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# Cloning of a Novel Member of the Reticulon Gene Family (RTN3): Gene Structure and Chromosomal Localization to 11q13

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**A novel member of the neuron-specific protein (NSP) or newly named reticulon (RTN) gene family was isolated during a subtraction cloning between macula and peripheral retina. The mRNA for this NSP/RTN-like gene is approximately threefold more abundant in macula than in peripheral retina. The cDNA is 2527 bp long and contains an open reading frame of 236 amino acids. The deduced peptide shows a strong similarity to the NSP/RTN and tropomyosin-like gene families but it is clearly a novel member. The gene contains seven exons and spans more than 15 kb. The gene was localized to chromosome 11q13 between markers D11S4535 and D11S4627 using somatic cell hybrid panels. Southern blot analysis identified the presence of a pseudogene(s) that was subsequently localized to chromosome 4. Multitissue Northern blot analysis found this gene to be widely expressed in human tissues with the highest expression in the brain. We are calling this gene RTN3 to reflect the newly proposed nomenclature.** 

## INTRODUCTION

The first members of the neuroendocrine-specific protein family were cloned by Roebroek *et al.* (1993) using monoclonal antibodies to proteins expressed specifically in small cell carcinomas and other neuroendocrine tissues (Broers *et al.*, 1991). Three transcripts of 3.4, 2.3, and 1.8 kb were identified and found to be alternatively spliced mRNAs originating from one gene (Roebroek *et al.*, 1996). The proteins were named NSP-A, NSP-B, and NSP-C with peptide lengths of 776, 356, and 208 amino acid residues, respectively. These proteins are known to be associated with the endoplasmic reticulum (ER) (van de Velde *et al.*, 1994) and have been called reticulons (Roebroek *et al.*, 1996). It has been suggested that these NSP-reticulons may be serving as ER-associated channel-like complexes (van de Velde *et al.*, 1994). These neuroendocrine-specific proteins or NSP-reticulons are of clinical importance as independent markers to distinguish small cell lung carcinoma (SCLC) from non-SCLC (Senden *et al.*, 1994; Senden *et al.*, 1997). NSP-A is present in the SCLC tumors while non-SCLC tumors are generally negative (Senden *et al.*, 1997a, b). The NSPs can also be used to distinguish non-SCLC tumors from non-SCLC with neuroendocrine differentiation (Senden *et al.*, 1997b). Recently, the original group that discovered this family of genes proposed changing the name from neuroendocrine-specific gene family to the reticulon, or RTN, gene family (Roebroek *et al.*, 1998).

The existence of additional members of the NSP/RTN-like gene family was previously recognized by Roebroek *et al.* (1996), using TBLASTN searches of the GenBank database. Two groups have recently cloned the cDNA and gene for the human NSP-like gene I (Roebroek *et al.*, 1998; Geisler *et al.*, 1998), and it is now named RTN2 (Roebroek *et al.*, 1998). These human sequences are available from GenBank (Accession Nos. AF038540, AF004222 to AF004224). Geisler *et al.* (1998) also published the mouse cDNA, and these sequences are available from GenBank (Accession Nos. AF038537 to AF038530).

In this study we describe the isolation and characterization of a novel member of the RTN gene family. Our interest in this novel RTN/NSP-like gene is due to its differential expression between the neural macula and the peripheral retina as well as its chromosomal localization to 11q13.1. This area of the genome is of interest to many investigators because several genetic diseases have been linked to this area, including two with ocular implications. We are calling this gene RTN3 to reflect the newly proposed nomenclature for this gene family (Roebroek *et al.*, 1998). RTN3 corresponds to formerly designated NSP-like gene II.

## MATERIALS AND METHODS

*Isolation of PAC clones.* Human genomic PAC clones for the RTN3 gene were isolated by PCR screening by Genome Systems Inc. (St. Louis, MO). Two different sets of primers were used, 6496/6497 (3′ end) and 33/6800 (5′ end) (Table 1), and three different PAC clones were isolated, p13545, p14563, and p14564.

*PCR amplification.* The PCR amplifications were performed in a 25-$\mu$l volume containing approximately 100 ng of genomic DNA (1 ng

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template when amplifying cDNA or PACs), 25 pmol of each primer, 200 $\mu$M dNTPs, 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 units of AmpliTaq polymerase (Perkin–Elmer-Applied Biosystems (PE-ABI), Foster City, CA).

Amplification was performed in a PTC-200 DNA Engine thermocycler (MJ Research, Watertown, MA) with one cycle at 95°C for 30 s, followed by 30 cycles of a 30-s denaturation step at 95°C, a 30-s annealing step at 60°C, and a 30-s extension at 72°C, with a final extension step of 5 min. The extension times varied depending on the size of the product amplified.

*Cloning of PCR products.* PCR products generated were cloned using the TOPO TA cloning kit (Invitrogen, San Diego, CA) following the manufacturer's protocol.

*5′ Rapid amplification of cDNA ends (5′ RACE).* The 5′ RACE was performed using a previously published technique (Rodriguez *et al.,* 1994) and RTN3-specific primers 36 and 40 (Table 1).

*DNA sequencing.* Automated fluorescence sequencing was performed in a Perkin–Elmer-Applied Biosystems Model 373 instrument. The sequencing reactions were performed using an ABI PRISM Dye Terminator cycle sequencing kit following the manufacturer's protocol. In general, 0.5 pmol of template and 3 pmol of primer were used per sequencing reaction. All other details are provided in the ABI manual included in the sequencing kit. The reactions were purified using Select-D G-50 columns purchased from 5Prime–3Prime (Boulder, CO).

*Chromosomal sublocalization by PCR.* The human monochromosomal somatic cell hybrid panel 2 and the DNA from the cell lines 11943, 11936, and 07298 were purchased from NIGMS Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). PCR amplification was performed using the RTN3-specific primers 43 and 44. The cell line J1-46 was a gift from Mr. David Geyer at the Eleanor Roosevelt Institute (Denver, CO). The Stanford G3 panel was purchased from Research Genetics (Huntsville, AL).

*Southern blot analysis of human genomic DNA.* Samples containing 10 $\mu$g of high-molecular-weight human genomic DNA and 300 ng of cosmid DNA were digested with *Bam*HI, *Eco*RI, and *Hin*dIII for 16 h at 30°C. The fragments were separated on a 0.7% (w/v) agarose (0.5× TBE) gel at 1 V/cm, stained in ethidium bromide (0.5 $\mu$g/ml), photographed, and transferred to a nylon membrane. Following the transfer, the blot was prehybridized in a solution containing 6× SSC, 10× Denhardt's, and 1.0% SDS for 1 h at 68°C. Hybridization was performed at 42°C for 18 to 24 h in Hybrisol I (Oncor, Gaithersburg, MD) using a PCR product from the 3′ untranslated end of RTN3. Following hybridization, the blots were washed in 2× SSC containing 0.1% SDS for 15 min at room temperature and transferred to a solution containing 0.2× SSC with 0.1% SDS at 65°C for 2× 15 min. The blots were exposed for 6–12 h and scanned in a Storm 860 PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA).

*RT-PCR.* RNA from different human tissues (Clontech Laboratories, Inc., Palo Alto, CA) was added in saturating amounts to Dynabeads Oligo(dT) (Dynal Inc., Oslo, Norway), and cDNA was synthesized immobilized to the magnetic beads as previously described (Rodriguez *et al.,* 1994). The Dynabead-bound cDNA (1 $\mu$l) was used as template. The amplification was performed for 20 cycles, multiplexing the RTN3 and GAPDH primers (27/6496, 2499/2500; see Table 1). The PCR was performed as described above except that AmpliTaq GOLD (PE-ABI) was substituted for regular AmpliTaq.

*Northern blots.* Total RNA from human tissues was either purchased from Clontech or isolated from the human tissues using the RNazol method (Cinna/Biotech Laboratory, Friendswood, TX). Macula and peripheral retina RNA was isolated from 5-mm punches from fresh monkey retina as previously described (Schoen *et al.,* 1995).

*Quantification of Northern blots and RT-PCR.* The Northern blots were quantified using a Storm 860 fluorescence PhosphoImager (Molecular Dynamics). The 28S bands were quantified by fluorescence using SYBR Green II (Molecular Probes, Eugene, OR), and the radioactive hybridization signal was quantified using the Europium screens. The RT-PCR products were quantified using SYBR Green II (Molecular Probes). The method used is very similar to a recently published technique (Spiess and Ivell, 1999).

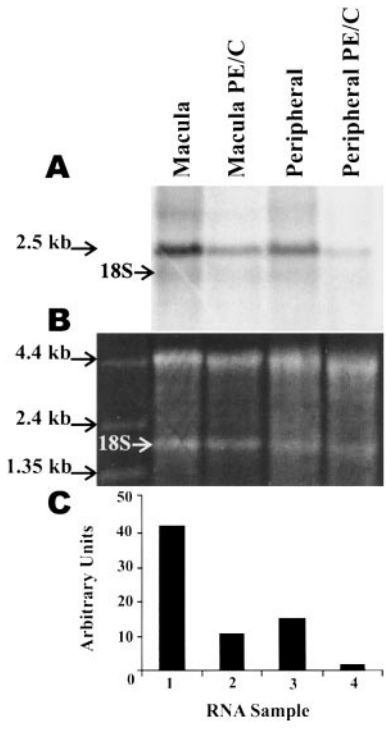



**FIG. 1.** Expression of RTN3 in monkey macula versus peripheral retina. Monkey RNA (5 $\mu$g) from the isolated macula, macula-pigment epithelium/choroid, peripheral neural retina, and peripheral retina pigment epithelium/choroid was separated by electrophoresis in a 1.2% agarose formaldehyde gel at 20–30 V for 6 h. The gel was stained with SYB green II (Molecular Probes) and then scanned using a STORM 860 apparatus. The gel was blotted and probed with a 1700-bp RTN3 probe generated by PCR using the primers 27/29. **A** shows the overnight PhosphoImager scan, and **B** shows the SYB green II stained gel. **C** shows the graphic representation of the relative signal between the RTN3 bands in **A** and the 18S band from **B.** The quantification was performed using Molecular Dynamics ImageQuant software and Microsoft (Seattle, WA) Excel software using a method similar to that of Spiess and Ivell (1999).

*Sequence alignment.* The coding sequence corresponding to exons 2 through 7 of RTN3 was aligned with NSP/RTN family members using the MEGALIGN program of the Lasergene DNAstar package (Madison, WI). Sequences shown are from the following accession numbers: NSP/RTN human, L10333, L10334, L10335; S-rex rat, U17603, U17604; S-rex chicken, U17605, U17606; and *Caenorhabditis elegans,* Z78066 (products W06a7.3a and W06a7.3b). The remaining peptide sequences were constructed from the EST database. Entries similar to NSPs or RTNs were identified by searching GenBank with BLASTN and TBLASTN (Altschul *et al.,* 1990) assembled with the SEQMAN program from Lasergene (DNASTAR) and are presented as deduced amino acid sequences. The mouse RTN3 ortholog is based on entries that include AA671994 and AA592329; human NLG-1 is from H13011 and AA354302; mouse NLG-1 is from W20705; human NLG-3 is from AA307342, AA081840, and N55351; the *Drosophila* sequence is from AA141366 and AA263788.

*Sequence comparison analysis.* A cladogram based upon the above alignment was produced with the Molecular Evolutionary Genetics Analysis (MEGA) program (Kumar *et al.,* 1994), version 1.1, using the neighbor-joining option of Saitou and Nei (1987). Genetic

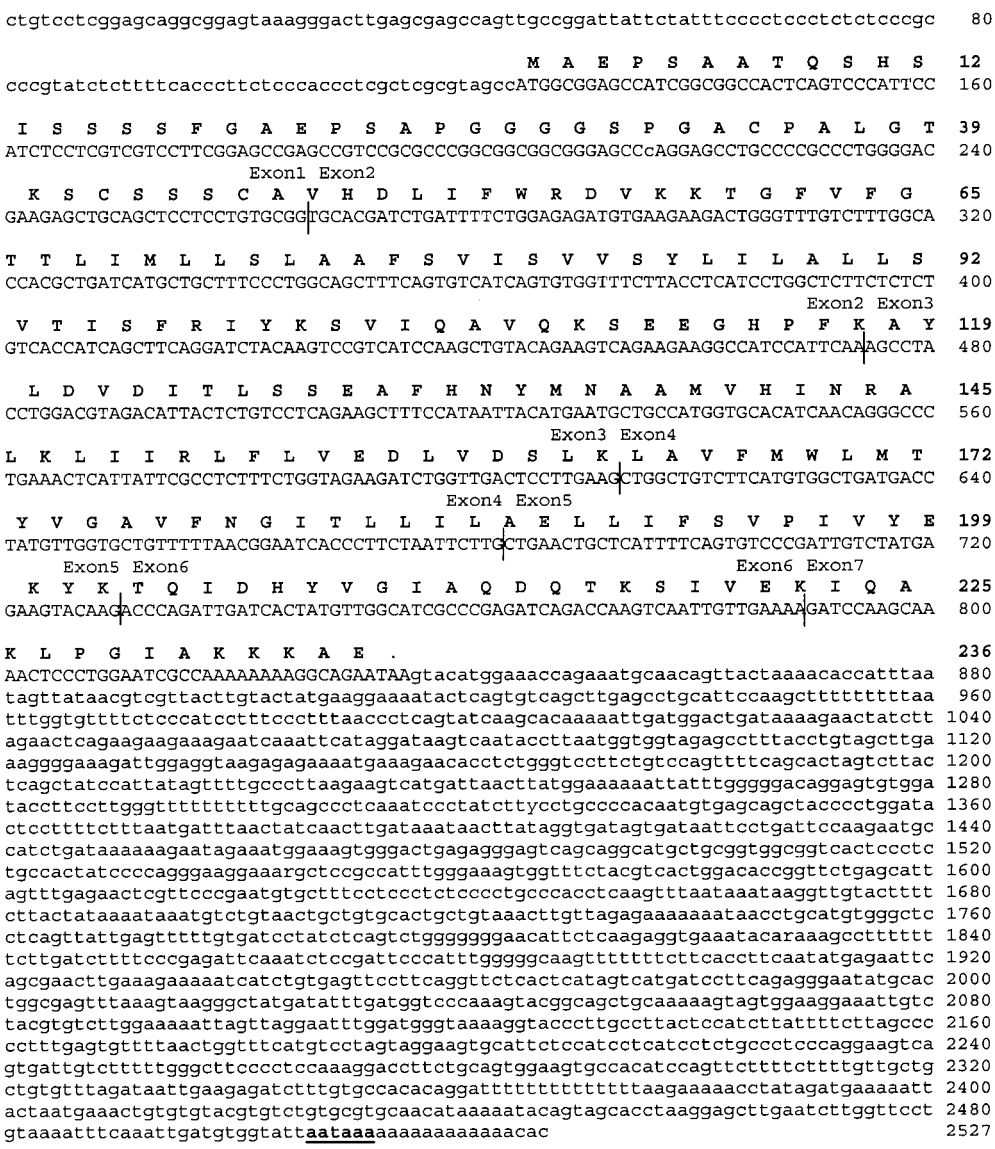



**FIG. 2.** Nucleotide and deduced amino acid sequence of the human RTN3 cDNA. The RTN3 cDNA sequence and the deduced amino acid sequence. The untranslated regions are shown in lowercase letters, and the coding region is shown in uppercase letters. The deduced amino acid sequence is shown in single-letter code above the second base of the codon. The intron locations are shown with a slash. The polyadenylation signal is underlined. Please note that the poly(A) tail is not shown and begins after the CAC.

distances were calculated from simple differences, and gaps were handled by pairwise deletion.

## RESULTS

### *Isolation of Macula-Enriched ESTs*

The RTN3 cDNA was initially isolated during a solid-phase subtraction between monkey macula and peripheral retina (Schoen *et al.*, 1995). This clone showed an approximately threefold increase in mRNA expression between macula and peripheral retina (Fig. 1). A BLAST (Altschul *et al.*, 1990) search of dbEST database identified a number of human ESTs. Several of the matching ESTs had been localized to 11q13 (Schuler *et al.*, 1996).

### *Cloning of the RTN3 cDNA*

Using primers designed from our initial isolate and the available human ESTs, we amplified the RTN3 cDNA from human retina. The human retina cDNA was synthesized on magnetic beads as previously described (Rodriguez *et al.*, 1994; Schoen *et al.*, 1995). The complete cDNA sequence was determined by sequencing the retina-derived products (Fig. 2). The retina cDNA was compared with the sequence of the gene (see below) to correct any possible sequence discrepancies introduced during the RT-PCR amplification. No sequence discrepancies were found between the cDNA and the gene. The transcription start was determined by 5′ RACE using a solid-phase protocol (Rodriguez *et al.*, 1994). The RTN3 cDNA is 2527 bp in length with

**TABLE 1**

**Oligonucleotide Sequences**

| Oligo number | Location | Sequence 5′ to 3′ |
|---|---|---|
| 22 | Exon 7 ← | TCACTGACTTCCTGGGAGGGC |
| 27 | Exon 7 → | AGGCAGAATAAGTACATGGAA |
| 29 | Exon 7 ← | CCACATCAATTTGAAATTTTACAG |
| 30 | Exon 7 → | GGAAAGATTGGAGGTAAGAGA |
| 31 | Exon 1 → | CTCGGAGCAGGCGGAGTAAAG |
| 36 | Exon 3 ← | CAGAAAGAGGCGAATAATGAG |
| 40 | Exon 4 ← | CAGCAAGAATTAGAAGGGTGA |
| 43 | Intron 2 → | ATCTTCTTCCATGTCAAATGTATG |
| 44 | Intron 3 ← | TCCTCCAAATTATAACCCAGT |
| 6496 | Exon 7 ← | AGAGGGAGGAAAGCACAT |
| 6497 | Exon 7 ← | ATAGAAATGGAAAGTGGGACT |
| 6779 | Exon 2 ← | GTGGTGCCAAAGACAAACCC |
| 2499 | GAPDH → | CCACCCATGGCAAATTCCATGGCA |
| 2500 | GAPDH ← | TCTAGACGGCAGGTCAGGTCCACC |

*Note.* This list does not include oligonucleotides used for sequencing.

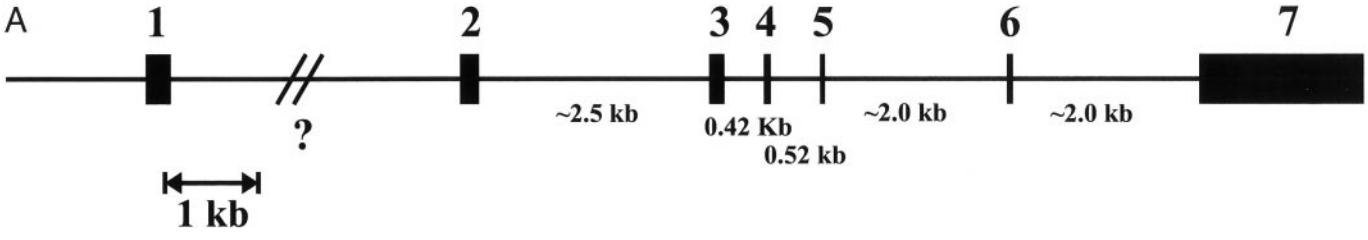
an open reading frame encoding 236 amino acids. The 3′ untranslated sequence spans from bp 836 to 2527, and a polyadenylation signal is present at basepair positions 2507–2512. The GenBank accession number for the human RTN3 cDNA is AF059524.

*Cloning and Mapping of the RTN3 Gene*

The RTN3 gene was cloned by PCR screening (Genome Systems, Inc.) using primers 6496 and 6497 (Table 1) within the 3′ end of the gene. Three PAC clones were isolated. The PAC clone p13545 was found to contain the full-length gene and was sequenced directly to determine the intron–exon junctions. The sizes of the introns were determined by direct sequence and/or by PCR amplification using exon to exon primers (Table 1). The map of the gene and the sequence across the junctions can be seen in Fig. 3. The RTN3 gene contains seven exons and spans approximately 15 kb in length. The GenBank accession numbers are as follows: AF059525 (5′ flanking, exon 1, partial intron 1), AF059526 (partial intron 1, exon 2, partial intron 2), AF059527 (partial intron 2, exon 3, intron 3, exon 4, intron 4, exon 5, partial intron 5), AF059528 (partial intron 5, exon 6, partial intron 6), and AF059529 (partial intron 6, exon 7, 3′ flanking).

The RTN3 gene was localized by PCR using a so-

A




B

| Exon No. | Size (bp) | Exon | Intron | Size (bp) | Intron | Exon | Exon No. |
|---|---|---|---|---|---|---|---|
| | | | | 5′ flanking | tgtcggagt | CTGTCCTCG | 1 |
| 1 | 266 | CCTGTGCGG | gtaaggcgc ... | ? ... | ttttgtcag | TGCACGATC | 2 |
| 2 | 208 | TCCATTCAA | gtgagttga ... | ~ 2.5kb ... | ttctggaag | AGCCTACCT | 3 |
| 3 | 139 | TCCTTGAAG | gttagttgt ... | 423 bp ... | ccttcacag | CTGGCTGTC | 4 |
| 4 | 70 | TAATTCTTG | gtaaggtgg ... | 523 bp ... | gtcttacag | CTGAACTGC | 5 |
| 5 | 47 | AAGTACAAG | gtaagcatt ... | ~ 2 kb ... | tccttaaag | ACCCAGATT | 6 |
| 6 | 59 | TGTTGAAAA | gtaagtaca ... | ~ 2 kb ... | tctcaatag | GATCCAAGC | 7 |
| 7 | 1740 | AAAAAACAC | aaactatga | 3′ flanking | | | |

C

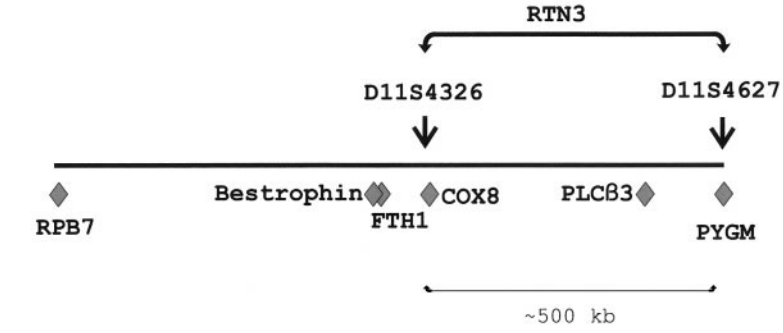



**FIG. 3.** Intron–exon junction sequence and scale map of the human RTN3 gene. (**A**) The map of the human RTN1 gene is shown to relative scale. Intron 1 is greater than 5 kb but its exact size was not accurately determined. (**B**) Sequence of the intron–exon junctions. The complete sequence is available from GenBank under Accession Nos. AF05925–AF05929. (**C**) Chromosomal sublocalization of the RTN3 gene based on somatic cell hybrids and the Stanford G3 panel.

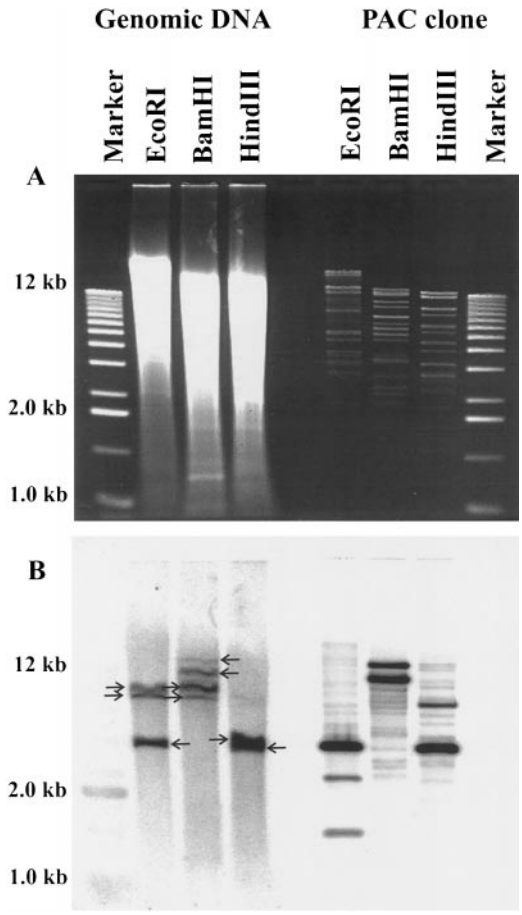



**FIG. 4.** Multitissue Northern blot analysis. Total RNA (~6 $\mu$g) from 12 human tissues was separated by agarose gel electrophoresis and blotted as described above. The blot was probed with a 1400-bp PCR product generated with oligos 30 and 22 encompassing most of the 3′ end of the cDNA. **A** shows the PhosphoImager exposure and **B** shows the graphical representation of the relative levels of the hybridization signal to the 28S SYBR Green II stained band (Spiess and Ivell, 1999).

matic cell hybrid panel (Coriell Institute), which confirmed its localization to chromosome 11, as reported for the matching ESTs in the Unigene database (Schuler *et al.*, 1996). The gene was sublocalized to 11q13.1 using the Research Genetics Stanford G3 Radiation Hybrid Panel and other somatic cell hybrids (data not shown). We used intron to intron primers across exon 3 to ensure that we were localizing the intron-containing gene and not a suspected pseudogene (data not shown). The results from the G3 panel indicate that RTN3 is located between markers D11S4535 and D11S4627 distal to the hsRPB7 gene (Schoen *et al.*, 1997). This area is approximately 500 kb in length. Our PACs suggest that RTN3 is located proximal to PLC$\beta$3 (Mazuruk *et al.*, 1995). The end sequence of one of our RTN3 PAC clones (p13545) matches a small area of the third fragment from the PAC pDJ519013 (Eugene McDermott Center for Human Growth and Development, http://gestec.swmed.edu), GenBank Accession No. AC004228. This would place the RTN3 gene at the extreme proximal end of the interval stated in Fig. 3. However, we have not been able to confirm this location with a known marker.

*Southern Blot Analysis and Pseudogene Localization*

To determine the complexity of the RTN3 gene, a Southern blot was performed using human genomic DNA and the p13545 PAC clone (Fig. 4). Since direct sequencing demonstrated that the p13545 PAC clone encompassed the intron-containing gene, the additional bands seen on the genomic DNA were suspected of originating from a pseudogene(s). Right-pointing arrows mark the bands corresponding to the suspected pseudogene(s), and left-pointing arrows mark the intron-containing gene. Other bands observed in the PAC are likely due to nonspecific hybridization due to the prolonged exposure required to obtain clear bands on the genomic DNA side of the blot. This was required because of the stoichiometrical difference between RNT3 in the genomic and PAC sides of the blot. A strategy for localizing the pseudogene(s) was devised using primers across intron 1. This intron is large and cannot be amplified with primers from exon 1 and exon 2. Using these primers, we performed the PCR analysis on the Coriell monochromosomal panel 2 that generated a product from the cell line containing chromosome 4 (data not shown). This product was purified and sequenced to verify its authenticity as RTN3. These

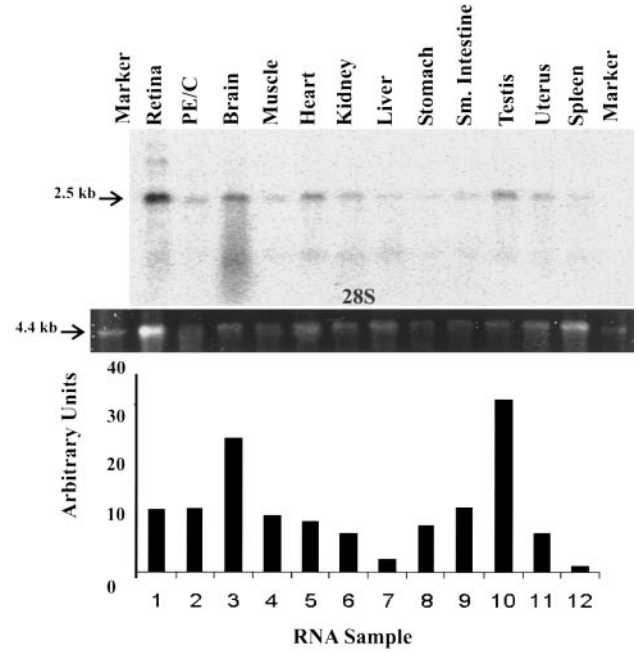



**FIG. 5.** Southern blot analysis of human genomic and PAC DNA. Human genomic DNA (10 $\mu$g) and the human PAC clone p13545 (300 ng) were digested with *Eco*RI, *Bam*HI, and *Hind*III. The digested DNA was separated by electrophoresis (200 V for 6 h) in 0.7% agarose using a CHEF DRII pulsed-field apparatus (Bio-Rad Laboratories, Hercules, CA). The right pointing arrows (→) mark the bands corresponding to the suspected pseudogene(s), and the left pointing arrows (←) mark the bands corresponding to the intron-containing gene.

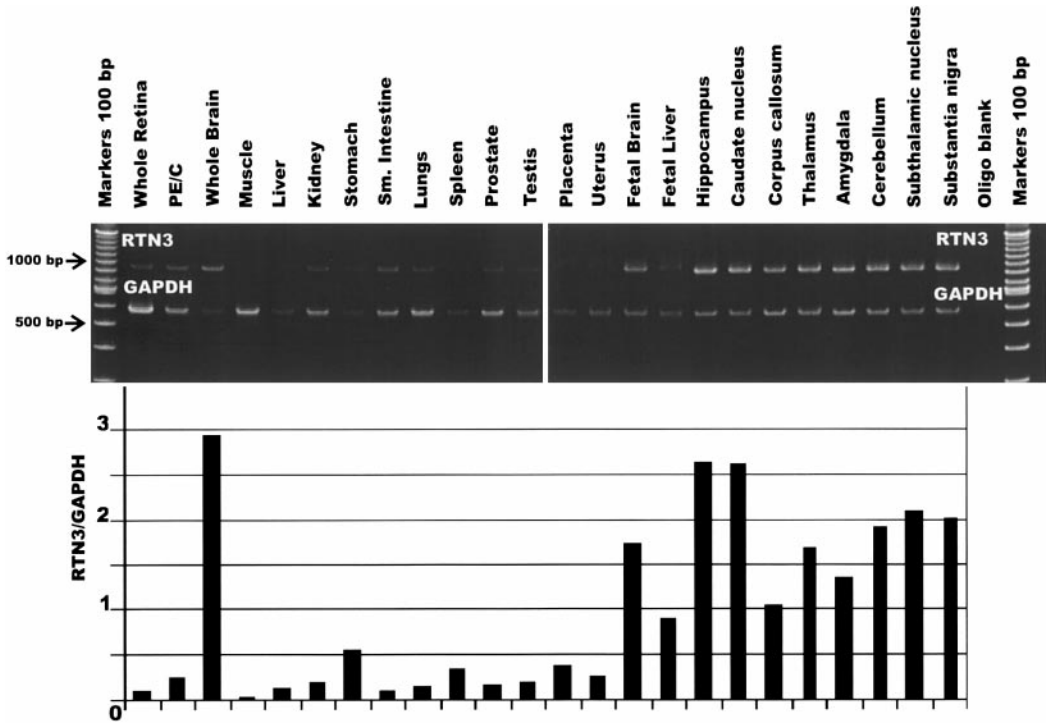



**FIG. 6.** Expression of RTN3 in human tissues by RT-PCR. The expression of RTN3 was measured by RT-PCR using cDNA from different human tissues as well as several areas of the human brain. The amplification was performed using solid-phase cDNA as template and multiplexing RTN3 (27/6496) and the GAPDH (2499/2500) primers. The products were separated in two 10% acrylamide TBE gels (Novex, San Diego, CA) that were run simultaneously at 150 V for 1.5 h in 0.5× TBE. The gels were stained with SYBR Green II, and the fluorescence intensity of the bands was measured using a Storm 860 PhosphoImager. The graph shows the ratio of the RTN3 to GAPDH.

results confirm those obtained from the Southern blot (Fig. 4) and demonstrated that RTN3 contains at least one pseudogene in chromosome 4.

*Multitissue Expression of the RTN3 Gene*

We performed Northern blot analysis on a variety of human tissues to determine the levels of expression of RTN3 (Fig. 5), using approximately 5 $\mu$g of total RNA from each tissue. The blot was probed with a 3′ untranslated RTN3-specific cDNA probe. The relative levels of expression were determined by normalizing the SYB green II stained 28S ribosomal RNA band to the signal generated by the probe using a STORM 860 instrument (Spiess and Ivell, 1999).

*Expression RTN3 in the Human Brain by RT-PCR*

RT-PCR was performed on different human tissues as well as different areas of the human brain to determine where RTN3 was expressed (Fig. 6). The primers for RTN3 and GAPDH were multiplexed, and the amplification was performed for 20 cycles. The levels of RTN3 were normalized to the levels of GAPDH based on the fluorescence measurement of the SYBR Green II stained bands on a PhosphoImager. GAPDH mRNA is known to be expressed in all human tissues at significant but somewhat variable levels. RTN3 was found widely expressed in human tissues but the levels relative to GAPDH were highest in the brain. All of the different areas of the brain show significantly greater relative expression of RTN3 (10- to 20-fold) than in the other nonneural tissues.

*Comparison of Homologs and Orthologs of the RTN/NSP-like Genes*

We constructed the sequences of the human and mouse NSP-like gene I/RTN2 (NLG-1) and human NSP-like gene III (NLG-3) from ESTs, as well as the mouse ortholog of RTN3 and a family member from *Drosophila* (distinct from another *Drosophila* family member, GenBank Accession No. DMU51048). The conserved portion of the RTN3 peptide sequence encoded in exons 2 to 7 is aligned with other family members in Fig. 7, illustrating striking similarities within the family. In this region, human RTN3 is 80% similar and 58% identical to human NSP/RTN1, and the rat (rS-Rex) and chicken (chS-Rex) orthologs of NSP/RTN1 also share 58% identity with RTN3. Among different family members, many of the substitutions are conservative, and frequently the differences define a particular peptide subfamily. For example, the human and mouse RTN3 sequences both have a phenylalanine at position 6 in the alignment, instead of the tyrosine found in every other family member. Of the vertebrate sequences, mouse and human NLG-1 peptides contain an additional sequence segment prior to the charged C-terminus. The pattern of substitutions is illustrated by the cladogram (Fig. 7B). The vertebrate NSP/RTN-like genes tend to separate from one an-

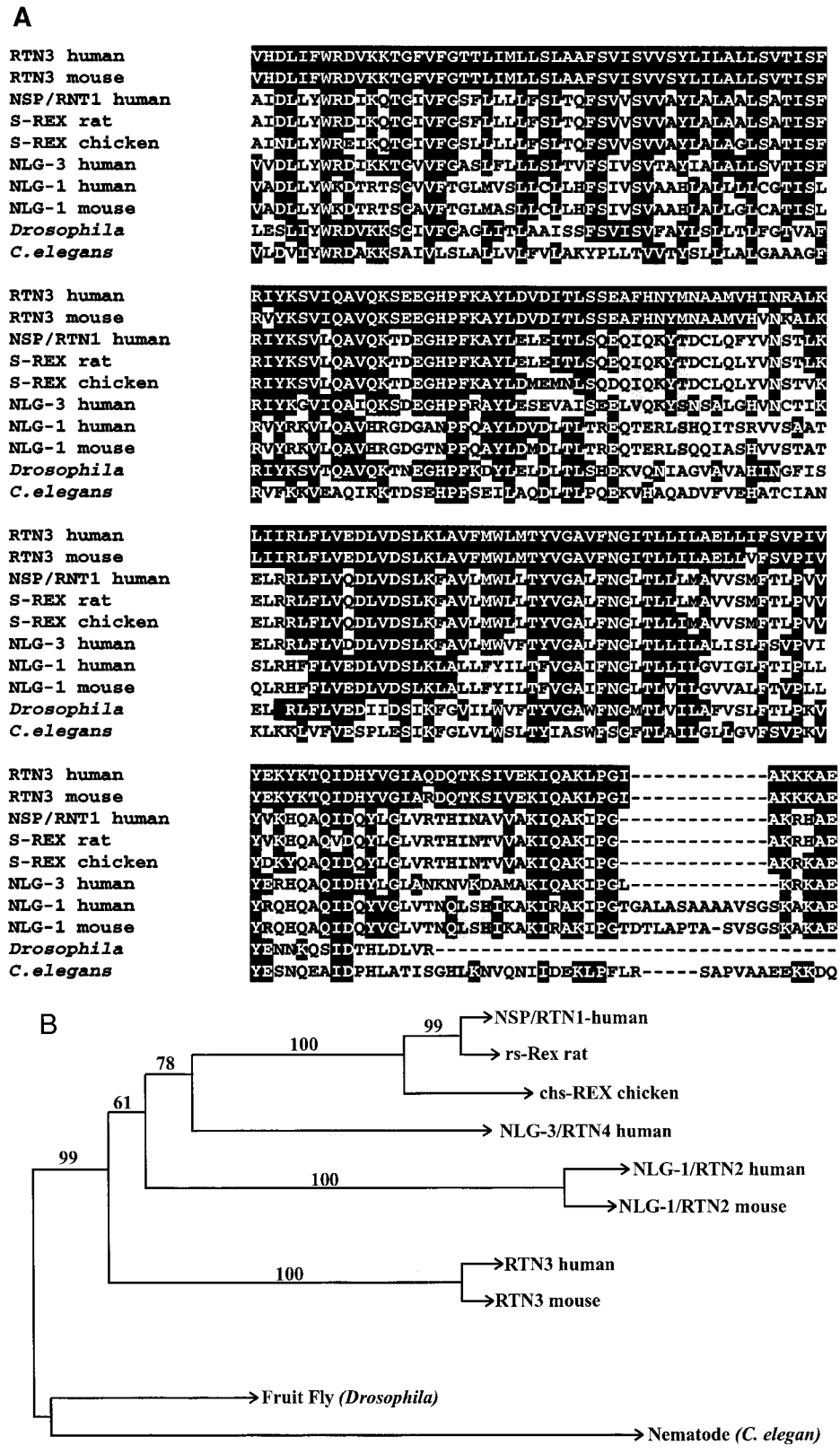



**FIG. 7.** Amino acid sequence alignment of the RTN3 and related peptides. (**A**) Alignment of C-terminus amino acid sequences of RTN3 and NSP orthologs with similar sequences of vertebrate and invertebrate origin. The portions of the peptides shown are limited to the regions of similarity that span the final six exons of coding sequences for RTN3 and the NSP/RTN genes and the final three exons of the *C. elegans* peptide. Black boxes indicate residues that are identical to the human RTN3 reference sequence. Conservative changes that are common to more than one peptide are shaded. (**B**) Cladogram of NSP-related peptide illustrates the relationships among the various family members. The numbers represent the results of 500 bootstrap replications.

other, while orthologous genes tend to associate. The mouse and human RTN3 peptides differ from each other at 7 of the 189 residues shown. The NSP/RTN-like genes from the fruit fly (*Drosophila*) and the nematode (*C. elegans*), having 63 and 50% similarity to the human RTN3, respectively, are clearly the outlying members of the group.

## DISCUSSION

The NSPs/RTNs are a group of highly conserved genes with preferential expression in neuroendocrine tissues. RTN3 is a member of this family that was of interest to us because of its differential expression between macula and peripheral retina as well as its chromosomal localization to 11q13 near the Best macular degeneration locus (Stone *et al.*, 1992). RTN3 is not mutated in Best macular dystrophy patients but a nearby gene named Bestrophin (Fig. 3C) was recently reported to be mutated in some Best macular dystrophy families (Petrukhin *et al.*, 1998).

The RTN3 cDNA is 2527 bp long with an open reading frame of 236 amino acids. The mRNA contains a relatively long 3′ untranslated region of 1692 bp. The gene is fragmented into seven exons and spans approximately 15 kb in length. Searches of the database reveal that RTN3 is related to the human neuroendocrine-specific proteins (NSPs) and the orthologous genes in rat (rS-Rex) and chicken (chS-Rex). This gene has alternatively spliced variants differing at the N-terminus; the C-terminal region is common to the variant forms and is the portion that resembles RTN3 (Roebroek *et al.*, 1993, 1996). No splice variants have been found for RTN3. Another peptide very similar to NSP/rex and RTN3 has been identified in translations of a genomic fragment from chromosome III of the nematode, *C. elegans* (Z78066). As in NSP/rex, there is alternative splicing of the N-terminal exons that results in either a short or a long N-terminal arm, and the region common to both transcripts is the portion conserved between it and the vertebrate NSP-like genes.

The gene structure of the NSP/RTN-like family of proteins is also well conserved throughout the region shown in the alignment in Fig. 7. The placement of introns, including the position of the junction within the codon, is identical in the genes for human RTN3 and NSP/RTN1 with exons 2 through 7 of RTN3 matching exons 4 through 9 of the human NSP/RTN1. The *C. elegans* gene has only three exons in this region, but two of these have junctions analogous to the mammalian junctions (the introns before exon 2 and after exon 6 of RTN3). Thus, RTN3 exon 2, NSP1/RTN1 exon 4, and the corresponding *C. elegans* exon all initiate at the second position of the codon that begins the conserved region. Similarly, the last exon of all three genes begins at the second position within the codon of the equivalent isoleucine residue.

The N-terminal segment of human RTN3, consisting of 47 (exon 1) amino acid residues, bears no resemblance to any of the N-terminal alternatives of the NSP1/RTN1 peptides, and it does not share recognizable functional motifs. It contains five sites that match the consensus pattern for N-myristoylation (Towler *et al.*, 1988) as defined by the PROSITE database. Similarly, the mouse ortholog for RTN3 contains six sites for N-myristoylation within the same 47-amino-acid-residue segment. Other PROSITE consensus sequences are conserved in the short arms of the NSP/rex orthologs: at position 7–11 of each is the overlapping of protein kinase C (Woodget *et al.*, 1986) and casein kinase (Pinna, 1990) consensus sites.

The function of the NSPs/RTNs remains elusive. While NSP1/RTN1 is an excellent marker for determining neuroendocrine differentiation in normal and malignant cells (Senden *et al.*, 1996), the precise biological role of these proteins remains unclear. In this study we show that the C-terminus sequence is well conserved between homologs and orthologs, while the N-terminus sequence corresponding to exon 1 is well conserved only among orthologs. This suggests that specific functional roles are likely maintained by each N-terminal sequence while the general conserved function is modulated through the C-terminus (Fig. 7). Possibly, the N-terminus may be serving to modify the interactions of the C-terminus sequence in the NSP/RTN family of proteins.

RTN3 is highly expressed in neural tissues, and all of the tested areas of the brain seem to show significant levels of expression (Figs. 5 and 6). RTN3 is expressed in whole retina at lower levels than in brain but a high level of expression is detected in the macular region of the retina. The relatively high expression of RTN3 in the macular region of the retina versus the peripheral retina is very interesting and may provide some insight into the fundamental functions of these two areas of the retina. Understanding the differences between these two areas of the retina is very important since the macula often degenerates with age, and macular degeneration is the leading cause of blindness in Western societies.

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