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# Molecular Characterization of Human and Mouse Photoreceptor Guanylate Cyclase-activating Protein (GCAP) and Chromosomal Localization of the Human Gene*

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**Guanylate cyclase-activating protein (GCAP) is a novel $Ca^{2+}$-binding protein that stimulates synthesis of cGMP in photoreceptors. Molecular cloning of human and mouse GCAP cDNA revealed that the known mammalian GCAPs are more than 90% similar, consist of 201–205 amino acids, and contain three identically conserved EF hand $Ca^{2+}$ binding sites. The sequence homology with recoverin, a related photoreceptor $Ca^{2+}$-binding protein, is less than 35%. *In situ* hybridization in primate retinas shows that the *GCAP* gene is expressed exclusively in photoreceptor inner segments. To investigate the *GCAP* gene structure, we probed 10 eucaryotic genomic DNAs with a bovine GCAP cDNA under stringent conditions. The results demonstrate that the *GCAP* gene has been well conserved during evolution of vertebrate species and that each gene is most likely present as a single copy. By genomic cloning, polymerase chain reaction, mapping, and direct sequencing, we show that the human *GCAP* gene spans approximately 6 kilobases of genomic DNA, and consists of four exons (>250, 146, 94, and 800 base pairs) separated by three introns (4.5 kilobases, 370 base pairs, and 347 base pairs). Using human/hamster hybrid panels and fluorescent *in situ* hybridization, the *GCAP* gene was localized to the short arm of chromosome 6 (p21.1).**

Light-detecting rod photoreceptors use cGMP as an internal messenger to gate cation channels and $Ca^{2+}$ ions to regulate synthesis of cGMP (1, 2). In dark-adapted photoreceptors, cGMP-gated channels are open, cGMP phosphodiesterase is inhibited, and the cytoplasmic cGMP and $Ca^{2+}$ concentrations are maintained at ~5 μM (3) and ~300 nM (4), respectively. Illumination of rhodopsin triggers an enzymatic cascade which rapidly hydrolyzes cytoplasmic cGMP (5). As a consequence, cGMP-gated cation channels close, disabling the influx of $Ca^{2+}$ (and other cations) into the outer segment, while the light-insensitive $Na^+/K^+$, $Ca^{2+}$ exchangers continue to extrude $Ca^{2+}$. The drop in cytoplasmic $Ca^{2+}$ is the signal for a photoreceptor guanylate cyclase (GC)[1] (6, 7) to accelerate the rate of cGMP synthesis, which ultimately enables the cell to return to the dark state. In cone photoreceptors, cytoplasmic $Ca^{2+}$ regulates a cone GC with features indistinguishable from those in rods (8).

GC activation is mediated by a membrane-associated $Ca^{2+}$-binding protein which is distinct from recoverin (9–11). The $Ca^{2+}$ dependence of GC activation is cooperative with a Hill coefficient of 2–4 (9, 12, 13), indicating the presence of several $Ca^{2+}$ binding sites in the GC activator. A $Ca^{2+}$-binding protein termed guanylate cyclase-activating protein (GCAP) has been biochemically isolated from bovine retinas, shown to activate GC in low $Ca^{2+}$, and to promote channel reopening when dialyzed into intact gecko rods (14). A second, less well characterized $Ca^{2+}$-binding protein termed p24, possibly closely related to GCAP, has been postulated to also activate GC in low $Ca^{2+}$ (15). In a previous report, we showed that a monoclonal antibody raised against bacterially expressed bovine GCAP blocks activation of GC by GCAP in low $Ca^{2+}$ and that an N-terminal peptide derived from the bovine sequence is a potent inhibitor of GC activation (16). *In situ* hybridization in bovine retinas suggested that GCAP mRNA is present in the inner segments of both rod and cone photoreceptors, but not in any other retinal cell type. To explore the possible involvement of the human *GCAP* gene in retinal dystrophies, we determined in this study its gene structure, its expression pattern in primate photoreceptor cells, and its chromosomal localization.

## MATERIALS AND METHODS

*Oligonucleotide Synthesis*—Primers were synthesized using a PCR-MATE (Applied Biosystems, Inc. model 391) DNA synthesizer. The oli-

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L36859 (human GCAP cDNA), L36860 (mouse GCAP cDNA), and L36861 (human GCAP gene).*

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[1] The abbreviations used are: GC, guanylate cyclase; GCAP, guanylate cyclase activating protein; PCR, polymerase chain reaction; RDS, retinal degeneration slow; bp, base pair(s); kb, kilobase pair(s).

gomers were deprotected in 700 μl of 14 M ammonium hydroxide at 55 °C for 8–12 h. An aliquot was treated with 1-butanol to remove ammonium hydroxide, and the precipitate was washed with isopropanol, lyophilized, and dissolved in 1 × TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The oligomers were quantitated by spectrophotometry (1 $OD_{260\ nm}$ = 33 μg).

*Southern Blotting*—Genomic DNA from nine eucaryotic species was isolated similarly as described previously (17). Except for yeast and human, all genomic DNAs were from kidneys. Human DNA was isolated from placental tissue. 8 μg of DNA exhaustively digested with *Eco*RI were loaded per lane. The size markers were *Hin*dIII-digested λ DNA. The probe was a 650-bp nick-translated fragment *a* (see Fig. 2) amplified with primers W231 (5′-GCC TGA GCG **ATG** GGG AAC ATT) and W224 (5′-GTA CAG AAA GAG TAG GCA GT) on a bovine cDNA template. Fragment *a* contains the translation start codon ATG (boldface), and W224 is located 10 bp downstream of the translation stop codon TGA. The hybridization conditions were 5 × SSPE, 10 × Denhardt's solution, 100 μg/ml denatured, sheared salmon sperm DNA, 2% SDS, overnight at 65–68 °C. The washing conditions were 2 × SSC (standard saline citrate), 0.1% SDS at room temperature, and 0.2 × SSC, 0.1% SDS at 65 °C, respectively, for 30 min each.

*Library Screening and Characterization of GCAP cDNA Clones*—The isolation of two bovine GCAP cDNA clones was described previously (16). The nick-translated fragment *a* was used to screen adult human retina λgt10 (J. Nathans, Johns Hopkins School of Medicine), and wild-type mouse retina λZapII (18) cDNA libraries under relaxed stringency (19). Of several positive clones identified in the primary screen, the following clones were fully characterized (see Fig. 2): two human λ-gt10 GCAP clones (HRG3, HRG7) containing the full coding sequence of GCAP, two truncated λzap mouse clones (MRG1, MRG2), and one full-length clone (MRG4). The human λHRG3 insert was amplified with λgt10 primers flanking the *Eco*RI cloning site and subcloned into the PCRII vector to yield HRG3. The inserts of mouse λzap clones were excised *in vitro*, according to the Stratagene protocol, and subcloned into pBluescript. The coding portions of all clones were completely sequenced on both strands, and PCR was performed as described previously (20).

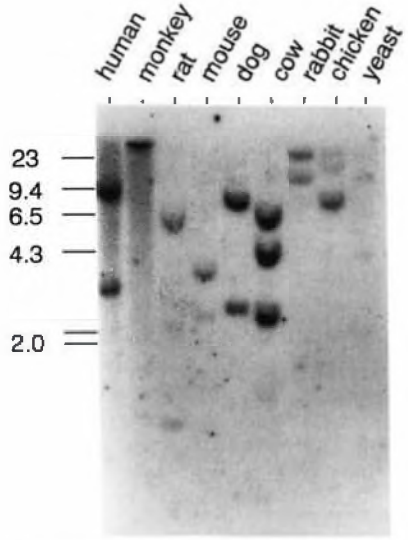


FIG. 1. **Genomic blot of various species.** *Eco*RI-digested genomic DNA from nine eucaryotic species (human; monkey (rhesus); rat (Sprague-Dawley); mouse (BALB/c); dog; bovine; rabbit; chicken; yeast (*S. cerevisiae*)) was probed with nick-translated bovine GCAP fragment *a* (Fig. 2*A*) under high stringency). The size markers (indicated on the *left*) are *Hin*dIII-digested λ DNA.

*DNA Sequencing*—Plasmid and λ DNA were purified using standard procedures (21). Supercoiled plasmid DNA was sequenced using the double-stranded procedure with universal or sequence-specific primers as described previously (20). λ DNA was directly sequenced using linear amplification DNA sequencing with 10 pmol of end-labeled primers and 750–1,500 ng of λ DNA (fmol sequencing kit, Promega, Madison WI).

*Human Genomic Clones*—A complete gene clone (λHG1) spanning all exons and introns was isolated from a human genomic library (λfixII, Stratagene, La Jolla, CA) using fragment *a* as a probe (see Fig. 2). A genomic fragment (HGTA1, 1013 bp) spanning parts of exon 2, intron 2, exon 3, intron 3, and parts of exon 4 was produced by PCR amplification with primers W238 (5′-GCT ACA TTG TTT CAT GGA GTA C) and W241 (5′-GCA TCT GGT CCT TCT GGA CGC) on a human genomic DNA template. Using λHG1 as a template, a second genomic fragment (HGTA2, 4.7 kb) spanning intron a was amplified with primers W242 (5′-TGA AGG CCT GAG CAA TGG GCAA) and W246 (5′-GCT GAG GCC TGC CAC GTA CTC C). A third fragment (HGTA3, 450 bp) containing mostly upstream sequence was amplified with antisense W245 (ACG TAC TGG CTG GCC GAC GG) and universal primer T7. All amplified fragments were cloned into the PCRII vector (TA cloning kit, Invitrogen) and partially or completely sequenced. Two *Eco*RI subclones, HGTA2a (2.2-kb insert) and HGTA2b (2.5-kb insert), were produced from HGTA2 by cleavage with *Eco*RI and religation (see Fig. 5*A*), and they were partially sequenced.

*In Situ Hybridization*—Monkey (*Macaca nemestrina*, female) retinas were obtained from the Regional Primate Research Center at the University of Washington, supported by National Institutes of Health Grant RR00166. Retinas were fixed and processed with sense and antisense RNA probe produced by transcription of the human GCAP clone HRG3 (see Fig. 2) as described previously (16).

*Chromosomal Localization*—The chromosomal location of the human *GCAP* gene was identified by PCR using human-hamster somatic cell hybrids and controls (BIOS Laboratories, New Haven, CT) as templates. For amplification of a 180-bp genomic fragment of exon 1 of the *GCAP* gene, sense primer W242 (5′-TGA AGG CCT GAG CAA TGG GCAA) and antisense primer W245 (5′-ACG TAC TGG CTG GCC GAC GG) were chosen. The PCR amplification mixture contained 50 ng of genomic DNA, 25 ng of each primer in 25 μl, and standard concentrations of *Taq* polymerase and substrate (22). The amplification cycles were 4 min at 94 °C; 30 times 1 min each at 60, 72, and 94 °C; and 10 min at 72 °C. The BIOS panel nomenclature (human chromosome content in parentheses) is as follows: 010 (10); 016 (16); 212 (5D, Y); 324 (18); 423 (3); 683 (1<30%, 5D, 12, 14, 19, 21, 22); 734 (5, 9, 18); 750 (5, 13, 14, 15, 19); **756** (5D, 6, 7, 12<30%, 13, 14, 19, 20, 21,Y); 803 (4, 5, 8, 22,X); 811 (8, 17, 18); 852 (2); 867 (1, 5, 13, 14, 18, 19); **909** (5D, 6, 8, 14,X); 937 (1, 5, 14, 15, 17, 21); 940 (5, 20); 1006 (4, 5, 7, 13, 15, 19, 21,Y<30%); 1049 (5, 11); 1079 (3, 5); 1099 (1, 5D, 13, 19, 21, 22). "D" denotes a partial deletion, "<30%" a partial presence of the respective chromosome. The two panels containing chromosome 6 are boldfaced.

*Fluorescent in Situ Hybridization*—For subchromosomal localization, both HRG3 and HRG-TA1 were labeled with biotin-14-dATP using the BioNick labeling system (Life Technologies, Inc.). A mixture of the two probes was hybridized to pro-metaphase chromosomes prepared from peripheral blood lymphocytes (23). The probe was either singularly or cohybridized with a digoxygenin-labeled chromosome 6 centromere-specific probe (D6Z1, Oncor) and detected as described previously (24). To determine specific band assignments, slides were first Q-banded, the position of the metaphase spreads were recorded, and

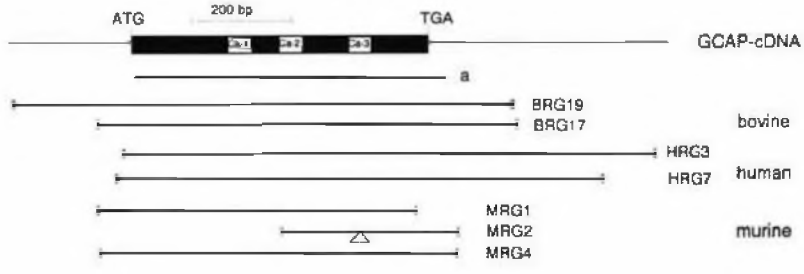


FIG. 2. **Physical map of mammalian cDNA clones.** The coding portion of GCAP cDNA is schematically represented as a *black box*. The $Ca^{2+}$ binding domains in GCAP (*Ca-1*, *-2*, *-3*) are highlighted. Clones isolated from bovine (*BRG17*, *BRG19*), human (*HRG3*, *HRG7*, and mouse (*MRG1*, *MRG2*, *MRG4*) cDNA libraries are shown as *lines*. A *triangle* in MRG2 indicates alternative splicing of this clone (see text). Fragment *a* was used as a probe for screening and genomic blotting.

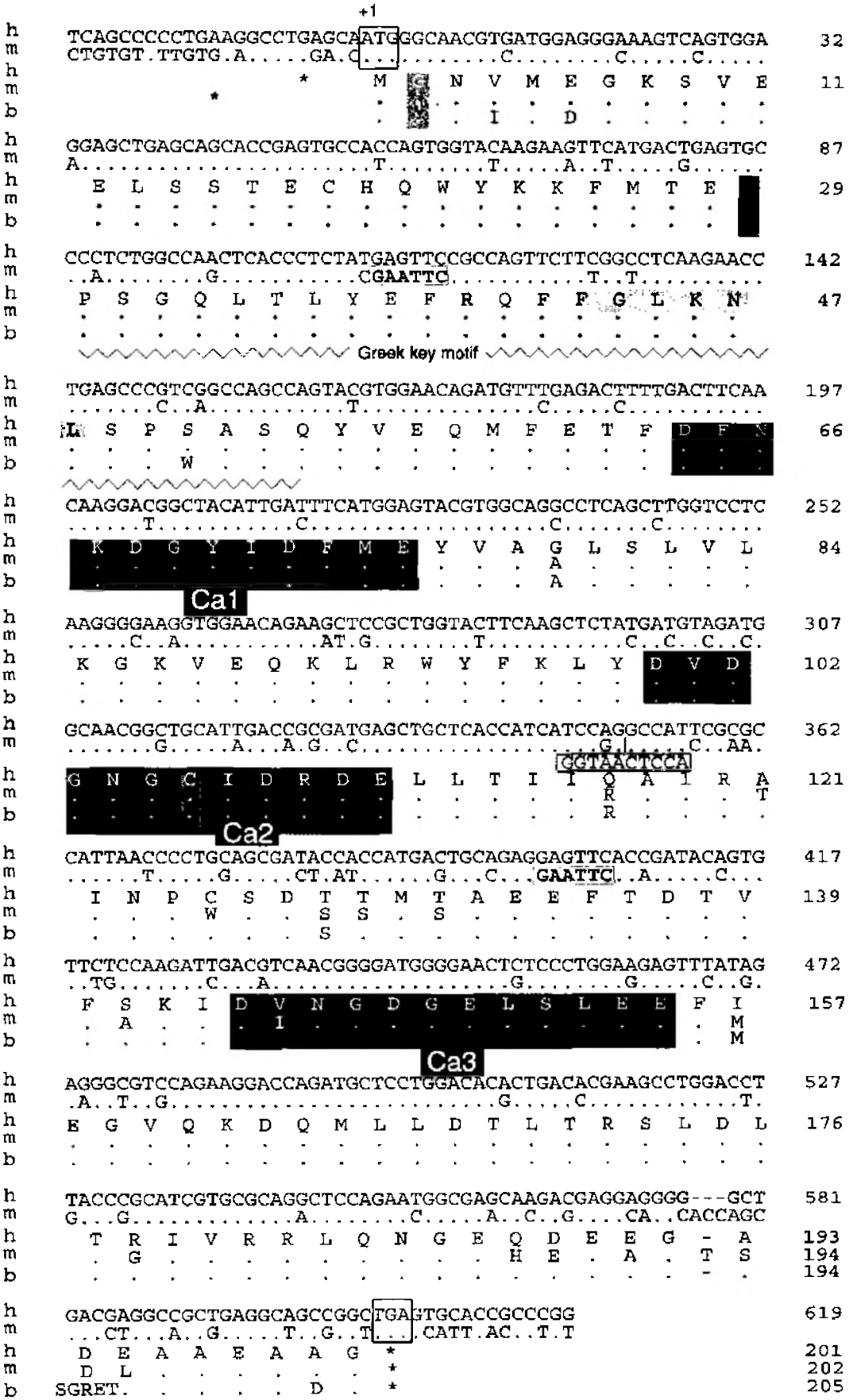


FIG. 3. **cDNA and deduced amino acid sequence of human and mouse GCAP.** Nucleotide numbering of the human (*h*) and mouse (*m*) sequence starts with the first nucleotide of ATG (*boxed*). In-frame stop codons that define the open reading frame are depicted by *asterisks*. The

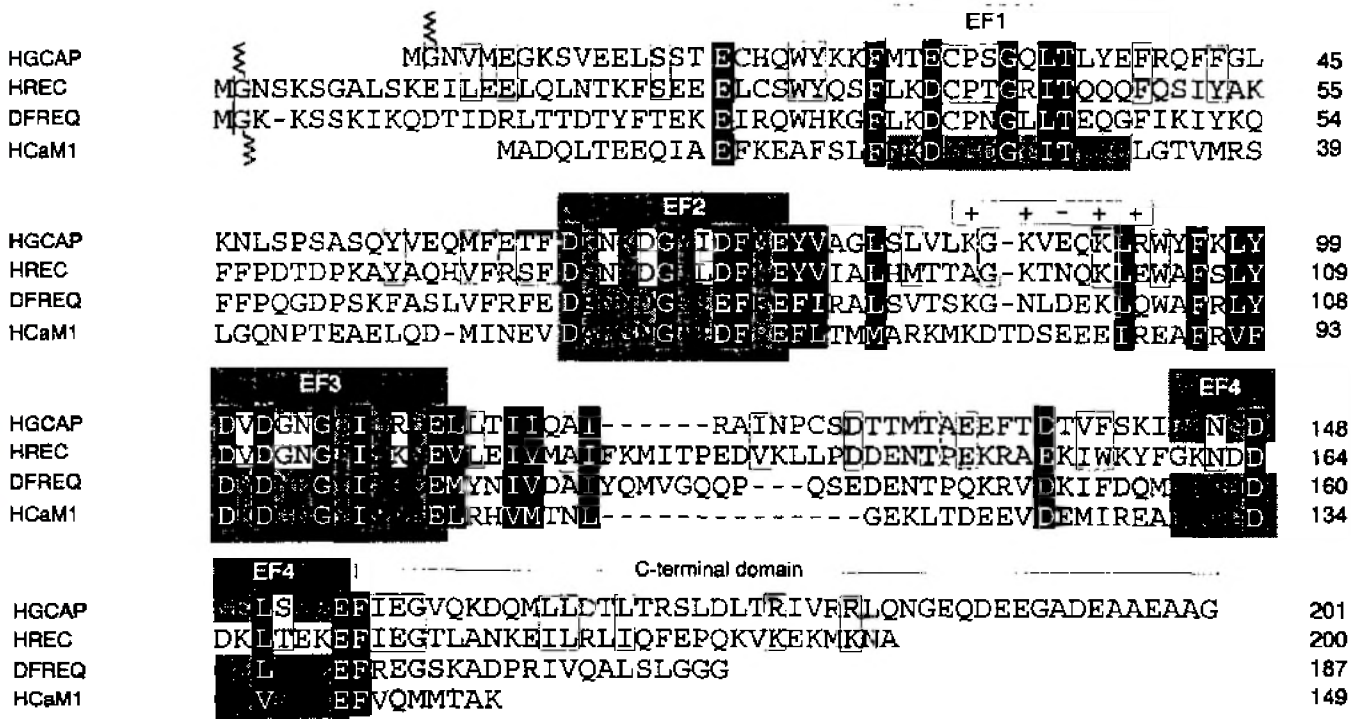


FIG. 4. **Amino acid sequence alignment of human GCAP with other $Ca^{2+}$-binding proteins.** The human GCAP sequence was aligned with human recoverin (*HREC* (9)), *Drosophila melanogaster* frequenin (*DFREQ* (54)), and human calmodulin (*HCaM1* (55)). Amino acids are depicted in *single-letter symbols*. L = I = V = M; Y = F; E = D; R = K; A = T = S are considered conservative substitutions. For best fit, several gaps were introduced (shown by *hyphens*). Conserved residues are shown in *black boxes*. Residues identical in GCAP and recoverin are *boxed* and *lightly shaded*. EF hand $Ca^{2+}$ binding domains (*EF1* to *EF4*) are shown as *dark-shaded boxes*. The myristoylation site at the second residue in GCAP, recoverin, and frequenin is indicated by a *vertical zig-zagged line*.

their images stored. The slides were then destained, fluorescent *in situ* hybridization analysis with the probe mixture was performed, and the position of the fluorescent signals relative to the Q bands were noted (25). At least 20–30 metaphases were examined per hybridization using a Zeiss Axiophot microscope equipped with a triple band pass filter (4,6-diamidino-2-phenylindole, fluorescein isothiocyanate, and rhodamine).

## RESULTS AND DISCUSSION

*GCAP Gene Homologies Revealed by Southern blotting*—Genomic Southern blotting is a valuable tool for identifying homologous genes in various species. We used a 610-bp bovine GCAP probe covering the coding sequence (see Fig. 2*a*) to detect *GCAP* genes in a variety of vertebrate species under stringent hybridization and washing conditions (Fig. 1). These conditions allow for approximately 10% base pair mismatches (17). The species tested included human, monkey, bovine, rat, mouse, dog, rabbit and chicken. Single fragments were detected in monkey (>23 kb), rat (6 kb), and chicken (6.5 kb) genomic DNA (Fig. 1). Two major fragments (9.4 and 3 kb) appear to harbor the human *GCAP* gene (Fig. 1, *lane 1*). In mouse, one major (3.5 kb) and several weakly hybridizing smaller fragment generated by internal *Eco*RI sites (see Fig. 3) indicate that the mouse *GCAP* gene is contained in less than 6 kb of genomic DNA. A weaker hybridization signal was also detected in the *Saccharomyces cerevisiae* (yeast) (Fig. 1, *lane 9*). The function of a calcium-binding protein in yeast related to GCAP is unknown. The results suggest that the *GCAP* gene structure is compact, that it is most likely present as a single copy per haploid genome, and that its sequence is well conserved among vertebrate species.

*Molecular Cloning of Human and Mouse GCAPs*—To elucidate the human and mouse *GCAP* gene structure, we first cloned the corresponding cDNAs. We previously identified bovine GCAP cDNA clones by screening a retina library with a degenerate oligonucleotide designed according to tryptic peptide sequences (16). Two overlapping cDNA clones (BRG17, BRG19) contained a poly(A) tail indicating complete sequences in the 3′-untranslated region. Fragment *a* (Fig. 2) was used as a probe to isolate homologous clones from human, and mouse retina libraries. The number of positively identified GCAP clones in these libraries was 1–5/25,000 plaque-forming units. At least one clone in each species containing a contiguous coding sequence was isolated and completely sequenced. The translation start codons of the GCAP cDNA sequences are preceded by in-frame stop codons (Fig. 3). The coding sequences are 603 to 615 nucleotides in length and contain no internal repeats as is typical for calmodulin (26) and troponin C (27). The lengths of the human (HRG3, 1.35 kb) and bovine (BRG19, 1.2 kb) cDNA clones are in agreement with the sizes (1.1–1.3 kb) of mRNAs seen on Northern blots (results not shown). The sizes of the human and bovine GCAP mRNA are consistent with a transcription start point located approximately 100 bp upstream of the translation start codon. Comparison of the size

insertion of 10 nucleotides at the putative splice junction of intron b and exon 3 (Fig. 5*B*) of the mouse gene are *boxed*. The two internal *Eco*RI sites (GAATTC) in the mouse sequence are *boldfaced* and *boxed*. The amino acid numbering starts with the first translation initiator (*M*) of the open reading frame. As a comparison, the amino acid residues deviating in the bovine (*b*) GCAP sequence (16) are also shown. The three predicted EF-hand motifs for $Ca^{2+}$ binding (*Ca1*, *Ca2*, and *Ca3*) are highlighted by a *black box*. The invariant cysteines are shown by a *shaded box*. The N-terminal region implicated in GC activation is *shaded*. The region predicted to be folded into a Greek key motif is *squiggly underlined*.

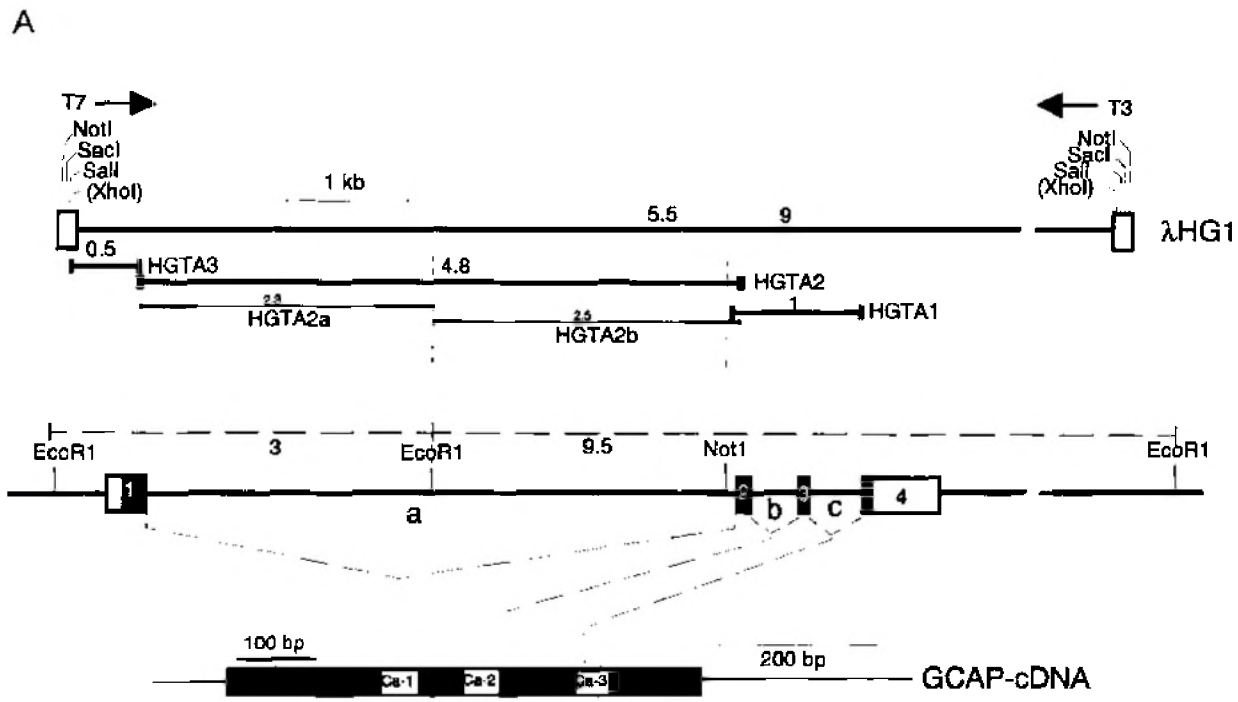


FIG. 5. **Human *GCAP* gene structure.** *A*, graphical representation of the gene and genomic clones. Exons 1–4 of the *GCAP* gene are depicted as *boxes*, the coding portions are filled. Introns (*a–c*) and flanking sequences are shown as *lines*. The extent of genomic subclones HGTA1–3 is shown by lines ending in *delimiters*. HGTA2 was further subcloned into HGTA2a and HGTA2b. The multiple cloning site of the λfixII vector flanking the genomic insert is *hatched*, and the unique restriction sites appropriate for excision of the insert are shown. Note that intron a has an internal *Not*I site. The two genomic *Eco*RI fragments identified in Fig. 1 (*lane 1*) are shown by broken lines. The approximate position of introns relative to the $Ca^{2+}$ binding domains in GCAP cDNA is indicated at the *bottom*. *B*, gene sequence. Exons are shown in *upper case letters*, introns in *lower case letters*. The 4.5-kb intron a sequence is shown only partially, introns b and c are complete. The polyadenylation site AATAAA (56) and the translation start codon ATG are *boxed*. The nucleotide numbering starts with A of ATG; only exon sequences are counted. Relevant primers used for amplification and/or sequencing are marked by *arrows*, with the sense or antisense strand indicated by *arrowheads*. The last residue of the *GCAP* gene sequence is the site of poly(A) attachment. The deduced GCAP amino acid sequence is shown as *single-letter* symbols. $Ca^{2+}$ binding sites are *shaded*.

of the mouse GCAP mRNA and the length of the three mouse GCAP clones (MRG1, MRG2, MRG4) indicates truncation at the 3′ end of at least 600 bp. The truncated clone MRG2 has an insertion of 10 bp at the splice junction of the last intron (Figs. 2 and 3) causing a reading frameshift. The resulting alternatively spliced version of mouse GCAP, if produced, would be truncated due to the presence of a stop codon three residues after the junction. The presence of an alternate splice site 10 bp downstream of the intron b/exon 3 junction of the mouse *GCAP* gene was verified by gene sequencing (not shown). The significance of alternative splicing of the mouse *GCAP* gene is not known.

*GCAP Is a Highly Conserved Protein*—As shown in Fig. 3, the predicted GCAP amino acid sequences consist of 201 (human), 202 (mouse), and 205 (bovine) residues. The sequence similarity among mammalian GCAPs is better than 90% at the amino acid level. The calculated molecular masses are 21–23 kDa. Amino acid and sequence analysis (hydropathy plot, results not shown) predicts that vertebrate GCAPs are soluble, acidic proteins ($pK_a \sim 4.2$) lacking hydrophobic domains. Native GCAP, however, is membrane associated and hydrophobic, indicating that presumably other structural elements contribute to GCAP's insolubility. One such feature is N-terminal heterogeneous acylation (16) at position 2 (Gly), a posttranslational modification which is at least in part responsible for membrane association of other proteins (28–30). Other features may include thioacylation (possible palmitoylation of a Cys in the N-terminal domain (position 18, Fig. 4), or clusters of alternating positive charges capable of interacting with negatively charged phospholipid head groups (30). Interestingly, such a cluster is present in GCAP at position 85–97 (between EF2 and EF3, Fig. 4), but not in recoverin which is more soluble than GCAP.

Residues 1–180 of the bovine, mouse and human GCAP sequences are unusually well conserved showing only 1 or 2 nonconservative amino acid substitutions. The N-terminal domain (residues 2–57, *shaded* in Fig. 3) is thought to be involved in GC activation (16). Sequence inspection reveals the presence of two invariant cysteines (*boxed* in Fig. 3) presumably indispensable for secondary structure, and predicts three EF hand structures involved in $Ca^{2+}$ binding (a fourth domain is most likely disabled, see next paragraph). The only divergent domain containing insertions/deletions is located at the C terminus. It consists of approximately 20 residues, and accounts for the variation in length of the human, mouse and bovine GCAPs.

*The GCAP Sequence Is Homologous to Other $Ca^{2+}$-binding Proteins*—GCAP is a novel member of the large and diverse superfamily of $Ca^{2+}$ binding proteins which includes the ubiquitous and promiscuous calmodulin, frequenin (found in *Drosophila* synapses), visinin (chicken retina), recoverin (vertebrate photoreceptors), and over 100 others (31). Sequence alignment (Fig. 4) with recoverin (HREC), frequenin (DFREQ), and calmodulin (HCaM1) which derive from a common ancestor with four EF hand $Ca^{2+}$ binding consensus sequences shows 31 identically conserved amino acid residues, most of them grouped around the four EF hand structures. The most pronounced differences occur in the variable C termini which range from seven (HCaM1) to 45 residues (GCAP). In addition,

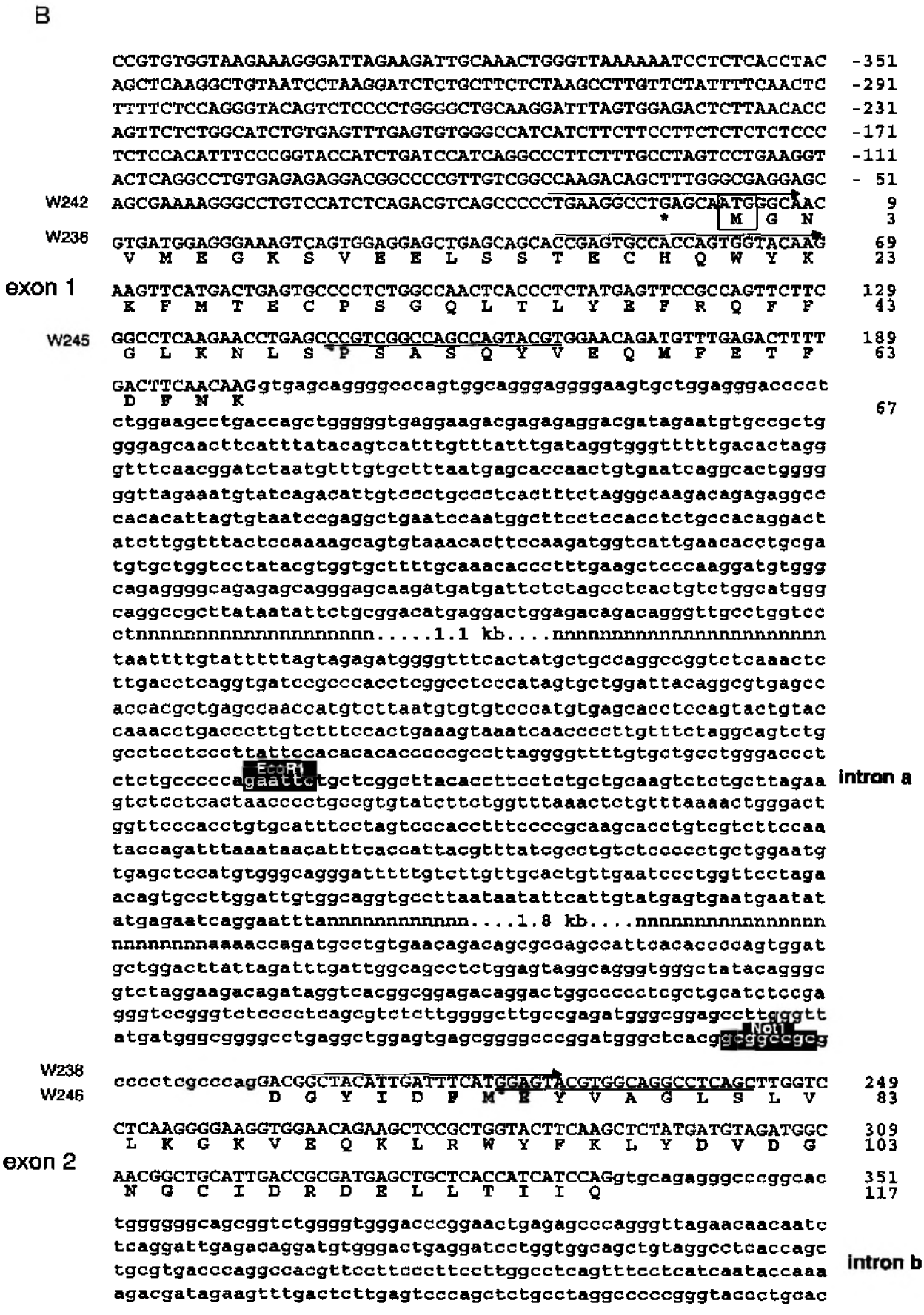


FIG. 5—*continued*

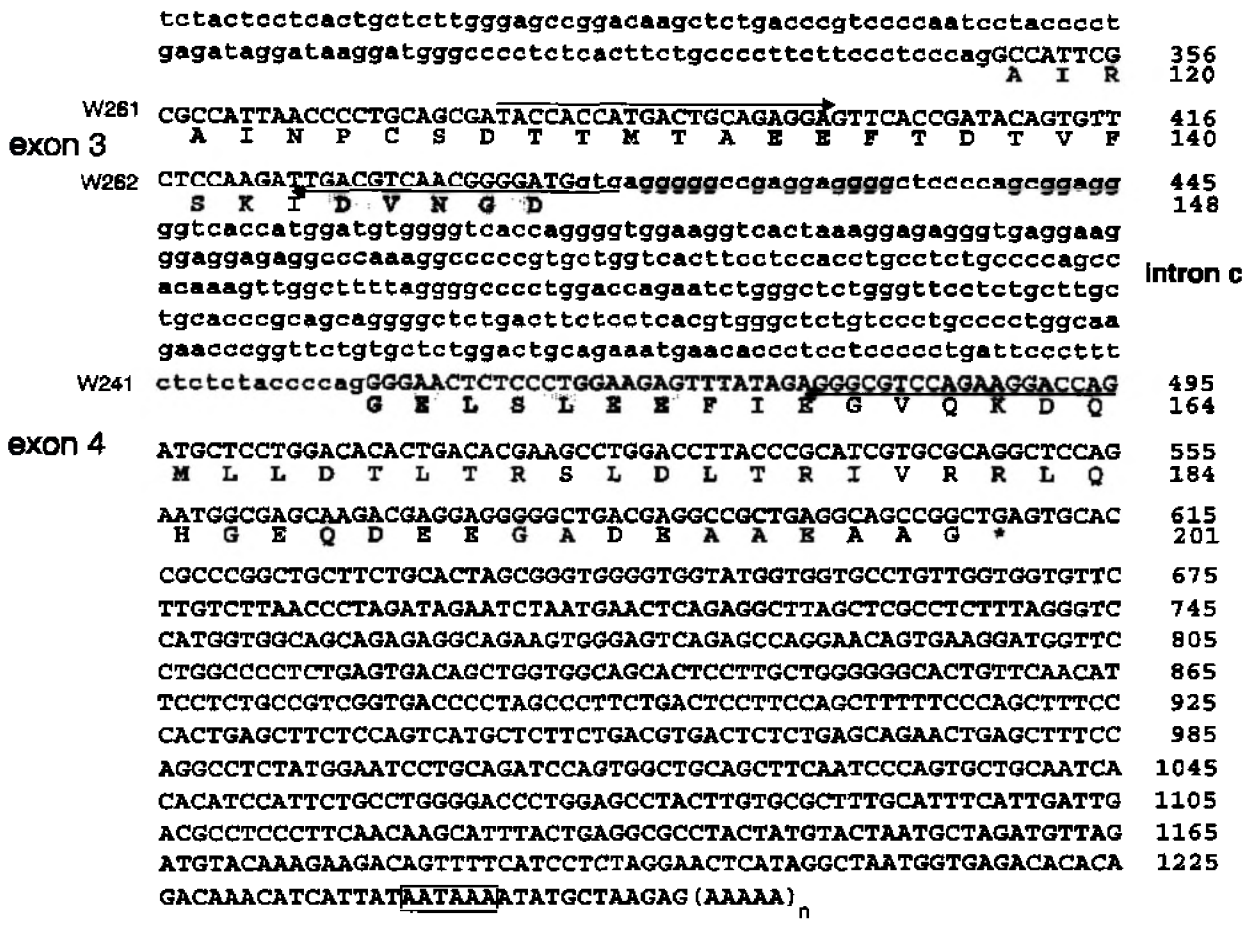


FIG. 5—*continued*

as noted for recoverin and calmodulin (32), the length of the linker between EF2 and EF3 is variable. The sequence similarity between the two photoreceptor $Ca^{2+}$-binding proteins recoverin and GCAP is only 36% even when conservative substitutions are taken into account. In recoverin, crystallographic data show the presence of four EF hand (helix-loop-helix) structures, but only two functional $Ca^{2+}$ binding sites (EF2 and EF3) (32). In GCAP and frequenin, the three domains EF2, EF3, and EF4 are well conserved, while insertion of a Cys and the presence of a rigid Pro render the first domain (EF1) incompatible with $Ca^{2+}$ binding. It is interesting to note that secondary structure predictions indicate the presence of a Greek key motif around the non-functional EF1 domain of GCAP (Fig. 3). Greek key motifs, common in the crystallins (components of the vertebrate lens) and protein S (a procaryotic calcium-binding protein lacking EF hand motifs) (33, 34), contain antiparallel $\beta$-strand structures. This suggests that the symmetric helix-loop-helix configuration (EF1, EF2, and EF3, EF4 separated by a groove) characteristic for recoverin tertiary structure may not be present in GCAP.

*Human GCAP Gene Structure*—To determine the intron/exon distribution of the *GCAP* gene, three overlapping genomic clones encoding GCAP (λHG1, λHG2, λHG3) were isolated by screening 500,000 plaque-forming units of a commercial λfixII genomic library. λHG1 containing all exons and introns of the *GCAP* gene as well as flanking regions was further characterized (Fig. 5*A*). Southern blotting of *Not*I-digested λHG1 revealed the presence of two fragments, 5 and 9 kb in length, predicting a 14-kb insert and the presence of an internal *Not*I site in the *GCAP* gene (Fig. 5, *A* and *B*). A contiguous physical map was produced by analysis of overlapping genomic subclones (HGTA1–3, Fig. 5*A*) representing various portions of the human *GCAP* gene. The gene structure was elucidated by complete sequencing of the resulting subcloned fragments and determination of the intron/exon splice junctions (Fig. 5*B*).

The results show that the gene is split into four exons distributed over 6 kb of genomic DNA. Intron a is 4.5 kb in length and contains the unique internal *Eco*RI site responsible for the two large genomic fragments seen in the genomic Southern blot (Fig. 1, *lane 1*). The lengths of introns b and c are 370 and 347 bp, and the two central exons are 146 and 94 bp each. The six splice junctions conform to the donor/acceptor consensus sequences (35). The positions of introns in the *GCAP* gene do not conform to the known domain structure of GCAP. The first EF hand $Ca^{2+}$ binding domain is split by intron a, the second is entirely encoded by exon 2, and the third $Ca^{2+}$ binding site is again split into two halves by intron c. In mouse, preliminary results indicate that the intron positions are exactly as in the human gene. There is no conservation of intron/exon boundaries between the *GCAP* gene and the human recoverin gene containing three exons (36) or the human calmodulin gene CaMI containing six exons (37).

*Expression of GCAP mRNA in Primate Retinas*—*In situ* hybridization with digoxygenin-labeled antisense and sense RNA was used to identify the retinal cell type which expresses the *GCAP* gene. As shown in Fig. 6*A*, no hybridization was observed with the sense probe (Fig. 6*A*), whereas the whole pho-

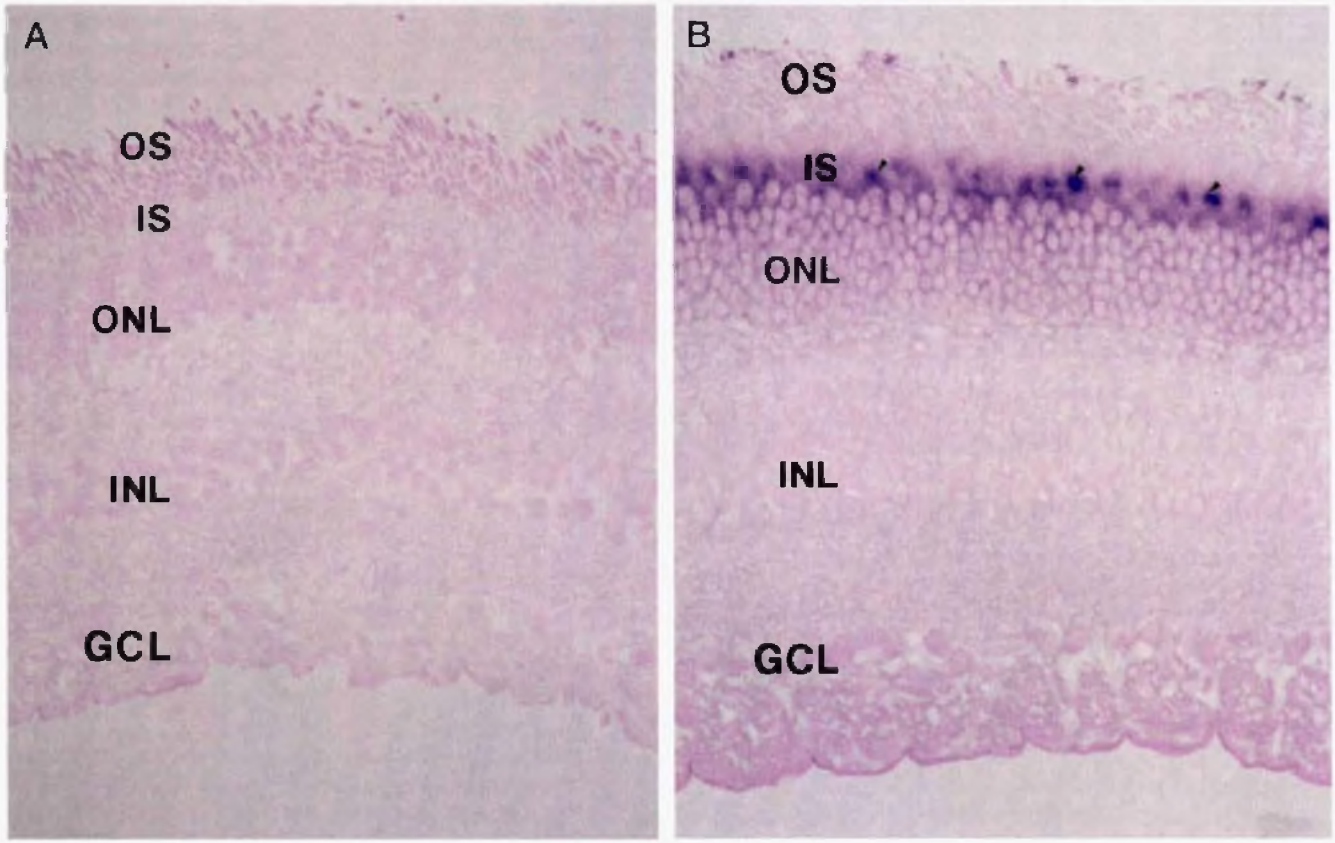


FIG. 6. **In situ hybridization of GCAP RNA to monkey retina sections.** Monkey retina sections were hybridized with digoxygenin-labeled sense (*A*) and antisense (*B*) human GCAP RNA. The retina layers are indicated as follows. *OS*, outer segments; *IS*, inner segments; *ONL*, outer nuclear layer; *INL*, inner nuclear layer; *GCL*, ganglion cell layer.

toreceptor layer was selectively stained with the antisense probe (Fig. 6*B*). The most intense staining was found in the myoid region of cone inner segments where protein synthesis takes place. No other cell type of the retina appears to express GCAP at comparable levels. These results indicate that GCAP is expressed specifically and discretely within photoreceptors cells, and that the distribution GCAP mRNA is very similar in primate and bovine retinas.

*Chromosomal Localization*—To identify the chromosome which contains the human *GCAP* gene (locus designation *GCAP1*), we performed PCR analysis on DNAs from 20 human-hamster somatic cell hybrids. We selected two primers (W242 and W246, Fig. 5*B*) which amplify a 180 bp human-specific intron-less portion of exon 1. Only hybrids 756 and 909 (see "Materials and Methods") yielded the expected product consistent with the *GCAP* gene residing on chromosome 6. Sublocalization to the short arm of chromosome 6 was unequivocally determined on banded metaphase spreads (Fig. 7, *A–D*). Assignment of the GCAP1 locus to region 6p21.1 was confirmed by co-hybridization of a chromosome 6 centromere-specific probe (Fig. 7*B*). Consistent signals were not observed on any other chromosomes. Analysis of interphase cells also showed only one copy of the probe present in the human genome consistent with the results of genomic Southern blotting (Fig. 1). The position 6p21.1 is almost identical to the position of another photoreceptor-specific protein involved in rod disc structure, "retinal degeneration slow" or *RDS*, on 6p21.1-cen. Defects in the *RDS* gene are responsible for the *rds* phenotype in mouse (38) and have been linked to retinitis pigmentosa (39, 40) and macular degenerations (41) in human. By synteny with *RDS* (42) and other loci near p21.1 (pim1 oncogene, *PIM1*; tumor necrosis factor, *TNFA*; cytochrome P450, *CYP21*) (43), the mouse *gcap1* locus is predicted to reside on chromosome 17.

*The GCAP Gene Is a Candidate for Causing Retinal Disorders*—Defects in genes expressed in photoreceptors have been linked to several retinal disorders and dystrophies in animal (20, 44) and human models (45, 46). Prominent among the human disorders are autosomal retinitis pigmentosa (rhodopsin, RDS/peripherin, phosphodiesterase-β) (45), retinitis punctata albescence (RDS) (47), tritanopia (blue cone opsin) (48), color blindness (red and green pigments) (49), macular degeneration (RDS) (41), and congenital stationary night blindness (rhodopsin, phosphodiesterase-β) (50, 51). Recently, a new locus for autosomal recessive retinitis pigmentosa, distinct from RDS, has been identified on 6p (52), and initial mapping studies indicate that this locus is within 2 cM of the *GCAP* gene and may be identical with the GCAP locus.[2] Since GCAP plays a key role in return of the photoreceptor to the dark state after illumination, a defect in the *GCAP* gene may disturb activation of cyclase and possibly disable accelerated cGMP synthesis essential for depolarization of photoreceptors. A predicted phenotype for a GCAP missense mutation may be non-progressive night blindness in which cGMP levels in dark adapted photoreceptors are abnormally low, as has been observed in families with defects in the rhodopsin gene causing constitutive activation of transducin (53) or in a large Danish pedigree in which a defect in the phosphodiesterase-β gene causes persistent hydrolysis of cGMP (51). More severe phenotypes like RP and macular de-

[2] P. Banerjee, J. Knowles, and C. Gilliam, personal communication.

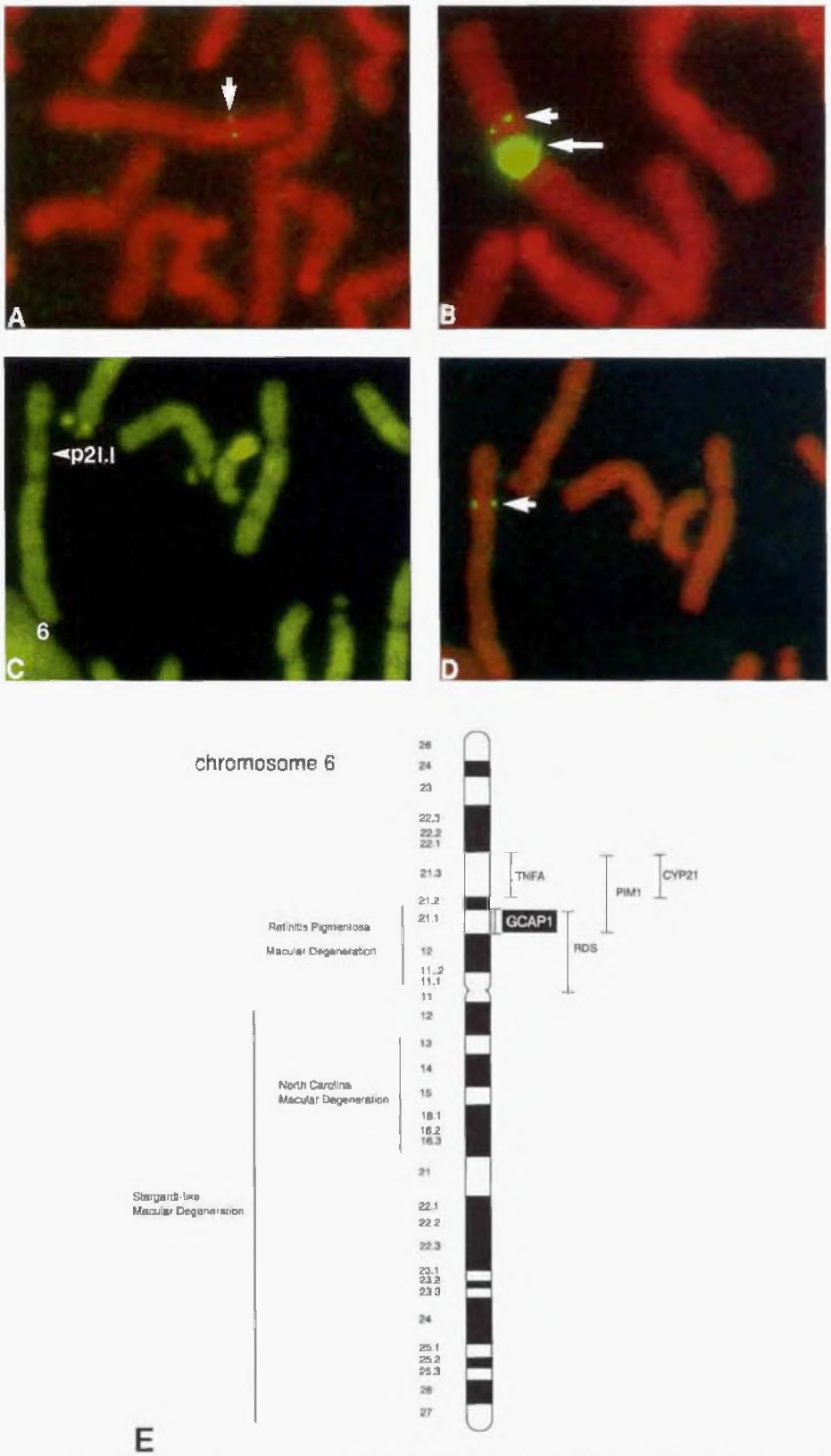


FIG. 7. **Subchromosomal localization by fluorescent *in situ* hybridization.** *A*, *GCAP* localization to chromosome 6 on propidium iodide stained metaphase spread. The arrow points to the GCAP1 locus. *B*, chromosome 6 co-hybridized with human GCAP DNA (*short arrow*) and FITC-labeled centromeric probe D6Z1 (*long arrow*). *C*, portion of a prehybridized, Q-banded metaphase chromosome spread. The *arrow* indicates the site of hybridization at the p21.1 band of chromosome 6. *D*, same metaphase as in *C*, now stained with propidium iodide and hybridized with the probe (*arrow*). *E*, idiogram of human chromosome 6 with the map position of the *GCAP* gene denoted by a *bar*.

generation in which photoreceptors progressively degenerate would be consistent with a null allele and loss of GCAP function.

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