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Human Rod Photoreceptor cGMP-gated Channel: Amino Acid Sequence, Gene Structure, and Functional Expression

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Phototransduction in retinal rods involves a G-protein–mediated signaling cascade that leads to cGMP hydrolysis and the closure of a cGMP-gated channel. This channel has recently been purified from bovine retina and molecularly cloned (Kaupp et al., 1989). We report here the cloning of cDNA and genomic DNA encoding the human rod cGMP-gated channel, based upon its homology to the bovine counterpart. The human mRNA structure differs from the bovine in containing an Alu repetitive element spliced into the 5′ untranslated region. The human cGMP-gated channel gene (CNCG) is located on chromosome 4 and contains at least 10 exons. One large exon encodes the carboxy-terminal two-thirds of the protein, whereas seven small exons encode the amino-terminal one-third of the protein. Alternative splicing removes one of the small exons in a subset of transcripts in the human retina, producing an internal in-frame deletion of 36 codons. When expressed in a human embryonic kidney cell line (293S), the full-length cDNA clone, but not the differentially spliced variant, produced functional ion channels broadly similar to the native channels in vertebrate rods.

Retinal photoreceptors respond to light with a membrane hyperpolarization, generated by the closure of cGMP-gated channels (for review, see Yau and Baylor, 1989). In darkness, a fraction of the channels are open due to a steady level of cytoplasmic cGMP, thus sustaining a continuous influx of cations into the outer segment of the cell. Light activates a G-protein–mediated signaling cascade that leads to the activation of a cGMP phosphodiesterase and hence the hydrolysis of cGMP, causing the channels to close (for review, see Pugh and Cobbs, 1986; Stryer, 1986). This cGMP-gated channel is unusual in that it is the first example of a ligand-gated channel that utilizes a cyclic nucleotide for activation (Fesenko et al., 1985; Haynes and Yau, 1985). Since its discovery, similar channels have been reported in other cell types, with a close relative being the cyclic nucleotide–gated channel involved in olfactory transduction (Nakamura and Gold, 1987; see also Dhallan et al., 1990; Ludwig et al., 1990).

A major step in understanding the biology of these channels was the successful isolation of cDNA clones encoding the bovine rod channel (Kaupp et al., 1989). The deduced primary structure of the bovine rod channel has 690 amino acids, and has a domain near the carboxy terminus that bears substantial homology to each of the cGMP-binding domains in cGMP-dependent protein kinases. In addition, a region homologous to the voltage-sensing domain in voltage-gated channels has been identified (Jan and Jan, 1990), even though the rod cGMP-gated channel shows no evidence of voltage gating in the absence of ligand. This feature suggests that the cGMP-gated channel and voltage-gated channels may share a common ancestry. When synthetic message derived from the bovine clone was injected into *Xenopus* oocytes, functional channels were observed with physiological properties quite similar to the native channel (Kaupp et al., 1989). Based on the Hill coefficient of activation by cGMP, which can be over 3, the number of subunits is likely to be at least four. Thus, it appears that an oligomer composed of several identical subunits can form a functional channel.

In this article, we report the cloning of cDNA and genomic DNA and the functional expression of cDNA encoding the rod cGMP-gated channel from human retina. Our motivation for this work is twofold. First, the intron–exon structure of the gene may provide clues to the evolution of this class of channels, and may direct a search for differentially spliced variants as seen in the *Shaker* potassium channels (Kamb et al., 1988; Schwarz et al., 1988). Second, recent work has shown that some inherited retinal diseases are caused by mutations in genes encoding phototransduction or structural proteins of the rod outer segment, including rhodopsin (Dryja et al., 1990, 1991; Sung et al., 1991), the β-subunit of cGMP phosphodiesterase (Bowes et al., 1990; Pittler and Baehr, 1991), and peripherin/*rds* (Travis et al., 1989; Farrar et al., 1991; Kajiwara et al., 1991). It seems reasonable to suppose that sequence variation in the rod cGMP-gated channel exists in the human gene pool, and that some variants may produce a functional alteration. The results reported here lay the groundwork for future experiments aimed at identifying and analyzing inherited defects in the human rod cGMP-gated channel.

Materials and Methods

cDNA clone isolation. PCR primers corresponding to nucleotides −49 to −27 and the reverse complement of nucleotides 2139–2166 of the

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bovine rod cGMP-gated channel (Kaupp et al., 1989) were used to amplify the coding region of the bovine channel from a bovine retina cDNA library (Nathans and Hogness, 1983) and the resulting fragment cloned in Bluescript KS (Dhallan et al., 1990). The coding region of the bovine channel was used as a probe to screen 5×10^5 recombinants from an adult human retina cDNA library (Nathans et al., 1986). Approximately 50 strongly hybridizing clones were identified and analyzed for insert size and structure, and the 5 longest clones were selected for detailed analysis. Comparison of the partial nucleotide sequences of these clones to that of the bovine channel cDNA showed that all five were lacking the 5′ end. An oligonucleotide, KY2, corresponding to nucleotides 348–371 in Figure 1 and derived from the 5′ end of one of the clones, was used as a probe to rescreen 10^6 recombinants from the retinal cDNA library by the method of Wood et al. (1985). Thirty clones were identified and the six largest were analyzed further. Although all six were longer than the previously isolated clones, none were full length based upon a comparison with the bovine sequence. Therefore, a second oligonucleotide, KY12, corresponding to nucleotides 68–97 in Figure 1 and derived from the 5′ end of one of the clones, was used to rescreen 2×10^6 recombinants from the retinal cDNA library as described above. Twelve clones were analyzed in detail, and one was found to contain the 5′ end of the human rod cGMP-gated channel cDNA. As this clone did not have the 3′ end, it was ligated to a 3′ proximal clone at the unique SphI site at nucleotide 100 (Fig. 1) to generate the full-length cDNA clone hRcG-1. Both cDNA clones, as well as the final ligation product, were sequenced on both strands.

Genomic clones. The full-length cGMP-gated channel cDNA clone hRcG-1 was used as a probe to screen a Sau3A partial digest human genomic library in bacteriophage λEMBL3 (Frischauf et al., 1983). Genomic DNA was obtained from peripheral blood leukocytes from J.N., a male with normal vision. Recombinants gJHN101–gJHN108 were obtained in an initial screen at high stringency [hybridization: 5× saline–sodium citrate (SSC), 50% formamide, 37°C; washing: 0.1× SSC, 0.1% SDS, 50°C]. Recombinant gJHN109, which encompasses exon 1, was identified by hybridization with a probe encompassing nucleotides −370 to −256 within the 5′ untranslated region of hRcG-1. Exons were mapped and sequenced using synthetic oligonucleotides derived from the hRcG-1 sequence as hybridization probes and as primers for PCR and dideoxy sequencing.

RNA blot hybridization. Total RNA was prepared using the guanidinium-phenol method (Chomczynski and Sacchi, 1987) from five human retinas obtained within 12 hr post-mortem. Formaldehyde/agarose gel electrophoresis and blotting were performed as described previously (Sambrook et al., 1989).

PCR amplification from cDNA. First strand cDNA was synthesized in a 20 μl reaction containing 1 μg of total RNA, 10 mM Tris, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl, 1 mM each dATP, dGTP, dCTP, and dTTP, 50 μg/ml random sequence hexamers, and 10 U of MoMLV reverse transcriptase (Bethesda Research Labs). The sample was incubated for 10 min at 23°C, 60 min at 37°C, 5 min at 99°C, and then cooled to 4°C. Following the addition of 80 μl containing 0.5 μM each PCR primer, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 2.5 U of Taq DNA polymerase, the sample was subjected to 35 rounds of PCR amplification. PCR products were resolved by alkaline agarose gel electrophoresis (Sambrook et al., 1989) and transferred to GeneScreen Plus membrane (Du Pont) for oligonucleotide hybridization.

Synthetic DNA primers and probes. KY12, TTGAAAAGGAAATACGAAGGATGGAAAATG [base pairs (bp) 68–97]; KY61, GACTAATGGATCTTATAA (bp 569–586); JM14, GATCAGAATTCTTCCAGTGGATAATGATGACGATA (bp 906–929); JM23, GTGGTTATTGATCCCTC (bp 444–460); JM51, AGAAAGAAGAAGCATGTTTT (bp 428–437 and 546–555); JM52, CCTGGATCCATTGCACTTTTTAATGTG (bp 245–264); JM53, TTTGAATTCTTCCTTTACCAGCAGTCC (bp 667–684); JM66, AGCAAGTCAGATAATAAAAACGAAAAT (bp 328–354).

Chromosome mapping. Thirty mouse–human hybrid cell lines were derived by cell fusion and characterized by karyotype analysis and by enzyme markers of known map location (Shows et al., 1978, 1982, 1984). The presence or absence of the human cGMP-gated channel gene was determined by Southern blot hybridization to EcoRI-digested DNA prepared from each hybrid cell line.

Expression and electrophysiology. The electrophysiological properties of the cloned channel were examined following transient transfection of the 293 human embryonic kidney cell line (American Type Culture Catalog CRL 1573; grown in 5% CO_2 in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum). A DNA fragment representing the complete channel clone was inserted into the polylinker site of pCIS, an expression vector containing a cytomegalovirus promotor, intron, polylinker, simian virus 40 (SV40), polyadenylation site, and SV40 origin of DNA replication (Gorman et al., 1990). To construct a cDNA corresponding to the differentially spliced transcript (i.e., lacking exon 8), a cloned PCR product derived from this transcript and encompassing part of exon 5, exons 6, 7, and 9, and part of exon 10, was annealed to single-stranded DNA encoding the full-length cDNA and used to prime DNA synthesis under standard *in vitro* mutagenesis conditions. Mutant clones were identified by DNA sequencing, and from one of them a 525 bp SphI to ClaI restriction fragment encompassing the differentially spliced region was excised and inserted in place of the corresponding fragment of the full-length channel clone. The 525 bp region derived from the *in vitro* mutagenesis reaction was sequenced to rule out spurious sequence changes. For channel production, 293 cells were transfected with a mixture of expression plasmid (5 μg), carrier DNA (Bluescript, 10 μg), and SV40 T-antigen expression plasmid (RSV-TAg, 0.5 μg) by the calcium phosphate method (Gorman, 1985).

Electrical recordings were performed 48 hr after transfection using a List EPC-7 patch-clamp instrument at a bandwidth of DC 5 kHz. The recording pipettes were fabricated from borosilicate glass and had tip lumens of ~1 μm. Seal resistance upon establishment of a membrane patch was typically of the order of 10 GΩ. Solutions were pH 7.6, and contained 10 mM glucose plus the following components. Ringer's solution contained 140 mM NaCl, 5 mM KCl, 10 mM Na HEPES, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. Ringer's solution without divalent cations contained 140 mM NaCl, 5 mM KCl, 10 mM Na HEPES, and 0.5 mM Na EDTA. cGMP was applied to the bath using a solenoid-controlled rotary valve system (Nakatani and Yau, 1988).

Results

Isolation and sequence analysis of cDNA clones. To identify cDNA clones encoding the human rod cGMP-gated channel, an adult human retina cDNA library in bacteriophage λgt10 (Nathans et al., 1986) was screened using as probe the coding region of the previously isolated bovine homolog (Kaupp et al., 1989). Two overlapping clones encompassing the entire coding region were identified, sequenced, and joined together to produce a cDNA, hRcG-1, containing the entire coding region, 367 bases of the 5′ untranslated region, and 420 bases of the 3′ untranslated region (see Materials and Methods).

The nucleotide sequence of hRcG-1 predicts an encoded protein sequence of 686 amino acids in length that is 91% identical to the bovine cGMP-gated channel (Fig. 1; Kaupp et al., 1989). The indicated initiator methionine codon corresponds to that assigned to the bovine channel, although the human sequence shows several in-frame ATG codons 5′ and 3′ of this position. Between nucleotides −217 and −15, in the 5′ untranslated region, the human sequence contains a member of the Alu family of repetitive elements (Britten et al., 1988; Jurka and Smith, 1988). The orientation of this Alu element, as defined by the direction of transcription of Alu elements by RNA polymerase III, is opposite that of the cGMP-gated channel transcription unit. In aligning the human and bovine sequences, the Alu element appears as a simple insertion in the human sequence. On either side of the Alu element, human and bovine 5′ untranslated sequences require two single nucleotide insertions for optimal alignment. In this 5′ untranslated region of 154 bases, the two nucleotide sequences are 80% identical.

Gene structure. To determine the structure of the human cGMP-gated channel gene, nine independent genomic clones were isolated from a Sau3A partial digest library in lambda phage EMBL3. Figure 2 shows the deduced chromosomal restriction map and the locations of the 10 exons identified within these clones, the precise boundaries of which are shown in Figure 1. With the exception of exon 2, the gene was characterized by


5'-GAATTCCGGATACACAAATCAGCATGTTTATTATTTTTAAAAAATTAGGGCCTGGAT -319
TTTGACAATGTCAAGATTTACCGTATATCCCTGTTTGTTTGGATACACCAGTGACGTCCACTTCTAGAAGACAAAGTTATATTACTTAAACAACCAAAGATGGAGTCTCGCTCTTCGCCCAGGCTGGAGTGCAGTGGCGCGATCTCGGCTCACTGCAGT -160
CTTCACCTCCCAGATTCAAGCGATTTTCAGCTAATTTTTGTATTTTTAGTAGAGATGGGATTTCACCATGTTGGCCAGGCTGGACTTGAACTCCTGATCTCGAGTGATCTGCCCACCTCAGCCTCCCAAAGTGCTGGTATTACAGATATGAAACTATCC -1
ATG AAG AAC AAT ATT ATC AAT ACA CAG CAG TCT TTT GTA ACC ATG CCC AAT GTG ATT GTA CCA GAT ATT GAA AAG GAA ATA CGA AGG ATG GAA AAT GGA GCA TGC AGC TCC TTT TCT GAG 120
Met lys asn asn ile ile asn thr gln gln ser phe val thr met pro asn val ile val pro asp ile glu lys glu ile arg arg met glu asn gly ala cys ser ser phe ser glu 40
lys val trp his asn ile val gly val thr gly
GAT GAT GAC AGT GCC TCT ACA TCT GAA GAA TCA GAG AAT GAA AAC CCT CAT GCA AGG GGT TCC TTT AGT TAT AAG TCA CTC AGA AAG GGA GGA CCA TCA CAG AGG GAG CAG TAC CTG CCT 240
asp asp asp ser ala ser thr ser glu glu ser glu asn glu asn pro his ala arg gly ser phe ser tyr lys ser leu arg lys gly gly pro ser gln arg glu gln tyr leu pro 80
(asp) met phe thr asp arg ser asn thr his gly ser gln
GGT GCC ATT GCA CTT TTT AAT GTG AAC AAC AGC AGC AAT AAG GAC CAG GAA CCA GAA GAG AAA AAG AAA AAG AAA AAA GAA AAG AAG AGC AAG TCA GAT AAT AAA AAC GAA AAT AAA AAC 360
gly ala ile ala leu phe asn val asn asn ser ser asn lys asp gln glu pro glu glu lys lys lys lys lys glu lys lys ser lys ser asp asn lys asn glu asn lys asn 120
glu lys pro asp lys
GAC CCA GAG AAG AAA AAG AAG AAA AAG GAC AAA GAG AAG AAA AAG AAA GAG GAG AAA AGC AAA GAT AAG AAA GAA GAC GAG AAG AAA GAA GTT GTG GTT ATT GAT CCC TCG GGA AAC ACA 480
asp pro glu lys lys lys lys lys lys asp lys glu lys lys lys lys glu glu lys ser lys asp lys lys glu glu glu lys lys glu val val val ile asp pro ser gly asn thr 160
(glu) asp gly
TAT TAC AAC TGG CTG TTT TGC ATC ACA TTA CCT GTT ATG TAC AAC TGG ACA ATG GTT ATT GCC AGA GCA TGT TTT GAT GAA CTT CAA TCT GAT TAC CTA GAA TAT TGG CTC ATT TTG GAT 600
tyr tyr asn trp leu phe cys ile thr leu pro val met tyr asn trp thr met val ile ala arg ala cys phe asp glu leu gln ser asp tyr leu glu tyr trp leu ile leu asp 200
ile ala phe
TAC GTA TCA GAC ATA GTC TAT TTA ATC GAT ATG TTT GTA CGA ACA AGG ACA GGT TAC CTA GAA CAA GGA CTG CTG GTA AAG GAA GAA CTT AAA CTC ATA AAT AAA TAT AAA TCC AAC TTG 720
tyr val ser asp ile val tyr leu ile asp met phe val arg thr arg thr gly tyr leu glu gln gly leu leu val lys glu glu leu lys leu ile asn lys tyr lys ser asn leu 240
leu val leu arg asp thr phe
CAA TTT AAA CTT GAT GTT CTG TCA CTG ATA CCA ACT GAT TTG CTG TAT TTT AAG TTA GGG TGG AAC TAT CCA GAA ATT AGA TTA AAC AGG TTG TTA CGG TTC TCT CGT ATG TTT GAG TTC 840
gln phe lys leu asp val leu ser leu ile pro thr asp leu leu tyr phe lys leu gly trp asn tyr pro glu ile arg leu asn arg leu leu arg phe ser arg met phe glu phe 280
val ile phe ile
TTC CAG AGA ACA GAA ACA AGG ACA AAC TAT CCA AAC ATC TTC AGG ATT TCC AAC CTT GTT ATG TAT ATC GTC ATC ATT ATC CAC TGG AAT GCA TGT GTG TTC TAC TCT ATT TCT AAA GCT 960
phe gln arg thr glu thr arg thr asn tyr pro asn ile phe arg ile ser asn leu val met tyr ile val ile ile ile his trp asn ala cys val phe tyr ser ile ser lys ala 320
ile tyr phe
ATT GGA TTT GGA AAT GAT ACA TGG GTC TAC CCT GAT ATT AAT GAT CCT GAA TTT GGC CGT TTG GCT AGA AAA TAC GTA TAC AGC CTT TAC TGG TCT ACA CTG ACT TTG ACT ACC ATT GGT 1080
ile gly phe gly asn asp thr trp val tyr pro asp ile asn asp pro glu phe gly arg leu ala arg lys tyr val tyr ser leu tyr trp ser thr leu thr leu thr thr ile gly 360
val asp
GAA ACA CCC CCT CCC GTG AGG GAT TCT GAG TAT GTC TTT GTG GTG GTT GAT TTC CTA ATT GGA GTG TTA ATT TTT GCT ACC ATC GTT GGT AAC ATA GGT TCT ATG ATT TCC AAC ATG AAT 1200
glu thr pro pro pro val arg asp ser glu tyr val phe val val val asp phe leu ile gly val leu ile phe ala thr ile val gly asn ile gly ser met ile ser asn met asn 400
phe ala
GCA GCC AGA GCA GAA TTT CAA GCA AGA ATT GAT GCT ATC AAG CAA TAT ATG CAT TTT CGA AAT GTA AGC AAA GAT ATG GAA AAG AGG GTT ATT AAA TGG TTT GAC TAC CTG TGG ACC AAC 1320
ala ala arg ala glu phe gln ala arg ile asp ala ile lys gln tyr met his phe arg asn val ser lys asp met glu lys arg val ile lys trp phe asp tyr leu trp thr asn 440
AAA AAA ACA GTT GAT GAG AAA GAA GTC TTA AAG TAT CTA CCT GAT AAA CTA AGA GCA GAA ATT GCC ATC AAC GTT CAC TTA GAC ACA TTA AAA AAG GTA CGC ATT TTT GCT GAT TGT GAA 1440
lys lys thr val asp glu lys glu val leu lys tyr leu pro asp lys leu arg ala glu ile ala ile asn val his leu asp thr leu lys lys val arg ile phe ala asp cys glu 480
arg
GCT GGT CTG TTG GTG GAG TTG GTC TTG AAA TTG CAA CCC CAA GTC TAC AGT CCT GGA GAT TAT ATT TGC AAG AAA GGG GAT ATC GGA CGA GAG ATG TAC ATT ATC AAG GAA GGC AAA CTC 1560
ala gly leu leu val glu leu val leu lys leu gln pro gln val tyr ser pro gly asp tyr ile cys lys lys gly asp ile gly arg glu met tyr ile ile lys glu gly lys leu 520
GCT GTG GTG GCA GAT GAT GGA GTC ACT CAG TTT GTG GTA TTG AGC GAT GGC AGC TAC TTC GGT GAG ATC AGC ATT CTT AAC ATT AAA GGG AGC AAA GCT GGC AAT CGA AGA ACG GCC AAT 1680
ala val val ala asp asp gly val thr gln phe val val leu ser asp gly ser tyr phe gly glu ile ser ile leu asn ile lys gly ser lys ala gly asn arg arg thr ala asn 560
ile
ATT AAA AGT ATT GGC TAC TCA GAC CTG TTC TGT CTC TCA AAA GAT GAC CTC ATG GAA GCT CTA ACT GAG TAC CCA GAT GCC AAA ACT ATG CTG GAA GAG AAA GGG AAG CAG ATT TTA ATG 1800
ile lys ser ile gly tyr ser asp leu phe cys leu ser lys asp asp leu met glu ala leu thr glu tyr pro asp ala lys thr met leu glu glu lys gly lys gln ile leu met 600
gly
AAA GAT GGT CTA CTG GAT CTA AAC ATT GCA AAT GCT GGC AGT GAT CCT AAA GAT CTT GAA GAG AAG GTT ACT CGA ATG GAG GGG TCA GTA GAC CTC CTG CAA ACC AGG TTT GCC CGA ATC 1920
lys asp gly leu leu asp leu asn ile ala asn ala gly ser asp pro lys asp leu glu glu lys val thr arg met glu gly ser val asp leu leu gln thr arg phe ala arg ile 640
ile ser
TTG GCT GAG TAT GAG TCC ATG CAG CAG AAA CTG AAA CAA AGA TTA ACC AAG GTT GAG AAA TTT CTG AAA CCG CTT ATT GAC ACA GAA TTT TCA AGT ATT GAG GGA CCT GGA GCG GAA AGT 2040
leu ala glu tyr glu ser met gln gln lys leu lys gln arg leu thr lys val glu lys phe leu lys pro leu ile asp thr glu phe ser ser ile glu gly pro gly ala glu ser 680
ala ser thr
GGG CCC ATC GAC TCT ACA TAG AACCGAAAAGCTGGTCATTAACAGGGACATGCCTCATGATCCTTTTGATCCTATGACTGACATCAACTAAAATTTAAAAGAAGAGGAAGACTCAGTTGGGAAATTTTTCCATGAGGAAAATGTGCTTTGGT 2192
gly pro ile asp ser thr * 686
thr (gln asp) *
GCAAGGTACAAGGCCCACACCCTCTCTGAGAGATACTATGATTAAAAAAGCTTTATATCTTGGGATTTTTCACAACTGATAATGTGCAAAGATATAAACTGATTAACTTGTCAGTGTCTGTATTTTCTGATTTTTTCACATACGCTCATTTTATGTAAT 2351
ATTCTTCATAAAAATGAATAAGTAGCCCTCACTTTCATGCCATTTCCATTGTTGAGTGAAGCGTATTTGAAGTAACTGAGAATTACCATGTACATCATATTTGGGATAACATTTTTAAAAATCGGAATTCC-3 2482

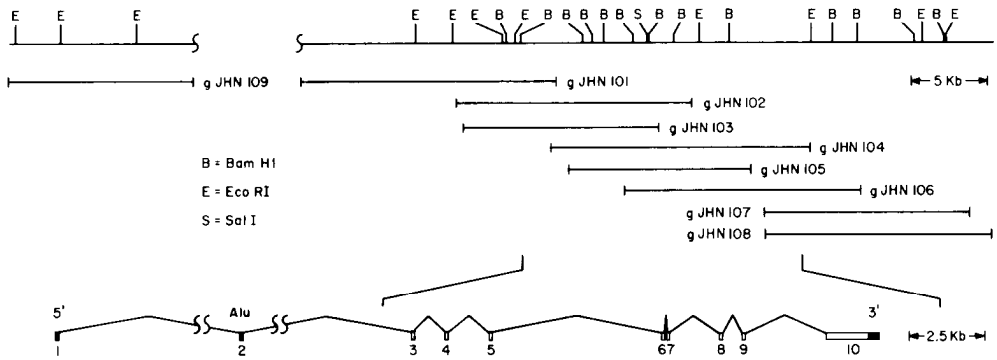

Figure 2. Human cGMP-gated channel gene structure. *Top,* Restriction maps of two discontinuous segments of chromosomal DNA encompassing exon 1 and exons 3–10. *Center, gJHN101–gJHN108* denote genomic fragments isolated as recombinant lambda clones by hybridization with the full-length human cGMP-gated channel cDNA clone. *gJHN109* denotes a genomic fragment isolated by hybridization with a probe extending from nucleotide −375 to nucleotide −255 in the 5′ untranslated region. *Bottom,* Intron–exon structure of the cGMP-gated channel gene. *Solid boxes,* 5′ and 3′ untranslated regions; *open boxes,* coding region. The chromosomal segment containing the Alu element (exon 2 in the 5′ untranslated region) has not been isolated.

determining the sequence of each exon and of the adjacent 50–100 bp of flanking intron sequence. The sequences abutting each exon conform to consensus splice junction sequences (Table 1; Mount, 1982). The coding region resides within exons 3–10. Whole genome Southern blot hybridization and sequence analysis of additional clones obtained by low-stringency screening of a human genomic library demonstrate that the chromosomal locus described here has considerably greater homology to the bovine rod cGMP-gated channel than does any other segment of human DNA (R. S. Dhallan, J. Macke, R. R. Reed, K.-W. Yau, and J. Nathans, unpublished observations), further evidence that the cDNA and genomic DNA we have isolated encode the rod cGMP-gated channel.

The putative first exon resides on genomic clone gJHN109, which does not overlap the chromosomal region defined by clones gJHN101–gJHN108. Its distance from the gJHN101–gJHN108 cluster is not known. This exon is tentatively assigned as the first exon in the gene because it contains the most 5′-proximal sequences found in the cDNA clone hRcG-1. However, the possibility exists that additional 5′ untranslated sequences could be located on exons upstream of this exon. These data imply that the transcription unit of the cGMP-gated channel genes is greater than 40 kilobases (kb) in length.

The region within the 5′ untranslated region containing the Alu element appears to derive from one (or possibly more than one) exon. The cDNA sequence immediately 5′ of the Alu element (exon 1) is followed in the genomic DNA by a consensus splice donor sequence, and the cDNA sequence immediately 3′ of the Alu element (exon 3) is preceded in genomic DNA by a consensus splice acceptor sequence. The Alu element is not present within the genomic clones shown in Figure 2, as determined by blot hybridization with oligonucleotide probes derived from it. Most likely, it resides within the gap between clone gJHN109 and clones gJHN101–gJHN108.

Chromosomal localization. To determine the chromosomal location of the cGMP-gated channel gene, we determined whether it was present or absent from each of 30 mouse–human hybrid cell lines that carry defined subsets of human chromosomes. Only human chromosome 4 showed perfect concordance for the Southern blot hybridization signal derived from the human gene (Table 2).

Analysis of transcripts. The gene structure described above reveals eight coding region exons, six of which (exons 4–9) reside entirely within the coding region. Interestingly, five of these six internal coding region exons are multiples of 3 nucleotides in length. The probability of this occurring by chance, assuming that each intron–exon junction is equally likely to fall within the three reading frames, is 0.0165. This pattern suggested the possibility that one or more of these coding region exons might be differentially spliced, a mechanism known to generate diversity in a number of other channel genes (Kamb et al., 1988; Schwarz et al., 1988; Timpe et al., 1988; Sommer et al., 1990).

As an initial step in examining transcript structure(s), RNA molecules homologous to the cGMP-gated channel were examined by Northern blotting. A single broad band centered at 3.5 kb was observed in total RNA from human retinas (Fig. 3). This result is consistent with the existence of a single major RNA species, but does not rule out rare variants of substantially different size or variants differing in size by less than 200 bp. With the latter possibility in mind, we PCR amplified segments of the cGMP-gated channel sequence using a first strand cDNA template synthesized by random priming of total human retina RNA and primers that were separated by several exons. In one reaction, using a primer pair derived from exons 5 and 10 (JM52 and JM14, respectively), two PCR products were obtained. The larger and more abundant product matched in size and sequence the corresponding region of cDNA clone hRcG-1. The smaller PCR product, which was typically present at 10% the abundance of the larger product, was found by DNA sequencing to be precisely missing exon 8, an in-frame deletion of 108 bases (Fig.

←

Figure 1. DNA and deduced amino acid sequence of the human rod photoreceptor cGMP-gated channel. The first nucleotide of the initiator methionine codon has been assigned position +1. The *third row of each line* displays the protein sequence of the bovine rod channel (Kaupp et al., 1989); only differences are indicated. The bovine channel has four additional residues compared to the human channel; these are enclosed in *parentheses.* The positions of the nine introns in the human channel have been marked by *solid triangles.* The Alu element between nucleotides −217 and −15 is *underlined.*

Table 1. Splice junction donor and acceptor sequences

	Acceptor		Donor
Consensus	...$(T/C)_{>11}$N(T/C)AG		GT(A/G)AGT...
		exon 1	GTAAGA...
	... Not determined	exon 2	Not determined...
	...CTTCTTTCTCCTCAG	exon 3	GTAAAT...
	...CCTATTGGACTGTAG	exon 4	GTGAGC...
	...TTTTTCATTTCCCAG	exon 5	GTAAGT...
	...TTATTTTTTCTTTAG	exon 6	GTAAGA...
	...GCTTAATTTTTTCAG	exon 7	GTAAGC...
	...TCTAATTGCCGCTAG	exon 8	GTGTCT...
	...TTTCTTTTTTTATAG	exon 9	GTAAAT...
	...TTTTTCTATTTTAG	exon 10	

This table shows sequences at the boundaries of coding region exons. Sequences at the 5′ and 3′ boundaries of each exon are denoted "acceptor" and "donor," respectively. The consensus acceptor and donor sequences are from Mount (1982).

4). To confirm the presence of the two spliced forms among the original PCR products, synthetic oligonucleotides were hybridized to gel fractionated PCR products (Fig. 5). Probe JM53, located within exon 10 hybridized to both major and minor PCR products (Fig. 5*A*); probe JM23, located within alternatively spliced exon 8, hybridized only to the major spliced product (Fig. 5*B*); and probe JM51, a 20-mer that straddles the novel exon 7–exon 9 splice junction, hybridized to the minor splice product. Interestingly, in the PCR reaction using total retina cDNA (Fig. 5*C*, lane 3), JM51 also hybridized to a larger molecular weight species that appears to be distinct from the major splice product, given that the cloned major splice product in lane 1 of Figure 5*C* does not hybridize under these conditions.

Table 2. Segregation of the human cGMP-gated channel gene with human chromosomes in mouse-human hybrid cell lines

Chromosome	No. of concordant hybrids (+/+)	(−/−)	No. of discordant hybrids (+/−)	(−/+)	% Discordancy
1	0	19	7	1	30
2	3	16	7	5	39
3	5	12	5	8	43
4	10	21	0	0	0
5	7	10	3	11	45
6	4	15	6	6	39
7	8	11	2	9	37
8	6	10	4	11	48
9	0	18	9	2	38
10	7	6	3	15	58
11	6	10	4	10	47
12	5	11	5	10	48
13	3	13	7	8	48
14	5	6	5	15	65
15	5	13	5	8	42
16	3	18	7	3	32
17	7	5	2	15	59
18	8	12	2	9	35
19	3	18	7	3	32
20	7	11	3	10	42
21	7	9	3	12	48
22	1	14	9	7	52
X	8	11	2	9	37

These data show chromosomal localization of the human cGMP-gated channel gene. The presence (+) or absence (−) of each human chromosome and the presence (+) or absence (−) of the Southern blot hybridization signal was scored for each of 30 mouse–human hybrid cell lines. Only chromosome 4 shows a 0% discordancy, indicating a matched segregation of the DNA probe with this chromosome.

Electrophysiology of the expressed channel. The electrophysiological properties of the channel protein encoded by the hRcG-1 clone were examined by transient expression in the 293 human embryonic kidney cell line (see Materials and Methods). Inside-out patches of plasma membrane were excised from transfected cells and tested for sensitivity to bath-applied cGMP. A cGMP-induced current could be observed from approximately 30% of these membrane patches 24–48 hr after transfection. Figure 6 shows the relation between normalized current activation and cGMP concentration obtained from one of these patches. It gives a Hill coefficient of 2.7 and a half-saturating cGMP concentration ($K_{1/2}$) of 80 μM. From five patches, the $K_{1/2}$ was 86 ± 18 μM (mean ± SD) and the Hill coefficient was 2.0 ± 0.6. These values are broadly consistent with those previously found for the native channel in amphibian and mammalian species (Luhring and Kaupp, 1989; Yau and Baylor, 1989), as well as with the cloned bovine channel expressed in *Xenopus* oocytes (Kaupp et al., 1989).

The current–voltage relation obtained at saturating cGMP concentration (1 mM) and with symmetrical Ringer's solutions without divalent cations is shown in Figure 7*A*. The relation is almost linear, with the slight upward curvature probably reflecting a small increase in the open probability of the liganded channel at positive voltages, as has been described for the native channel (Karpen et al., 1988; Haynes and Yau, 1990). Figure 7*B* shows the current–voltage relation, from a different patch, with Ringer's solution containing divalent cations in the patch pipette and Ringer's solution without divalent cations in the bath. Again, as with the native rod channel, the relation shows pronounced outward rectification under these conditions, reflecting a voltage-dependent block by divalent cations (see Yau and Baylor, 1989). In the absence of divalent cations, single-channel openings induced by cGMP could also be observed (Fig. 7*C*). The prominent openings showed a conductance of approximately 30 pS, which is broadly similar to that found for the native channel in other vertebrate species (Yau and Baylor, 1989). However, as with the expressed bovine channel (Kaupp et al., 1989), the distribution of open times appeared to have more than one mode. Some of the openings were very brief,

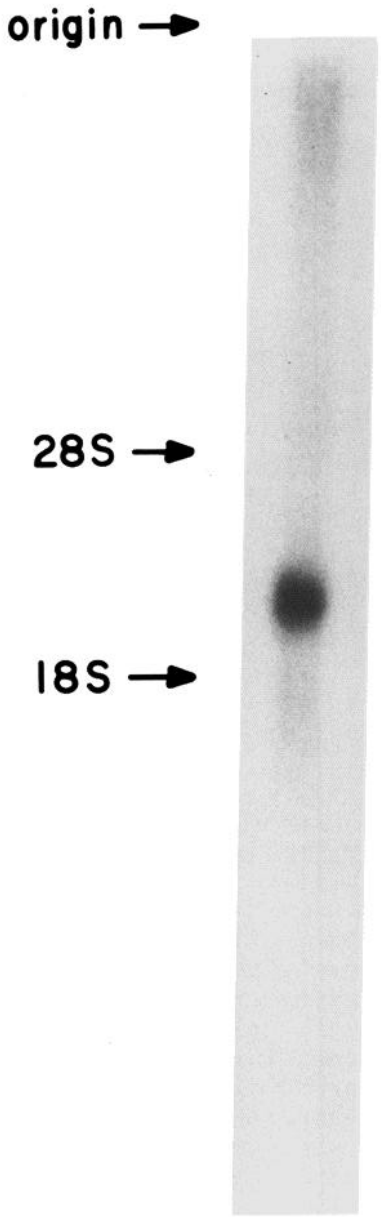

Figure 3. Blot hybridization of cGMP-gated channel mRNA from human retina. Ten micrograms of total human retina RNA were fractionated on a formaldehyde/agarose gel, and hybridized with a probe extending from nucleotide 854 to nucleotide 1368 of the cGMP-gated channel cDNA clone. The electrophoretic mobility of 28S (6.3 kb) and 18S (2.4 kb) ribosomal RNAs are indicated. The hybridizing species is centered at 3.5 kb.

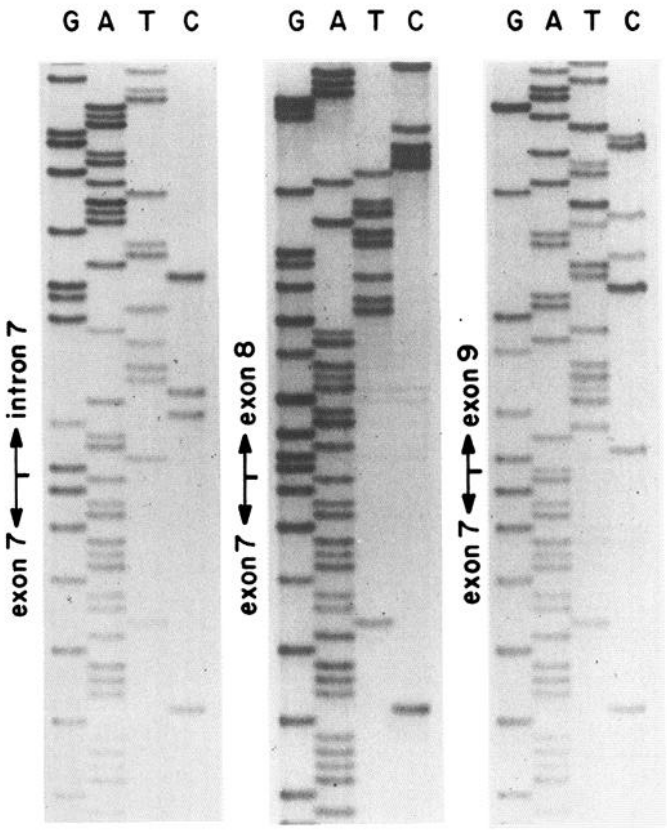

Figure 4. Nucleotide sequences across the differentially spliced exon 7 3′ boundary. *Templates: left,* a 3.5 kb EcoRI–SalI subclone derived from genomic clone gJHN105; *center,* cloned cDNA phCG1 corresponding to the major spliced RNA species; *right,* cloned cDNA corresponding to the minor spliced RNA species generated by PCR amplification of first strand cDNA using primers KY12 and KY61, which prime in exons 3 and 9, respectively. The template used for sequencing of the minor splice product (*right*) was produced by first incorporating the minor spliced PCR product into the expression plasmid (see Materials and Methods). The sequencing primer for each reaction was JM66 (in exon 7).

lasting on the order of 1 msec like the native channel, while other openings were extremely long, lasting many tens of milliseconds, a feature not observed with the native channel. The reason for this striking difference between native and expressed channels remains to be examined. We have also examined 293 cells transfected with the cDNA clone corresponding to the minor transcript that is missing exon 8. From 30 excised membrane patches from transfected cells, no cGMP-induced current was observed.

Discussion

The experiments reported here establish the amino acid sequence of the human rod cGMP-gated channel, the chromosomal localization and the intron–exon arrangement of the gene that encodes it, the existence of a variant RNA generated by differential splicing, and the functional properties of the recombinant channel. Three lines of evidence support the identification of this cloned channel as that of human rod photoreceptors: first, the expressed protein has the appropriate physiological properties; second, the corresponding gene is more homologous to the bovine rod channel cDNA than is any other segment of human DNA; and third, mRNA transcribed from this gene is present in human retinas.

The human cGMP-gated channel and its bovine homolog are 91% identical at the amino acid level, with the greatest divergence residing in the amino terminal 92 amino acids that are posttranslationally cleaved from the bovine channel (Molday et al., 1991). Within the mature polypeptide (amino acids 93–690 of the bovine channel), the two share 93% amino acid identity, with most differences being conservative substitutions. Two single amino acid insertions are required to align the two sequences.

An unexpected finding is the presence of an Alu repetitive element within the 5′ untranslated region of the human mRNA. This element appears to be encoded in the genome as one (or more than one) separate exon that becomes intercalated between two preexisting exons in the mature transcript. This unusual

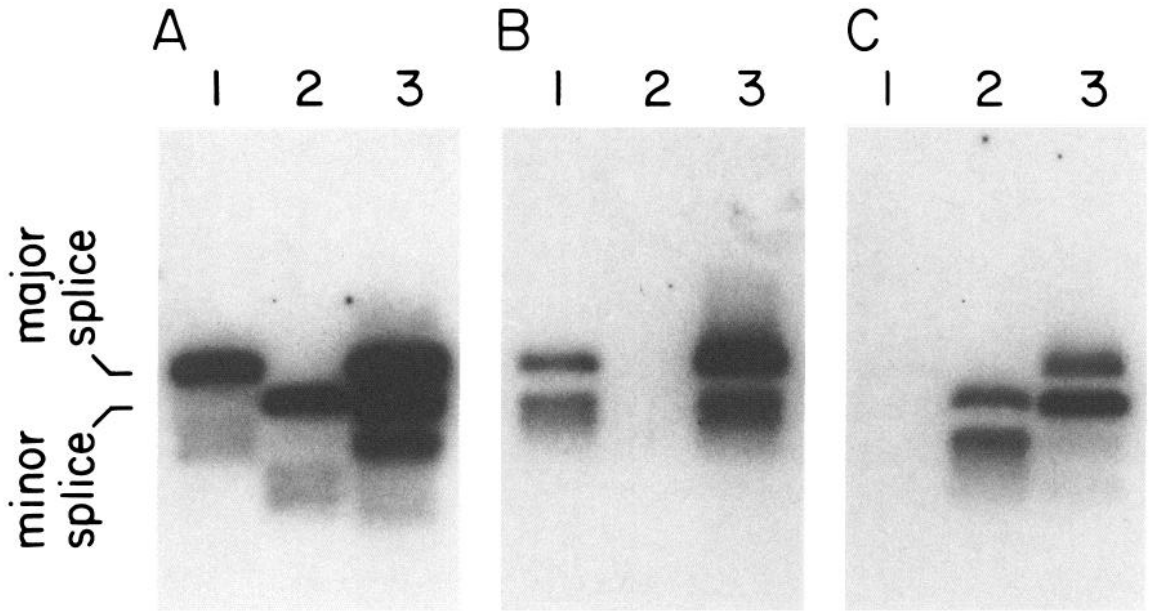

Figure 5. Blot hybridization of PCR products derived from alternatively spliced transcripts. *A–C* show identical blots of PCR products separated by alkaline agarose gel electrophoresis. All PCR products were amplified using primers JM52 and JM14, which prime in exons 5 and 10, respectively. *Lanes 1* and *2*, control PCR products derived from amplification of cloned full-length and alternatively spliced cDNA templates, respectively. *Lane 3*, PCR products derived from amplification using first strand human retina cDNA as template. *A*, Probed with JM53, located in exon 10, 5′ of PCR primer JM14, and therefore common to both major and minor spliced forms. *B*, Probed with JM23, located within alternatively spliced exon 8. *C*, Probed with JM51, a 20-mer that spans the alternative exon 7–exon 9 splice junction. The source of the lower molecular weight species in all lanes is presumed to be incomplete or aberrant PCR products. The higher molecular weight band in *lane C3* may represent a splicing product different from the two described here.

sequence arrangement may have some effect on channel production. Given that many Alu elements are present in cellular RNA—in both orientations within 3′ untranslated regions and in RNA polymerase III transcripts in the orientation opposite to that found in the cGMP-gated channel—it is reasonable to suppose that RNA heteroduplexes may form *in vivo* between the Alu sequence in the cGMP-gated channel mRNA and other Alu sequences of complementary strandedness. It is reasonable to suppose that such heteroduplexes might alter the stability and/or translational efficiency of the mRNA.

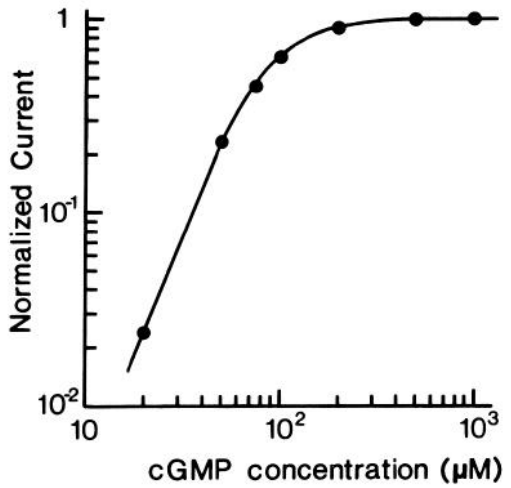

Figure 6. Dose–response relation between membrane current activation and cGMP concentration measured on an excised membrane patch from a 293 cell transfected with the cloned cDNA. Ringer's solution without divalent cations was present on both sides of the membrane, and the transmembrane potential was held at +40 mV. The *smooth curve* is the Hill equation, $j/j_{max} = C^n/[C^n + K_{1/2}{}^n]$, where C is the cGMP concentration, $K_{1/2}$ is the cGMP concentration that produces half-maximal activation, and n is the Hill coefficient. The fit to the data was made with $K_{1/2} = 80$ μM and $n = 2.7$. j_{max} in this experiment was 445 pA.

The arrangement of coding region exons in the human cGMP-gated channel shows an intriguing asymmetry: the amino-terminal 229 amino acids are encoded by seven exons, whereas the carboxy-terminal 457 amino acids are encoded by a single large exon. This arrangement is reminiscent of the pattern observed among intron-containing genes in the yeast *Saccharomyces cerevisiae*, in which most introns occur near the 5′-ends of transcription units. Fink (1987) has suggested that this pattern may reflect loss of introns by homologous recombination with incomplete reverse transcripts of mRNA molecules. An alternative scenario to account for the observed gene structure would be that the large 3′-proximal exon is directly descended from an ancestral gene, whereas the small 5′-proximal exons evolved more recently. This scenario fits well with the observation that five of the six small internal coding region exons are multiples of three nucleotides in length. These five exons could have intercalated into a preexisting channel gene without changing the reading frame.

The pattern of exon lengths described above prompted us to look for differentially spliced variants in the 5′ coding region of the mRNA. Thus far, one variant has been identified in which exon 8 is absent, resulting in an in-frame deletion of 36 codons. The exon 8 donor and acceptor splice junction sequences are among the most divergent from the consensus (Table 1), suggesting that the splicing variant could result from a lower efficiency of RNA cleavage adjacent to this exon. Exon 8 encodes

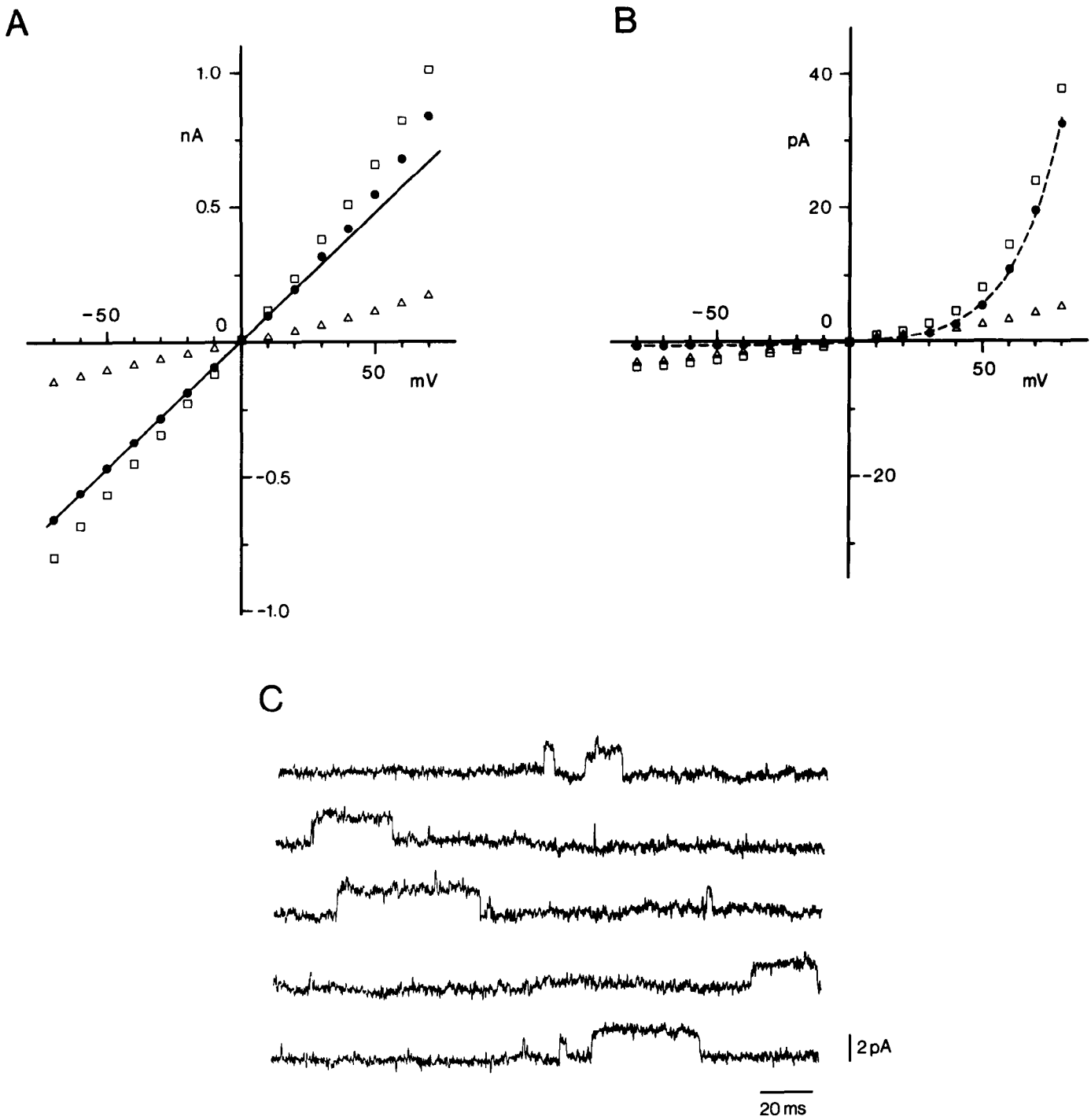

Figure 7. *A* and *B*, Steady-state current–voltage relations for the expressed channel in the absence and the presence of divalent cations. Two separate patches are shown, with 1 mM cGMP in both cases. The experiment consisted of making 1 sec voltage pulses at ±10 mV increments from a holding potential of 0 mV. The current at each voltage was measured at the end of the voltage pulse. *Open triangles*, Relation obtained in the absence of divalent cations; *open squares*, relation obtained in the presence of 1 mM cGMP; *solid circles*, difference between the preceding two conditions, giving the relation for the cGMP-gated channel. In *A*, Ringer's solution without divalent cations was present on both sides of the membrane patch. A *straight line* is fitted to the points at negative voltages and extrapolated to positive voltages. In *B*, Ringer's solution with divalent cations was present in the pipette, and Ringer's solution without divalent cations was present in the bath solution. Symbols have the same significance as in *A*. The *broken curve* is a scaling of the equation $j(V) = \exp[(V - 4)/17.5] - 1$, where V is membrane potential in mV. *C*, Single-channel openings recorded from another patch. Ringer's solution without divalent cations was present on both sides of the membrane. The transmembrane potential was held at +60 mV in the presence of 5 μM cGMP.

a stretch of amino acids that constitutes a proposed first transmembrane domain of the channel (Kaupp et al., 1989). Transfection of the variant cDNA did not produce functional cGMP-gated channels (see Results). This suggests that a homooligomer formed from the protein encoded by this variant RNA is either unstable or not functional (or both). The possibility that a heterooligomeric complex could form between this variant and the major protein species remains to be examined.

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