The β subunit of human rod photoreceptor cGMP-gated cation channel is generated from a complex transcription unit

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Abstract Human and bovine rod photoreceptor cGMP-gated cation channel consists of two subunits: α (63 kDa) and β (240 kDa). The human β subunit was shown to consist partly of sequence encoded by the cDNA clone hRCNC2b. Here we present the complete sequence of the human β subunit and demonstrate that the previously reported human *GAR1* gene encoding a glutamate-rich protein (hGARP) encodes its N-terminal portion. Using PCR, RNA blot and genomic DNA analysis, we provide evidence that the β subunit is produced from a complex locus on chromosome 16 which is also capable of generating independent transcripts corresponding to *GAR1* and the C-terminal two-thirds of the β subunit. The results indicate that the β subunit of the cGMP-gated cation channel is produced from an unusual locus consisting of more than one transcription unit.

Key words: Non-selective cGMP-gated cation channel; Gene structure; Alternative splicing; Retina; Photoreceptor; Beta subunit; (Human)

1. Introduction

Cyclic GMP-gated cation channels (CNCG) are members of a diverse family of non-selective cation channels present in a variety of tissues including testis, kidney, heart, olfactory and retina [1,2]. The channel from mammalian rod outer segments (ROS) has been studied extensively due to its essential role in visual transduction where it controls the flow of ions across the plasma membrane in response to light-induced alterations in the levels of intracellular cGMP (reviewed in [3,4]). In the dark, sufficient amounts of cGMP are present to maintain the channel in a partially open state. Adsorption of light leads to hydrolysis of free cGMP resulting in channel closure and hyperpolarization of the cell.

Partial purification of the bovine channel from ROS identified 63 and 240 kDa proteins [5,6]. Cloning and expression of the 63 kDa protein yielded a channel activity similar to that observed with the native rod photoreceptor cGMP-gated channel [7]. A highly homologous human counterpart showed comparable properties [8], thus suggesting that the photoreceptor channel was a homo-oligomer of 63 kDa subunits [4]. More recently, however, two alternatively spliced human retina cDNAs, hRCNC2a (2a) and hRCNC2b (2b), differing only at their 5′ ends were reported [9]. While expression of the encoded proteins alone does not result in channel activity, coexpression of the human 63 kDa protein (now termed α) with the protein encoded by clone 2b is required to confer sensitivity to the channel blocker L-*cis*-diltiazem, to produce rapid channel flickering, and for Ca^{2+}/calmodulin regulation of cGMP affinity [10], all characteristics of the native channel. This led to the proposal that the 2b clone encoded the second subunit of the CNCG. Chen et al.[10] later showed by partial amino acid sequence analysis that the bovine 240 kDa protein, now termed the β subunit, shares high homology with the sequence of clone 2b and also indicated the presence of additional unrelated sequences [10]. Recent cloning of bovine β subunit cDNA [11] has shown that it consists of an unusual bipartite structure containing an N-terminal glutamate-rich segment which is nearly identical to a previously reported glutamate-rich protein (GARP) [12], and a C-terminal portion that is 86% identical to the polypeptide encoded by the human 2b cDNA clone [9]. While no mechanism was proposed to account for the generation of the bipartite structure, this work did suggest that the 2a and 2b clones previously reported [9] do not represent the entire human β subunit.

We previously reported the characterization of cDNA and genomic clones encoding a human homologue of bovine GARP [13] (referred to here as hGARP and its gene as *GAR1*), that may represent the unidentified component of the human β subunit. The *GAR1* gene generates a predominant transcript of 2.2 kb predicting a 299 aa protein that is only 61% identical to bovine GARP [12]. In this work, we have deduced the primary structure of the human β subunit demonstrating that it is comprised of an hGARP N-terminal and 2b C-terminal portion. We also show that the locus encoding the β subunit resides on chromosome 16 and is unusually complex encompassing two non-overlapping transcription units.

2. Materials and methods

2.1. Materials

Human/rodent somatic cell hybrid DNAs were from BIOS Inc., human retina poly (A^+) mRNA and a human leukocyte genomic DNA library prepared in λ-EMBL3-sp6/T7 bacteriophage was from Clontech. A DECAprime DNA labeling kit was from Ambion. Primer sequences were chosen using Gene Runner primer analysis software (Hastings Software, Inc.), and the oligonucleotides generated in house using a Beckman Oligo 1000 DNA synthesizer. Sequencing reactions were carried out on both strands with the fmol cycle sequencing system (Promega) as described previously [14]. Sequence analysis was performed with the Wisconsin GCG DNA analysis programs running in UNIX on a SUN workstation.

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2.2. Chromosome localization

The generation of the somatic cell hybrids used in this study and chromosome localization by PCR analysis has been reported [15,16]. Unless otherwise stated, numbering is according to [9] for clone 2b, and [13] for *GAR1*. Primers used for amplification were: 2b, 2823–2844/2b, 3054–3033); hGARP, 460–481/5′-CGAGTCGCTAACAGGACTGAAT-3′ (*GAR1*, intron gh); 5′-CAGAGACTCACCTAGTGTCTTG-3′ (*GAR1*, intron gc)/5′-GTCAGTCAGGTGAGAAGATCC-3′ (*GAR1*, intron ge).

2.3. Identification of a hybrid mRNA

Poly (A^+) from human retina was reverse-transcribed as previously described [17], and then amplified for 30 cycles with primer pairs representative of different regions of 2b and hGARP cDNAs. Primer sequences were: 1 (hGARP, 662–683); 2 (2b, 411–390); 3 (2b, 2416–2437); 4 (hGARP, 127–106); 5 (hGARP, 460–481); 6 (hGARP, 389–411); 7 (hGARP, 43–64); 8 (hGARP, −60–36); 9 (2b, 672–651); 10 (2b, 935–914); 11 (2b, 1106–1085); 12 (2b, 1298–1280); 13 (2b, 1614–1593); 14 (2b, 1964–1943); 15 (2b, 2437–2416); 16 (2b, 2844–2823); 17 (2b, 192–213); 18 5′-GCAGACCATCAGCATCCTTCC-3′ (IS region, Fig. 2a); 19, 5′-CTTCTCCACAGCTTTGTT-CCCT-3′ (IS region); 20 (hGARP, 689–668); 21 (hGARP, 764–785); 22 (2b, 3054–3033). The 672 bp PCR product shown in Fig. 2b, lane 1, was amplified using Pfu DNA polymerase, purified, and subcloned into pBSII-KS (Stratagene) for sequence analysis.

2.4. RNA blot analysis

Human retina poly (A^+) RNA was fractionated in a 0.8% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane as previously described [17]. Individual blot strips were hybridized with decamer-primed radiolabeled PCR fragments as indicated. Hybridization and washings were as described previously [17].

2.5. Analysis of cDNA and genomic clones

A human retinal cDNA library was screened with radiolabeled PCR products (2b, 192–935 and 2a, 1–341) as previously described [13]. A human leukocyte genomic DNA bacteriophage library was screened with radiolabeled PCR products representative of the IS region (amplified with primers 18, 19; Fig. 2a), and a portion of the 2b cDNA (primers 17, 9) as described [13]. Hybridizing phage were purified to homogeneity, DNA isolated and sequenced. The identity of the genomic clones was verified by sequence analysis of the exons and some of the flanking intron sequence.

3. Results

3.1. Chromosome localization

As a first step to examine the structure of the human cGMP-gated channel β subunit (locus designation CNCG2), we mapped the location of the genomic region encoding cDNA clones 2a and 2b [9]. Primers were designed from the sequence that amplify a 232 bp segment of the 3′-untranslated region. Amplification of somatic cell hybrid DNAs indicated that the gene maps to chromosome 16. Fine localization using the same primers for amplification of somatic cell hybrid DNAs localizes the gene to 16q13 (Fig. 1). We previously reported the fine mapping of the *GAR1* gene encoding hGARP (locus designation CNCG3), to chromosome 16q13 using fluorescence in situ hybridization [13]. Using two primer pair combinations that amplify different portions of the *GAR1* gene, we verified this location and placed the two loci within a 600 kb segment of chromosome 16 between the hybrid breakpoints CY18A(P1) and CY126.

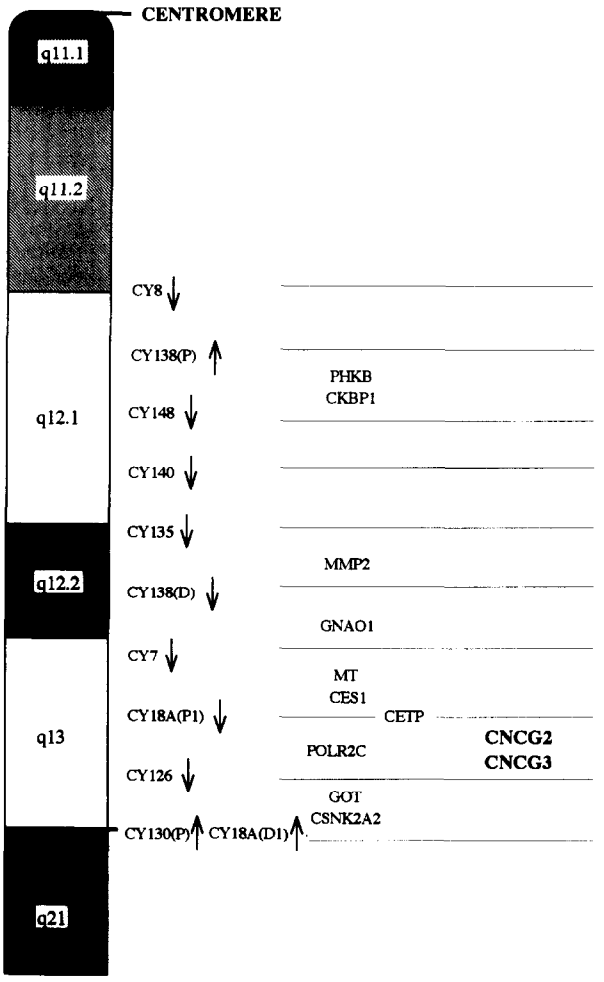

Fig. 1. Ideogram of the proximal portion of the long arm of chromosome 16 showing the location of CNCG2 and CNCG3. PCR analysis of somatic cell hybrids assigned both loci to an ~600 kb region of 16q13 delimited by the hybrid breakpoints CY18A(P1) and CY126. Symbols for other loci located in this region [15,22] are shown for comparison. Breakpoints in mouse/human somatic cell hybrids are indicated by horizontal lines. The vertical arrows indicate the orientation of retained segments of chromosome 16 at each breakpoint.

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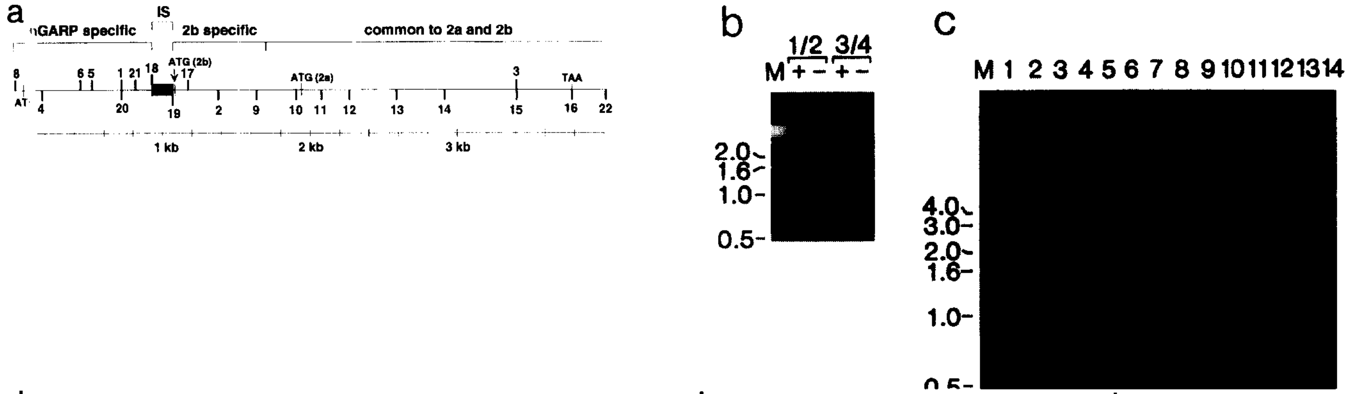
Fig. 2. Characterization of hybrid mRNA. (a) Schematic representation of the location of primers used for PCR amplification and DNA sequencing. The horizontal line represents the hGARP/2b hybrid cDNA sequence beginning in the 5′ untranslated region of the hGARP cDNA [13] and ending in the 3′ untranslated region of 2b [9]. Primers (numbered 1–22) marked above the line are sense and those below are antisense. The filled rectangle represents the inserted 152 nt intergenic (IS) segment which is not present in the reported sequences for hGARP and 2b cDNAs. (b) RT-PCR analysis of a hGARP/2b hybrid transcript in human retina. RT-PCR was performed with primers 1 and 2 (lanes 1/2) or primers 3 and 4 (lanes 3/4). The plus and minus signs indicate that the PCR reaction was done with or without template DNA. (c) Composition of the hybrid mRNA. Different primer combinations (see panel a) representative of the entire 2b and hGARP cDNAs were used to amplify human retina first strand cDNA: lane 1 (primers 8/2); lane 2 (7/2); lane 3 (6/2); lane 4 (5/2); lane 5 (21/2); lane 6 (1/9); lane 7 (1/10); lane 8 (1/11); lane 9 (1/12); lane 10 (1/13); lane 11 (1/14); lane 12 (1/15); lane 13 (18/16); lane 14 (18/22). Lane m shows the 1 kb ladder (BRL). (d) Deduced amino acid sequence of the unique portion of the human β subunit and comparison to bovine β subunit. Numbering is according to the published sequences of hGARP [13] for the human β subunit (hum) and the bovine β subunit (bov) [11]. The inserted sequence not present in the published 2a, 2b or hGARP cDNAs is shown boldface. Vertical arrows mark the approximate positions where introns split the protein coding regions of the *GAR1* and *2a* genes, and refer to Fig. 4. Vertical lines denote identity. Gaps (−) were introduced to optimize alignments. Amino acids encoded by the primer sequences (primers 1 and 2) used for amplification are underlined.

3.2. Identification of a hybrid mRNA

The close proximity of the two genes led us to speculate that a hybrid mRNA might account for the expression of the β subunit. PCR was performed to determine if a hybrid message consisting of hGARP and 2a or 2b cDNA sequence exists in human retina. It was assumed that if a hybrid did exist transcription of both genes would proceed in the same direction, and therefore primer combinations (Fig. 2a) were used that would amplify either possible arrangement. Indeed, a PCR product was observed (Fig. 2b, lanes 1/2) using a sense primer near the 3′-end of the protein coding region of hGARP cDNA [13] (Fig. 2a, primer 1), and an antisense primer near the 5′ end of the clone 2b-specific cDNA sequence [9] (Fig. 2a, primer 2), indicating the existence of a



d

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                   [g9]                   gi          [g10]        gj
hum  221 LQPKEEPKEAPAPEPQPGSQAQTSSLPPTRDPARLVAWVLHRLEMALPQPVLHGKIGEQE
           | |||     |||||  ||  |||||  | ||| || ||||||||||||  || ||||
bov  227 PEPEEEPI----PEPQPTIQA--SSLPPPQDSARLMAWILHRLEMALPQPVIRGKGGEQE

              [g11] gβ                           [β1]
hum  281 PDSPGICDVQTISILPGGQVEPDLVLEEVEPPWE-DAHQDV--STSPQGTEVVPAYEEEN
          | |  ||||||||||| | |  | |||| | || | ||    ||||   |  || ||
bov  281 SDAPVTCDVQTISILPGEQEESHLILEEVDPHWEEDEHQEGSTSTSPRTSEAAPADEEKG

                βa                       [β2]
hum  338 KAVEKMPRELSRIEEEKEDEEEEEEEEEEEEEE---------------------------
           ||  |||| ||  ||||||||| |  ||||||
bov  341 EVVEQTPRELPRIQEEKEDEEEEKEDGEEEEEEGREKEEEEGEEKEEEEGREKEEEEGEK

hum      ------------------------------------------------------------

bov  401 KEEEGREKEEEEGGEKEDEEGREKEEEEGRGKEEEEGGEKEEEEGRGKEEVEGREEEEDE

               βb            [β3]           βc
hum  371 ---EVTEVLLDSCVVSQVGVGQSEEDGTRPQSTSDQKLWEEVGEEAKKEAEEKAKEEAEE
                |||||  | |    ||||     ||             |    ||       |
bov  461 EEEQDHSVLLDSYLVPQSEEDQSEESETQDQSEVGGAQTQGEVGGAQALSEESETQDQSE

                                  [β4]                     βd
hum  428 ---------------------VAEEEAEKEPQDWAETKEEPEAEAEAASSGVPATKQHPE
                                |       ||      |   || | |||||||  |||
bov  521 VGGAQDQSEVGGAQAQGEVGGAQEQDGVGGAQDQSTSHQELQEEALADSSGVPATEEHPE

                           [β5]                       βe
hum  467 VQVEDTDADSCPLMAEENPPSTVLPPPSPAKSDTLIVPSSASGTHRKKLPSEDDEAEELK
          |||| |||| || ||||||| |  | |||||||| || || |  || ||| ||||||||
bov  581 LQVEDADADSRPLIAEENPPSPVQLPLSPAKSDTLAVPGSATGSLRKRLPSQDDEAEELK

                                  βf   [2a2]
hum  527 ALSPAESPVVAWSDPTTPKDTDGQDRA
          |||| |||||||||| |  || ||||
bov  641 MLSPAASPVVAWSDPTSPQGTDDQDRA
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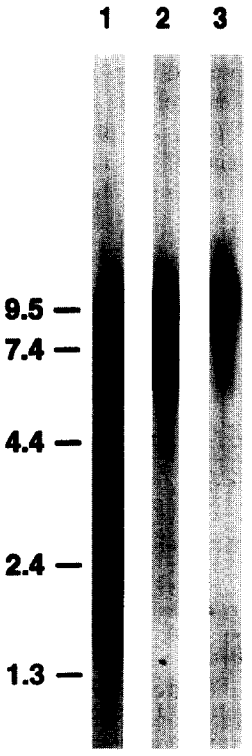

Fig. 3. Human retina RNA blot analysis. Blots were probed with ^{32}P-labeled PCR products corresponding to nt 43–689 of hGARP cDNA [13]; (lane 1, 1 μg), nt 192–672 of human 2b [9] (lane 2, 1 μg); and the unique 5′ untranslated region of 2a (lane 3, 3 μg). Exposure times were 24 h for lanes 1 and 2, and 6 days for lane 3. The positions of molecular weight standands in kb are shown on the left.

5′-hGARP/2b-3′ hybrid mRNA. However, the PCR product appeared to be slightly longer than that predicted from the published cDNA sequences. No product was observed (lanes 3/4) in a PCR amplification reaction using a sense 2b (primer 3)/antisense hGARP (primer 4) primer pair, consistent with the *GAR1* gene being upstream of the 2b region. Use of several additional primer pairs representative of different regions of the two genes also yielded PCR products that were slightly longer than that predicted (Fig. 2c), indicating an inserted DNA segment (IS). Control amplifications for each primer set without template yielded no observable product (not shown). The 672 bp PCR product observed in Fig. 2b, lane 1, was cloned into pBluescript and its entire sequence determined. As shown in Fig. 2d, the 5′ end of the sequence was identical to that encoding amino acids 221–291 of our previously published hGARP cDNA sequence, diverging from the hGARP sequence at the intron k splice site [13]. The next 152 nt encoding amino acids 292–342 are not present in the published hGARP [13]or 2b [9] cDNA sequences, and the last 302 nt encoding amino acids 343–445 correspond to amino acids 1–101 of the 2b cDNA [9]. All of the PCR products generated in Fig. 2c hybridize to a radiolabeled probe representative of the unique 152 nt segment (M.D.A. and S.J.P., unpublished observations).

3.3. Verification of RT-PCR products

To verify that the PCR products represent an actual mRNA, we screened a human retinal cDNA library with a PCR-amplified segment (primers 17/10, Fig. 2a) representative of the 5′ end of the protein coding region of cDNA clone 2b. Surprisingly, of the 58 clones isolated, none contained the first 104 nt reported for clone 2b [9]. Fifty-five of the clones contained the unique 152 nt segment found in the hybrid PCR products (Fig. 2b,c). The remaining three clones did not extend far enough 5′ to include the unique segment. We were also unable to amplify by RT-PCR of human retina mRNA a product consisting of the 5′ untranslated region of clone 2b and any portion of the remainder of the sequence. From these results it was concluded that the clone 2b likely represents a truncated portion of the mRNA encoding the β subunit with a 5′ cloning artifact of 104 nt. To verify that the 2a clone represents an actual mRNA present in the retina, and not merely another truncated β subunit cDNA clone, we screened 300,000 plaque-forming units of the human retina cDNA library with a probe representative of the published 5′ untranslated region of clone 2a [9]. Seven primary isolates were identified, thus suggesting that 2a transcripts are present in low abundance in the retina. Sequence analysis of three representative clones indicated that these clones contained the reported 5′ untranslated region of 2a, and that this sequence matches that found in human genomic clones covering this region (see below). Using similar RT-PCR analysis (not shown) as was performed with the 2b clone, it was determined that the entire reported sequence of clone 2a is part of a contiguous mRNA from human retina.

3.4. RNA blot analysis

To further characterize the coding capacity of the genes, probes for RNA blot analysis were designed reflecting the unique sequences of the β subunit, 2a, and hGARP cDNAs. The hGARP probe identifies five transcripts ranging in size from 9.5 to 1.8 kb (Fig. 3, lane 1), with the predominantly hybridizing 1.8 kb transcript similar to that previously reported [13]. The β subunit-specific probe identifies at least two transcripts of 9.5 and 6.0 kb (Fig. 3, lane 2) corresponding to transcripts of the same size identified with the hGARP probe. The 2a-specific cDNA probe identified one transcript of 10 kb that was significantly less abundant than the hGARP or β subunit mRNAs (Fig. 3, lane 3), consistent with the low abundance of clones obtained from the cDNA library screen. These results indicate that the genes encoding 2a and hGARP each produce an independent transcript, as well as contributing to the production of at least two β subunit transcripts, and that additional hGARP transcripts remain to be characterized.

3.5. Genomic DNA analysis

To examine potential mechanisms to account for the complex transcription pattern we have partially characterized the intron/exon organization of the genomic region encompassing both genes (Fig. 4). The *GAR1* gene organization has previously been reported [13], and consists of 12 exons (g1–g12), with the open reading frame for hGARP ending in exon g12. The hybrid transcript encoding the channel β subunit (β subunit) is composed of the first 11 exons of the *GAR1* gene, six exons contained only in the β subunit transcript, and all but the first exon encoding the 2a transcript. The first exon encoding the 5′ untranslated region of clone 2a is located within a 2.7 kb intron (βf) between exons β6 and 2a2. Sequence analysis of the genomic region containing the 2a1 exon confirms the published sequence of the unique 5′ un-

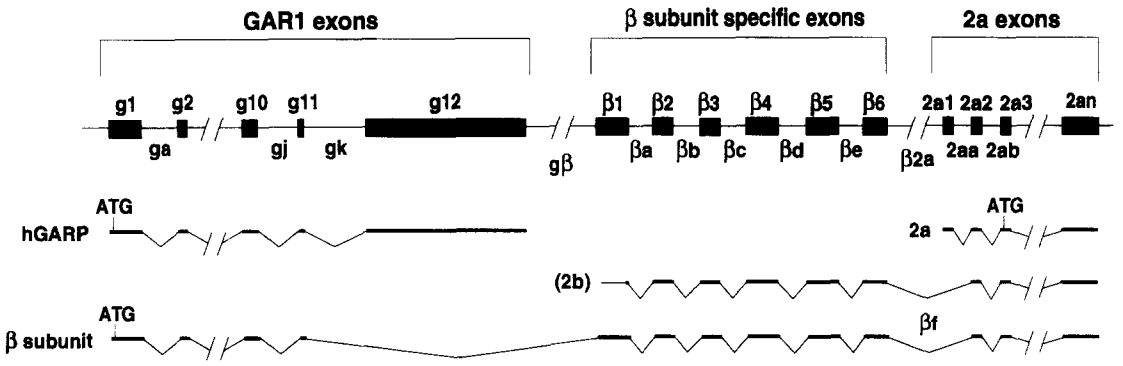

Fig. 4. Intron/exon organization of the genomic region encoding the β subunit shown above is the gene organization determined from analysis of overlapping genomic clones. Filled rectangles represent exons. The *GAR1* gene consisting of exons g1–g12 and introns ga–gk spans ~11 kb, and has been completely characterized [13]. The location of the exon containing the unique 152 nt sequence (β1) is ~7 kb downstream of exon g12, making intron gβ ~10 kb. Only the β subunit-specific and 5′ regions of the 2a portion have been characterized thus far. Exon 2a1 consists of the first 340 nucleotides of the reported 2a cDNA [9], and is separated from the last β subunit-specific exon (β6) by intron β2a, which is ~2.5 kb in length. Shown below the genomic structure are the transcripts identified thus far. Characterization of cDNA clones representing mRNAs produced from the *GAR1* gene (hGARP) and 2a region (2a) have been previously reported [9,13]. The 2b cDNA contains an artifact of cloning at its 5′ end (see text) and otherwise does not differ from the β subunit transcript (β) which consists of the first 11 exons of the *GAR1* gene (g1–g11), six exons unique to the β subunit transcript (β1–β6), and all but the first exon (2a1) of the 2a region.

translated region of the 2a cDNA as well as that obtained from our cDNA library screen with a 2a-specific probe (see above). Thus, splicing of the 2a transcript occurs at the same acceptor splice site but a different internal donor splice site forming intron 2aa. The remainder of the 5′ untranslated region of 2a is encoded by exons 2a2 and 2a3, which also encode part of the open reading frame for the β subunit.

4. Discussion

In this report, we show that the human rod photoreceptor CNCG β subunit mRNA encodes the first 291 amino acids of hGARP [13], 337 amino acids present only in the β subunit, and the entire 623 amino acids predicted from the 2a cDNA sequence [9]. However, this 1251 aa protein has a calculated molecular weight of ~140 kDa, significantly smaller than the SDS-PAGE observed molecular weight for the β subunit of 240 kDa [10]. This difference seems to be due to a concentration of acidic residues in the β subunit that significantly reduces SDS binding. Western analysis of purified ROS proteins and cell-free translation of hGARP identifies a 67 kDa product, not the predicted 32 kDa (M.D.A. and S.J.P., unpublished observation). Similar anomalies of migration in SDS-PAGE gels have been observed recently for the bovine β subunit [11] and for other acidic proteins [10,18–20].

Further evidence to support the identity of the hybrid mRNA encoded protein as the human β subunit is apparent from the recently reported bovine β subunit sequence [11]. The human and bovine sequences in the C-terminal 2a portion (encoded by exons β5–β6, 2a2-n, Fig. 4) are 86% identical, and in the N-terminal hGARP portion (exons g1–g11, and β1) 65% identity is found. The least homology is contained within exons β2–β4 that consist in bovine mostly of the glutamate repeat segments found in bovine GARP (Fig. 2d, [12]). The β subunit is the first integral component of phototransduction that contains internal protein sequence that is not highly conserved among mammals.

At least three gene products are predicted to be generated from this complex locus encoding the β subunit. The hybrid protein consisting of amino acid sequence encoded by both genes and by additional intergenic exons is the β subunit of the channel. The *GAR1* gene encodes a soluble protein (hGARP) that is highly enriched in photoreceptor ROSs (M.D.A. and S.J.P., unpublished results) whose cDNA and gene we have previously characterized [13]. The RNA blot shown in Fig. 3 indicates that an independent transcript is also generated from a separate transcription unit encoding the 2a mRNA. Using a probe that is specific to the apparent first exon (Fig. 4, exon 2a1) of this transcript a 10 kb mRNA was identified. This transcript is clearly distinct from the transcripts identified with the β subunit- and hGARP-specific probes (Fig. 3, lane 2) since 3 times as much RNA and significantly longer exposure times were required to observe the 10 kb transcript. RT-PCR analysis using transcript-specific primers further confirmed that this transcript is present in human retina RNA. Additionally, several clones were identified in a human retina cDNA library, and genomic DNA analysis confirmed the presence of the unique first intron and exon (Fig. 4, intron 2aa, exon 2a1). While we have not demonstrated that the predicted 70 kDa protein is expressed in the retina, co-expression in a heterologous system with the α subunit of the CNCG modifies the properties of α to closely mimic that observed with the native channel [9], except that it does not confer Ca^{2+}/calmodulin regulation of cGMP affinity [10,11]. Thus, the 2a cDNA does contain the necessary sequences to be translated into a functional regulatory subunit.

From the intron/exon organization determined (Fig. 4) we predict that the generation of the 2a transcript most likely would occur via use of an internal promoter present in the intron downstream of exon β6 (β2a), while the β subunit transcript is produced by alternative splicing that excludes exons g12 and 2a1, and allows readthrough of transcription termination signals downstream of the g12 exon. A similar differential use of transcription termination has been observed during B-lymphocyte maturation [21]. Future studies will be directed towards characterization of the function of the independent *GAR1* and *2a* gene products

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