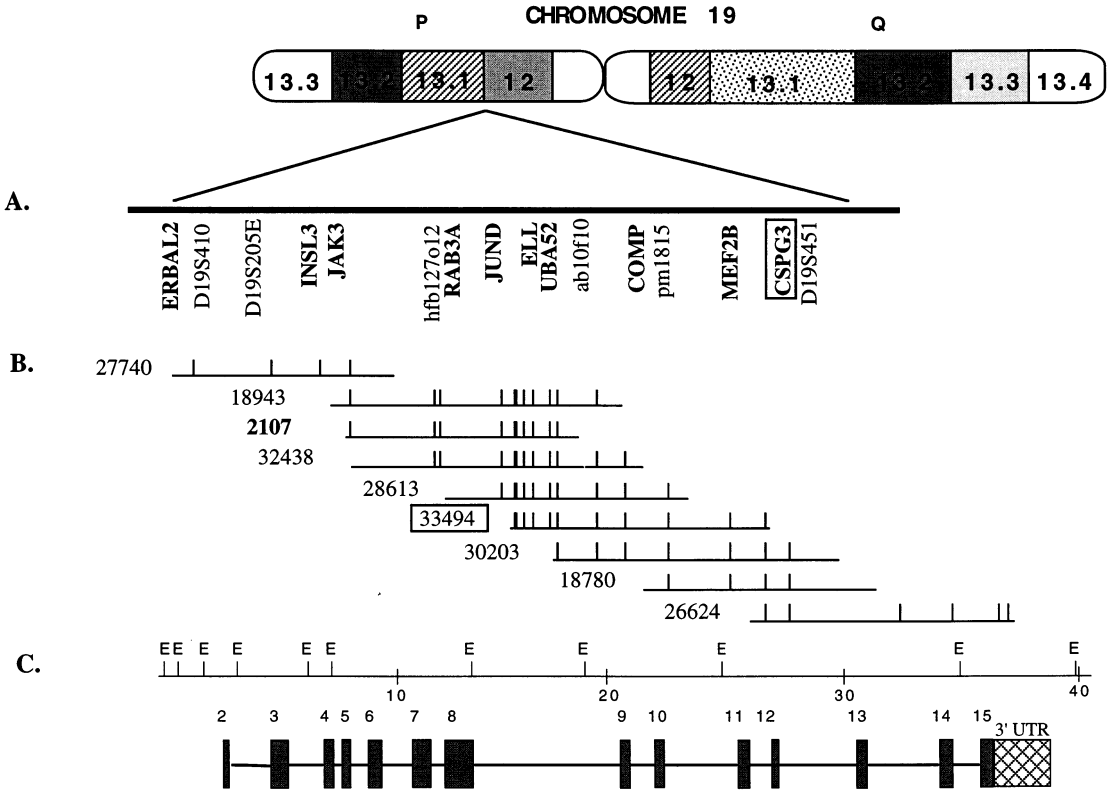

Fig. 3. (A) Map location of the neurocan gene (boxed) with respect to other genes in the region (shown in bold type) as well as ESTs and anonymous markers. (B) View of partial *Eco*RI map containing the neurocan gene. The numbers on the left side represent the cosmid name, vertical lines represent *Eco*RI sites as determined previously. Cosmid 2107 (shown in bold type) has been FISH-mapped to 19p12-13.1. Cosmid 33494 (boxed) contains the complete neurocan gene and was partially sequenced to determine the exon/intron boundaries. The complte map spans 948 kb from ELL to D19S451; this map contains approx. 140 kb of sequence from the centromeric portion of the map. (C) Exon organization of the neurocan gene in relation to the *Eco*RI map of cosmid 33494. The *Eco*RI map represents 41 kb of genomic sequence, with distance (in kb) shown along the bottom of the diagram. Transcriptional orientation is from telomere to centromere.

of binding to hyaluronic acid (Rauch et al., 1991, 1992). This binding, thought to prevent cell migration, is interrupted by the proteolytic processing of neurocan, resulting in a truncated protein containing only the C-terminal region which is unable to bind hyaluronic acid. It has been shown previously that conservation of key amino acids necessary for this binding is exhibited in all proteoglycans (Perkins et al., 1991; Rauch et al., 1992), supporting the evolutionary importance of this function in the process of neuronal differentiation. The human neurocan protein displays the same amino acid conservation, suggesting that it too plays a similar role in cell migration and development of nervous tissue.

The brain- and nervous-tissue specificity displayed by *CSPG3* marks it as a potential candidate for neurological disease. Although no diseases have been mapped to human chromosome 19p12-13.1 for which the gene remains uncloned, there are several mouse mutants which localize to mouse chromosome 8 in the region conserved with human 19p12-13.1. The mouse neurocan

gene has previously been mapped between the microsatellite markers *D8Mit29* and *D8Mit78* (Rauch et al., 1995). As of release 1.0 of Jackson Laboratory's Mouse Genome Informatics [The Jackson Laboratory, Bar Harbor, ME, February 1998. World Wide Web (URL: http://www.informatics.jax.org/)], the mutant showing the best correlation with the reported position of mouse neurocan is referred to as *quinky* (*Q*), a semi-dominant mutation characterized by circling and/or shaking behavior, as well as skeletal, tail, and inner-ear defects. In addition to *quinky*, other mutants mapping close to this region include *proportional dwarf* (*pdw*), *oligosyndactylism* (*os*), *adrenocortical dysplasia* (*acd*), and *hook* (*Hk*). Further mapping of these mouse loci relative to the mouse neurocan gene as well as mutation analysis will be needed to test the possible role of neurocan in these disorders.

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