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Cloning and characterization of the human gene for the α-subunit of G_{i2}, a GTP-binding signal transduction protein

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We isolated and characterized human genomic clones encompassing the gene for the α-subunit of G_{i2}, a GTP-binding signal transduction protein abundantly expressed in myeloid cells. The gene is divided into 9 exons and spans 23.5 kb. Exons 2, 6 and 7 encode putative guanine nucleotide-binding domains that are highly conserved among GTP-binding proteins. A polyadenylation signal located within exon 9 predicts an mRNA size (~2.3 kb) several hundred bases longer than that of published cDNAs, and consistent with the size seen on RNA blot hybridization. Primer extension and S_1 nuclease analysis determined a major and several minor transcriptional start sites. The first exon and 5′ flanking region are highly G+C rich, contain several GC boxes (SP1 transcription factor binding sites), a CAAT box, and lack a TATA box. The presumptive promoter region is thus similar to that of *ras* and other widely expressed genes.

G-protein; Signal transduction; Genomic clone; GC box

1. INTRODUCTION

Heterotrimeric GTP-binding proteins (G-proteins) convey extracellular signals from cell-surface receptors to effector enzymes and ion channels [1,2]. G-protein α-subunits bind guanine nucleotide, serve as substrates for bacterial toxin-catalyzed ADP-ribosylation, and may confer specificity in receptor and effector coupling. cDNAs for seven α-subunits, each distinct gene products, have been cloned to date [1,2]. These include two forms of transducin, the retinal photoreceptor G-protein, G_s, the adenylyl cyclase stimulatory protein, G_o, a G-protein pertussis toxin substrate abundantly expressed in brain, and three other pertussis toxin substrates, G_{i1}, G_{i2}, and G_{i3} (arbitrarily numbered in order of cloning) whose functions have not been defined.

G_{i2} is expressed, at least at low levels, in essentially all tissues and cells examined [3,4], but the level of expression is particularly high in myelomonocytic and glial cells [5]. G_{i2} may act in neutrophils to couple chemotactic factor receptors to stimulation of phospholipase C [6]. Expression of G_{i2}, measured by protein and RNA analyses, is greatly enhanced when HL-60 and U-937 cells are induced to differentiate by agents such as dibutyryl-cAMP and dimethylsulfoxide [7]. In order to examine the regulation of the G_{i2} gene, and to define the exon structure of a G-protein α-subunit gene, we have isolated and characterized human genomic DNA clones encompassing the $G_{i2\alpha}$ subunit gene and its 5′ and 3′ flanking regions.

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2. MATERIALS AND METHODS

2.1. *Screening of human genomic library*

A phage EMBL3 genomic library constructed from human leukocyte DNA (Clonetech Laboratory, Inc.) was screened. The *Eco*RI insert of G18, a plasmid containing a full length rat $G_{i2\alpha}$ cDNA [4] (kindly provided by R. Reed and D. Jones), was ^{32}P labelled by nick translation [8] and used to screen the library by hybridization to replicate filters [9]. Nitrocellulose filters were

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prehybridized at 65°C for 2 h in 10% dextran sulfate, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidine, 0.1% Ficoll, 0.9 M NaCl, 90 mM Na citrate, 0.5% SDS and heat denatured salmon sperm DNA (200 μg/ml), and hybridized with labelled probe, 1×10^6 cpm/ml for 16 h at 65°C. Filters were washed with 0.3 M NaCl, 30 mM Na citrate, 0.1% SDS three times for 5 min at 25°C, then washed with 15 mM NaCl, 1.5 mM Na citrate, 0.1% SDS three times for 15 min at 50°C.

2.2. *Restriction mapping and Southern blot analysis*

DNA of isolated clones was digested with restriction enzymes under conditions specified by the suppliers and capillary blotted to nitrocellulose filters [9]. Full length rat Gi2α cDNA and restriction fragments of the cDNA were ^{32}P labelled by random priming [10] and hybridized to filters as described above.

2.3. *Subcloning and DNA sequencing*

Relevant fragments were subcloned into either plasmid pUC19 or pGEM3-Z (Promega-Biotech) and sequenced by the dideoxy nucleotide chain termination method [11], using either the universal M13 or pGEM primer or specifically synthesized primers (Midland Certified Reagent Co., Midland, TX). 7-Deaza dGTP was substituted for dGTP to sequence very G+C rich regions [12].

2.4. *Primer extension analysis*

RNA was isolated from dibutyryl-cAMP induced HL60 cells by the method of Chirgwin [13] and poly(A) selected. A 17-mer oligonucleotide primer complementary to exon 1 genomic sequence (Midland Certified Reagent Co.) was 5′ end-labelled with ^{32}P using T_4 polynucleotide kinase. The primer extension experiment was performed by a modification of the method of Williams and Mason [14]. 150000 cpm of primer was hybridized to 2.5 μg of RNA in 1 M NaCl, 10 mM Pipes, pH 6.4, for 6 h at 55°C. The primer extension reaction was performed as described except that 40 units of AMV reverse transcriptase (Boehringer, Mannheim) was used.

2.5. *S_1 nuclease analysis*

Single-stranded DNA probe was obtained by primer extension of a phage M13 subclone containing the *Bam*HI genomic fragment containing exon 1 (see fig.1), followed by digestion with *Pst*I. Total fibroblast RNA (5 μg) previously isolated [15] and ^{32}P-labelled probe (3000–5000 Cerenkov cpm) were hybridized and digested with S_1 nuclease as described [15].

2.6. *RNA blot hybridization analysis*

Unselected RNA from dibutyryl-cAMP induced HL60 cells was separated on a formaldehyde-agarose gel and blotted to

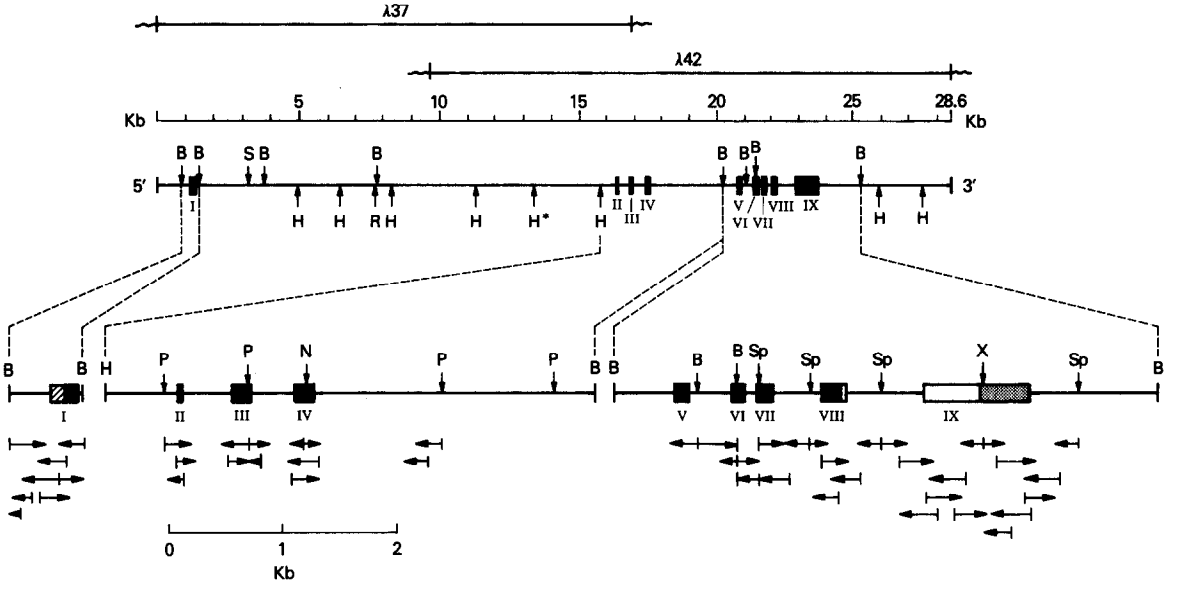

Fig.1. Physical map and sequencing strategy of human $G_{i2}\alpha$ gene. At the top, the position of the genomic inserts of 2 overlapping clones, λ37 and λ42, obtained by screening with cDNA G18 [4] are shown. The restriction map of the represented DNA region is shown beneath a scale in kilobases. The positions of exons 1–9 (in Roman numerals) are indicated. Relevant subcloned regions containing the exons are shown in expanded scale below with further restriction sites. The exons are displayed on the expanded scale with the hatched area representing the 5′ nontranslated region, the solid area representing the coding region, the open area representing the 3′ nontranslated region to the 3′ extent of previously isolated cDNAs [4,17], and the stippled area representing the proposed extent of exon 9 to the first polyadenylation signal (see text). Below is the dideoxy sequencing strategy with arrows representing the direction and extent of each reaction. B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; S, *Sal*I; P, *Pst*I; N, *Nco*I; Sp, *Sph*I; X, *Xba*I. The order of the adjacent *Hin*dIII fragments separated by the asterisked *Hin*dIII site was not unequivocally determined. The *Bam*HI fragments containing exon 5 and the 5′ end of exon 6 were not analyzed for further restriction sites.

nylon filters [7]. DNA fragments were labelled by random priming [10] and filters were hybridized and washed as described [3]. The filters were exposed for 20 h with an intensifying screen.

3. RESULTS

3.1. *Isolation and characterization of the human $G_{i2}\alpha$ gene*

We screened a human genomic library with a full-length rat $G_{i2}\alpha$ cDNA [4]. Of approx. 5×10^5 clones screened, two positive clones, designated λ37 and λ42, consistently hybridized strongly to $G_{i2}\alpha$ cDNA. These clones were selected for further analysis. Hybridization analyses were performed on restriction endonuclease digests of these clones using the ^{32}P-labelled 1755 bp full length rat $G_{i2}\alpha$ cDNA, a 427 bp *Eco*RI-*Pst*I cDNA fragment encompassing the most 5′ end (5′ probe), and an approx. 400 bp *Xba*I-*Eco*RI cDNA fragment encompassing the 3′ end (3′ probe). The λ37 clone hybridized only with the 5′ probe, while λ42 had distinct fragments hybridizing with the 5′ and 3′ probes. Further restriction, cross hybridization, and nucleotide sequence analysis of subcloned fragments showed that the λ37 and λ42 inserts overlap, and span a total distance of approx. 28.6 kb (fig.1).

Fragments hybridizing with ^{32}P-labelled cDNA were subcloned into pGEM 3-Z or pUC19 plasmid vectors for higher resolution mapping and sequencing (fig.1). The human $G_{i2}\alpha$ subunit gene spans approx. 23.5 kb and is composed of 9 exons. All of the splice junctions conform to the GT-AG rule for intron-exon borders (table 1) [16]. Exon 1 contains 133 bp of 5′ nontranslated region (determined by S_1 nuclease and primer extension analysis) and the first 118 bp of coding sequence.

Table 1

Position and sequence of intron-exon junctions in human $G_{i2}\alpha$ gene

Intron	Position[a]	Splice junction sequence and intron size		
		Exon[b]	Intron[c]	Exon[b]
1	118/119	38 39 CTG TTG G leu leu g	gtgaggcccg.....~15 kb.....tctgttccag	40 41 42 GT GCT GGG ly ala gly
2	161/162	52 53 54 CAG ATG AA gln met ly	gtaagtgctg.....~0.35 kb.....tggctatcag	55 56 GATC ATC s ile ile
3	303/304	100 101 AGA GCG arg ala	gtatgtgccc..... ~0.47 kb .....tgtgcccag	102 103 GAC GAC asp asp
4	464/465	153 154 155 GCT GCC TA ala ala ty	gtgagtgct.....~3.1 kb.....cccag	156 157 CTAC CTG r tyr leu
5	593/594	196 197 198 CAC TTC AA his phe ly	gtgagcga..... ~0.30 kb.....catctgcag	199 200 GATG TTT s met phe
6	723/724	240 241 GAG ATG glu met	gtgagaggat..... ~0.90 kb .....tacccccag	242 243 AAC CGC asn arg
7	877/888	291 292 TAC ACA G tyr thr g	gtgtggggac..... ~0.41 kb .....ctccccccag	293 294 295 GG GCC AAC ly ala asn
8	1092/1093	GGGATG 3′NT	gtgagccaga..... ~0.65 kb .....ccacctccag	GGCCACC 3′NT

[a] Base pair number starting with base pair no.1 of initiator codon

[b] Exon nucleotide sequence in upper case and amino acid translation (with residue number) or 3′ nontranslated (3′NT) region are shown

[c] Intron nucleotide sequence in lower case and approximate size in kilobases (kb) are shown

Exons 2–7 all encode coding region. Exon 8 contains the last 188 bp of coding nucleotides and the first 27 bp of the 3′ nontranslated region. Exon 9 contains the remainder of the 3′ nontranslated region. The sequence we determined for the portions of the genomic clones corresponding to the coding region is identical to that obtained for a cDNA isolated from human lymphocytes [17] and from human U937 monocytoid cells [18], except for a silent C to A transversion at position 267 relative to the translational start site in the latter.

3.2. *Characterization of exon 1 and the 5′ flanking region*

The transcriptional start sites for exon 1 were mapped by primer extension and S_1 nuclease analysis. Extension of a primer complementary to the cDNA about the translational start site resulted in a major extended product of 149 bp (heavy arrow, fig.2) and several bands of lower molecular mass. Hybridization with yeast tRNA yielded no extended products. A uniformly labelled single stranded probe representing a *Pst*I-*Bam*HI genomic fragment that spans approx. 330 bp upstream of the translational start site to the first portion of intron 1 was hybridized to fibroblast RNA and digested with S_1 nuclease. This resulted in a major band at about 252 (heavy arrow, fig.2), indicating that to be the length of the first exon. This maps the major transcriptional start site to the same location determined by the primer extension. Several minor bands of smaller size are also present in the S_1 experiment. A minor band (thin arrow, fig.2) 55 bp above the major band may represent an upstream minor start site for transcription. This latter band was not confirmed by our primer extension results. This discrepancy is not due to the difference in cell-type from which the RNA was isolated since similar results were obtained when the S_1 analysis was performed with HL60 RNA (not shown).

The sequence of 354 bp of 5′ flanking region and of exon 1 is shown in fig.3 with the major and probable minor transcriptional start sites indicated. The major RNA transcript has 133 bp of 5′ nontranslated region and extends 20 bp further upstream than the previously isolated cDNA from U937 cells [18]. The rat $G_{i2}\alpha$ cDNA from olfactory tissue [4] remains homologous to the sequence shown and extends further upstream than the major human transcriptional start site. Our 5′ nontranslated sequence from exon 1 differs by 12% and 9%, respectively, from the sequences obtained for the same region in the human lymphocyte cDNA [17] and the cDNA from U937 cells [18].

Examination of the 5′ flanking and 5′ nontranslated sequence reveals a G+C content of

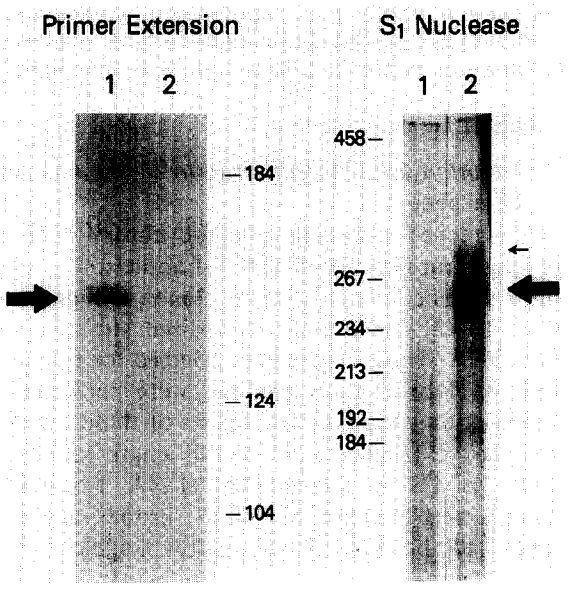

Fig.2. Primer extension and S_1 analysis of the 5′ end. For primer extension, the 17-mer oligonucleotide 5′-TCACGGTGCAGCCCATC-3′, a primer complementary to exon 1 about the translational start site (see fig.3) was 5′ end labelled with ^{32}P and hybridized to 2.5 μg RNA isolated from dibutyryl-cAMP induced HL60 cells (lane 1) or 2.5 μg yeast tRNA (lane 2) at 55°C for 6 h. Primer extension was performed as described [14], and the reaction run on a polyacrylamide-urea gel. Molecular mass markers are noted to the right. In lane 1 a major band of 149 bp is noted by a heavy arrow. Several smaller minor bands below are also noted below the major band. This reaction was repeated besides a sequencing ladder to estimate the size of the major band. For the S_1 nuclease experiment, a uniformly ^{32}P-labelled complementary *Pst*I to *Bam*HI genomic DNA fragment spanning the region from 330 bases upstream of the translational start site to the first *Bam*HI site of intron 1 was prepared. This labelled probe was hybridized to human fibroblast RNA (5 μg) at 72°C overnight, digested with S1 nuclease as described [15], and run on a polyacrylamide-urea gel (lane 1, no RNA; lane 2, RNA, 72°C). Molecular mass markers are noted on the left. A major band at 252 bp is seen in lane 2 (heavy arrow). Several minor bands are noted below the major band as well as a higher molecular mass band (thin arrow) mapping a potential start site about 55 bp upstream of the major start. The locations of the major and minor transcriptional sites are indicated in fig.3.

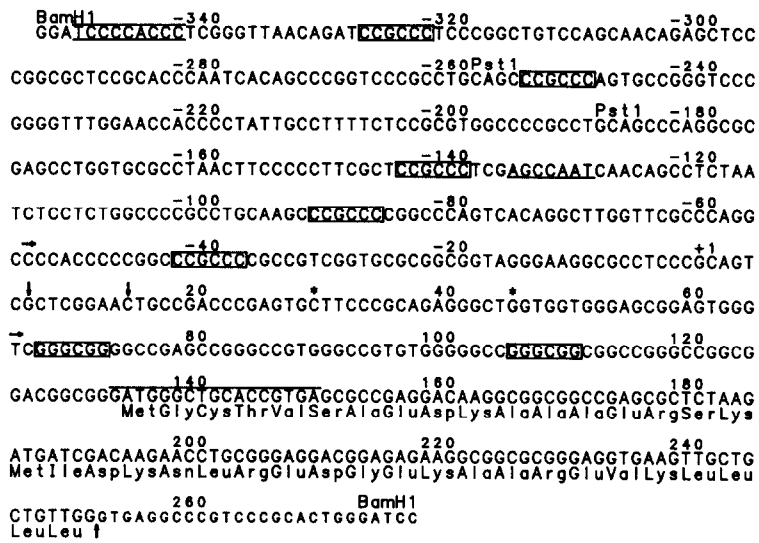

Fig.3. Sequence of 5′ flanking region and exon 1. The sequence of 354 bp of 5′ flanking region and exon 1 are shown with +1 representing the position of the major transcription start site. Amino acid sequence below shows the translation in the open reading frame. The end of exon 1 is shown by an upwards arrow and the first several bases of intron 1 are shown. Minor transcriptional start sites confirmed by primer extension and S_1 nuclease analysis are marked with asterisks. Those clearly shown by primer extension alone are marked with downward arrows and those shown by S_1 nuclease digestion alone are marked with rightward arrows. A CAAT box consensus sequence is underlined, 7 GC boxes (putative Sp1 DNA binding domains) [20] are boxed, and a candidate DNA binding domain for the AP-2 transcription factor [21] is underlined and overlined. The position of the oligonucleotide primer used in the primer extension experiment is indicated with an overline.

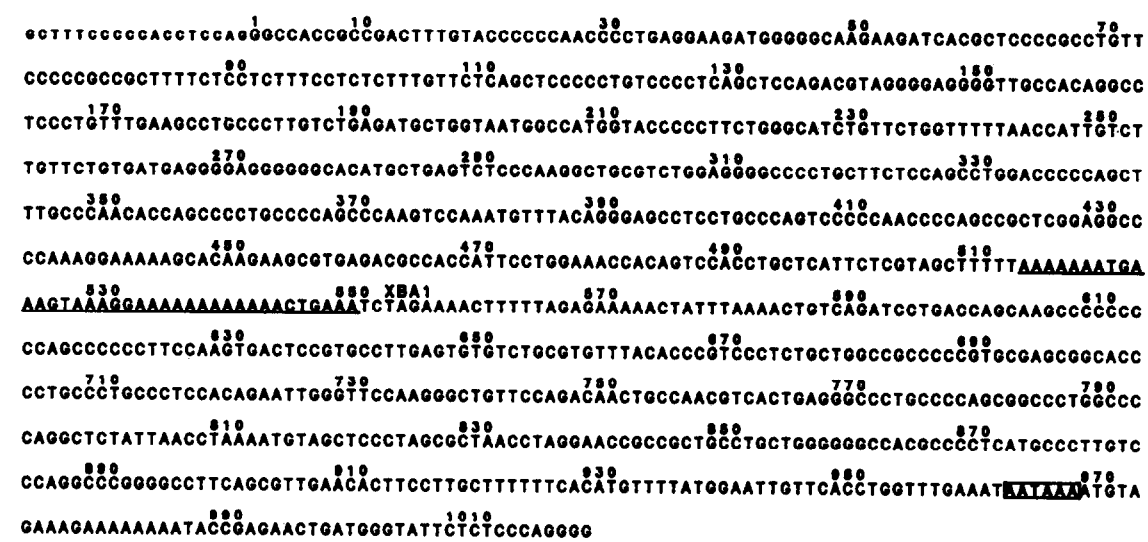

Fig.4. Sequence of exon 9. The sequence of exon 9 is shown with position 1 representing the first base of the exon. The last several bases of intron 8 are shown in small characters. The A-rich genomic region corresponding to the poly(A) tails in $G_{i2}\alpha$ cDNAs is underlined. The *Xba*I site indicates the 5′ end of the fragment used in the RNA hybridization analysis (fig.5). The downstream AATAAA polyadenylation signal is boxed.

>80% with a high frequency of CpG dinucleotides [19]. The 5′ flanking sequence reveals no TATA box consensus sequence but a CAAT box at positions −130 and possibly −283. There are 5 GC boxes (sequence CCGCCC or GGGCGG) in the 5′ flanking region and two additional GC boxes located in the 5′ nontranslated region of exon 1. These are potential DNA binding sites for the transcriptional factor Sp1 [20]. A search for other important DNA binding domains reveals a candidate AP-2 binding domain [21] at −350 and a candidate AP-1 binding domain [22] at −450 (not shown).

3.3. *Characterization of exon 9*

The sequence of the proposed ninth exon is shown in fig.4 to the polyadenylation signal. Sequence of the 871 bp *Sph*I to *Xba*I fragment from λ42, representing the 5′ aspect of exon 9 (see fig.1) reveals a 512 bp region homologous with the last several hundred bases of the 3′ nontranslated region of previously sequenced $G_{i2}\alpha$ cDNAs [4,17]. The sequence of the first 512 bases of exon 9 is identical to that reported for the human cDNA isolated from U937 cells except for single base changes at positions 13, 137, 155, and 387 [18]. This same region shows a greater number of differences in sequence (9% total) compared to the cDNA from human lymphocytes [17].

An A-rich region containing 28 As over a span of 37 bases begins at base 513 of exon 9, just upstream of the *Xba*I site. Human [17] and rat [4] cDNAs reveal a poly(A) tract coincident with the A-rich region in our genomic sequence. Consistent with the lack of polyadenylation signals in reported cDNA sequences, no polyadenylation signals are found upstream of the A-rich region. Sequence downstream of the *Xba*I site reveals an AATAAA polyadenylation signal 405 bases away (fig.4).

The 3′ *Xba*I to *Sph*I genomic fragment was ^{32}P labelled and used as a probe for RNA hybridization analysis (fig.5). This probe hybridized strongly to an mRNA of approx. 2.2 kb, identical in size to that seen using the *Eco*RI-*Pst*I cDNA fragment as a probe. Therefore genomic sequence downstream of the region of homology to the 3′ end of isolated cDNA clones is represented in the $G_{i2}\alpha$ mRNA. Continuation of exon 9 to the downstream polyadenylation signal would explain

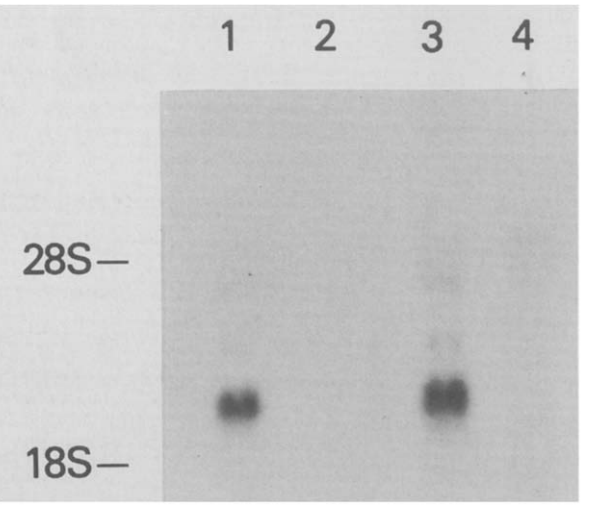

Fig.5. RNA blot hybridization analysis of HL60 RNA with genomic and cDNA probes. HL60 cells were induced to maturation with dibutyryl-cAMP and RNA isolated [7]. 15 μg unselected HL60 RNA (lanes 1 and 3) were size separated on an agarose gel adjacent to ribosomal RNA, 5 μg (lanes 2 and 4), and then transferred to nylon (Nytran) filters. The filters were hybridized with either the approx. 850 *Xba*I to *Sph*I genomic fragment from λ42 which is just downstream of the region homologous to the 3′ end of isolated cDNAs [4,17] (lanes 1 and 2) or the 427 bp *Eco*RI to *Pst*I fragment from the 5′ end of the rat $G_{i2}\alpha$ cDNA (lanes 3 and 4). Conditions were as described [3]. The positions of 18 S and 28 S ribosomal RNA markers are noted on the left.

the approx. 0.5 kb discrepancy between reported cDNA length (about 1.7 kb) [4,17,18] and $G_{i2}\alpha$ mRNA size (about 2.2 kb) as seen on RNA hybridization analyses by us and others [3,4,7,18]. It is likely that during cDNA synthesis, oligo(dT) priming at the A-rich region instead of the poly(A) tail resulted in cDNAs incomplete at the 3′ end by approx. 440 bases.

4. DISCUSSION

The cloning and characterization of a human G protein α-subunit gene, $G_{i2}\alpha$, reveals it to be a complex gene containing 9 exons and spanning 23.5 kb. Genomic exon structure often correlates to functional protein domains [23]. X-ray crystallographic analysis of the related GTP-binding protein EF-Tu [24] and comparison of its primary sequence with those of the ras and G pro-

tein α-subunits has allowed a model of the tertiary structure of these proteins to be derived. According to this model four protein domains, designated 'A', 'C', 'E', and 'G', highly conserved in all GTP-binding proteins, combine to form an internal guanine nucleotide binding site [25]. Regions at the amino and carboxy-terminus and between the domains A and C that show considerable variation in sequence between the G protein α-subunits may be involved in interactions with effectors, receptors, and the β/γ subunits [25]. The sequence coding the A domain of $G_{i2}\alpha$ is split between the first and second exon with the short second exon encoding the majority of the A region. Exon 6 of $G_{i2}\alpha$ encodes the conserved region including both the C and E domains while exon 7 encodes the G domain. The variable region possibly involved in effector binding is represented in exons 3, 4, and 5. The presumed carboxy-terminal receptor binding domain is encoded by exon 8.

The related H, K, and N-*ras* genes, GTP-binding protooncogenes of undefined physiologic function, each contain 4 exons [26]. *ras* exons 2 and 3, encoding the most conserved region including domains C, E, and G, closely align with exons 6 and 7 of $G_{i2}\alpha$. The *ras* exon 2–3 splice site aligns well with the $G_{i2}\alpha$ exon 6–7 splice site. R-*ras* is a gene with 6 exons that appears evolutionarily distinct from H, K, and N-*ras* [27]. However, comparison of this gene with $G_{i2}\alpha$ reveals a similar correlation of exons 3 and 4 of R-*ras* with exons 6 and 7 of $G_{i2}\alpha$, respectively. Also, the exon 2–3 splice site of R-*ras* aligns precisely with the exon 5–6 splice site of $G_{i2}\alpha$. The regions forming the internal guanine nucleotide binding pocket are crucial in defining the tertiary structure of GTP-binding proteins and therefore exons encoding these regions are conserved through evolution.

A partial genomic clone of human $G_o\alpha$ including exons 1 and 2 was recently isolated [28]. Comparison to our $G_{i2}\alpha$ clone reveals an identical splice site between exons 1 and 2. The second exons of both of these genes are almost identical in length with exon 2 of $G_{i2}\alpha$ containing 43 bases compared to 42 for $G_o\alpha$. Comparison of the first intron of $G_o\alpha$ and $G_{i2}\alpha$ reveals a striking size difference with the G_o intron spanning 217 bases versus that of G_{i2} spanning approx. 15 kb.

Several characteristics of the $G_{i2}\alpha$ promoter, such as high G + C content, high frequency of GpC dinucleotides, multiple GC boxes, and lack of a TATA box are common to the promoters of *ras* [29,30] and 'housekeeping' genes, such as the hypoxanthine phosphoribosyl transferase [31], EGF receptor [32], HMG-CoA reductase [33], and adenosine deaminase [34] genes. These promoter characteristics are presumably important for allowing these genes to be ubiquitously expressed. It is unclear whether the presence of 2 GC boxes in the 5′ nontranslated region of exon 1 indicates a regulatory role of this region.

Similar to other genes lacking a TATA box [31–34], the $G_{i2}\alpha$ gene has more than one transcriptional start site as determined by two methods. Results of S_1 nuclease analysis using fibroblast and HL60 RNA indicate the start sites to be consistent between at least two cell types. Unlike most 'housekeeping' gene promoters [31–34], the $G_{i2}\alpha$ promoter has a CAAT box consensus sequence located 130 bases upstream of the major transcriptional start site. Our S_1 nuclease results suggest a minor transcriptional start site may be located about 55 bases upstream of the major site. The CAAT box is located 80 bases upstream of this minor start site, a location more typical for CAAT boxes [35]. However, this site was not confirmed by primer extension, perhaps due to the lower sensitivity in this assay.

G_{i2} is highly expressed in myeloid cells [5,7] and is inducible when monocytic cell lines such as U937 and HL60 are differentiated by agents such as dibutyryl-cAMP [7,18]. These cell lines also differentiate upon treatment with phorbol esters [36]. The $G_{i2}\alpha$ promoter contains possible DNA binding domains for AP-1, a factor that mediates the transcriptional effects of phorbol esters [22], and AP-2, a factor that may mediate the transcriptional effects of cAMP and phorbol esters [21]. It is unknown whether induction of G_{i2} upon treatment with agents such as cAMP (and potentially phorbol esters) is due to direct transcriptional stimulation mediated through factors such as AP-1 and AP-2 or is related to other mechanisms which are enhanced upon differentiation. Deletional analysis of this 5′ region in transfection assays will help in defining the role of these potential domains as well as other promoter regions in regulation of this gene in an inducible and tissue-specific manner.

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