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Structure of Human G Protein $G_{\gamma 5}$ Gene *GNG5*

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Heterotrimeric G-proteins consist of α, β and γ subunits that together function in numerous signal transduction pathways. G_{γ} subunits form dimers with G_{β}-subunits which together can significantly contribute to signal transduction. So far 11 G_{γ} subunit isoforms have been found. $G_{\gamma 5}$ appears to be the most prominently occurring G_{γ}-subunit and we now report the genomic structure of human *GNG5*. The gene spans 6 kb and consists of four exons that range from 92 bp to 290 bp and three introns that range from 134 bp to 3.3 kb. Intron-exon boundaries conform to *GT-AG* consensus splice junctions. Alternative transcriptional start sites that result from two separate, closely linked promoters in the 5′-flanking region of the gene were confirmed by luciferase reporter-gene assay.

***Key Words: GNG5;* G-proteins; $G_{\gamma 5}$; luciferase reporter; alternative transcriptional initiation.**

Heterotrimeric G-proteins are membrane-associated proteins consisting of $\alpha\beta\gamma$ subunits that together regulate flow of information from extracellular signals to a variety of metabolic effectors inside cells. Upon interaction of a G-protein with its activated receptor there is exchange of GTP for GDP that is bound to the α subunit. This causes a conformational change in "switch" regions of the G_{α}-GTP complex which weakens the affinity of G_{α} and $G_{\beta\gamma}$ for each other. Separation of G_{α} from $G_{\beta\gamma}$ allows them both to interact with and modulate effector molecules (1). So far 11 G_{γ}-subunit isoforms have been found (2). The precise role of individual G_{γ}-subunits is not known, but the $G_{\beta\gamma}$ dimers are not functionally separable. The dimers have at least three functions: first, they are required for formation of each $G_{\alpha\beta\gamma}$ trimeric G_{α}-protein which stabilizes the GDP-bound form of the subunit. Secondly, they are required members of the receptor-trimeric G-protein complex, which can lead to the formation of a high affinity agonist binding state as in the case of A1 adenosine receptors (3). $G_{\beta\gamma}$-dimer can also directly interact and recruit the beta-adrenergic receptor kinases to the membrane and cause increased agonist-dependent phosphorylation and desensitization of receptors (4). Thirdly, $G_{\beta\gamma}$-dimers can function as direct activators of certain effectors; for example, the dimer can active the muscarinic K^+ channel (5), adenyl cyclase subtypes II and IV with $G_{\alpha s}$(6, 7), phospholipase A2 (8) and phospholipase C subtypes β 1, 2 and 3 (9).

Genomic structures of several G_{γ}-subunits have been characterized. Bovine (10), as well as the human (10, 11) transducin γ-subunit gene (*GNGT1*) consists of three exons and two introns. Murine $G_{\gamma 4}$-subunit has the same genomic structure with a much longer intron 2. Tissue distribution of some G-protein γ-subunits and their mRNAs have also been analyzed. Northern blotting indicates that $G_{\gamma 1}$, $G_{\gamma 2}$ and $G_{\gamma 3}$ are mainly distributed in the brain (10, 12), where as $G_{\gamma 4}$, $G_{\gamma 5}$, $G_{\gamma 7}$, $G_{\gamma 10}$ and $G_{\gamma 11}$ have wide tissue distribution (2, 12, 13). Among these isoforms $G_{\gamma 4}$ and $G_{\gamma 11}$ appear to have alternative transcripts, and the mRNAs for $G_{\gamma 4}$ occur in varied amounts in different tissues (2).

$G_{\gamma 5}$ has been colocalized with vinculin and may function at cell focal adhesions (14). $G_{\gamma 5}$ is also the major gamma subunit component of $G_{\alpha\iota 2}$ and G_q in human platelets (15). Human, bovine, rat, and mouse are four species known to have $G_{\gamma 5}$ (13, 14, 16). Both cDNA and genomic sequence (*GNG5*) of bovine $G_{\gamma 5}$ have been characterized (13), and human *GNG5* gene has been mapped to chromosome 1p22 by PCR analysis of somatic cell hybrids and fluorescence in situ hybridization with a YAC clone that contained the $G_{\gamma 5}$ gene. In this report we have elucidated the previously uncharacterized structure of human *GNG5* which expresses the most widely occurring G_{γ}-subunit.

MATERIALS AND METHODS

RT-PCR. Template cDNA was synthesized from total RNA using M-MLV reverse transcriptase and random hexanucleotide primer. The first strand cDNA made from 2-4 μg of total RNA was used for a 25 μl PCR reaction. Each reaction was performed with 200 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.4 μM of each

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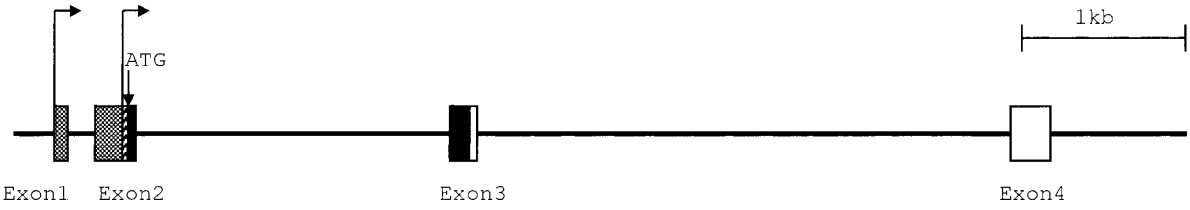

FIG. 1. Genomic structure of human $G_{\gamma 5}$. Hatched regions in Exons 1 and 2 contain 5′UTR of a minor, long transcript. The striped area in Exon 2 is the 5-untranslated end of a major short transcript. Arrows represent the two alter-native transcriptional start points. The translational initiation ATG codon and Exons 3 and 4 are common to both transcripts. Solid areas are coding regions; open areas are 3′UTR.

sense and anti-sense primer, and 1.25 units of Taq DNA polymerase. Thermal cycling was done in a MJ Research thermal cycler, programmed for the first reaction cycle for 3 min at 94°C, 1 min at 50°C and 3 min at 72°C, with the final cycle extending the 72°C step to 10 min.

Southern blot analysis of lambda DNA. Approximately 1 μg of lambda phage DNA that harbored the insert of interest was digested by an appropriate restriction enzyme and resolved on an 0.8% agarose gel. DNA was electroblotted from the gel to a nylon membrane and the resulting blot was hybridized with a corresponding probe that had been random-primed labeled with biotinyl-11-dATP. Hybridization with 2 ng/ml of labeled probe was performed at 42°C in 4× SSC, 5× Denhardt's, 0.2% SDS/50μg/ml sheared herring sperm DNA. Post-hybridization washes were conducted in the order: 2× SSC/0.2% SDS for 15 min at room temperature, four times; and 0.1× SSC/0.5% SDS for 10 min at 50°C.

Genomic library screening by hybridization. A human λ-EMBL-3 bacterial phage library was plated and grown at a density of approximately 10^5 pfu/150-mm plate for 8 h at 37°C. After cooling the plates at 4°C for at least 1 h, they were overlaid with a nylon filter for 2 min and the phage DNA denatured. Membranes were auto-crosslinked and prehybridized in 4× SSC, 1.0% SDS and 50% formamide at 42°C for 2-24 h, and hybridized with 2ng/ml labeled probe at 42°C overnight in 4× SSC, 5× Denhardt's, 0.1% SDS and 0.2mg/ml sheared herring sperm DNA. Post-hybridization washes were performed in the order: 2× SSC, 0.1% SDS, four times for 15 min at room temperature; 0.2× SSC, 0.1% SDS for 1-4 times at 42-65°C.

DNA sequencing. Nucleotide sequences were determined by the dideoxynucleotide chain termination method using Circum-Vent DNA polymerase (New England Biolabs) according to the instructions of the manufacturer. Primers were end-labeled with [γ-^{32}P] ATP and each sequencing reaction was performed on a thermal cycler, programmed for the first reaction cycle for 3 min at 95°C, 1 min at 60°C and 1 min at 72°C, and for the second to 20th cycles for 1 min at 95°C, 1 min at 60°C and 1 min at 72°C. The product was separated on a 5% polyacrylamide, 7.5 M urea gel.

5′ RACE. Transcription initiation sites were determined by rapid amplification of cDNA ends utilizing a Human Placenta Marathon cDNA RACE kit from Clontech according to instructions of the manufacturer using gene specific primers: NA121: 5′-ACAAACTGGGAGGAAAACTGAG-3′; NA124: 5′-CTAAGAGGGGGTCTGAAGAGGACC-

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bovine      1 ATGTCTGGTTCATCCAGCGTCGCGGCTATGAAAAAAGTGGTTCAACAGCT 50
              |||||||| || |||||||||| |||||||| ||||||||||||||||||
human       1 ATGTCTGGCTCCTCCAGCGTCGCCGCTATGAAGAAAGTGGTTCAACAGCT 50
                M  S  G  S  S  S  V  A  A  M  K  K  V  V  Q  Q  L

bovine     51 CCGGCTGGAGGCCGGGCTCAATCGGGTGAAGGTTTCCCAGGCAGCTGCAG 100
              ||||||||||||||| ||||| || || || ||||||||||||||||||||
human      51 CCGGCTGGAGGCCGGACTCAACCGCGTAAAAGTTTCCCAGGCAGCTGCAG 100
                 R  L  E  A  G  L  N  R  V  K  V  S  Q  A  A  A

bovine    101 ATTTGAAACAGTTCTGTCTGCAGAATGCTCAACATGACCCTCTGCTGACT 150
              | |||||||||||||||||||||||||||||||||||||||||||||||
human     101 ACTTGAAACAGTTCTGTCTGCAGAATGCTCAACATGACCCTCTGCTGACT 150
                D  L  K  Q  F  C  L  Q  N  A  Q  H  D  P  L  L  T

bovine    151 GGAGTATCTTCAAGTACAAATCCCTTCAGGCCCCAGAAAGTCTGTTCCTT 200
              ||||||||||||||||||||||||||||| ||||||||||||||||||||
human     151 GGAGTATCTTCAAGTACAAATCCCTTCAGACCCCAGAAAGTCTGTTCCTT 200
                 G  V  S  S  S  T  N  P  F  R  P  Q  K  V  C  S  F

bovine    201 TTTGTAG 207
              |||||||
human     201 TTTGTAG 207
                L  ***
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FIG. 2. Human and bovine *GNG5* coding region sequence comparison. There is 95% DNA sequence identity between these two species and the amino acid sequences are identical. The vertical line represents the common position of an intron for both human and bovine *GNG5*.

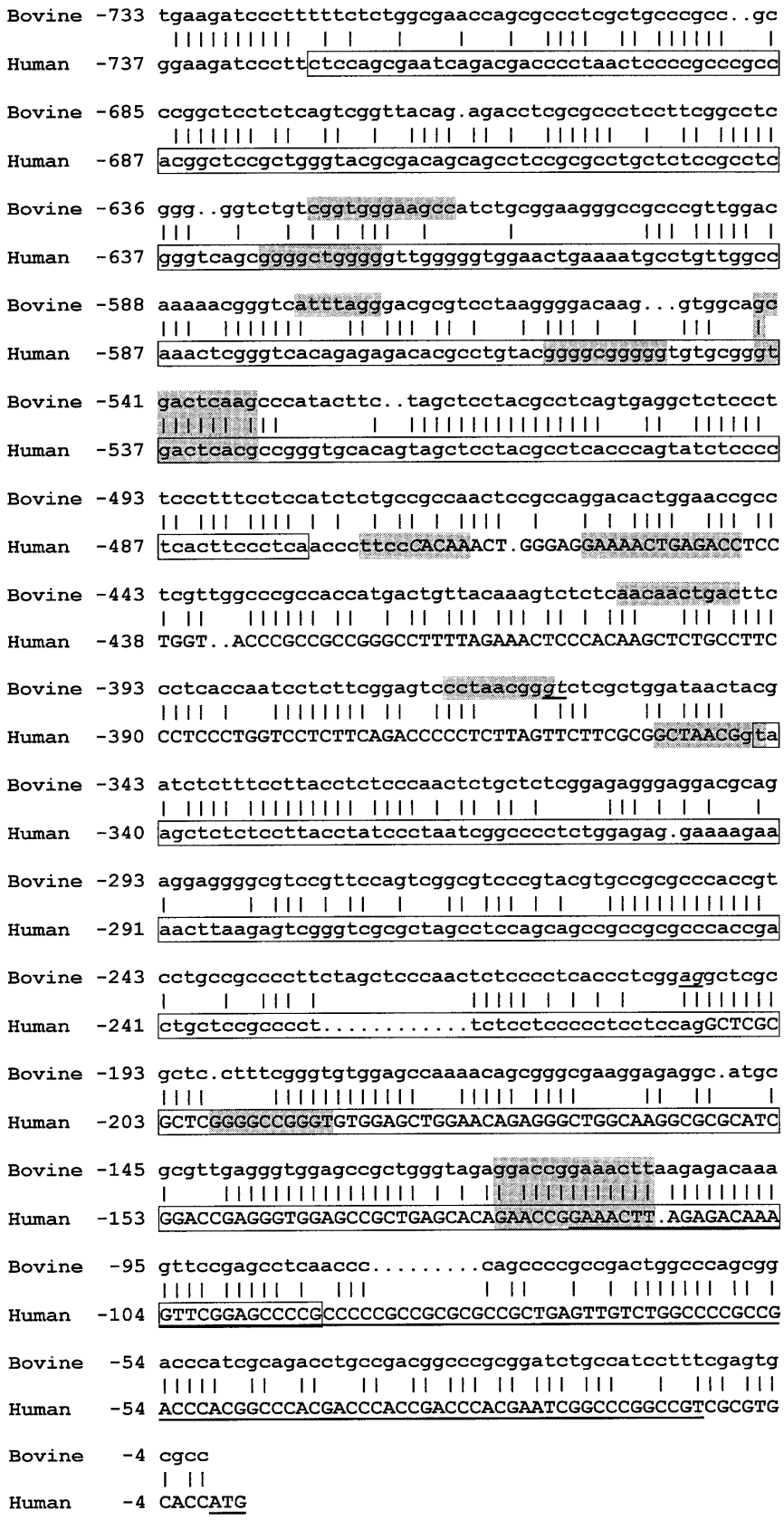

FIG. 3. Comparison of the 5′-flanking regions of human and bovine $G_{\gamma 5}$ genes. Consensus sequences for DNA-binding proteins are highlighted: AP-l(Human, -539; bovine, -543); v-Myb (human, -350; bovine, -370) and c-Ets-(human, -126; bovine, -118) are three consensus

AGGGAG-3′; NA148: 5′-GTTGAACCACTTTCTTCATAGCGGCGACG-3′; and NA158: 5′-CCAGTCAGCAGAGGGTCATGTTGAGCATTC-3′.

Human GNG5 promoter-luciferase constructs. *pGL3* and *pRL* vectors (Promega) were employed to analyze promoter activity of the 5′-flanking region of *GNG5*. Three PCR fragments of *GNG5* 5′-flanking region were inserted upstream of the luciferase gene in the *pGL3*-Basic vector. They were amplified by PCR using the following primers: NA168 (sense), 5′-CACACACCGAGCTCAAGATCCCTTCT-CCAGCGAATC-3′; NA169 (antisense), 5′-CACACTGAAGATCTT-AGCCGCGAAGAACTAAGAGGG-3′; NA170 (sense): 5′-CACTGT-ACGAGCTCAACTGAGACCTCCTGGTACCCG-3′; and NA171 (antisense): 5′-GAAGCCAGCCATGGTGCACGCGAC-3′. Gene-specific primers were made with either Sac I and Bgl II or Sac I and Nde I adapters at the 5′ end and flanked by an extra eight bases to facilitate restriction enzyme digestion. Antisense primer *NA 171* was designed to include the translational initiation site ATG overlapped by a Nco I *CCATGG* sequence that was used to subclone the *pGL3*-Basic vector with the firefly luciferase gene in frame. Plasmid p1689, whose insert contained the 389 nt PCR fragment amplified by using *NA168* and *NA169,* represented the distal promoter construct (*pGL3*-distal). Plasmid p1701, whose 452 nt insert contained the PCR fragment amplified by *NA 170* and *NA 171,* represented the proximal promoter construct (*pGL3*-proximal). Plasmid p1671, whose insert contained the fragment amplified by *NA168* and *NA171,* represented a construct having both distal and proximal promoters (*pGL3*-entire). The resulting three constructs were confirmed by PCR analysis using both gene specific and vector primers and by sequencing. The *pGL3*-Promoter vector (Promega) and the *pGL3*-Control vector (contained both SV40 promoter and enhancer) (Promega) were used as positive controls. The *pGL3*-Basic vector that has no promoter or enhancer, was used as a negative control. The *pRL-TK* vector (Promega) carrying *herpes simplex virus* thymidine kinase was cotransfected with the experimental DNA constructs to normalize the transfection efficiency. Plasmid DNA was isolated and purified by alkaline lysis and Quiagen tip separation. Two independent preparations of each construct were used in the promoter function studies, and three luciferase assays were performed on each experimental sample.

Transfection. MDA-MB-231 breast cancer and A549 lung adenocarcinoma cells were separately plated in a 24-well tissue culture plate at densities of 8×10^4 cells/well and incubated 20 h. A 400 ng sample of each DNA construct to be transformed was mixed with 4 μl *plus reagent* (Gibco BRL) for 15 min to form a complex before 1 μl LipofectAMINE (Gibco BRL) was added followed by a 15 min incubation at room temperature. The formed lipososomes were then put on the cells with 200 μl serum free medium. Following a 3 h incubation, complete medium with 13% serum was added to the culture. After a 36 h incubation cells were scraped from the plates and extracted using 100 μl PLB (passive lysis buffer) (Promega). Transfection efficiency was monitored by using *pRL-TK* encoding *Renilla* luciferase at a concentration of 8 ng/well.

Dual luciferase assays. A luminometer (Turner Designs Model TD-20e) was programed to perform a 2-second remeasurement delay, followed by a 10-second measurement period for each reporter assay. A 20 μl aliquot of the cell extract was added to 10 μl of LAR II (luciferase assay reagent II) (Promega), and firefly luciferase activity was measured followed by the addition of 100 μl of Stop & Glo Reagent (Promega) which quenched the previous firefly luciferase and simultaneously activated the *Renilla* luciferase (*pRL-TK* vector). The *pGL3* firefly luciferase activity was normalized by pRL-TK *Renilla* luciferase activity to minimize experimental variability. The relative luciferase activity is defined as: [*pGL3*(proximal, distal or entire)/*pRL-TK*]/[*pGL3*-Basic/*pRL-TK*]. All statistical analyses were done using Microsoft Excel.

RESULTS

Genomic Organization of Human G Protein γ-5

By a combination of hybridization screening of a human λ-EMBL-3 genomic library and PCR analysis of a YAC clone (λ1171) that harbored *GNG5* (17) the human $G_{\gamma 5}$ gene was found to consist of four exons (Fig. 1). The λ-EMBL3 library was first screened with a G-protein $G_{\gamma 5}$-subunit hybrid-cDNA probe (clone *HPCB 10a;* ref. 13) that encompassed the last two exons of $G_{\gamma 5}$ which include half the protein coding sequence plus the complete 3′UTR. A clone *G4* was obtained, and a 2 kb Xho I fragment of *G4* was identified by Southern blotting with an exon 1 probe from a human $G_{\gamma 5}$ pseudo gene (13) and deduced by sequencing to contain exon 1 of $G_{\gamma 5}$. The complete coding region of human $G_{\gamma 5}$ was then obtained by further sequencing on this Xho I subclone. Human $G_{\gamma 5}$ cDNA is 95% identical to that of the previously determined bovine cDNA (Fig. 2). Predicted amino acid sequences of the human and bovine proteins are identical, consistent with the high identity of individual γ-subunit isoforms observed across species.

Restriction mapping and PCR analysis showed clone *G4* to contain the first three of the four exons of the human $G_{\gamma 5}$ gene. Introns 1 and 2 were then characterized by further restriction and PCR analysis of *G4*. In order to examine the 3′ end of the $G_{\gamma 5}$ gene, a YAC clone (Y1171) that harbors *GNG5* (17) was analyzed by PCR with a sense primer from exon 3 and an antisense primer from exon 4. The PCR product was subcloned and sequenced using the exon primers to obtain the intron-exon border sequences (Table I). All 5′ donor and 3′ acceptor splice sites conform to the consensus *GU-AG* rule (18). Exon 1 is entirely non-coding region while exon 2 contains 209 nt of 5′-untranslated sequence and codons 1-27 for the amino half of the protein. Exon 3 encodes the remaining 41 amino acids plus 22nt of the 3′UTR, while exon 4 consists of the remaining 234 nt of 3′UTR. Human *GNG5* spans 6 kb between the transcriptional start site and its AATAAA RNA polyadenylation cleavage site.

sequences common to both bovine and human. Several other consensus sequences: IK-2 (-626), CdxA (-577) and v-Myb (-406) are present in bovine, whereas Sp1 (-629, -556, -199), STATx (-471) and IRF-1 (-454) are present in human (20). Two predicted promoter regions of human *GNG5* using Proscan (version 1.7) (19) are boxed. A minor transcriptional start site, C(-467); a major transcriptional start region from nt -120 to -11; and the translational initiation ATG are underlined. Human exons are in upper case, and 5′-flanking region and intron 1 sequence are in lower case. Consensus *gt-ag* splice sites of a putative intron in the bovine sequence are underlined and italicized.

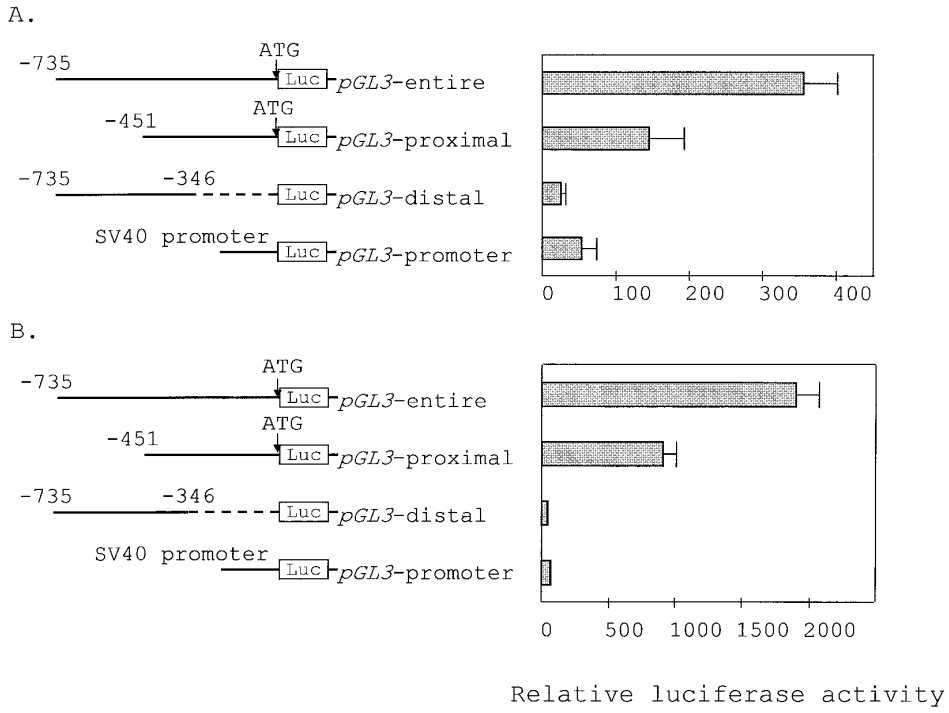

FIG. 4. Promoter analysis of human *GNG5*. A) Promoter activity of 5′-flanking region of *GNG5* in MDA-MB-231 cells. B) Promoter activity of 5′-flanking region of *GNG5* in A549 cells. Schematic representation of each construct is shown at the left. Values are normalized for transfection efficiency and represent the mean ± S.E.M. for three independent transfection experiments that were performed from two preparations of plasmid DNA.

Alternative Transcriptional Initiation Sites

Transcriptional initiation sites were studied by 5′RACE using Marathon cDNA from Clontech. This method determined two distinct initiation sites. A longer transcript (793 bp) begins at exon 1 and is the minor mRNA, while a shorter, approximately 560 bp transcript arises from the major start region which is in exon 2 (Fig. 1). A search of the current EST database provided four cDNA clones (H72187; H63312; H63815 and AA135650) that match the long transcript with three beginning at its *CACAA* start site. Northern blot, however, previously showed only the short transcript band to be visible (13). This relative level of transcription from the two start sites is also confirmed by the limited occurrence of the long sequence in the current EST databank. So far, 33 out of 106 human $G_{\gamma 5}$ ESTs are from 5′-sequence clones that likely represent "full length" $G_{\gamma 5}$ cDNAs. Only 4 (12%) of these 5′-clones highly match this farthest extended 5′ region of the human $G_{\gamma 5}$ sequence. The major short transcript appears not to arise from a single defined start point, since 28 clones derived from both 5′RACE and the EST cDNA library analysis showed multiple transcriptional start points from nt −120 to −11 (Fig. 3).

Characterization of the 5′-Flanking Region

The 5′-flanking regions at both predicted transcriptional initiation sites are highly GC rich (Fig. 3). The computer program PROSCAN (version 1.7) (19) was used to analyze the 5′-flanking region plus the 5′ end of the cDNA clone, predicting that the region from −725 to −456 and −342 to −92 may contain functional promoters of transcription. This prediction agrees with our two-transcript observation derived from both experimental sequencing and database analyses.

To confirm the existence of functional promoters in the 5′-flanking region, reporter analyses were done on three nested constructs (Fig. 4): *pGL3*-distal; *pGL3*-proximal; and *pGL3*-entire. The promoter activity of these constructs was analyzed with a firefly luciferase reporter enzyme in transient transfection assays in MDA-MB-231 (human breast cancer) and A549 (human lung carcinoma) cells. All three gamma-5 gene constructs showed promoter activities at least comparable with that of the SV40 promoter (Fig. 4). The relative firefly luciferase activity of the *pGL3*-proximal and *pGL3*-entire constructs in MDA-MB-231 cells were 145 and 356, respectively, and 908 and 1904 in A549 cells. In contrast, the relative promoter activity of distal promoter *pGL3*-distal was much lower, 25 and 39 in MDA-MB-231 and A549 cells, respectively. The latter activities, however, were significant considering that the relative promoter activity of SV40 alone is 53 and 65 in MDA-MB-231 and A549 cells, respectively. The diminished promoter activity of the distal promoter compared to the proximal promoter region is consistent

TABLE 1

Sequences at Intron–Exon Splice Junctions for Human *GNG5*

Donor	Acceptor	Intron size	Exon size
			124 bp
TAACG gt aagctctc	... cctcctcc ag GCTCG	134 bp	290 bp
TAAAA gt gagcgggg	... ttatgtat ag GTTTC	1.75 kb	145 bp
CAAAG gt gagtttcc	... cccatctt ag GTTTC	3.3 kb	234 bp

Note. Exon sequences are shown in uppercase letters and intron sequences in lowercase letters. The sizes of introns and exons are given in kilobases or base pairs. All intron–exon borders match the consensus *gt-ag* rule.

with the low abundance of the longer transcript, while the proximal promoter region is responsible for the expression of the more abundant, shorter transcript. Bovine and human 5′UTR and 5′-flanking regions have relatively high 63% identity. In addition, several transcription factor binding sites which are found in human: AP-1, V-Myb and c-Ets- are also found in the corresponding region of bovine *GNG5*. A CAAT box found in bovine, however, is not present in the human gene.

DISCUSSION

Human $G_{\gamma 5}$ gene consists of 4 exons and 3 introns with putative alternative transcriptional start sites in exons 1 and 2. Exon 1 consists entirely of 5′UTR and exon 4 consists of 3′UTR. The coding region is located at the 3′-end of exon 2 and in most of exon 3. Exon 2 contains 5′-flanking sequence and the transcriptional start site of the major human $G_{\gamma 5}$ transcript. This transcript was previously observed by Northern blotting as the only detectable $G_{\gamma 5}$ mRNA in placenta (13). Similar structures have been observed for other human $G_{\gamma 5}$ genes and related genes from other species (Fig. 5).

A unique aspect of the human $G_{\gamma 5}$ gene is that it appears to have at least two transcriptional initiation sites, which suggests there may be regulation of this gene either in development or tissue distribution. We have shown that both distal and proximal promoters or the entire promoter region are sufficient to drive transcription of the luciferase gene (Fig. 4). Relative activities of the promoters are high because they are comparable to, or much greater than, the activity induced by the SV40 promoter, which is considered to be a strong promoter. The proximal promoter activity as well as that of the entire 5′ untranslated region increased dramatically between the two cell lines MDA-MB-231 and A549 (relative luciferase activity increasing from 145 to 908 for *pGL3*-proximal and from 356 to 1904 for *pGL3*-entire), which implies that there might be some tissue-specific *cis*-elements in the proximal promoter region in addition to its basal transcriptional elements. The rare long transcript that begins in exon 1 would appear to be constitutively expressed at a constant low level while the major, short transcript that initiates in exon 2 could be regulated by tissue-specific factors (or at different developmental stages). A putative intron with a consensus *gt-ag* was also noted in bovine *GNG5* 5′-flanking region that corresponds to the one present in the human gene (Fig. 3). This suggests a similar rare longer transcript might also exist in bovine. Futher experiments need to be done to verify this possibility. Multiple transcripts from a gene that encode the same polypeptide could

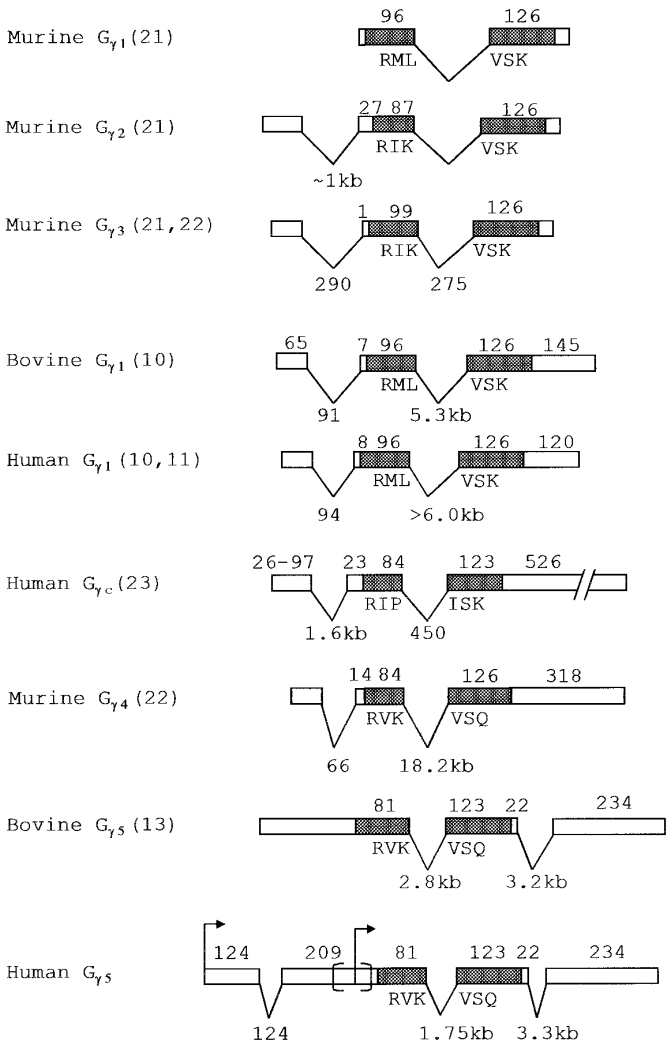

FIG. 5. Genomic structures of G_{γ}-subunit genes and their comparison with human *GNG5*. Shaded bars represent exon sequences, open bars represent 5′- and 3′-UTR. Intron positions and sizes (when known) are also shown in bp unless indicated as kb. Amino acid sequence of the coding region that is divided by an intron is indicated in single letter format below the exons. Two arrows represent the minor and major transcriptional initiation sites of human *GNG5*; with the multiple transcriptional start region of the major transcript bracketed.

exist to control tissue expression of that protein. Regulation of such a gene could be at the level of DNA transcription, or during mRNA metabolism where one transcript is more stable than others, or where translation of each message is controlled by different trans factors binding variable non-coding nucleotide sequence. It will be important to determine and compare how the genes for the many isomeric G_{γ}-subunits of the heterotrimeric G-proteins are regulated. This information combined with biochemical studies of the proteins should allow us to better understand the biological roles of the G_{γ}-subunit family in G-protein signaling.

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