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Structure, alternative splicing, and expression of the human *RGS9* gene ☆

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Abstract

An isoform of *RGS9* was recently identified as the GTPase activating protein in bovine and mouse rod and cone photoreceptors. To explore the potential role of the *RGS9* gene in human retinal disease, we determined its exon/intron arrangement, and investigated its expression in human retina. The results show that the gene, located on 17q24, consists of 19 exons and spans more than 75 kb of genomic DNA. The entire gene was found to be contained on a single BAC clone with an insert size of 170 kb. The major transcripts of the gene are alternatively spliced into a 9.5 kb retina-specific transcript (*RGS9-1*) and a brain specific 2.5 kb transcript (*RGS9-2*). Exons 1–16 are constitutive and present in both variants. Exon 17 contains the 3' end of the open reading frame and the 3'-UTR of the *RGS9-1* variant. In *RGS9-2*, exon 17 is alternatively spliced and joined to exons 18 and 19 that are not present in the retina variant. Immunolocalization with a monoclonal antibody recognizing the retina and brain variants shows abundant expression in photoreceptors and possibly very low levels in cell types of the inner retina. Owing to the specific expression of *RGS9-1* in photoreceptors the *RGS9* gene is a candidate gene for RP17, a form of autosomal retinitis pigmentosa, located on the long arm of chromosome 17. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Chromosome 17q; Phototransduction; Regulators of G protein signaling (RGS); Retinitis pigmentosa

1. Introduction

Hydrolysis of GTP bound to GTP-binding proteins (G proteins) is a key event in intracellular signal trans-

duction (Angleson and Wensel, 1993). GTP hydrolysis, accelerated by GTPase activating proteins (GAPs), down-regulates the signal transduction pathway by deactivation of G protein subunits. GAP activity can be exerted by diverse and unrelated proteins, like phospholipase C β 1, or neurofibromin (NF1) [for review, see Scheffzek et al. (1998)]. A missense mutation in NF1, the GAP of the GTP binding protein *ras*, causes one of the most common neurodegenerative diseases, neurofibromatosis type 1 (Klose et al., 1998). Recently, a novel group of GAP proteins, termed RGS (regulators of G protein signaling) has been discovered by genetic screening in yeast and nematodes (Dohlman et al., 1995), and by homology screening in mammalian systems [for review, see De Vries and Gist (1999)]. At least 20 distinct *RGS* genes have so far been identified. The GAP in mammalian photoreceptors was recently identified as an *RGS9* variant (Hem et al., 1998), in the following

Abbreviations: BSA, bovine serum albumin; DEP, dishevelled, EGL-10 pleckstrin domain; FISH, fluorescent in situ hybridization; GAP, GTPase activating protein; GGL-domain, G protein γ -subunit-like domain; ivs, intervening sequence (intron); NF1, neurofibromin 1; NGS, normal goat serum; ORF, open reading frame; PB, phosphate buffer; RGS, regulators of G protein signaling; ROS, rod outer segment; RP, retinitis pigmentosa; UTR, untranslated region.

☆ The symbol 'RGS9' has been approved by the HUGO/GDP nomenclature committee.

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termed RGS9-1 (Rahman et al., 1999). Of multiple RGS proteins expressed in the retina, only RGS9-1 met both criteria of accelerating GTPase activity of transducin and co-localizing with other members of the phototransduction cascade (He et al., 1998). Moreover, the acceleration of GTP hydrolysis by RGS9 is enhanced by the PDE γ subunit, whereas the GAP activity of all other RGS proteins tested is insensitive to or inhibited by PDE γ . RGS9 was recently found to be tightly associated with the long splice variant of G β 5 (Makino et al., 1999).

The *RGS9* gene is expressed in the brain, and in the retina/pineal, in alternatively spliced forms (Gold et al., 1997; Granneman et al., 1998; Thomas et al., 1998). In rat, the major RNA species are a large form of 9 kb encoding the (smaller) retina RGS9-1, and a shorter version of 2.5 kb encoding the larger RGS9-2 brain variant (Granneman et al., 1998). The retina-specific bovine and mouse RGS9-1 contain 484 amino acids, whereas the larger human and rat brain forms contain approximately 675 residues. The major distinction is in the C-terminal part of the polypeptide. The retina form has a distinct 18 amino acid C-terminus, whereas the brain form has an approximately 200 residue C-terminal extension. Both variants contain the RGS domain responsible for GAP activity. To identify the molecular basis for the splice variants, and to establish the *RGS9* gene as a candidate gene for retinal disease, we cloned the human RGS9 isoforms, determined the gene structure, and characterized the expression pattern of RGS9-1 in the retina, and of RGS9-2 in the brain.

2. Materials and methods

2.1. cDNA cloning of human *RGS9*

Human RGS9-1 variants were cloned from a λ gt10 human retina cDNA library (obtained from Dr Jeremy Nathans, Johns Hopkins) using a bovine cDNA as a probe. The inserts of eight clones were amplified with λ gt10 forward and reverse primers flanking the insertion site, and cloned into pCR2.1 (Invitrogen), two of which (hrgs#1, and hrgs#10) are shown in Fig. 1B. Inserts were sequenced on both strands using a LI-COR 4000L automatic DNA sequencer with infra-red tagged universal primers. The RGS9-2 form was amplified from the library using an N-terminal primer 5'-GAA TCC ATC CAG GGG CCA GGA TGA CA (translation start bold-faced) and a C-terminal primer 5'-GGC ATC TGT CAG AGC ATC TTC CTGG, to yield the PCR9.2 clone (Fig. 1B). The short retina form was amplified with the same N-terminal primer and an RGS9-1-specific C-terminal primer 5'-GTG CAT GAC CCC ACC CCC AGG AAC GCAG, to yield the PCR9.1 clone.

2.2. Gene cloning

Introns of the *RGS9* gene were amplified with exon-specific primers (Table 1) from human genomic DNA with *Taq* (Promega) or *Expand* (Boehringer Mannheim) DNA polymerases. Amplified introns were either directly sequenced using an ABI310 capillary sequencer, or after cloning into PCR2.1 vector. *Ivs1* was too large to be amplified using genomic DNA as a template. The GenomeWalker Kit (Clontech) was used to identify the position, and the 5' and 3' ends of *ivs1* of the *RGS9* gene. Briefly, three human genomic libraries digested separately with *DraI*, *PvuII* and *SspI*, and ligated to the specially designed GenomicWalker adaptor were used as a template of two consecutive PCR reactions with adaptor primers (AP1: 5'-GTA ATA CGA CTC ACT ATA GGC and AP2: 5'-ACT ATA GGG CAC GCG TGGT) and *RGS9*-specific primers. The 5'-end of *ivs1* was amplified with APs and exon 1 sense primers 5'-GCT TCT CCT CTT GTC TCC CAC TGG and 5'-GAA TCC ATC CAG GGG CCA GGA TGA CA. The 3'-end of *ivs1* was amplified with APs and exon 2 antisense primers 5'-GGA GGT GGG CCG TAT TGG GCC TGT and 5'-CTG TCA TGG CAT GAG GAA CGC TGG TGA CCAG. The secondary PCR fragments were sequenced directly using an ABI310 capillary sequencer. The accuracy of the sequence was confirmed by amplification and sequencing of exon 1 and exon 2 with primers located in *ivs1*, *ivs2* and 5'-UTR. Intron amplification primers shown in Fig. 1A, but not listed in Table 1, are as follows: Kz34, 5'-CTT GGG GTC TTG CAG GGG GTA AATG; Kz-intron, 5'-CTC TGC ACT CCC AGG TCT TCA TGG AGA TGC; Kz37, 5'-GAG GAT CTG AAG TAT GGA GAT CAG; Kz6, 5'-TGA GCA TGT AAA TGT GGG TTT GTG CG; Kz16, 5'-TAT GCT CGC TAT TTA AAA TCT CCG. To isolate human BAC clones containing the *RGS9* gene, two synthetic oligonucleotides hrgs-e1: 5'ATG ACA ATC CGA CAC CAA GGC CAG CAG TAC AGG CCG AGG ATG (42 mer, derived from exon 1) and hrgs9-e2: 5'GAA GCG CTC GTG AAG GAC ATG CAG AAC CCA GAG ACA GGG GTC CGAA (46 mer, derived from exon 2) were labeled and used to screen a human BAC library CITBI-E1. Two BAC clones were identified: hRGS9BAC-1 with an insert size of 170 kb and hRGS9BAC-2 with an insert size of 210 kb. The hRGS9BAC-1 contained the entire *RGS9* gene.

2.3. Southern blot analysis

Hybridization with a mixture of [32 P]-labeled human hrgs#1 cDNA (1.1 kb) and hrgs#10 cDNA (2.1 kb) was performed in 40% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl, 35 μ g/ml of herring sperm DNA in 50 mM Tris, pH 7.4 at 37°C. The blots were washed at room temperature in 2 \times SSC, 0.1% SDS for

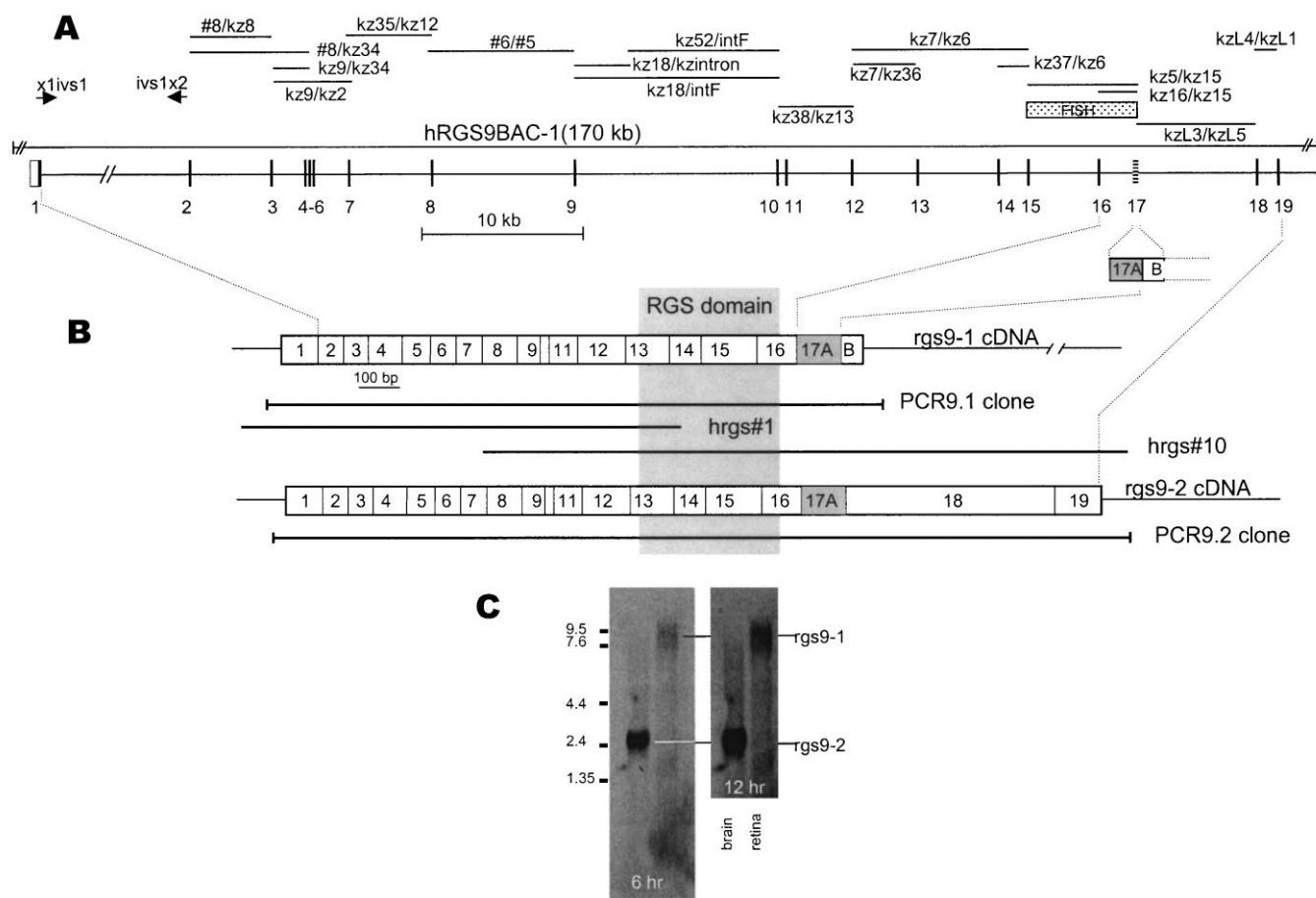


Fig. 1. Schematic gene structure and cDNA clones of the human *RGS9* gene. (A) Graphical depiction of the gene. Exons 1–19 are shown as vertical black lines, connected by introns (ivs1–18). The extent of genomic PCR fragments, amplified from human genomic DNA, and primers used for amplification are indicated above the kb scale as horizontal lines. Ivs1 was too large to be amplified with long-range PCR methods. Arrows extending from exon 1 and exon 2 indicate short fragments amplified with the Genome Walker method. The 7 kb C-terminal genomic probe used for subchromosomal localization of the *RGS9* gene is indicated as a dotted bar. The alternatively spliced exon 17 and its two parts, exon 17A and exon 17B, are shown enlarged. A single BAC clone, hRGS9BAC-1, was identified and shown to contain the entire gene. (B) Schematic representation of the two major splice variants of the *RGS9* gene. The RGS9-1 and RGS9-2 variants share exons 1–16, and exon 17A (hatched). The RGS domain is shaded. Clones hrgs#1 and hrgs#10 are partial cDNA clones. (C) Northern blot of human brain (caudate nucleus) and retina RNA. The major retina transcript is RGS9-1; the major brain transcript is RGS9-2. No expression of the retina transcript can be seen in the brain, and vice versa, by this method.

30 min followed by 30 min at 50°C in 0.1 × SSC, 0.1% SDS. A Southern blot containing *EcoRI*-digested DNA from various species was purchased from BIOS Laboratories (New Haven, CT). The hybridization and washing procedures were performed according to the manufacturer's protocol.

2.4. Northern blot analysis

mRNA was isolated from human retinal tissue, obtained from the Utah Lions Eye Bank, Moran Eye Center, using the FastTrack 2.0 mRNA kit (Invitrogen). PolyA mRNA from human caudate nucleus was purchased from Clontech. Brain and retinal mRNAs (~3 μg each) were resolved by agarose gel electrophoresis in the presence of 0.66 M formaldehyde and transferred to a nylon membrane. A [³²P]-labeled N-terminal

847 bp PCR amplified product (sense primer 5'-GAA TCC ATC CAG GGG CCA GGA TGA CA located in exon 1, and antisense primer 5'-GTC CCA GAA CTG GGT GTC ATC GGT GAT CC located in exon 12) was used to probe the northern blot. After hybridization at 37°C overnight, the blot was washed with 1 × SSC/0.1% SDS at 55°C and exposed to Biomax film.

2.5. Antibodies

The monoclonal antibody D7 was raised against bacterially expressed RGS9 antigen (Cowan et al., 1998). It recognizes an epitope encompassing the carboxyl terminal end of the RGS domain and the amino terminal end of the C-terminal. The anti-peptide antibody was raised in rabbits against the peptide CT0, CVDITQVM-SKLDRRS, corresponding to residues 462–475 of

Table 1
RGS9 gene amplification primers. Columns 2 and 4: nomenclature of primers used in Fig. 1A. Columns 3 and 5: sense and antisense primers, respectively. Columns 6 and 7: sizes of amplified PCR fragments in bp and kb, respectively. Sizes of exons are precise, sizes of introns are approximate. Sizes of exons are 1, 17A, and 19 concern coding sequences only. Column 8: GenBank accession numbers

	2	3	4	5	6	7	8
Exon 1		5'-GGCTGCCTTTCGAACTTG		5'-TCCCAGGGTCCAGGTGAGGGGCCA	0.166	57	AF178056
Exon 2		5'-GCCCTGCACAACTTTCAAAATTGTTTC		5'-GGAGGTGGCCGTATTGGCCCTGT	0.226	97	AF178057
Exon 3		5'-GGAAGGAGACCAGTCAGGC		5'-CTCCATGGAGCTTACACAAAGC	0.194	51	AF178058
Exon 4		5'-ATGAGCTCCTGCTCAGC		5'-AATCCAACCACTTGGGCC	0.279	107	AF178059
Exon 5		5'-GGGCCAAGTGGTTGGAT		5'-GACATGCCCACTACAGA	0.278	52	AF178059
Exon 6		5'-GCTTAGGGAGGCTGCAGATGG		5'-GTAGTGTGTGGAAATGAGG	0.212	59	AF178059
Exon 7		5'-GCACATGGCATAATGCTTCG		5'-ACCGAGAGGACAAGCATG	0.166	77	AF178060
Exon 8		5'-AGACACAAGTCTTCTGGC		5'-GAAAGAGACAGAGGACACC	0.174	82	AF178061
Exon 9		5'-CAGATTAAACCAAGACCCACAG		5'-GAATCCGACCTAGGCCCTAC	0.196	63	AF178062
Exon 10		5'-GGCATTCTTCTCATGGGAAG		5'-GTTTGAACATTGCACTGGTTTTC	0.248	30	AF178063
Exon 11		5'-GTGTGTAGTGGACACTGAAG		5'-TCTTCAGTCTGCAGCCC	0.230	62	AF178064
Exon 12		5'-ACAGCCTGTCAGTTGAC		5'-CTGTATGCTTTGAAGGGGTG	0.294	114	AF178065
Exon 13		5'-GGAGGAGGATTGAATTGAG		5'-GCTCTGAAACACATCTGCTGAGA	0.336	116	AF178066
Exon 14		5'-GGAATCCATCCCGTTGA		5'-CAGCCAGTACCAGGCTGTTGT	0.296	88	AF178067
Exon 15		5'-CCTCGGTAAACCGATTCTGT		5'-CTCTATCCACGTCATCTCTC	0.349	139	AF178068
Exon 16		5'-CCATTCTACTGCCAAGGG		5'-GAGACCCCTCACTTCTCTG	0.251	86	AF178069
Exon 17A		5'-GTCAGTGTGGGCAAGCT		5'-GTGCATGACCCACCCAGGAACG	0.264	172	AF178070
Exon 18		5'-GCAACTCACCACTGAGCT		5'-CATGCATACGGCACGTC	0.557	485	AF178071
Exon 19		5'-CATCTCAAGGATGTCAGGATG		5'-GGCATCTGTCAGAGCATCTTCTGG	0.273	130	AF178072
Ivs1		(not amplifiable)			>10		
Ivs2	#8	5'-GAATGCAGAACCCAGAGGGTCTCTG	Kz8	5'-GACTGGAGATCCAAAAGCCGCTG	5		
Ivs3-6	Kz9	5'-CAGCGGCTTTGGATCTCCAGTC	Kz2	5'-TGCATAATGACAAAAGTCCCACCTTATAG	5		
Ivs7	Kz35	5'-CTTTGAAACC AAAAATGAACTATAAG	Kz12	5'-GCATCGGTGCACCCAGCCAGTATGCC	5		
Ivs8	#6	5'-GGAAGGAGAGAAACAAAGCAG	#5	5'-GAATCCGACCTAGGCCCTAC	8		
Ivs9	Kz18	5'-CCTTGGAAATGGACAATGTG	intF	5'-GACAGCAAGACTGTTTCTCTGTAA	>10		
Ivs10-11	Kz38	5'-AAACAACAACCTCGTTGCTGTC	Kz13	5'-GTCCCAAGAACTGGTGTCACTGGGTGATCC 3			
Ivs12-14	Kz7	5'-CAACGATGCCATCATGCAGGCTGCCT	Kz6	5'-TGAAGCATTAATGTGGTTTTGTGCG	11		
Ivs15-16	Kz5	5'-GGGAGGAGCGCTGGATCAACATAGATGG	Kz15	5'-GTGCATGACCCACCCAGGAAGCCAG	8		
Ivs17	KzL3	5'-GCCAACAACCTGTGGACATCAGCCAG	KzL5	5'-CGGCTGCGAGGCCAGGAGGTGTCG	>8		
Ivs18	KzL4	5'-GTCACCGAGAGCAGGAGGCTCCC	KzL1	5'-GGCATCTGTCAGAGCATCTTCTCTGG	2.3		

RGS9-1, with a cysteine residue added to the N-terminus for coupling. The CT0 peptide was synthesized and purified at the Protein Chemistry Core Facility of Baylor College of Medicine. At Bethyl Laboratories, the peptide was conjugated to hemocyanin and used to prepare antibodies by standard immunization and peptide-affinity purification procedures.

2.6. Western blot analysis

Human eyes were obtained from the Utah Lions Eye Bank, Moran Eye Center, University of Utah. Both retina and RPE were carefully excised from each eye and sonicated in $1 \times$ PBS/1 mM PMSF. Total human brain protein was obtained from Clontech. Brain and retina proteins were electrophoresed on a 12.5% gel and electroblotted onto nitrocellulose. RGS9 proteins were detected with the RGS D7 monoclonal antibody (1:500), which recognizes both the retina and brain variants, or the RGS9-1 polyclonal antibody anti-CT0 (1:500) using the ECL detection kit (Amersham Pharmacia Biotech).

2.7. Immunocytochemistry

After corneal excision, human eyes were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (PB), pH 7.3, overnight at 4°C. The retina was removed after fixation, and small sections were embedded in 4% low melting point agarose in 0.1 M PB for vibrotome sectioning. 100 μ m sections were preincubated for 1 h in 10% normal goat serum (NGS) diluted in ICC buffer (PB containing 0.5% BSA, 0.2% Triton X-100, and 0.05% sodium azide, pH 7.3). ICC buffer was used for the dilution of all antibodies and for all subsequent washes. Sections were incubated with either the RGS D7 monoclonal antibody (1:1000) or the anti-CT0 RGS9-1 polyclonal antibody (1:1000) overnight at room temperature. Negative control sections were also run in which the monoclonal RGS D7 or polyclonal RGS9-1 antibodies were preincubated with 25 μ g/ml of their respective peptides or recombinant proteins. After washing, the sections were incubated in FITC-conjugated goat anti-mouse or FITC-conjugated goat anti-rabbit antibody (Vector Laboratories) at a concentration of 1:100 in ICC buffer. For the double labeling experiment, an FITC-conjugated goat anti-rabbit secondary antibody was used for detecting RGS9-1 and Rhodamine-conjugated goat anti-mouse secondary antibody was used to detect RGS9 in sections that were previously hybridized with both primary antibodies at the above concentrations. Sections were mounted on slides and immunofluorescence was recorded by LSM 510 confocal microscopy (Zeiss, Germany). Human brain (striatum) slices were obtained at autopsy and fixed as described for retina.

2.8. Chromosomal localization and FISH

Using human–hamster somatic cell hybrids (BIOS Laboratories) as templates, the chromosomal location of the human *RGS9* gene was identified by PCR with primer 5'-GGG CGA GGC GCT GGA TCA ACA TAG ATGG and antisense primer 5'-CAC AGC TCT ACA GAG TTC TCT AGCC, specific for the 5' end of *ivs17*). The PCR amplification was performed as previously described (Li et al., 1998). For subchromosomal localization, a 7 kb genomic fragment comprising exons 16 and 17 (Fig. 1) was labeled with biotin-14-dATP using the BioNick labeling system (Life Technologies). The probe was hybridized to pro-metaphase chromosomes prepared from chromosomal normal peripheral blood lymphocytes as previously described (Li et al., 1998).

3. Results

3.1. *RGS9-1* cDNA

Only two of eight isolated partial cDNA clones (*hrgs#1* and *hrgs#10* in Fig. 1B) isolated from the retina library carried uninterrupted exon sequences. All others (not shown) had significant amounts of incomplete intron sequences at various positions indicative of incomplete splicing of the mRNA and a complex gene structure. Although the exon/intron distribution conforms to the proposed gene structure (see below), the physiological significance of these variants is unknown. To verify that both *hrgs#1* and *hrgs#10* clones originated from the same transcript, a complete *RGS9-1* coding fragment (PCR9.1 clone, 1.5 kb, Fig. 1B) was amplified with N-terminal and C-terminal primers. The sequence of this fragment corresponded exactly to *RGS9-1*. Northern blotting (Fig. 1C) indicated that the major transcript in the retina is a 9.5 kb mRNA encoding *RGS9-1*, and in the brain a 2.5 kb mRNA encoding *RGS9-2*. To explore whether the retina library contains the brain variant, we amplified and sequenced the complete coding region of *RGS9-2* (2.1 kb) with an N-terminal primer and a C-terminal primer. We found that the brain variant was present, but at much lower levels than the retina variant, and that the sequence of the amplified fragment corresponded to *RGS9-2*, except for a splice variation of 9 bp at the 3' end of exon 6 (Fig. 2 and Table 2) present in some retina and brain variants (Granneman et al., 1998). These results indicate that the primary *RGS9* transcript is processed into several mRNA variants. In retina, the major variant is *RGS9-1*. *RGS9-2* is not detectable by western and northern blotting in this tissue, suggesting that it is present at very low levels.

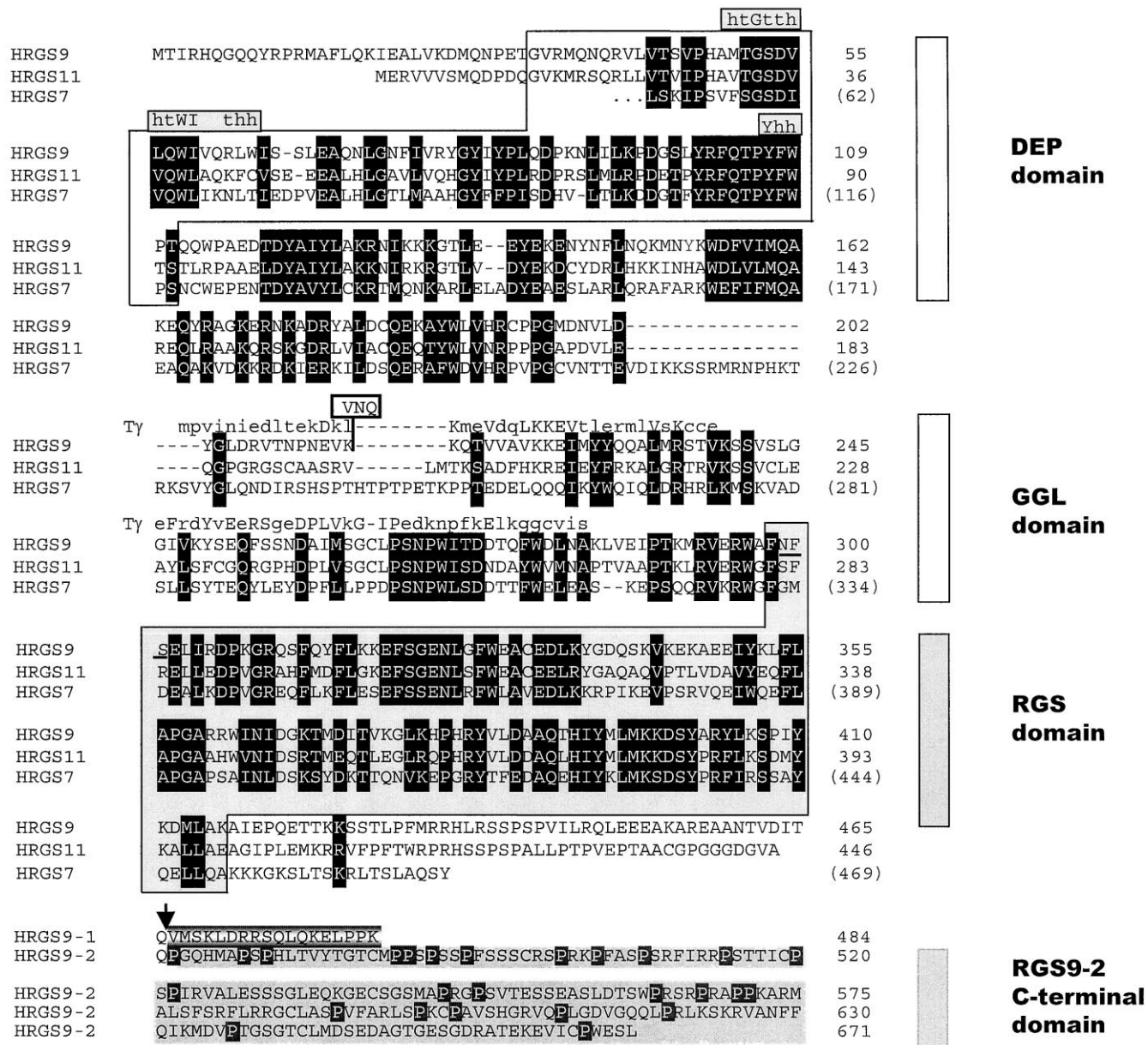


Fig. 2. Sequence alignment of human RGS9-1, RGS11, and RGS7 amino acid sequences. The sequences were retrieved from the GenBank using the following accession numbers: hRGS7, U32439 (Koelle and Horvitz, 1996); hRGS9-2, AF071475 (Granneman et al., 1998); hRGS11, (Snow et al., 1998b). The alignment was generated by PC/Gene, exported and manually processed. The RGS9-1 and RGS9-2 sequences are identical upstream of the junction of exon 17A and exon 17B (vertical arrow). A box 'VNQ', located in the GGL domain, shows the point of insertion of nine nucleotides at the 3' end of exon 6 in some splice variants (Granneman et al., 1998). The RGS9-1 C-terminus is both underlined and overlined. The specific RGS9-2 C-terminus is shaded, and proline residues in this region are marked white on grey. The DEP domain (Ponting and Bork, 1996; Shaw, 1996) in the N-terminal region of RGS proteins is boxed. Key residues conserved in a variety of other proteins involved in signaling pathways are indicated above the RGS9 sequence [nomenclature according to Ponting and Bork (1996)]. The GGL domain is identified by sequence similarity to the transducin γ subunit ($T\gamma$), shown in lower case letters above RGS9 (matches to RGS sequences in caps). The RGS domain is shaded gray, and the putative NFS glycosylation site is underlined.

3.2. The RGS9 gene is alternatively spliced

The structure of the gene encoding the retina and brain variants was elucidated by amplification of introns with exon-specific primers, generating overlapping fragments throughout the gene. The proposed gene structure (Fig. 1A) was verified by amplification of all exons with

intronic primers (Table 1), and by identification of a single BAC clone containing the entire gene. The results show that the RGS9 gene (Fig. 1A) is split into 19 exons. The sizes of the 18 introns vary from 196 bp (ivs4) to over 10 kb; ivs1 was found to be too large to be amplified. The splice junctions conform to the donor/acceptor consensus sequences except for the splice

Table 2

RGS9 gene splice junctions. The three amino acids (VNQ) contained in some splice variants are shaded. The rare GC splice donor sequence in ivs16 is printed white on black. The junction of exons 17A/17B is identified by an arrow

splice donor	splice acceptor	intron size (kb)
1 TTTCTCCAAAAGgtaaacctggcc.....ivs1.....tctgtcttgcagATTGAAGCGCTC F L Q K I E A L		>10
2 ATGCCATGACAGgtgtgatagctt.....ivs2.....ctcttttttcagGAAGTGATGTTTC H A M T G S D V		5
3 TCTCCAGTCTGGgtgagagctcat.....ivs3.....tgcttttccagAGGCACAGAACT I S S L E A Q N		2
4 TACAGATTCAGgtgagtcttggc.....ivs4.....tttcttttgcagACACCGTATTTTC Y R F Q T P Y F		0.196
5 ATACCGATTACGgtaaatacttca.....ivs5.....tttcttttccagCCATCTATCTGG D T D Y A I Y L		0.263
6 GAATATGAAAAGgtatggaggtgc.....ivs6.....tttctcttttagGAAAATTACAAT E Y E K E N Y N		2.2
7 AGAGCAGTACAGgtgagtgaagg.....ivs7.....ttggctttccagGGCTGGAAGGA E Q Y R A G K E		5
8 CACCGATGCCCTgtgagtatcctc.....ivs8.....tattccatttagCCTGGAATGGAC H R C P P G M D		8
9 AATGAAGTCAAGgtaaacaggtatgtctctgc.....ivs9.....tttgtcttacagAAAACAAACAGTC N E V K v n q K Q T V		>10
10 GTCAAAAAAGAGgtaattagtctt.....ivs10.....tgctctttccagATCATGTATTAC V K K E I M Y Y		0.85
11 GTCCCTGGGAGgtatgtccctta.....ivs11.....tttcttttccagGATTTGTGAAATA S L G G I V K Y		4
12 AAATGCCAAATTgtatgtatttta.....ivs12.....tcatccttgcagGGTGGAAATCCC N A K L V E I P		3.8
13 AAGAATTCAGTgtgggtctttgt.....ivs13.....ctcaccatgcagGAGAGAATCTGG K E F S G E N L		5
14 GGAGATTTACAagtgagcatcagc.....ivs14.....ctcccacccagGCTGTTCTGGC E I Y K L F L A		2
15 CTCATGAAGAAGgtagggtgggtcc.....ivs15.....tggtcccactagGATTTCTTATGCT L M K K D S Y A		4
16 CACCAAGAAAAGgcagtggaatt.....ivs16.....ccttcttccagCTCCACCTCCC T K K S S T L P		2.8
17A GACATCACCCAGgtcatgagcaag.....ivs17.....ccttctctacagCCGGGCCAGCAC D I T Q v m s k P G Q H		>8
18 GAGAGTAGCAAAGtaagaaccgca.....ivs18.....ttgcacctgaagCTTTTCCAGAT R V A N F F Q I		2

donor site of ivs16, which contains a GC instead of GT (Table 2). Although rare, over ten GC splice donors have been identified among 1800 donor sites, and the retina-specific ABCR gene contains two examples among 49 donor sites (Allikmets et al., 1998). The sequence of the 5' end of ivs9 revealed the presence of an alternate internal donor site that gives rise to an insertion of 9 bp (encoding VNQ, see Table 2, Fig. 2, and Section 4) in some mRNAs. The 19 exons of the *RGS9* gene, ranging in size from 30 bp (exon 10) to several hundred bp (exons 1, 18, and 19), are distributed over more than 75 kb, but less than 170 kb of genomic DNA, as limited by the insert size of the hRGS9BAC-1 clone. Gene sequencing showed that the retina variant *RGS9-1* is encoded by exons 1–17 (Fig. 1), whereas the brain variant *RGS9-2* is encoded by exons 1–16, a short 112 bp segment of exon 17 (17A, Fig. 1), and exons 18 and 19. Thus, exons 1–16 encoding the N-terminal half and the RGS domain of the polypeptides are constitutive and present in both variants. Exon 17 is alternatively spliced in a cell-specific manner and its two parts, exon 17A and exon 17B, are distinctly used in different neurons to generate the two different mRNA variants. In photoreceptors, both parts of exon 17 serve as a terminal coding exon, whereas in brain neurons 17B is

considered as an intronic sequence and removed by splicing. The junction of exons 17A and 17B (Table 2) conforms to the donor/acceptor consensus sequences for splicing (Breitbart et al., 1987).

3.3. *RGS9-1* and *RGS9-2* polypeptides

The ORFs of *RGS9-1* and *RGS9-2* cDNAs predict polypeptides of 484 and 671 amino acids respectively (Fig. 2). The two polypeptides share the DEP (dishevelled, EGL-10, pleckstrin) (Ponting and Bork, 1996), GGL ('G protein γ -subunit like') (Snow et al., 1998a), and RGS domains (Fig. 2) encoded by exons 1–16. Owing to alternative splicing of the gene, the *RGS9-1* and *RGS9-2* variants are distinct in their C-termini. The *RGS9-1* variant has an 18 residue C-terminus (which is a part of the epitope for the anti-CT0 antibody) specific for the retina form. The *RGS9-2* variant has 205 residues of extended C-terminus that are rich in prolines (Fig. 2). The alternative splice donor site of exon 6 generates an insertion of three residues VNQ in some retina and brain *RGS9* variants. There is no known post-translational modification in either *RGS9* isoforms, although there is an NXS consensus sequence for N-glycosylation at position 299–301 that is conserved in mammalian

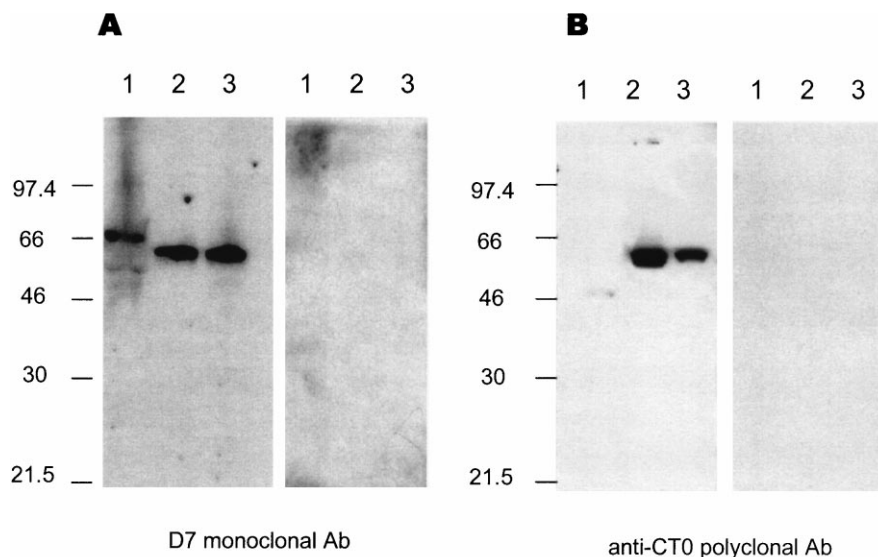


Fig. 3. Western blots of whole brain protein and human retina lysate. Lane 1: brain protein medley (Clontech); lane 2: 72 years old retina; lane 3, 5 years old retina. (A) The two blots were probed with a monoclonal antibody D7 specific for both RGS9 variants (left) or antibody preabsorbed with 25 $\mu\text{g/ml}$ of recombinant RGS9 polypeptide (right). (B) Western blot identical to (A), but probed with the polyclonal anti-CT0 antibody (left), or with antibody pre-absorbed with CT0 peptide. Size standards (BioRad) for polypeptides are indicated on the left.

species (Fig. 2). The RGS9-1 protein carries a large excess of positively charged amino acids, and the predicted isoelectric point pI is greater than 9.5. The native protein is membrane associated, but the amino acid sequence lacks known transmembrane or lipid modification motifs. It is likely that clusters of positive charges promote membrane binding by direct interaction with negatively charged phospholipid head groups (Resh, 1994), as suggested by the finding that some RGS9-1 can be removed from membranes by high salt, and nearly all can be removed by sodium carbonate pH 12. Preliminary results suggest that the carboxyl terminal may play a critical role in membrane binding (W.H. and T.G.W., unpublished results).

3.4. Expression of RGS9-1 and RGS9-2 in the retina and brain

Western blots of human retina tissue (5 years old and 72 years old individuals) and human brain extracts were carried out with a monoclonal antibody specific for both variants, and a polyclonal antibody specific for the retina variant. The results show the predominant RGS9-1 polypeptide of the expected size (58 kDa) in retina but no detectable trace of the brain variant (size about 70 kDa) (Fig. 3). Conversely, western blots with whole brain extracts detected the brain variant of approximately 70 kDa, which is predominantly expressed in the striatum. In brain extracts, the 58 kDa retina variant was not detectable by this method, and no 70 kDa RGS9 protein was detectable when the anti-peptide polyclonal antibody was used. For both antibodies, prior incubation with the respective antigen

(25 $\mu\text{g/ml}$) resulted in complete loss of signal on western blots.

3.5. Immunolocalization of RGS9 variants in human retina and striatum

Further characterization of RGS9 variants within the retina was possible through immunocytochemical analysis of human retinal tissue utilizing both polyclonal and monoclonal RGS9 antibodies. A monoclonal antibody raised against bacterially expressed RGS9, specific for both the retina and the brain variant, was used for immunostaining. In bovine retina, this antibody stained exclusively rods and cones, predominantly the outer segments, the perinuclear cytoplasm, and the axon and synaptic terminals (Cowan et al., 1998). The immunostaining in human rods and cones was similar to that of the bovine retina, cones being strongly stained and rods much more weakly stained (Fig. 4D and E). Cell bodies also showed some intense punctate staining. In addition to photoreceptors, we also observed weak staining in cell types of the inner retina, possibly dopamine-rich amacrine cells (Fig. 4I). When an anti-peptide antibody specific for the retina variant was used, RGS9-1 was more specifically immunolocalized to the outer segments of cones and rods (Fig. 4A and B), suggesting that staining of RGS9 within the INL may, in fact, represent the brain variant isoform. Double labeling experiments using both antibodies on human retinal tissue clearly showed cross-staining (Fig. 4G and H) predominantly within the photoreceptor outer segments, especially cones. Immunostaining with D7 mAb was observed in both putamen (Fig. 4J and K) and caudate basal ganglia (not

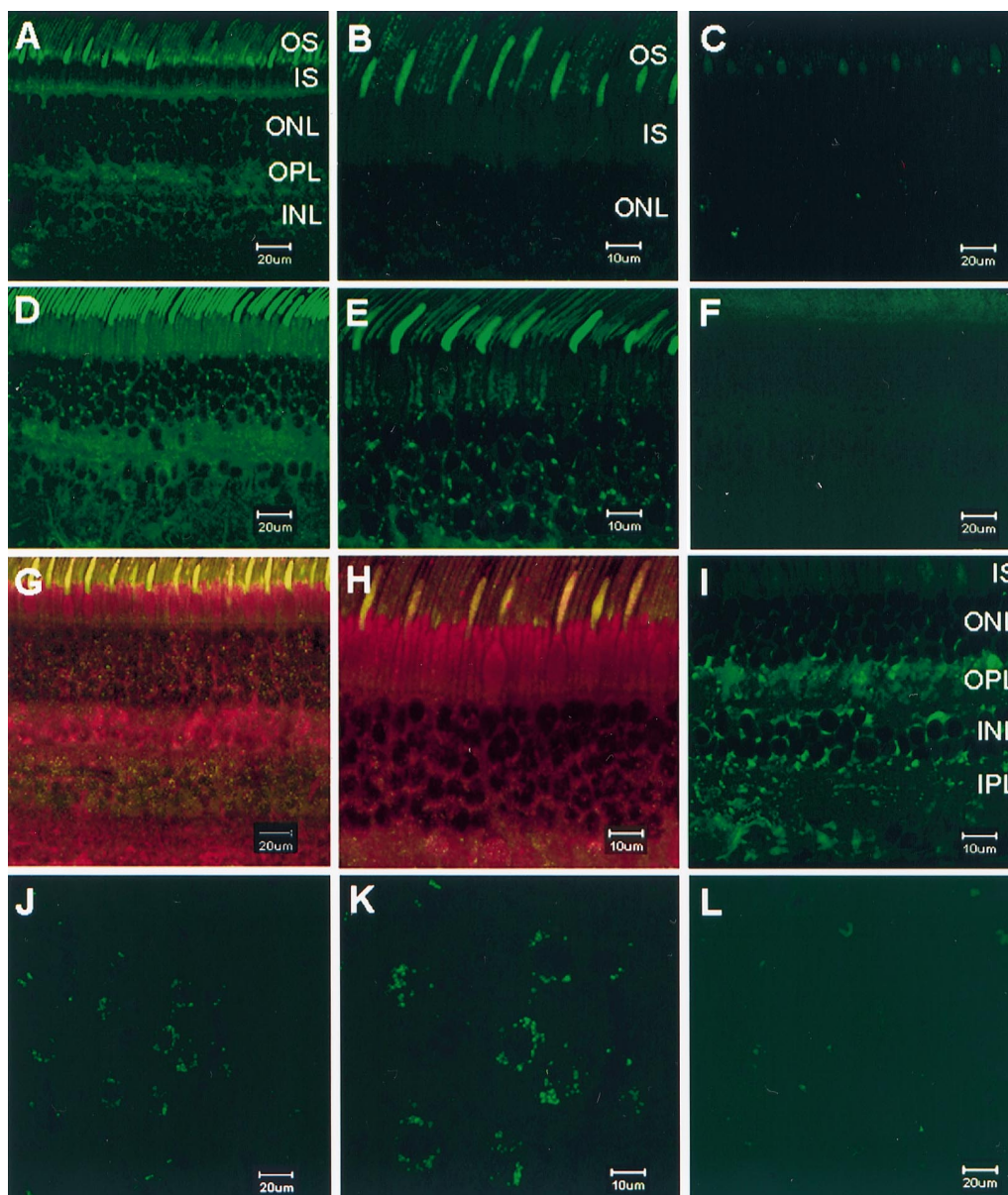


Fig. 4. RGS9 immunostaining in the human retina (A)–(I) and striatum (J)–(L). (A)–(C) Immunolocalization of the RGS9-1 isoform (anti-CT0 polyclonal antibody) in rod and, more intensely, in cone outer segments of photoreceptor cells in the human retina. (D)–(F) Immunolocalization of RGS9 in the human retina using the D7 monoclonal antibody, which recognizes both variants of RGS9. Staining is apparent in both photoreceptor and inner nuclear layers. (G)–(H) Colocalization to photoreceptor outer segments of the RGS9-1 isoform by both antibodies. RGS9-1 is detected only in the outer segments when double labeled (yellow signal) with FITC (RGS9-1) and rhodamine (D7) (red signal). (B), (E) and (H) are higher magnifications of immunolocalization with the RGS9-1 antibody, D7 monoclonal antibody and double labeling with both antibodies respectively. (C) and (F) are negative controls in which the RGS9-1 and D7 antibodies were first incubated with their respective antigens (25 μg/ml) prior to immunostaining. (I) Immunolocalization of an RGS9 isoform with the monoclonal D7 antibody in the inner nuclear layer. (J) Immunolocalization to vesicles associated with interneuron cell bodies of the putamen using antibody D7. (K) Higher magnification of this region. (L) No staining for the RGS9-1 isoform is apparent using the anti-CT0 RGS9-1 antibody.

shown) as clusters of vesicles surrounding interneuron cell bodies. However, immunostaining for the RGS9-1 variant (CT0 antibody) was absent (Fig. 4L), suggesting that the staining in brain is exclusively due to the RGS9-2 variant. These results show that the RGS9-1 retinal isoform resides specifically within outer segments of rods and cones of the human retina, consistent with its known function in phototransduction.

3.6. Genomic Southern blot and chromosomal localization

The *RGS9* gene was found present in diverse mammalian species, as judged by genomic Southern blotting under stringent hybridization conditions (Fig. 5A). Under these conditions, the *RGS9* gene was only weakly detectable in some species, e.g. hamster, dog and cow.

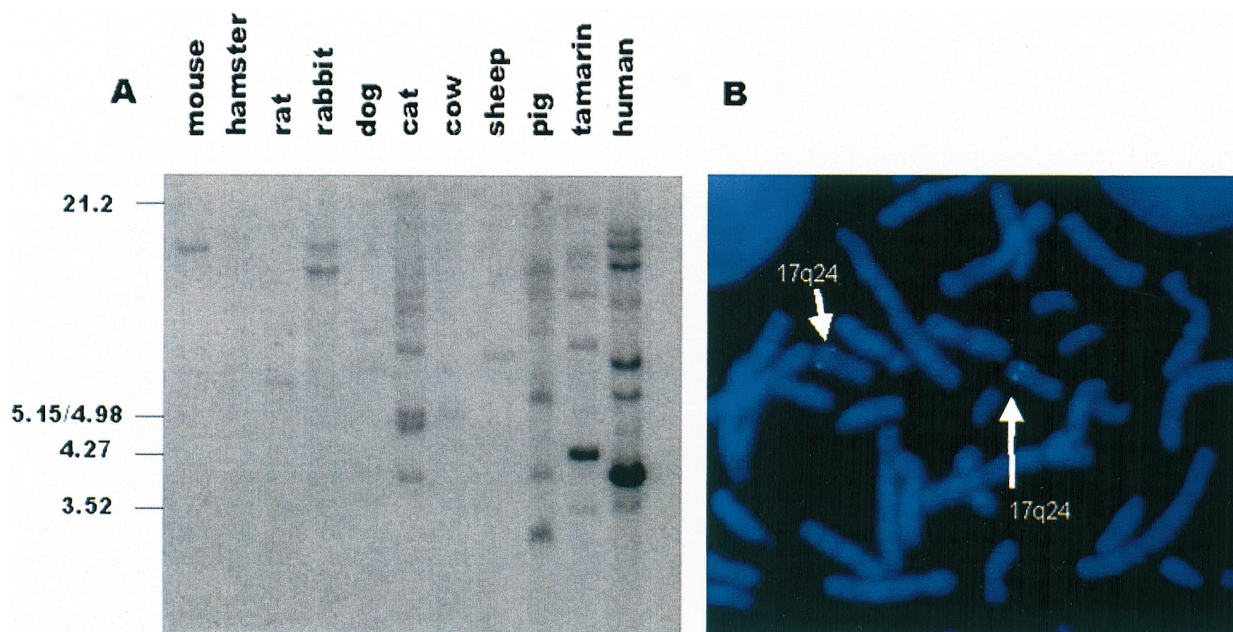


Fig. 5. Multiple species Southern blot and chromosomal location of the human gene. (A) Southern blot of mammalian genomic DNA digested with *EcoRI*. The hybridization probe was a mixture of radiolabeled *hrgs#1* and *hrgs#10*. Size markers are shown on the left. (B) FISH using a 7 kb genomic probe comprising exons 15, 16 and 17 (Fig. 1A).

The mouse *RGS9* gene appeared to be contained in a single *EcoRI* fragment of less than 15 kb in size. In human, the banding pattern of *EcoRI*-digested DNA is complex, consisting of at least ten fragments, consistent with the large size of the gene (over 75 kb). Amplification of a portion of exons 16 and 17 of the *RGS9* gene from human–hamster somatic hybrid panels located the gene to chromosome 17. The *RGS9* gene locus was fine-mapped by FISH on banded metaphase chromosomes to 17q24 (Fig. 5B). This result is identical to a recently published locus for the *RGS9* gene on 17q23–q24 (Granneman et al., 1998).

4. Discussion

The *RGS9* gene spans more than 75 kb of genomic DNA; thus, it is much larger than another *RGS* gene expressed in the retina (*RGS-r* of *RGS16*), which consists of only five exons contained in less than 5 kb (Snow et al., 1998b). By gene analysis, we provide a mechanism for the generation of two major variants, *RGS9-1* and *RGS9-2*, by alternative splicing. The two mRNAs generated in different tissues are very different in size, the retina version of 9.5 kb and the brain version of 2.5 kb (Fig. 1), similar to sizes reported in tissues of other species. Since the coding sequences are only 1452 and 2013 bp in size, at least the retina mRNA variant must carry significant portions of untranslated sequences to account for the size of 9 kb. Inspection of the 3'-UTR of exon 17 revealed the presence of a polyadenylation

signal approximately 1 kb downstream of the translational stop of *RGS9-1*, and inspection of exon 19 identified a signal approximately 300 bp downstream of the *RGS9-2* stop codon. The polyA signal on exon 19 is consistent with the length of the *RGS9-2* mRNA (2.5 kb), whereas the large *RGS9-1* RNA is most likely generated by other signals further downstream. A large 5'-UTR is much less likely, but it cannot be excluded since the transcription start point has not been determined. The use of an alternative site of polyadenylation precluding inclusion of downstream exons in the *RGS9* gene is reminiscent of mechanisms used to generate calcitonin and calcitonin gene-related peptides in the brain (Lou et al., 1998), or the membrane spanning and secreted forms of IgM (Colgan and Manley, 1997), among others.

PCR amplification suggests the existence of small and large forms of *RGS9* variants in the retina. Comparing our sequences and sequences obtained for *RGS9* by other groups (Granneman et al., 1998), both forms appear to occur again in two variants, with or without an insertion of three amino acids (VNQ) due to alternative splicing at the 5' end of *ivs9* (Fig. 2). None of the retina cDNA clones shown in Fig. 1 contained this insertion, and functional consequences of this exon expansion are not known. The three amino acids are inserted in the GGL domain (between DEP and RGS, Fig. 2) that has recently been associated with sequence similarity to G protein γ -subunits (Snow et al., 1998a). This domain has been suggested to mediate binding to specific G β subunits, and appears relatively variable

among different RGS proteins (Fig. 3), perhaps reflecting different cell-specific GAP activities.

The full-length human RGS9-1, as cloned from the retina, consists of 484 amino acids encoded by 17 exons, the larger RGS9-2 variant by 19 exons. Exon 17 is alternatively spliced in a cell-specific manner. In photoreceptor RGS9-1 mRNA the entire exon 17 is present, and the RGS9-1 polypeptide is terminated at a translation stop codon 54 bp downstream of the junction between exons 17A and 17B. The exon/intron arrangement and cell-type specific RNA processing of exons 16–19 we have found in human are very similar to that in mouse (Rahman et al., 1999). Use of the brain intron as a retina exon gives rise to a specific C-terminus VMSKLDLRRSQLQKELPPK for RGS9-1 not contained in RGS9-2. An antibody raised against a peptide containing the first nine residues of this sequence was found to recognize RGS9-1, but not the RGS9-2 polypeptide in western blots (Fig. 3). Immunolocalization (Fig. 4) of RGS9-1 with this antibody shows the exclusive expression of RGS9-1 in photoreceptors. The staining is much stronger in cones, consistent with results in bovine (Cowan et al., 1998). It is thought that the higher concentration of RGS9-1 in cones may in part be responsible for the faster response time of this photoreceptor cell type.

The RGS9-2 variant in rats was found expressed in the striatum, hypothalamus and other brain regions rich in dopaminergic neurons (Gold et al., 1997). Similarly, we found that the human RGS9-2 isoform is expressed in putamen and caudate basal ganglia, whereas the RGS9-1 variant is not present in these striatal regions. Interestingly, the staining pattern for the RGS9-2 variant is similar to that shown for the D2 long isoform of the dopamine receptor as prominent clusters surrounding interneuron cell bodies of the striatum (Khan et al., 1998). The full-length polypeptide, as cloned from the retina, contains 471 amino acids, shares the DEP, GGL, and RGS domains with RGS9-1 (exons 1–16), and carries an extended C-terminal region of approximately 200 amino acids encoded by two distinct exons (exon 18 and exon 19, Fig. 1). This region is rich in prolines (Fig. 2), and is suspected in neuron-specific functions in the brain. When expressed transiently in tissue culture, RGS9-2 was found to suppress G_i mediated signaling in whole cell recordings, probably by acting as a GAP (Granneman et al., 1998). When a monoclonal antibody that does not distinguish the two RGS9 variants was used for immunolocalization in the retina, weak staining was observed in neurons of the inner retina (Fig. 4). This staining may reflect RGS9-2 in a subset of neurons, possibly dopaminergic cells as observed in the brain (Granneman et al., 1998), or cross-reactivity specific for this antibody.

The location of the *RGS9* gene on chromosome 17q24, and the specific expression of the RGS9-1 variant

in photoreceptors, together with the importance of RGS9-1 for down-regulation of the phototransduction cascade, suggest the *RGS9* gene as a potential candidate gene for inherited retina disease. Several disease loci implicated with RP have been identified in this region. A locus for adRP (RP17) has been mapped to 17q22 in a South African kindred for which PDE γ and TIMP2 were excluded (Bardien et al., 1997), and seven out of 13 small arRP families from Sardinia gave positive lod scores with 17q markers (0.6–2.1) (Wright et al., 1997). A gene causing an autosomal disorder (mulibrey nanism) with unknown basic metabolic defect affecting growth and showing yellowish dots in the ocular fundi was found located on 17q (Avela et al., 1997). Interestingly, an autosomal recessive progressive rod–cone degeneration (*prcd*) in canine was recently found syntenic with RP17 (Acland et al., 1998). The disease phenotype in canines is late onset, with rods and cones degenerating after apparently normal postnatal development.

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