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The Human GCAP1 and GCAP2 Genes Are Arranged in a Tail-to-Tail Array on the Short Arm of Chromosome 6 (p21.1)

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GCAP1 and GCAP2 are related Ca^{2+}-binding proteins that activate photoreceptor guanylate cyclase(s). We showed previously that the human GCAP1 gene, consisting of four exons, is located at 6p21.1 (locus designation GUCA). To identify the chromosomal location of the GCAP2 gene, we first cloned its cDNA and determined its intron–exon distribution by PCR analysis. The results show that the introns of the GCAP2 gene are positioned exactly as in the GCAP1 gene and are nearly double in size. Sequence similarity between the two genes, however, is limited to portions of exons 1 and 2. The GCAP1 and GCAP2 genes are transcribed into single mRNA species (1.7 and 2.2 kb, respectively) and are detectable only in the retina by Northern blotting. The GCAP2 gene was found by somatic human–hamster hybrid panel analysis and FISH to reside at GUCA in a region indistinguishable from that of GCAP1. PCR analysis with exon 4-specific primers showed that the genes are in a tail-to-tail array less than 5 kb apart and altogether span less than 20 kb of genomic DNA. The identical gene structures and loci of GCAP1 and GCAP2, and the identical function of the gene products, are consistent with a gene duplication event.

INTRODUCTION

In vertebrate rod photoreceptor outer segments (ROS), light triggers changes in concentrations of cGMP and Ca^{2+}. In dark-adapted outer segments, cytoplasmic cGMP and free Ca^{2+} levels are relatively high (Pugh and Lamb, 1990; Lagnado and Baylor, 1992). After photobleaching and activation of a cGMP phosphodiesterase, cGMP concentrations drop, causing closure of cGMP-gated cation channels in the plasma membrane. As a result, free Ca^{2+} in outer segments is reduced, which in turn activates guanylate cyclase (GC) (Koch and Stryer, 1988; Koch, 1991). This feedback mechanism is mediated by a membrane-associated Ca^{2+}-binding protein termed GCAP (guanylate cyclase activating protein) (Gorczyca *et al.*, 1994). To date, two retina GCAPs (GCAP1 and GCAP2) have been characterized (Palczewski *et al.*, 1994; Dizhoor *et al.*, 1994, 1995; Gorczyca *et al.*, 1995; Frins *et al.*, 1996). Both GCAPs were biochemically isolated from bovine retinas, cloned and expressed in heterologous systems, and shown to activate ROS membrane associated GC in low concentrations of free Ca^{2+}.

Like GCAPs, two guanylate cyclases (GC1 and GC2) have been cloned from the retina (Shyjan *et al.*, 1992; Lowe *et al.*, 1995; Goraczniak *et al.*, 1994; Yang *et al.*, 1995), indicating the presence of a diverse Ca^{2+}-dependent modulation system of GCs in this tissue. By virtue of their retina-specific expression, GC and GCAP genes are candidates for being involved in vertebrate retinal dystrophies. In human, the GC gene (GUC2D) is located on 17p13.1 (Oliveira *et al.*, 1994), and the GCAP1 gene (GUCA) is located on 6p21.1 (Subbaraya *et al.*, 1994). Potential defects in the GCAP1/GC1 modulatory unit have been suggested to cause autosomal recessive retinal degeneration. In the *rd* chicken (Semple-Rowland *et al.*, 1996), cGMP levels are very low prior to the onset of degeneration. Recently, the gene responsible for autosomal recessive retinitis pigmentosa (arRP) in

Sequence data reported in this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. U82537.

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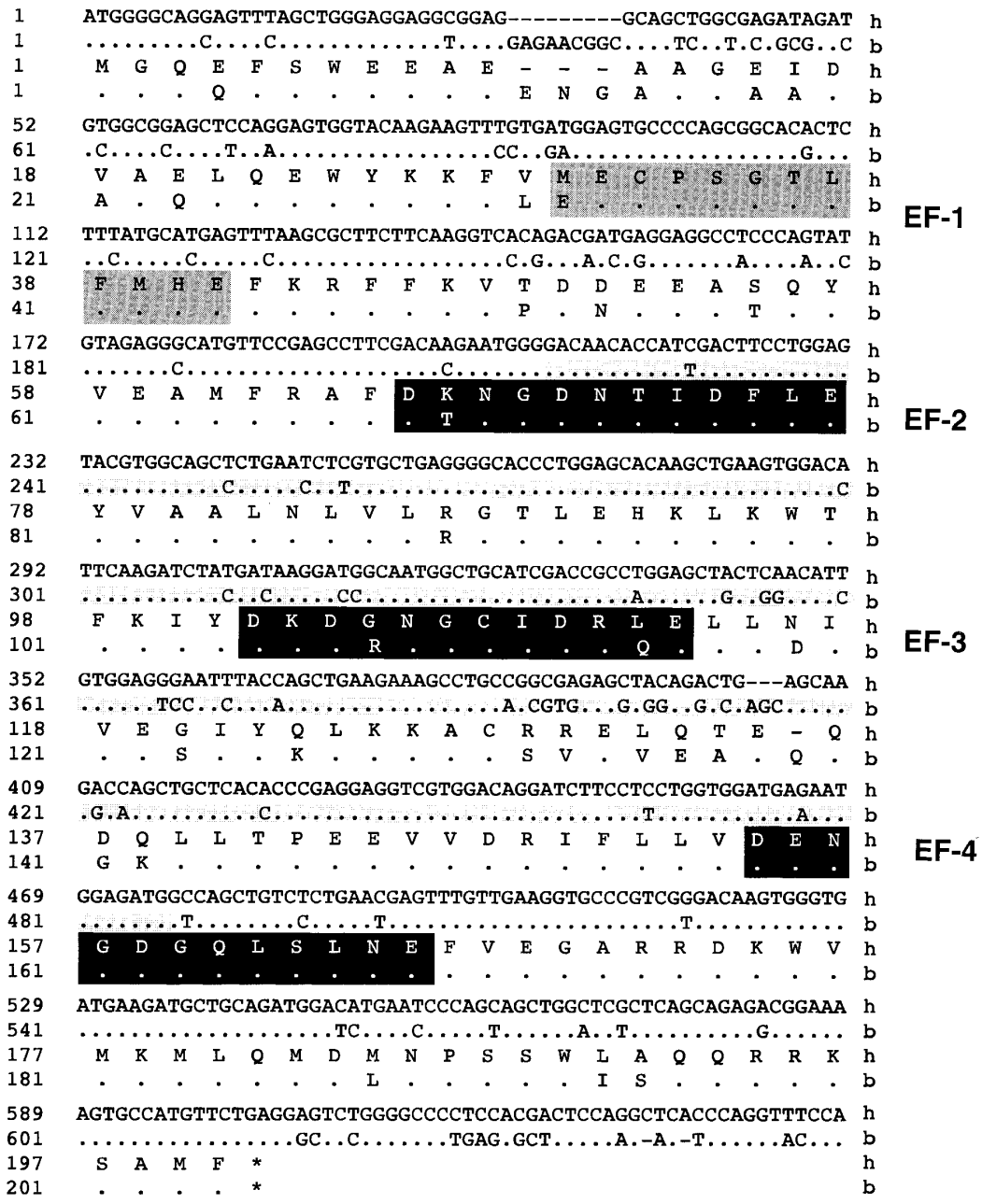

FIG. 1. GCAP2 cDNA and predicted amino acid sequence. The human and bovine GCAP2 cDNA coding sequences and predicted amino acid sequences were aligned. For optimal alignment, several gaps were introduced (shown by hyphens). Identical residues are indicated by dots. The EF-hand domains EF2-4 that are presumed to bind Ca^{2+} are shown as black boxes. The EF-1 domain that does not bind Ca^{2+} is shaded. The lightly shaded area in the bovine cDNA (positions 206 to 487) was found deleted in a cloned splice variant. The endpoints of the shaded area coincide with splice junctions of introns a and c, respectively. Lowercase h and b on the right indicate human and bovine sequences, respectively.

a Dominican pedigree has been mapped by linkage analysis to 6p21, a locus indistinguishable from that of GCAP1 (Knowles *et al.*, 1994; Subbaraya *et al.*, 1994; Shugart *et al.*, 1995). In this paper, we elucidate the GCAP2 gene structure and describe our finding that the GCAP1 and GCAP2 genes are arranged in a tail-to-tail array at the short arm of chromosome 6 (locus designation GUCA1A and GUCA1B, respectively). To identify a possible link between the arRP disease locus and the GCAP gene array, we fine-mapped the GCAP locus relative to the disease locus using radiation hybrid panels.

MATERIALS AND METHODS

Library screening, identification and characterization of clones. A nick-translated bovine GCAP2 fragment was used to screen adult human retina λgt10 cDNA libraries (J. Nathans, Johns Hopkins

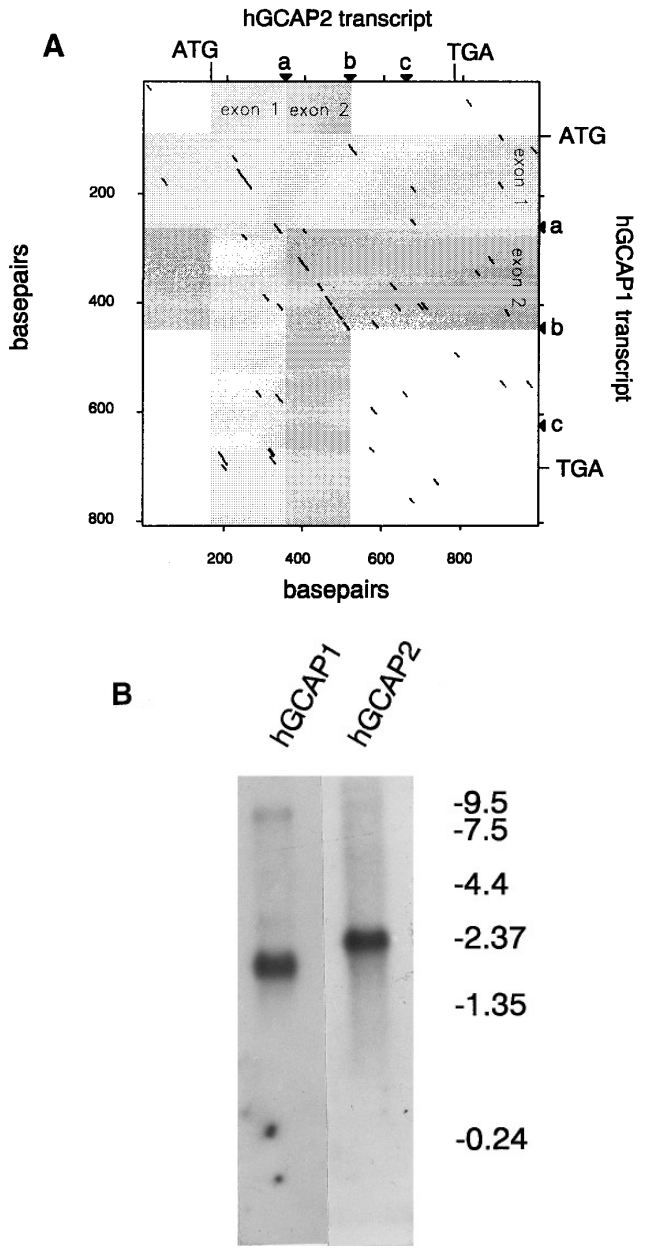

FIG. 2. Human GCAP2 and GCAP1 mRNA. (**A**) Dot matrix analysis of the human GCAP2 (hGCAP2) and GCAP1 (hGCAP1) transcripts. Positions of introns are depicted by a–c, positions of translation start and stop are indicated by ATG and TGA. The window of comparison was 10 nucleotides at a stringency of 90%. The areas of exons 1 and 2 containing sequence similarity are 5 and 10% shaded, respectively. (**B**) Northern blot of human retina RNA. 2 μg of human retina was loaded per lane. The blot was probed with GCAP1 cDNA (left) and reprobed with GCAP2 cDNA (right). Known size standards are indicated on the right in kb.

School of Medicine) under relaxed stringency (Pittler *et al.*, 1990). The inserts of λgt10 phage clones were amplified with λgt10-fwd and λgt10-rev primers and cloned into PCRII vector (Invitrogen). PCR was performed as described previously (Pittler and Baehr, 1991) in a MJ Research cycler or in a Perkin–Elmer Model 9600 using the protocol of the manufacturer. Plasmids were purified using standard procedures (Sambrook *et al.*, 1989). Supercoiled plasmid DNA was sequenced using the double-stranded procedure as described previously (Pittler and Baehr, 1991) or with a LI-COR Model 4000L automatic sequencer using universal primers labeled with an infrared fluorescent tag. The coding portions of all clones were completely sequenced on both strands.

Northern blotting. Retina RNA was separated on 0.43 *M* formaldehyde agarose gels (Ausubel *et al.*, 1987) and transferred to maximum strength Nytran (Schleicher and Schuell). A multiple tissue RNA blot (Clontech Laboratories, Inc.) was probed with random-primed (Feinberg and Vogelstein, 1984), linearized PCRII plasmid containing the human GCAP2 coding sequence.

Southern blotting. A genomic Southern blot containing *Eco*RI-digested DNA from 10 mammalian species was purchased from BIOS Laboratories (New Haven, CT). The probe was a 650-bp nick-translated human GCAP2 cDNA fragment containing the complete coding region. The hybridization and washing were performed according to the manufacturer's protocol.

Gene characterization and DNA sequencing. Introns of the GCAP2 genes were amplified with exon-specific primers from human and bovine genomic DNA and with *Taq* polymerase, according to the Cetus/Perkin–Elmer protocol, or with *Taq*/Pwo polymerase (Expand Long PCR System, Boehringer Mannheim). Amplified introns were cloned into PCRII and sequenced with a Model LI-COR 4000L automatic DNA sequencer, or they were directly sequenced with amplification primers and fluorescently tagged chain terminators on a capillary sequencer (Perkin–Elmer Model 310). Most intron sequences were determined in only one direction. Deletions of sequenced portions were generated by PCR amplification of internal fragments.

Chromosomal localization. The chromosomal location of the human GCAP2 gene was identified by PCR using human–hamster somatic cell hybrids and controls (BIOS Laboratories) as described previously (Subbaraya *et al.*, 1994). For amplification of a 180-bp genomic fragment of exon 1 of the GCAP2 gene, sense primer W318 and antisense primer W319 (Fig. 5) were chosen. For subchromosomal localization, HGCAP2 cDNA and a genomic fragment comprising intron c were labeled with biotin-14–dATP as described previously (Subbaraya *et al.*, 1994).

Radiation hybrid mapping. The Genebridge 4 radiation hybrid panel was obtained from Research Genetics. PCR amplification was carried out using *Taq* polymerase and AmpliTaq Gold (Perkin–Elmer) as described previously (Knowles *et al.*, 1994). PCR primers 5′-GAG GCA GAA GTG GGA GTCA and 5′-CTG TGT GTC TCA CCA TTA GCC were designed using PRIMER V0.5 (S. E. Lincoln, M. J. Daly, and E. S. Lander) and used for the amplification of a portion of exon 4 of GCAP1. GCAP1 was mapped using the RHMAPPER program provided by the Whitehead Institute Center for Genome Research (WICGR) on the World-Wide Web.

RESULTS

GCAP2 cDNA

Using bovine GCAP2 cDNA as a probe, we isolated a 980-bp human cDNA clone (HG2) containing the complete GCAP2 coding sequence (Fig. 1). The open reading frame consisted of 600 nt predicting a human GCAP2 of 200 amino acids. The bovine and human GCAP2 cDNA sequences are more than 90% similar, while the human GCAP2 and GCAP1 cDNA coding regions show only 57% overall sequence similarity. Strong sequence similarity between the two human genes is limited to the N-terminal region encoded by exons 1 and 2, as shown by dot-matrix comparison (Fig. 2A) performed at 90% stringency.

Expression of GCAP2 mRNA

Northern blot analysis of bovine and human retina RNA showed single transcripts for GCAP1 and GCAP2

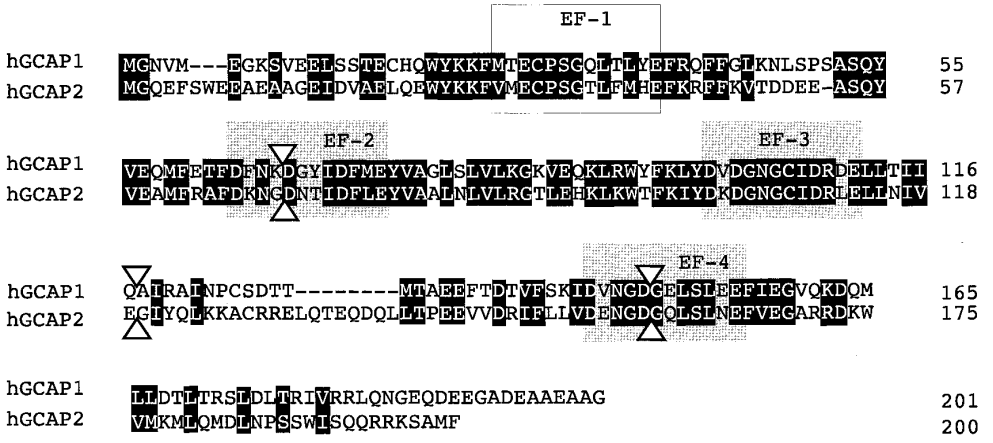

FIG. 3. Alignment of human GCAP2 and GCAP1 amino acid sequences. Amino acids are depicted as single-letter symbols. L=I=V=M, Y=F, E=D, R=K, and A=T=S are considered conservative substitutions. For best fit, several gaps were introduced (shown by hyphens). Residues conserved in both GCAP sequences are shown on a black background. EF-hand consensus sequences (EF-1 to EF-4) are shaded. EF-1 (not binding Ca^{2+}) is boxed. The positions of introns interrupting the coding sequence are indicated by open triangles.

genes (1.7 and 2.2 kb for human; 1.3 and 2.2 for bovine) in both species (Fig. 2B). No cross-reactivity of GCAP1 probes with GCAP2 mRNA (and vice versa) was observed, consistent with the low sequence homology of the cDNA probes (Fig. 2A). Analysis of multiple tissue blots containing RNA from various human tissues (heart, brain, lung, liver, skeletal muscle, kidney, and pancreas) showed that GCAP2 is not expressed at detectable levels in nonretina tissues. Thus we conclude that, like GCAP1, GCAP2 is expressed at high levels only in the retina. GCAP1 mRNA could be detected by *in situ* hybridization in both rod and cone photoreceptor cells of bovine and primate retinas (Palczewski *et al.*, 1994; Subbaraya *et al.*, 1994). GCAP2 has been postulated to be also present in the outer and inner segments of photoreceptor cells (Dizhoor *et al.*, 1995). Biochemical data (Gorczyca *et al.*, 1995), however, suggest that GCAP2 is only a minor component of rod photoreceptor cells and may be present mostly in other parts of the retina.

GCAP1 and GCAP2 Are Members of the Calmodulin Superfamily

GCAPs are members of the large and diverse superfamily of calmodulin-like Ca^{2+}-binding proteins (Nakayama *et al.*, 1992) that evolved from a common ancestor containing four EF-hand consensus sequences. The amino acid sequences of GCAP1 and GCAP2 predict three EF-hand Ca^{2+}-binding consensus sequences (Fig. 3). A fourth domain near the N-terminus is thought to be unable to chelate Ca^{2+} due to a lack of the key residues involved in ion coordination. Within the GCAP1 subfamily, the N-terminal domains are well conserved, while the C-termini are divergent (Palczewski *et al.*, 1994). In GCAP2, the N-terminal domain appears more divergent and the C-terminal region is conserved (Semple-Rowland *et al.*, 1996). The most conserved domains in human GCAPs (Fig. 3) and GCAPs from other species are located between EF2 and EF3 encoded by exon 2, indicating an important role of this domain in the structure and function of GCAPs.

GCAP2 Gene Homologies among Mammalian Species

We used a 610-bp human GCAP2 cDNA probe to detect GCAP2 genes in a variety of mammalian species under stringent hybridization and washing conditions (Fig. 4). The species tested included human, tamarin, pig, sheep, bovine, cat, dog, rabbit, rat, and mouse. A single fragment was detected only in mouse genomic DNA (18 kb). All other species yielded two or more fragments. In human, two major fragments (Eco-a, 16 kb; Eco-b, 2 kb; Fig. 4, lane 1) harbor the

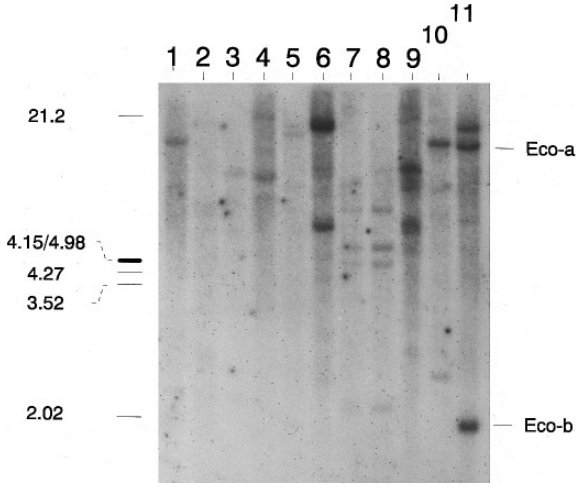

FIG. 4. Genomic blot of various species. *Eco*RI-digested genomic DNA from 10 mammalian species (lane **1,** mouse; lane **2,** hamster; lane **3,** rat; lane **4,** rabbit; lane **5,** dog; lane **6,** cat; lane **7,** cow; lane **8,** sheep; lane **9,** pig; lane **10,** tamarin; lane **11,** human) was probed with nick-translated bovine GCAP2 cDNA under high stringency. The size markers are *Hin*dIII/*Eco*RI double-digested λ DNA.

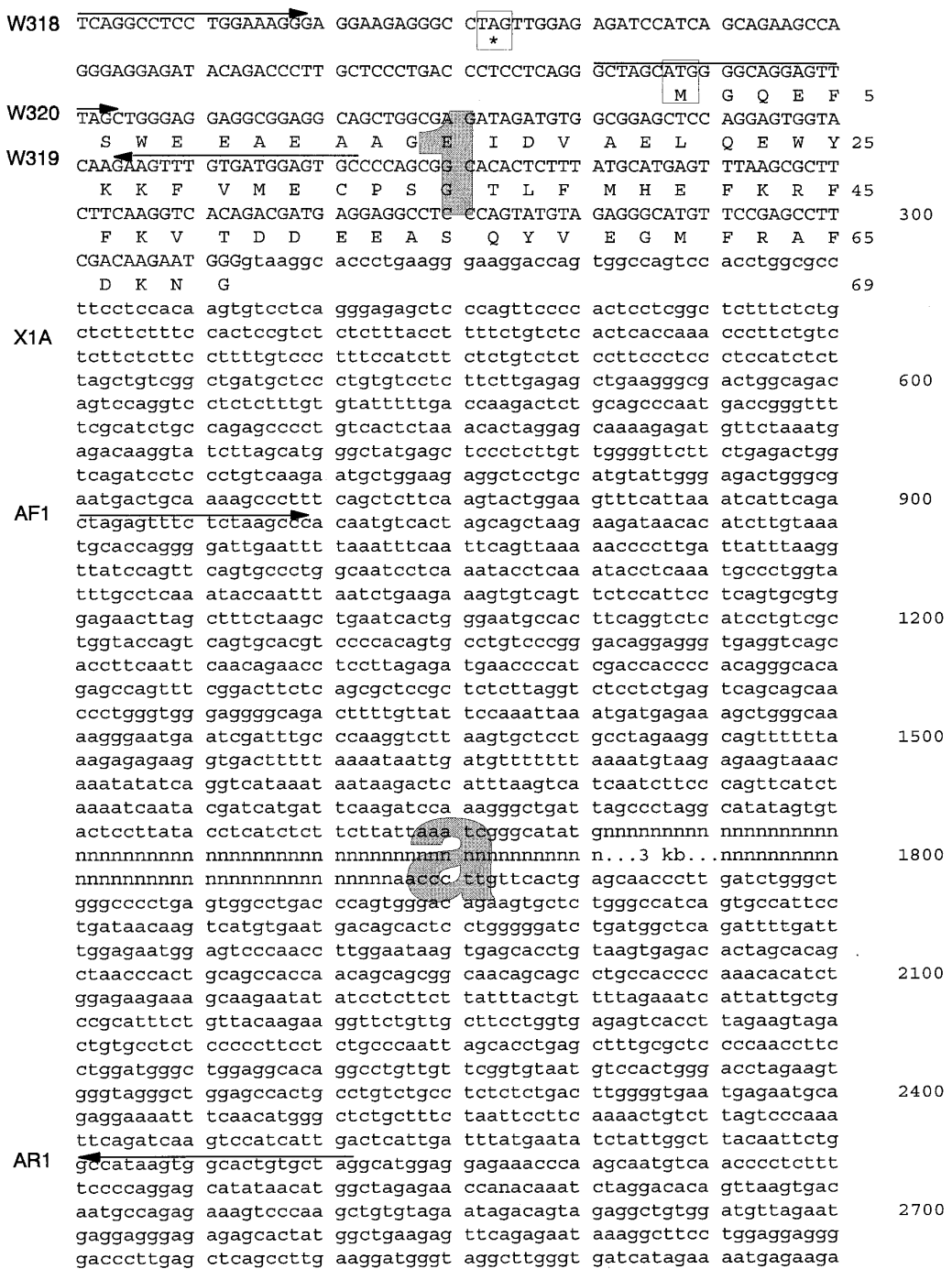

FIG. 5. Nucleotide and predicted amino acid sequences of the human GCAP2 gene. Exons are shown in uppercase letters and are identified by large background numbers. Introns are shown in lowercase letters and are identified by large background letters a, b, or c. Intron sequences a and b are shown only partially. The putative polyadenylation site AATAAA (Proudfoot, 1989) is shown white on a black background. *Eco*RI restriction sites, in-frame stop codons, and translation initiator ATG are boxed. The nucleotides are numbered on the right, starting with the first residue shown. Primers used for amplification of exons and introns are indicated by arrows pointing to the left (antisense) or to the right (sense). Numbering of amino acids is shown on the right, starting with the translation initiator M. The CA microsatellite repeat in intron b is hatched. Sequences homologous to *Alu* repeats (Jurka and Milosavljevic, 1991) are shaded.

complete human GCAP2 gene. A third fragment of 18 kb appears to be incompletely digested and composed of fragments a and b. The size of fragments Eco-a and Eco-b is consistent with *Eco*RI sites identified in the human GCAP2 gene (Fig. 5). The results of genomic Southern blotting suggest that the

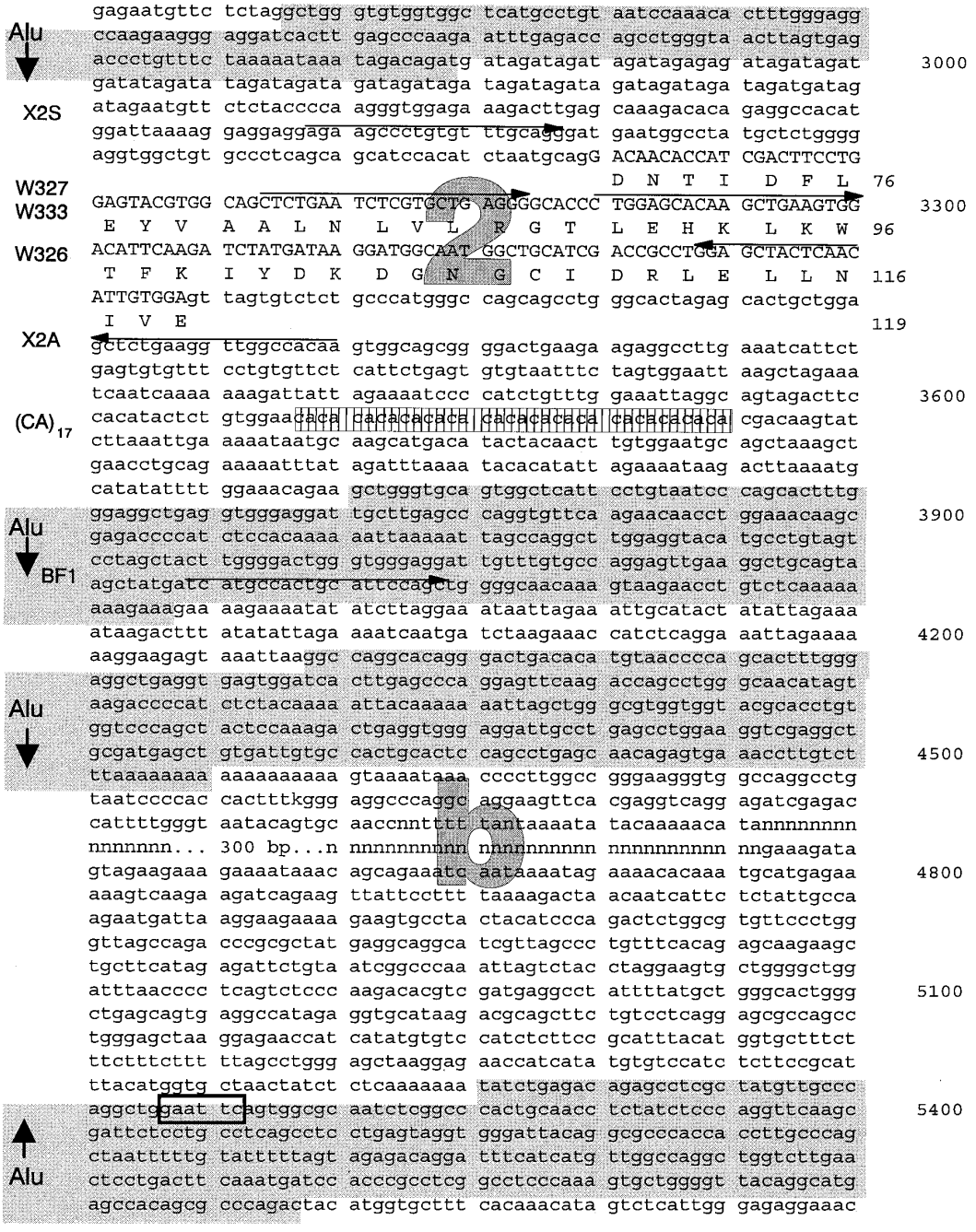

FIG. 5—*Continued*

GCAP2 gene structure is most likely present as a single copy per haploid genome and that its sequence is well conserved among vertebrate species.

Gene Structure

While cloning bovine GCAP2 cDNA we identified a low-copy splice variant (not identifiable in Northern blots) in which a central portion of GCAP2 cDNA was deleted (EF-2 to EF-4, Fig. 1). The borders of the deletion suggested that the GCAP2 intron–exon arrangement was very similar or identical to that of the GCAP1 gene. To verify the GCAP2 gene structure, we amplified the introns of the human and bovine gene with exon-specific primers and determined their full or partial sequences. Introns were found at positions identical to those of the human and mouse GCAP1 genes (Subbaraya *et al.*, 1994). The three introns a, b, and c (6, 3, and 0.7 kb in human (Fig. 5) and 8, 1.6, and 0.9 in bovine (not shown)) revealed no interspecies sequence similarity and no similarity to the corresponding introns of the GCAP1 gene. As found commonly in human

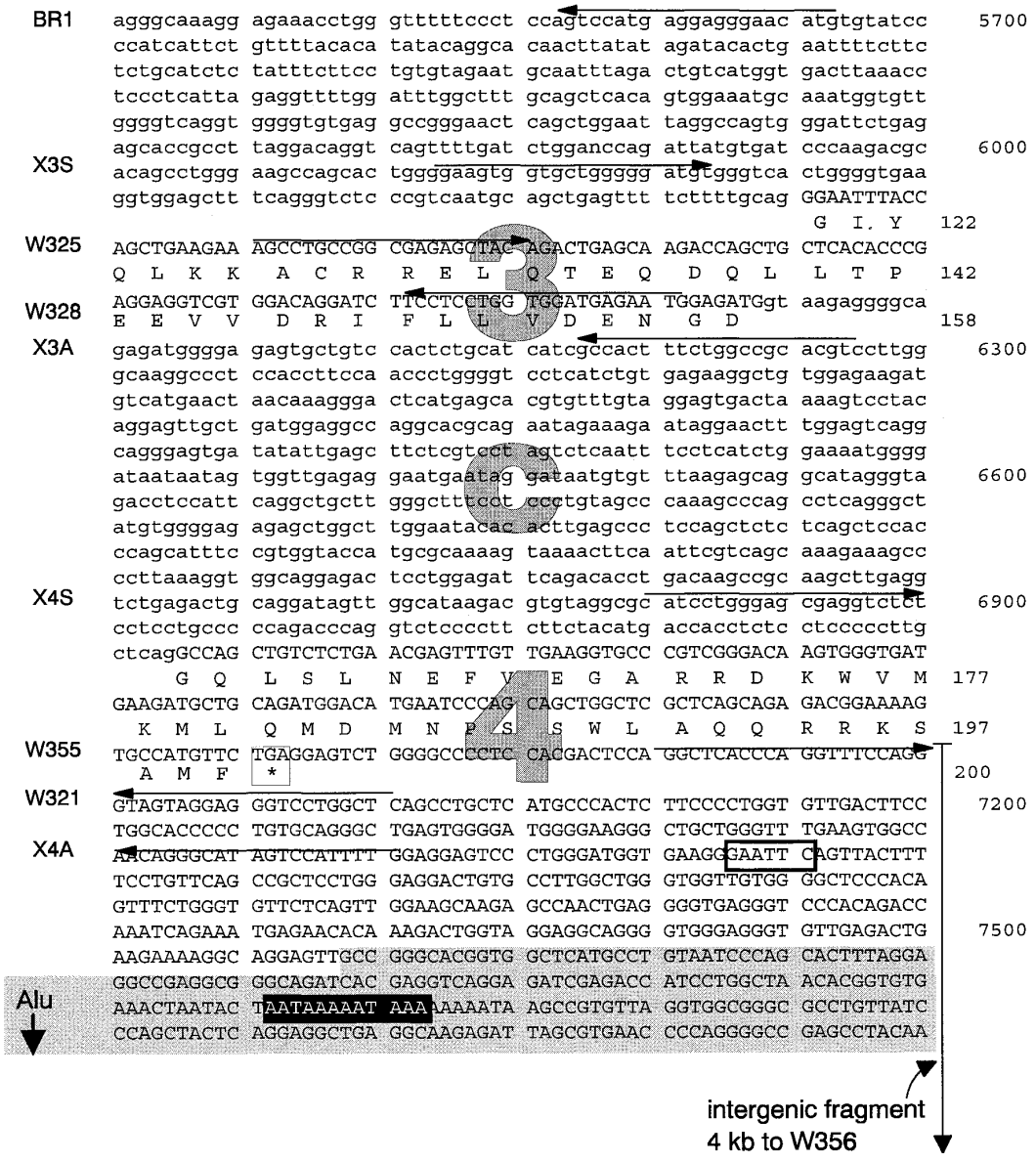

FIG. 5—*Continued*

genomic DNA, introns of the human GCAP2 gene have several *Alu* repeats in the sense or antisense direction. In addition, intron b contains a $(CA)_{17}$ microsatellite sequence. The six splice junctions conform to the donor/acceptor consensus sequences (Padgett *et al.*, 1986). Due to the larger introns, the four exons of the GCAP2 gene are distributed over more than 10 kb of genomic DNA (GCAP1 gene less than 6 kb). Identical gene structure and sequence similarity in exons 1 and 2, and identical function of the gene products, suggest that the GCAP1 and GCAP2 genes are the product of a gene duplication event.

Tail-to-Tail Arrangement of the GCAP1 and GCAP2 Genes

Amplification of exon 1 of the human GCAP2 gene from human–hamster somatic hybrid panels located the gene to chromosome 6 (not shown). Sublocalization was unequivocally determined on banded metaphase spreads (not shown) to 6p21.1, a locus indistinguishable from that of GUCA1A. To estimate the size of the intergenic region and to identify the respective orientation of the two genes, we performed PCR analysis with exon-specific primers. As shown in Fig. 6, PCR amplification with exon 4-specific primers resulted in a 4.5-kb intergenic fragment. Partial sequencing of this fragment showed that it contained portions of both GCAP1 and GCAP2 3′-UTRs including polyadenylation signals located on opposite strands. This result shows that the GCAP1 and GCAP2 genes are in close proximity and are arranged in a tail-to-tail array such that the intergenic distance between respective translational stop codons is less than 5 kb. In mouse, the two GCAP genes are interdigitating; i.e., the polyadenylation signals of

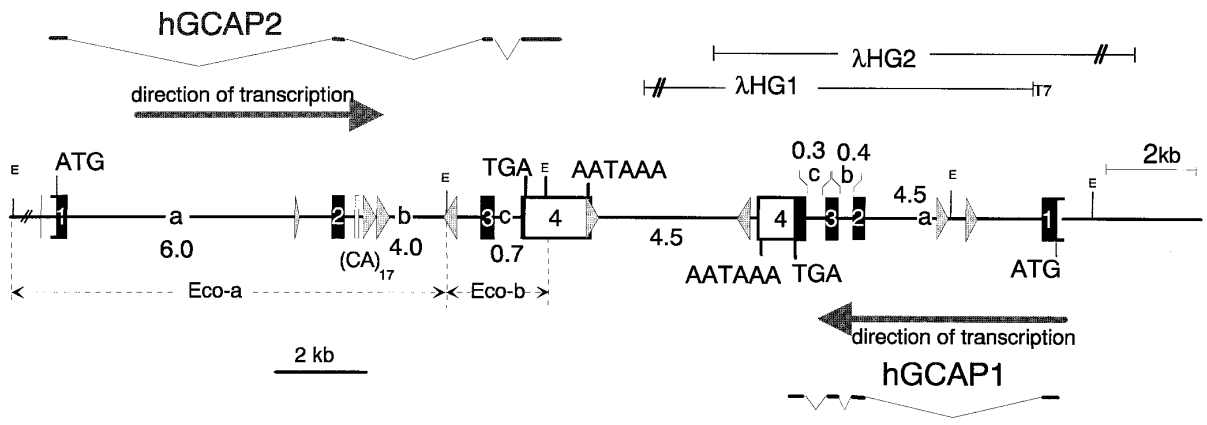

FIG. 6. Tail-to-tail arrangement of GCAP2 and GCAP1 genes. The human GCAP1 and GCAP2 gene structures are depicted graphically. Exons are shown as boxes; the coding portions are filled. Introns (length given in kb) and flanking sequences are shown as lines. A triangle pointing left or right indicates an *Alu* repeat in the sense or antisense direction, respectively. *Eco*RI (E) restriction sites identified in the gene sequence and the lengths of the fragments (Eco-a, Eco-b) are shown. Large arrows indicate the direction of transcription. The extent of cloned cDNA for both genes (hGCAP1 and hGCAP2) is indicated by bars above (hGCAP2) or below (hGCAP1) the respective genes. λHG1 and λHG2 are genomic clones described previously (Subbaraya *et al.*, 1994).

one gene are located on the noncoding strand in the 3′-UTR of the other gene (Bronson and Baehr, manuscript in preparation).

Map Positions of GCAP1/GCAP2 on 6p

The GCAP gene array is located at 6p21 (Subbaraya *et al.*, 1994). One form of arRP, which segregates in two large Dominican pedigrees, has been localized to the same chromosomal band (Knowles *et al.*, 1994; Shugart *et al.*, 1995). To map more precisely the position of the GCAP gene array relative to the disease locus, we used the Genebridge 4 radiation hybrid panel, which has a resolution of approximately 300 kb. As shown in Fig. 7, exon 4 of the GCAP1 gene mapped centromeric to the microsatellite markers D6S943 and D6S1560. These markers constitute the centromeric and telomeric boundaries of the disease gene in the Dominican pedigrees. Based on the resolution of the Genebridge 4 radiation hybrid panel and the fact that the GCAP array spans no more than 20 kb, GCAP2 would be expected to map to the same position as GCAP1 (approximately 16.7 Mb centromeric to D6S943). Therefore, since the GCAP gene array lies outside the minimal genetic region defined by the microsatellite markers, both GCAP genes have been ruled out as potential disease genes for arRP in the Dominican pedigrees.

DISCUSSION

GCAPs are members of the calmodulin multigene family that are characterized by the presence of multiple (2 to 8) EF-hand motifs enabling Ca^{2+} binding. This multigene family is very heterogeneous and comprises Ca^{2+}-binding proteins expressed ubiquitously (e.g., calmodulin) or cell-specifically (e.g., GCAP1). The GCAPs are among the very few Ca^{2+}-binding proteins for which a specific function, activation of GCs in the Ca^{2+}-free form, has been unambiguously assigned (for review see Polans *et al.*, 1996). GCAP1 is present predominantly in photoreceptor outer segments where phototransduction occurs, and it modulates photoreceptor GC by interacting with an intracellular domain (Duda *et al.*, 1996). The function of GCAP2 is identical to that of GCAP1 (Laura *et al.*, 1996). Previous immunocytochemical experiments (Dizhoor *et al.*, 1995) showed that GCAP2 is expressed in photoreceptors, but may also be present in other retinal cell types.

We found that the GCAP1 and GCAP2 genes reside at the same chromosomal locus. Members of the same gene family are thought to originate from an ancestral progenitor by gene duplication. The mechanism of gene duplication is not known, but it is assumed that the genes arose, as an unpredictable and rare event, by homologous recombination between repeated elements or by nonhomologous breakage and reunion (Maeda and Smithies, 1986). While duplicated genes can be dispersed to a different location or to a different chromosome, some have been shown to be arranged tandemly head-to-tail on the same chromosome or to be organized in irregularly spaced clusters in any orientation on the same chromosome (for review see Graham, 1995). Examples of gene clusters are the α-globin and β-globin gene clusters (Karlsson and Nienhuis, 1985), the homeobox gene clusters (Krumlauf, 1992), the Ca^{2+}-binding proteins of the S100 subfamily (Schäfer *et al.*, 1995), and the histone gene cluster in mammals (Osley, 1991). An example of tandem repeats of related genes are the red and green visual pigment genes on the X chromosome (Nathans *et al.*, 1986; Neitz and Neitz, 1995).

PCR analysis and sequencing indicated that the GCAP1 and GCAP2 genes are organized in a tail-to-tail array on the short arm of chromosome 6. To date,

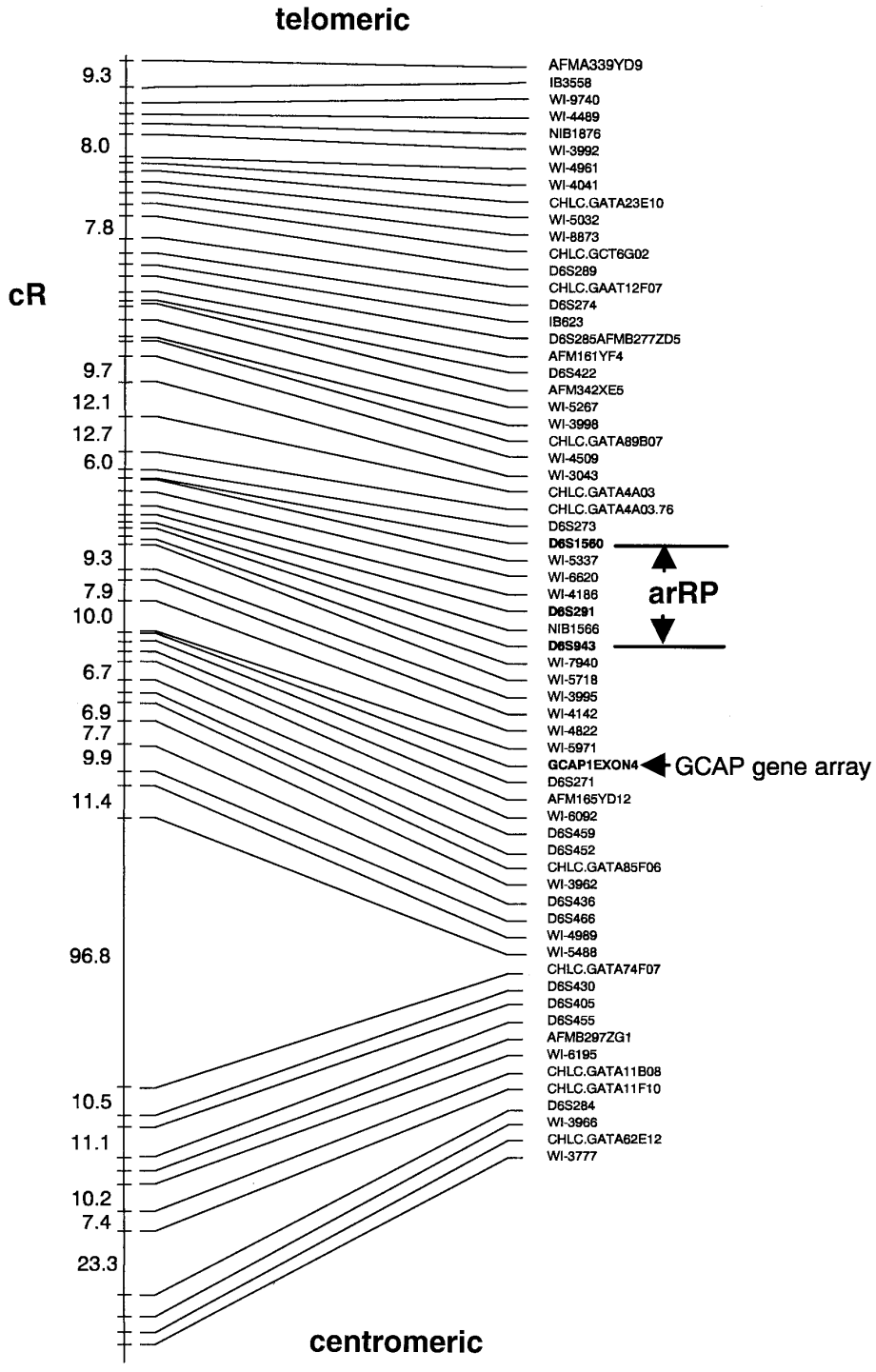

FIG. 7. Radiation hybrid map of a portion of chromosome 6p. D6S1560, D6S291, D6S943, and GCAP1EXON4 (printed boldface) are placed in the context of the Whitehead Institute framework map. The GCAP gene array is 55.6 centirays (cR) or 16.7 Mb (1 cR is approximately equal to 300 kb) centromeric to D6S943, which represents the centromeric boundary of the disease locus in the Dominican pedigree.

only two mammalian GCAP genes have been cloned; it cannot be ruled out that other related GCAP-like genes are located in close proximity at the same locus. Tail-to-tail arrangements have been shown for the human and rat apolipoproteins AI and CIII (Karathanasis, 1985), the human and mouse insulin-like growth factor binding proteins (IGFBP) (Ehrenborg *et al.*, 1991), and the human MAO-A and MAO-B genes (Chen *et al.*, 1992), to name a few. The intergenic distance varies: it is 50, 20, 5, and 3 kb in MAO genes, human IGFBP, mouse IGFBP, and human Apo AI and CIII, respectively. Gene duplication mechanisms that lead to tail-

to-tail orientations are unknown, but obviously require an inversion in addition to nonhomologous breakage. An immediate consequence of a tail-to-tail arrangement is that the genes must be transcribed from different strands and that the 5′ regulatory elements are on opposite ends of the gene arrangement, allowing for differential evolution and possibly divergent tissue specificity. In the three cases referenced here (IGFBP, MAO, and Apo AI/CIII), the expression patterns differ. The presence of GCAP tail-to-tail arrangements in human and mouse suggests that the rearrangements that led to the evolution of distinct forms of GCAPs occurred before the mammalian diversification. A prediction would be that the genes are also in a tail-to-tail array in other mammalian species.

The location of the human GCAP genes at GUCA (6p21.1) is nearly identical to a locus for autosomal recessive RP in a large Dominican pedigree (Knowles *et al.*, 1994; Shugart *et al.*, 1995). Down-regulation of GCAP1 was recently shown to be involved in an animal model for recessive Lebet's congenital amautosis (Semple-Rowland *et al.*, 1996). Based on the colocalization of the GCAP gene array with the disease locus, the retina-specific expression of the GCAPs, and the involvement of GCAP1 in an animal model for arRP, we reasoned that both GCAP genes may be candidates for the arRP disease gene in this pedigree. To evaluate the role of GCAP1 as the disease gene, the coding regions of all four exons were sequenced in two homozygous affected and two homozygous unaffected individuals. No sequence differences were found between the affected and the unaffected individuals. In addition, no differences were observed at the intron–exon splice site junctions (P. Banerjee, manuscript in preparation). Instead of proceeding to determine the noncoding sequences of GCAP1 and the complete DNA sequence for GCAP2 in these individuals, we decided to map the GCAP gene array relative to the minimal genetic region in the Dominican pedigrees. The results (Fig. 7) show that the GCAP gene array maps approximately 17 Mb centromeric to the disease locus, essentially ruling out an involvement of the GCAPs in the arRP disease phenotype.

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Note added in proof. Defects in the photoreceptor guanylate cyclase gene (GUC2D on 17p13.1) have recently been shown (Perrault *et al.*, *Nature Genetics* **14:** 461–464) to cause Leber's congenital amaurosis (LCA), a genetically heterogeneous blinding eye disease. Since GCAP(s) is required for photoreceptor cyclase activity, defects in the GCAP gene array could account for LCA phenotypes unlinked to chromosome 17p13.

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