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Multiple Forms of $G_{o\alpha}$ mRNA: Analysis of the 3′-Untranslated Regions[†,‡]

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ABSTRACT: G_o, a guanine nucleotide binding protein found predominantly in neural tissues, interacts in vitro with rhodopsin, muscarinic, and other receptors and has been implicated in the regulation of ion channels. Despite the virtual identity of reported cDNA sequences for the α subunit of G_o ($G_{o\alpha}$), multiple molecular weight forms of mRNA have been identified in tissues from all species examined. To investigate the molecular basis for the size heterogeneity of $G_{o\alpha}$ mRNAs, four cDNA clones were isolated from the same retinal λgt10 cDNA library that was used earlier to isolate λGO9, a clone encompassing the complete coding region of $G_{o\alpha}$. These clones were identified as $G_{o\alpha}$ clones based on nucleotide sequence identity with λGO9 in the coding region; they diverge, however, from λGO9 in the 3′-untranslated region 28 nucleotides past the stop codon. An oligonucleotide probe complementary to a portion of the 3′-untranslated region of λGO9 that differs from the newly isolated clones hybridized with 3.0- and 4.0-kb mRNAs present in bovine brain and retina whereas a similar probe for the unique region of the new clones hybridized with a 4.0-kb mRNA in both tissues and with a 2.0-kb mRNA found predominantly in retina. A similar hybridization pattern was observed when brain poly(A+) RNA from other species was hybridized with the different 3′-untranslated region probes. It appears that differences in the 3′-untranslated regions could, in part, be the basis for the observed heterogeneity in $G_{o\alpha}$ mRNAs.

Structurally related guanine nucleotide binding proteins (G proteins)[1] couple many membrane-bound receptors to their intracellular effector proteins. This family includes the transducins (G_t), which link photoexcited rhodopsin in the retina to the cyclic GMP phosphodiesterase, G_s and G_i, which are responsible for stimulation and inhibition, respectively, of adenylyl cyclase, and G_o, a protein originally isolated from bovine brain (Sternweis & Robishaw, 1984; Florio & Sternweis, 1985; Stryer, 1986; Gilman, 1987; Moss & Vaughan, 1988). G_o interacts in vitro with rhodopsin, muscarinic, and other receptors (Kurose et al., 1986; Tsai et al., 1987) and has been implicated in ion channel regulation (Hescheler et al., 1987).

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[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02900.

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[1] Abbreviations: G protein, guanine nucleotide binding protein; G_t, retinal G protein transducin; $G_{t\alpha}$, α subunit of G_t; $G_{t\alpha 1}$, $G_{t\alpha}$ found in retinal rod outer segments; G_s and G_i, stimulatory and inhibitory G proteins coupled to adenylyl cyclase, respectively; $G_{s\alpha}$, α subunit of G_s; $G_{i\alpha}$, α subunit of G_i; G_o, G protein that may regulate ion flux; $G_{o\alpha}$, α subunit of G_o; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction.

The G proteins are heterotrimers composed of α, β, and γ subunits. The α subunits, which are immunologically distinct, define the specificity of interactions with receptors and effectors (Stryer, 1986; Gilman, 1987; Moss & Vaughan, 1988). They range in size from 39 to 52 kDa and bind and hydrolyze GTP, and some are substrates for ADP-ribosylation by cholera or pertussis toxins (Stryer, 1986; Gilman, 1987; Moss & Vaughan, 1988). The β subunits, which are similar in all G proteins, are found as 35- and 36-kDa species (Manning & Gilman, 1983; Gilman, 1987). The γ subunits are approximately 8 kDa. The transducin γ subunits apparently differ from those of other G proteins (Hildebrandt et al., 1985; Gilman, 1987).

Originally, G proteins like G_s, G_i, and G_t were named on the basis of their presumed function. It has become clear that each of these classes of G proteins includes more than one type of subunit. Rod and cone cells in the retina contain different forms of $G_{t\alpha}$ (Lochrie et al., 1985; Medynski et al., 1985; Tanabe et al., 1985; Yatsunami & Khorana, 1985; Grunwald et al., 1986; Lerea et al., 1986; Raport et al., 1989). At least four species of $G_{s\alpha}$ arise by alternative splicing of transcripts from a single gene (Bray et al., 1986; Robishaw et al., 1986; Kozasa et al., 1988). Different members of the $G_{i\alpha}$ family, designated $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$, arise from distinct genes (Itoh et al., 1988; Weinstein et al., 1988). Most of the G-protein α subunits are encoded by a single mRNA (Nukada et al., 1986; Sullivan et al., 1986; Beals et al., 1987; Bray et al., 1987; Didsbury et al., 1987; Jones & Reed, 1987; Suki et al., 1987). Multiple mRNAs, however, have been consistently reported for $G_{o\alpha}$ despite the high degree of identity among bovine, rat, and human $G_{o\alpha}$ cDNA sequences (Itoh et al., 1986; Brann et al., 1987; Jones & Reed, 1987; Ovchinnikov et al., 1987; Van Meurs et al., 1987; Lavu et al., 1988; Luetje et al., 1988; Price et al., 1989). We have investigated the molecular basis for the heterogeneity of $G_{o\alpha}$ mRNA and describe in this report $G_{o\alpha}$ cDNA clones that differ significantly from other $G_{o\alpha}$ nucleotide sequences in the 3′-untranslated regions.

Experimental Procedures

Materials. Restriction endonucleases (*Bgl*II, *Pst*I, *Hin*dIII, and *Eco*RI), including buffers, AMV reverse transcriptase, and random prime labeling kits, were purchased from Boehringer Mannheim; bacteriophage T4 polynucleotide kinase, terminal deoxynucleotidyl transferase, and all other restriction endonucleases were from BRL; *Taq* DNA polymerase was from Perkin-Elmer Cetus; [γ-^{32}P]ATP (6000 Ci/mmol), [α-^{32}P]dCTP and dATP (5000 Ci/mmol), [α-^{35}S]dATP ($\geq$800 Ci/mmol), and Plaquescreen membranes were from New England Nuclear; Nytran membranes were from Schleicher & Schuell; Bluescript phagemid vector was from Stratagene; and kits for Maxam–Gilbert and dideoxynucleotide sequencing using Sequenase were from New England Nuclear and United States Biochemical Corp., respectively.

Screening of the Retinal cDNA Library. A bovine retinal cDNA library in λgt10, kindly provided by Dr. Jeremy Nathans, was screened by using two different protocols. First, cDNA clones were selected on the basis of hybridization with a 24-nucleotide probe complementary to nucleotides 607–630 of a bovine rod transducin sequence (Yatsunami & Khorana, 1985) or nucleotides 793–816 of a bovine cone transducin (Lochrie et al., 1985) as described previously (Van Meurs et al., 1987). Alternatively, clones were selected by hybridization with a 1339-base *Bss*HII–*Eco*RI restriction fragment of λGO9 (Van Meurs et al., 1987) labeled with ^{32}P by using the random oligonucleotide primer method (Feinberg & Vogelstein, 1983) in 40% formamide/4× SSC (1× = 0.15 M NaCl/15 mM sodium citrate)/5× Denhardt's solution [1× = 0.02% Ficoll/0.02% poly(vinylpyrrolidone)/0.02% bovine serum albumin]/0.5% SDS/10 mM Tris-HCl (pH 7.4)/10% dextran sulfate containing denatured salmon sperm DNA, 100 μg/mL, at 42 °C.

Sequencing of cDNA Clones. Although the cDNA library was prepared with *Eco*RI linkers, two of four clones chosen for subcloning contained inserts that could not be excised from λgt10 by using *Eco*RI alone. These inserts were excised by using a combination of *Eco*RI and *Hin*dIII. Inserts were subcloned into the Bluescript phagemid vector and sequenced by using dideoxynucleotide chain termination (Sanger et al., 1977) with synthetic oligonucleotides and Sequenase or by the method of Maxam and Gilbert (1980). Sequences were verified by sequencing both cDNA strands entirely. Sequence alignments were performed by using the ALIGN program of Myers and Miller (1988) with a gap penalty of 20.

Oligonucleotide Probes. The following probes were either synthesized on an Applied Biosystems Model 380B DNA synthesizer or purchased from Pharmacia: GO-COMM, 5′GAAGGGCTCCGTGTCTTCCATCCGACTCACCACGTCACACACCATCTT3′ (complementary to bases 313–360 of λGO9); GO9, 5′AGGCTGTGTGTCTTAACAAAGGCCAAAAGGTCATGCTACCAGGAGATC3′ [complementary to bases 60–107 of λGO9 (Figure 2B)]; GO3.1, 5′TTTGTTATGCCTTTTTGGAATTTGTTTACCAACTTGCATTTGTTAGTG3′ [complementary to bases 92–139 of λGO3.1 (Figure 2B)]; GO-ILE, 5′AATGATGGAGATATCGATGAA3′ (complementary to bases 778–798 of λGO9); GO-THR, 5′AATGATGGAGGTATCGATGAA3′ (complementary to bases 778–798 of λGO9 except for substitution of C for T at base 788); PCR-FOR, 5′ATCTGTAACAACAAGTTCTTC3′ (corresponds to bases 760–780 of λGO9); PCR-REV, 5′GGTCAGTACAAGCCGCAGCCC3′ (complementary to bases 1047–1067 of λGO9).

RNA Hybridization. Total cellular RNA was isolated from bovine brain or retina (Chirgwin et al., 1979), and poly(A+) RNA was separated by oligo(dT)–cellulose chromatography. The RNAs were separated by electrophoresis in a 1% agarose/2.2 M formaldehyde gel and transferred to nitrocellulose for hybridization. Hybridizations were performed overnight at 42 °C with a *Sty*I–*Eco*RI fragment of λGO9 (nucleotides 262–1276), as described earlier for the λGO9 *Bss*HII–*Eco*RI fragment, or 48-base oligonucleotides complementary to regions specific for the $G_{o\alpha}$ coding sequence (probe GO-COMM) or the 3′-untranslated regions of λGO9 (probe GO9) or λGO3.1 (probe GO3.1). Blots were washed for 30 min in 5× SSC/0.5% SDS at 42 °C followed by two washes in 0.5× SSC/0.5% SDS for 20 min each at 65 °C for the cDNA probe or at 55 °C for the oligonucleotides. Hybridization and washes of blots containing brain poly(A+) RNA from different species were performed as described for bovine brain and retina except that a 412-nucleotide *Hin*dIII–*Eco*RI fragment from the 3′-untranslated region of clone λGO3.1 was used instead of probe GO3.1.

DNA Hybridization. Calf thymus DNA was digested overnight at 37 °C with the appropriate enzyme. Reactions were terminated by phenol/chloroform extraction followed by ethanol precipitation. The DNA fragments were separated by electrophoresis in a 0.8% agarose gel, transferred to a Nytran membrane, and hybridized with the random-primed ^{32}P-labeled (Feinberg & Vogelstein, 1983) *Bam*HI–*Eco*RI fragment of λGO9 or 3′-end-labeled (Eschenfeldt et al., 1987) oligonucleotide probes GO3.1 and GO9. Hybridizations were

performed at 42 °C overnight in 5× SSPE [1× = 0.15 M NaCl/10 mM NaH_2PO_4, (pH 7.4)/1 mM EDTA]/5× Denhardt's/0.5% SDS/10 mM Tris-HCl (pH 7.4)/10% dextran sulfate containing denatured salmon sperm DNA (100 μg/mL) and either 20% formamide for oligonucleotide probes or 40% formamide for the cDNA probe. Blots probed with oligonucleotides were washed for 20 min at room temperature in 2× SSC/0.5% SDS followed by two washes for 60 min each at 55 °C in 0.5× SSC/0.5% SDS. Blots probed with the cDNA were washed once at room temperature in 2× SSPE/0.5% SDS, followed twice for 60 min each in 0.5× SSPE/0.5% SDS at 55 °C, and twice for 60 min in 0.25× SSPE/0.75% SDS at 55 °C. Blots were exposed to Kodak XAR-5 film at −70 °C with intensifying screens either overnight (for cDNA probe) or for 12 days (for oligonucleotide probes).

DNA Amplification by the Polymerase Chain Reaction. Bovine retinal poly(A+) RNA (5 μg) was reverse-transcribed using AMV reverse transcriptase. Following alkaline hydrolysis of RNA, the single-stranded cDNA was phenol-extracted and ethanol-precipitated. An estimated 1 pg of the total cDNA pool or 25 ng of plasmid DNA was subjected to amplification with *Thermus aquaticus* DNA polymerase (2 units) as described by Saiki et al. (1988). Reaction mixtures including 400 pmol each of forward (PCR-FOR) and reverse (PCR-REV) primer were subjected to 35 cycles of 30 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C, using a Perkin-Elmer Cetus DNA thermal cycler.

DNA Dot Blot Analysis. Following amplification of DNA by the polymerase chain reaction (PCR) and dilution with 10 volumes of 10 mM Tris-HCl (pH 7.5)/1 mM EDTA, samples (20 μL) were spotted onto a Nytran membrane, and the DNA was cross-linked to the membrane by UV irradiation. Hybridization of the blots with GO-ILE and GO-THR oligonucleotides was as described for oligonucleotides under RNA Hybridization. The blots were washed once in 5× SSC/0.5% SDS at room temperature for 20 min and twice for 20 min each in 5× SSC/0.5% SDS at 45 °C followed by autoradiography. Subsequent washes were performed in 2× SSC/0.5% SDS for 20 min each with temperature increments of 5 °C followed by autoradiography.

Results

Isolation of $G_{o\alpha}$-Related cDNA Clones. To identify $G_{o\alpha}$-related cDNAs, a bovine retinal λgt10 cDNA library was screened under low-stringency conditions using a 24-base oligonucleotide probe complementary to a region (5′GAGCGCAAGAAGTGGATCCACTGC3′) of $G_{t\alpha l}$ cDNA that codes for amino acids 203–210, which are adjacent to part of the presumptive guanine nucleotide binding site. The library was screened again under low-stringency conditions using a combination of the λGO9 cDNA and an 18-base oligonucleotide probe complementary to the 3′-terminus of the $G_{o\alpha}$ coding region (5′TCCGGGGCTGCGGCTTGT3′). In several G proteins, this region contains the site of modification by pertussis toxin and is highly conserved. From a total of $\sim 2 \times 10^6$ clones screened by these two methods, 4 clones were isolated and subcloned into Bluescript for sequencing.

All of the clones analyzed appear to be of the same type which is nearly identical with λGO9 except for a distinctly different 3′-untranslated region. Clone λGO1, a 2.2-kb insert, contained a complete coding sequence identical with that of λGO9 except for the presence of a C rather than T at nucleotide position 788 (+1 = A of the initiation codon) resulting in replacement of isoleucine at position 263 in the deduced amino acid sequence of λGO9 with threonine. To clarify the base ambiguity at position 788, an approach was used that couples DNA amplification using the polymerase chain reaction with subsequent differential hybridization using codon-specific oligonucleotides that are identical except for one base. A 308 base pair fragment of $G_{o\alpha}$ from nucleotides 760–1067 was amplified from total cellular cDNA using primers PCR-FOR and PCR-REV. The amplified DNA, including that from clones λGO9 and λGO1, was hybridized with oligonucleotide probes GO-ILE, specific for DNA containing a T at nucleotide position 788 of λGO9, or GO-THR, specific for DNA containing a C at the corresponding position of λGO1. At low stringency, probe GO-THR hybridized with both control DNAs as well as all retinal cDNA samples (Figure 1A); similar results were obtained with probe GO-ILE (Figure 1B). At high stringency, probe GO-THR hybridized with DNA amplified from the λGO1 clone as well as the different cDNA samples but not with DNA amplified from λGO9 (Figure 1C). Under identical stringency conditions, probe GO-ILE hybridized with only DNA amplified from clone λGO9 (Figure 1D). Thus, the large majority of molecules amplified and analyzed from the total pool of retinal cDNA by this method had a C in the second position of $G_{o\alpha}$ codon 263. It appears that the thymidine in this position in λGO9 either is the result of a cloning artifact or represents a small population of message expressed in this tissue and that, at least, a majority of $G_{o\alpha}$ molecules contain a threonine at position 263.

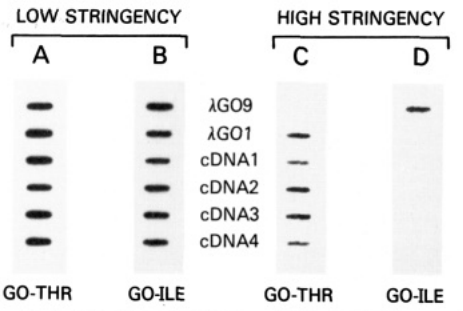
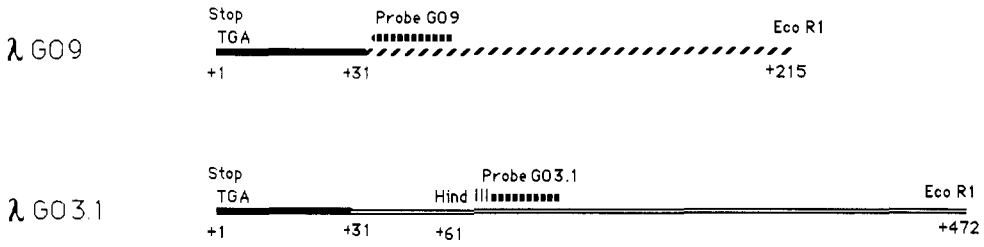


FIGURE 1: Hybridization of PCR-amplified cDNA samples with codon-specific oligonucleotides. DNA fragments corresponding to bases 760–1067 of λGO9 (Van Meurs et al., 1987) were amplified by the PCR from several different bovine retinal cDNA preparations and from plasmids containing λGO9 and λGO1 cDNA as described under Experimental Procedures. Samples (20 μL) of 1:10 dilutions of each amplified DNA sample were spotted on a Nytran membrane and hybridized as described under Experimental Procedures. Low-stringency washes for GO-THR (A) and GO-ILE (B) were performed in 5× SSC/0.5% SDS at 45 °C; high-stringency washes for GO-THR (C) and GO-ILE (D) were in 2× SSC/0.5% SDS at 60 °C.

The 5′-untranslated region of λGO1 contains an extra G residue at position −65 that was lacking in the reported sequence of λGO9. Resequencing of this region of λGO9, however, indicates the presence of the extra G as found in λGO1. In the 3′-untranslated region of λGO1, the first 31 nucleotides (including the termination codon) are identical with those of λGO9 whereas the downstream 32-base sequence is different and ends in a *Hin*dIII restriction site not present in λGO9. The 3′-untranslated region of λGO1 was apparently truncated during subcloning as a result of using *Hin*dIII to excise the insert from λgt10. Partial sequencing of this region directly in the λgt10 clone confirmed this interpretation (data not shown).

The three other new clones (λGO3.1, λGO3.21, and λGO3.X) appeared to be variations of λGO1. Each contains a *Hin*dIII restriction site in the 3′-untranslated region and an incomplete coding region with various degrees of cloning artifact at the 5′-terminus. In this region, at different points

A



B

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GO3.1   TGACCTCTTGTCCTGTATAGCAACCTATTTGGTAATGATTCCAGCACTCA -50
        ********************************  * ** ***  * ****
GO9     TGACCTCTTGTCCTGTATAGCAACCTATTTG--ACTGCTTCACGGACTCT -48

GO3.1   CAGAAAAGCTTGCACACATACACACACACCCCACCCCTCCCCACTAACAA -100
            ****
GO9     ---------TTGCT----------------------------------- -53

GO3.1   ATGCAAGTTGGTAAACAAATTCCAAAAAGGCATAACAAACCTTATATATA -150
              **  ** *  ***    ** *** **
GO9     ----------GTTGACGATCTCCTGGTAG-CATGACC------------- -79

GO3.1   TAGACAAATATATATTAAAGTTTTTTAGTCTGTACTAGAAAGAGCTTCAG -200
                          **** * ** *  **   ***    **
GO9     ----------------------TTTTGGCCTTTGTTA---AGA----CAC -100

GO3.1   ACAGAACTGACCACCATTCCATTGCTCATCAATTTCCTGGGACAGCACCT -250
        ****              ** **    *****   **  *   *  ****
GO9     ACAG--------------CCTTTCTGTATCAAGCCCCCTGTCTA--ACCT -134

GO3.1   GAGCGTGCGCTTACGCGCGTACACACACATAGACACGCACTGCGATACAA -300
              ***          ** ** **  **  ** ** **  *
GO9     ------------ACG---------ACCCAGAGTGACTGACGGCTGT---- -159

GO3.1   GTCCTGATTTGGGAGTCCGTCCTTTTAAAAACAGCCACATGCTTTCACGC -350
                        ** *** *  * *       ***** *
GO9     ------------------GTACTTCTCTAGA-------ATGCTAT----- -179

GO3.1   TCTGAGACCCACCCGTTTCTGTGAGCAGGGGGAGGGCAAGGAAAGCCCTG -400
            ***  *   * ** * ****
GO9     ----AGAATCCGGCTTTAGTTTGAG------------------------- -200

GO3.1   GCCTCAGTCCAGCCTTTTCTCTGCTTCCACCTGCTCAGGCTGTGTGCTCT -450
                                                      ***
GO9     ----------------------------------------------TCT -203

GO3.1   TGGTTCTGTCCTGCACTTGTGT -472
        *          * ** **
GO9     T----------TACATTTAGAA -215
```

FIGURE 2: Sequences of the 3′-untranslated regions of $G_{o\alpha}$ cDNA clones. (A) Schematic representation of the 3′-untranslated regions of clones λGO9 and λGO3.1. Numbering begins with the first base of the termination codon. The solid black line indicates the region of sequence identity among all clones; open and hatched bars signify regions of sequence difference. The positions of oligonucleotide probes GO9 and GO3.1 as described under Experimental Procedures are indicated above the respective sequences. Positions of restriction sites are also indicated above the sequence. (B) Nucleotide sequences of clones λGO3.1 and λGO9. Gaps in sequence, indicated by hyphens, were introduced to optimize alignment. Identical nucleotides are indicated by asterisks. Numbers on the right indicate the nucleotide number beginning with the termination codon.

in each clone, the nucleotide sequence yields a deduced amino acid sequence that is not similar to those of the other clones or to any G-protein α subunits. Oligonucleotide probes complementary to parts of λGO9 that encode the site of cholera toxin catalyzed ADP-ribosylation and the GTP hydrolysis site failed to hybridize with these clones. Clones λGO3.1 (Figure 2A) and λGO3.X, which were excised with *Eco*RI alone, extend 469 nucleotides beyond the termination codon, ending in an *Eco*RI site. They contain a *Hin*dIII site 61 nucleotides downstream of the stop codon. Their 3′-untranslated regions between the termination codon and *Hin*dIII site are identical with those of λGO1 with the exception that they lack two nucleotides (GT) at the point of divergence from λGO9. The 3′-untranslated region of λGO3.21 is identical with that of λGO3.1, except that it terminates at the *Hin*dIII site as a result of using this enzyme to excise the insert from λgt10. Like λGO9, all of these clones lack a consensus polyadenylylation signal, AATAAA, and are presumably incomplete at the 3′-terminus.

The PCR/codon-specific oligonucleotide hybridization approach was utilized to determine whether the extra GT dinucleotide present in clone λGO1 at the point of divergence

Table I: Densitometric Analysis of the Hybridization of RNA with 3′-Untranslated Region Probes[a]

size (kb)	cDNA	ratio[b]	GO-COMM	ratio	GO9	ratio	GO3.1	ratio
brain 2.0	[c]		5.4 ± 1.7 (2)	0.08			22.5 ± 3.7 (2)	0.29
3.0	23.7 ± 5.3 (3)	0.31	31.4 ± 9.7 (2)	0.45	30.3 ± 3.5 (2)	0.43		
4.0	75.9 ± 6.0 (3)		68.6 ± 9.7 (2)		69.8 ± 3.5 (2)		77.6 ± 3.7 (2)	
retina 2.0	35.1 ± 7.8 (3)	0.83	8.1 ± 2.2 (4)	0.14			11.5 ± 5.0 (3)	0.13
3.0	25.2 ± 4.4 (3)	0.63	32.2 ± 2.1 (4)	0.53	36.4 ± 5.7 (5)	0.59		
4.0	40.9 ± 5.6 (3)		59.9 ± 3.8 (4)		62.8 ± 4.8 (5)		88.6 ± 5.0 (3)	

[a] Blots of bovine brain and retinal poly(A+) RNA were hybridized with cDNA or oligonucleotides specific for the coding or 3′-untranslated regions as described under Experimental Procedures. Densitometric analyses of the autoradiographs were performed by using an LKB Model 2202 UltroScan densitometer. Peak area for the indicated mRNA is reported as a percentage of the total peak areas. Data are the mean (±standard deviation) for the number of blots indicated in parentheses. [b] Ratio of indicated peak area to that of the 4.0-kb mRNA in the same tissue. [c] Not observed in blots used for densitometric analysis.

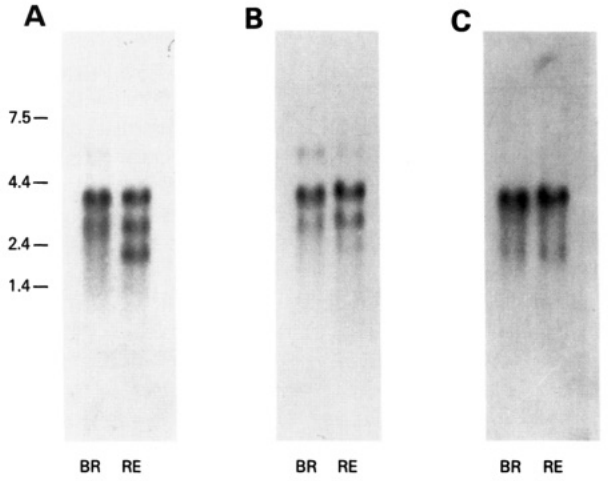

FIGURE 3: Hybridization of RNA from bovine brain and retina with the 3′-untranslated region probes. Poly(A+) RNA (5 μg) from bovine brain (BR) or retina (RE) was separated by electrophoresis, transferred to nitrocellulose, and hybridized with (A) the *Sty*I–*Eco*RI fragment of λGO9, (B) oligonucleotide probe GO9, or (C) probe GO3.1 as described under Experimental Procedures. Positions of RNA standards (nucleotides × 10^{-3}) are indicated on the left.

might be artifactual. An oligonucleotide complementary to bases 26–39 of λGO3.1 (Figure 2B) with bases inserted to account for the extra GT failed to hybridize with DNA amplified from several retinal cDNA preparations, whereas a probe matching the λGO3.1 sequence hybridized with each amplified DNA sample (data not shown). These data indicate that the predominant $G_{o\alpha}$ mRNAs in bovine retina do not contain the additional GT dinucleotide found in λGO1 at the point of the divergence from λGO9.

Except for the 28 bases most proximal to the translational termination codon, the nucleotide sequence of the 3′-untranslated region of the 4 new clones does not exhibit any degree of identity with that of λGO9 (Figure 2B) and appears unrelated to those of other G proteins. A search of all sequences in the GenBank database failed to yield sequence related to the 3′-untranslated region of λGO3.1.

Northern Blot Analysis of $G_{o\alpha}$ mRNA. Poly(A+) RNA from bovine brain and retina contained multiple mRNAs that hybridized with a *Sty*I–*Eco*RI fragment encompassing 75% of the coding region and all of the 3′-untranslated region of λGO9 (Figure 3A). As we reported (Price et al., 1989), the cDNA probe hybridized with mRNAs of 2.0, 3.0, and 4.0 kb in retina and 3.0 and 4.0 kb in brain. It hybridized only weakly and inconsistently with a 2.0-kb mRNA in brain. Occasionally, a mRNA species of approximately 5.6 kb whose identity is unclear was observed in both tissues. These $G_{o\alpha}$ mRNAs were initially identified on the basis of their hybridization with oligonucleotide and cDNA probes specific for the $G_{o\alpha}$ coding region (Price et al., 1989). Probe GO9 which is specific for

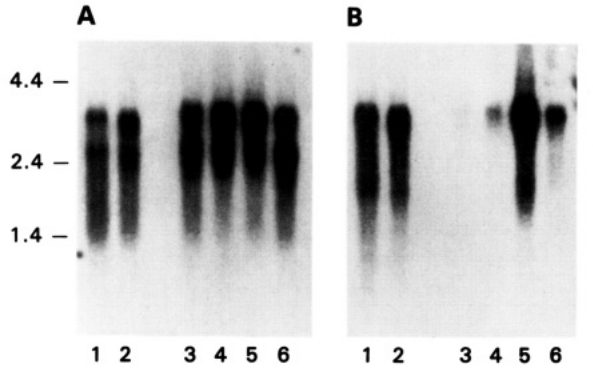

FIGURE 4: Hybridization of RNA from different species with the 3′-untranslated region probes. Poly(A+) RNA (10 μg) from the designated species was separated by electrophoresis, transferred to nitrocellulose, and hybridized as described under Experimental Procedures with (A) probe GO9 or (B) the *Hin*dIII–*Eco*RI fragment of clone λGO3.1. Lane 1, bovine retina; 2, bovine brain; 3, rat brain; 4, mouse brain; 5, rabbit brain; 6, human brain. Positions of RNA standards (nucleotides × 10^{-3}) are indicated on the left.

the 3′-untranslated region of λGO9 hybridized with the 3.0- and 4.0-kb mRNAs in both brain and retina but not with the retinal 2.0-kb species (Figure 3B). Densitometric analyses of autoradiograms from multiple mRNA blots indicated that the ratio of the percentage of total area represented by the 3.0-kb band relative to the 4.0-kb mRNA in both brain and retina is the same for the cDNA fragment, and probes GO-COMM and GO9 (Table I).

Probe GO3.1 which is specific for the 3′-untranslated region of type λGO3.1 hybridized with the 4.0-kb, but not the 3.0-kb, mRNA in both brain and retina (Figure 3C). It also hybridized with the 2.0-kb mRNA in both brain and retina. Densitometric analysis indicated that the amount of 2.0-kb mRNA detected in retina by probe GO3.1 relative to the 4.0-kb mRNA is approximately the same as determined for probe GO-COMM; however, this amount is much lower than that detected with either *Bss*HII–*Eco*RI or *Sty*I–*Eco*RI cDNA fragments (Table I). One potential explanation for this discrepancy is that cross-hybridization occurred between the cDNA fragments and a non-$G_{o\alpha}$ mRNA that comigrates with the 2.0-kb $G_{o\alpha}$ mRNA even under moderately stringent wash conditions. On the basis of tissue distribution and reported message size, a candidate for such cross-hybridization might be $G_{t\alpha 1}$ in retina. The amount of 2.0-kb mRNA detected by probe GO3.1 in brain, relative to the 4.0-kb species, is much higher than that observed with probe GO-COMM (Table I). This inconsistency may reflect differences in the amounts of each message in the various poly(A+) preparations used for these studies.

Hybridization of brain poly(A+) RNA from different animal species with probes specific for each 3′-untranslated region resulted in patterns similar to those observed for bovine brain

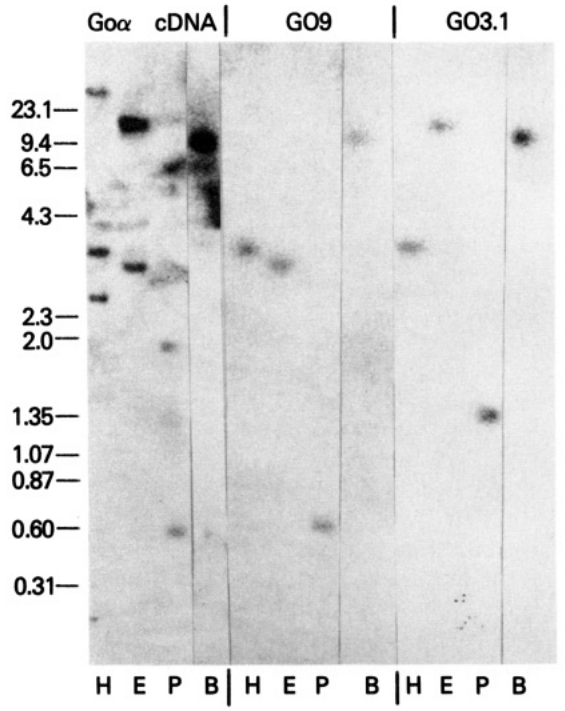

FIGURE 5: Hybridization of calf thymus genomic DNA digests with $G_{o\alpha}$-specific cDNA and oligonucleotide probes. Calf thymus DNA (~19 μg/lane) was digested with (H) *Hin*dIII, (E) *Eco*RI, (P) *Pst*I, or (B) *Bgl*II. DNA fragments were separated by electrophoresis, transferred to Nytran, and hybridized with a *Bam*HI–*Eco*RI fragment of λGO9 (nucleotides 408–1276) or oligonucleotide probes GO9 or GO3.1 as described under Experimental Procedures. Positions of DNA standards (nucleotides × 10^{-3}) are indicated on the left.

and retina. Probe GO9 hybridized with mRNAs in the 3- and 4-kb range in rat, mouse, rabbit, and human (Figure 4A) whereas the *Hin*dIII–*Eco*RI fragment of clone λGO3.1 hybridized predominantly with ~4-kb mRNA in mouse, rabbit, and human and weakly with a 2.0-kb mRNA in rabbit (Figure 4B). The extent of hybridization of mRNA with the *Hin*dIII–*Eco*RI fragment varied among species and may indicate a lack of conservation of this portion of the 3′-untranslated region. Consistent with this, probe GO3.1 did not hybridize with mRNA from these species (data not shown).

Southern Blot Analysis of Bovine Genomic DNA. To determine whether the two 3′-untranslated regions are the products of one or more $G_{o\alpha}$ genes, bovine genomic DNA was digested with several restriction enzymes, and Southern blots were hybridized with oligonucleotide probes specific for the untranslated regions (Figure 5). In two of the digests, both untranslated region-specific probes hybridized with identical DNA fragments, consistent with the conclusion that there is one $G_{o\alpha}$ gene and that sequences representing the two 3′-untranslated regions are relatively close to each other.

Discussion

From the high degree of interspecies conservation of nucleotide sequence in 3′-untranslated regions of individual G-protein cDNAs, it can be inferred that this region may have an important role in the expression of specific G proteins. We have identified a family of $G_{o\alpha}$ cDNA clones with 3′-untranslated regions that differ distinctly from those of published $G_{o\alpha}$ sequences. Four clones were isolated and identified as $G_{o\alpha}$-like based on the correspondence of their sequences to that of λGO9, a $G_{o\alpha}$ cDNA clone isolated from the same library (Van Meurs et al., 1987).

Clone λGO1 contains an open reading frame that is identical with that of λGO9 except for a single nucleotide difference at base +788 (+1 = start ATG codon). The C residue in this position changes an isoleucine codon in λGO9 to a threonine codon in λGO1. A threonine has been reported in this position in all other deduced $G_{o\alpha}$ amino acid sequences (Itoh et al., 1986; Jones & Reed, 1987; Ovchinnokov et al., 1987; Lavu et al., 1988) and was also found in a partial clone, λGO3.21. An approach combining DNA amplification by PCR with codon-specific oligonucleotide hybridization was utilized to investigate the basis for the nucleotide difference at position 788. This approach has been used to detect point mutations in a number of genes (Ireland et al., 1988; Morel et al., 1988; Weisgraber et al., 1988; Fucharoen et al., 1989). These studies indicated that the thymidine in λGO9 probably resulted from a base misincorporation during cDNA synthesis rather than from a different mRNA arising from nonidentical alleles.

The 5′-untranslated region and the first 31 bases of the 3′-untranslated region of λGO1 are also identical with λGO9. The remaining 32 nucleotides of the 3′-untranslated region do not display homology with the corresponding regions of λGO9 (Van Meurs et al., 1987) or other G-protein cDNAs (Lochrie et al., 1985; Medynski et al., 1985; Tanabe et al., 1985; Yatsunami & Khorana, 1985; Bray et al., 1986; Itoh et al., 1986; Nukada et al., 1986; Robishaw et al., 1986; Sullivan et al., 1986; Beals et al., 1987; Bray et al., 1987; Didsbury et al., 1987; Jones & Reed, 1987; Suki et al., 1987; Itoh et al., 1988; Kozasa et al., 1988; Weinstein et al., 1988). λGO1 was truncated in the 3′-untranslated region by the use of *Hin*dIII during subcloning. Other partial clones that were subcloned without using *Hin*dIII contain the unique 3′-untranslated region and extend 411 nucleotides beyond the *Hin*dIII recognition sequence. All of these are members of a group of $G_{o\alpha}$ cDNAs that are identical with reported $G_{o\alpha}$ clones except for the 3′-untranslated region. The point of divergence of the two classes of cDNAs is 28 nucleotides downstream from the termination codon, consistent with the possibility that the first 28 bases may differ in function (e.g., translational control, mRNA stability) from the rest of the untranslated sequence.

As only one type of $G_{o\alpha}$ cDNA had been reported, it may seem surprising that all four clones contained this newly recognized form of $G_{o\alpha}$ 3′-untranslated region. Expression of mRNA containing a λGO3.1-like 3′-untranslated region is not restricted to bovine retinal tissue. Northern blot analysis demonstrated that both types of 3′-untranslated regions are present in poly(A+) RNA from bovine brain as well as in brains of other species. Furthermore, in the tissues surveyed, the λGO3.1-specific probe hybridized with ~2.0-kb mRNA whereas the λGO9-specific probe hybridized with ~3.0-kb mRNA; mRNA in the 4.0-kb range hybridized with both probes.

The observed variations in the relative amounts of the different forms of $G_{o\alpha}$ mRNA may reflect differences in the extent of expression of the individual transcripts in specific cell types (regions). Consistent with this idea, in situ mRNA hybridization (Brann et al., 1987; Largent et al., 1988) and immunohistochemical (Worley et al., 1986; Chang et al., 1988) studies in brain have demonstrated that $G_{o\alpha}$ expression is limited to specific cell types.

The occurrence of $G_{o\alpha}$ mRNAs with different 3′-untranslated regions could reflect the presence of multiple genes for $G_{o\alpha}$ that are nearly identical in the 5′-untranslated and coding regions but differ in the 3′-untranslated regions. The results of Southern blotting of restriction enzyme digests of bovine genomic DNA are, however, consistent with the presence of a single $G_{o\alpha}$ gene. In that case, the multiple mRNAs may

result from alternative splicing of transcripts from this gene. The fact that, on Southern blots, oligonucleotides specific for both 3′-untranslated regions hybridize with identical fragments is consistent with the conclusion that sequences representing the two types of 3′-untranslated regions are located close to one another within the genome. Hybridization of clone-specific oligonucleotide probes with either the 2.0- or the 3.0-kb mRNA, but not both, is consistent with generation of messages containing different exons in their 3′-untranslated regions by alternative splicing. The hybridization studies could not, however, distinguish between the presence of two different 4.0-kb $G_{o\alpha}$ mRNAs or a single 4.0-kb message that contains both exons.

It is unlikely that the 3′-untranslated region of $G_{o\alpha}$ reported here is artifactual. Four independent clones appear to have identical sequences in this region. Moreover, the mRNAs that hybridize with the λGO3.1 probe correspond to those detected previously with the coding region of the λGO9 cDNA and with other probes specific for the $G_{o\alpha}$ coding region (Price et al., 1989). Thus, a family of $G_{o\alpha}$ cDNA clones has been described that are unique among G proteins in that they appear to encode identical (or virtually identical) forms of $G_{o\alpha}$ protein but have different 3′-untranslated regions. Further studies will provide information important for understanding the physiological significance of these multiple $G_{o\alpha}$ transcripts.

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NMR Comparison of Prokaryotic and Eukaryotic Cytochromes *c*†

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ABSTRACT: ^{1}H NMR spectroscopy has been used to examine ferrocytochrome *c*-551 from *Pseudomonas aeruginosa* (ATCC 19429) over the pH range 3.5–10.6 and the temperature range 4–60 °C. Resonance assignments are proposed for main-chain and side-chain protons. Comparison of results for cytochrome *c*-551 to recently assigned spectra for horse cytochrome *c* (Wand et al. (1989) *Biochemistry 28*, 186–194) and mutants of yeast iso-1 cytochrome (Pielak et al. (1988) *Eur. J. Biochem. 177*, 167–177) reveals some unique resonances with unusual chemical shifts in all cytochromes that may serve as markers for the heme region. Results for cytochrome *c*-551 indicate that in the smaller prokaryotic cytochrome, all benzoid side chains are rapidly flipping on the NMR time scale. In contrast, in eukaryotic cytochromes there are some rings flipping slowly on the NMR time scale. The ferrocytochrome *c*-551 undergoes a transition linked to pH with a p*K* around 7. The pH behavior of assigned resonances provides evidence that the site of protonation is the inner or buried 17-propionic acid heme substituent (IUPAC-IUB porphyrin nomenclature). Conformational heterogeneity has been observed for segments near the inner heme propionate substituent.

Cytochrome *c* is a well-known component of respiratory electron transport chains where it transfers electrons to a terminal oxidase. Its structure is highly conserved in eukaryotes. It has usually 102–103 residues in higher eukaryotes and up to 116 in lower eukaryotes and plants, which can have a variable N-terminal extension. The ca. 100-residue core has high sequence homology, and the crystal structures from horse, tuna, bonito, and yeast show a tightly conserved tertiary structure. In prokaryotes, a functionally equivalent electron-transport protein is found, but there can be large variations in size and sequence (Dickerson & Timkovich, 1975; Timkovich, 1979; Meyer & Kamen, 1982). Cyt *c*-551[1] from *Pseudomonas aeruginosa* is an example of a bacterial cytochrome of smaller size (82 residues). It donates electrons to membrane oxidases during the aerobic respiration of *Pseudomonads* or to a dissimilatory nitrite reductase during anaerobic respiration. The amino acid sequence (Ambler, 1963a,b) and X-ray crystal structure (Dickerson et al., 1976; Almassy & Dickerson, 1978; Matsuura et al., 1982) have been determined. Critical structural differences compared to eukaryotic cytochromes include deletions around the bottom of the heme crevice, a major reorientation of the invariant tryptophan found hydrogen bonding to the buried heme propionate groups, and a novel polyproline-type helix that positions the sixth ligand methionine for iron coordination. Cyt *c*-551 also demonstrates functional differences compared to cyt *c*. Its reduction potential is sensitive to pH and linked to a pH-controlled transition with a p*K* around 7 (Moore & Williams, 1977; Moore et al., 1980), while that of eukaryotic cytochromes *c* is independent of pH for several units around neutrality. Its rate of electron self-exchange is more than 3 orders of magnitude faster than for cyt *c* (Timkovich et al., 1988, and references therein). There is evidence that its association with redox partners may rely more on hydrophobic interactions than for cyt *c* (Timkovich, 1986).

Recently, extensive ^{1}H NMR studies have been reported for the eukaryotic horse cyt *c* (Wand et al., 1989), and the Thr 102 and Phe 82 mutants of eukaryotic yeast iso-1 cyt *c* (Pielak et al., 1989a,b). Investigation of the spectrum of *P. aeruginosa* cyt *c*-551 in its ferrous oxidation state affords an opportunity to compare structure and spectra between the larger eukaryotic cytochromes and the smaller prokaryotic version. *Pseudomonas aeruginosa* cyt *c*-551 has been studied previously by ^{1}H NMR and select assignments have been made (Keller & Wüthrich, 1976; Moore et al., 1977; Keller & Wüthrich, 1978; Chao et al., 1979; Senn et al., 1980). This article proposes extensive assignments for the ferrocytochrome

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[1] Abbreviations: cyt, cytochrome; DQF-COSY, double-quantum filtered correlation spectroscopy; NOESY, nuclear Overhauser enhancement correlation spectroscopy; NOE, nuclear Overhauser enhancement; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; TPPI, time proportional phase incrementation.

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