Volume 221, number 1, 81-86 FEB 05042 August 1987

Detection of multiple forms of $G_{i\alpha}$ in HL60 cells

Philip M. Murphy, Brock Eide*+, Paul Goldsmith*, Mark Brann*, Peter Gierschik†, Allen Spiegel* and Harry L. Malech

*Bacterial Diseases Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, *Molecular Pathophysiology Section, Metabolic Diseases Branch, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA* and †*Pharmacology Institute, Heidelberg University, FRG*

Received 7 July 1987

Comparison of cDNA sequences from multiple sources predicts a genus of highly homologous but structurally distinct G protein α-subunits, designated as $G_{i\alpha}$, that may include the α-subunit of the functionally defined adenylate cyclase inhibitory G protein. Using specific oligonucleotide probes on Northern blots, we show that $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$, but not $G_{i\alpha\text{-}1}$, are expressed in HL60 cells. Antisera raised against synthetic peptides from regions predicted to be conserved (AS6) and divergent (LE3) among $G_{i\alpha}$ subtypes bind to a 40 kDa protein in Western blots of HL60 membranes. AS6 identifies an additional protein at 41 kDa. Thus, Northern blot and immunoblot results show that at least two $G_{i\alpha}$ subtypes, a 40 kDa protein likely to correspond to $G_{i\alpha\text{-}2}$ and a 41 kDa protein possibly representing $G_{i\alpha\text{-}3}$, may be expressed in a single cell type.

Guanine nucleotide regulatory protein; mRNA; Differentiation; Signal transduction; Pertussis toxin; (HL60 cell)

1. INTRODUCTION

Guanine nucleotide binding regulatory proteins (G proteins) are a heterogenous, but related, group of membrane-associated proteins which functionally link surface receptors with effector moieties such as enzymes or ion channels [1,2]. They are all heterotrimers composed of a structurally distinct α-subunit and very similar or identical β- and γ-subunits. Within the α-subunit reside functional domains for guanine nucleotide binding, GTPase activity, pertussis and/or cholera toxin catalyzed ADP-ribosylation, and probably for interaction with specific receptor and effector proteins. Four different G protein classes, designated as G_s, G_i, G_o and transducin, were originally distinguished on the basis of modulation of receptor and effector activity by guanine nucleotides, the molecular mass of the α-subunit, and susceptibility to toxin catalyzed ADP-ribosylation.

Comparison of nucleotide sequences of cDNA clones encoding the α-subunits supports the concept that G_s, G_i, G_o and transducin comprise G protein genera containing one or more highly homologous but structurally distinct subtypes [3-6]. In particular, structural evidence for multiplicity within the $G_{i\alpha}$ genus has been provided by the isolation of three distinct clones from human cDNA libraries [4,7-9], all of which have homologues in other species ([5,10,11] and Reed, R. and Jones, D., personal communication). Immunochemical and toxin studies [12-16] have also pointed toward the likelihood of multiple distinct G_i-like proteins. In the present study, we use oligonucleotide probes and antisera raised against synthetic peptides to show than human myeloid cells

Correspondence address: H.L. Malech, Building 10, Room 11N110, National Institutes of Health, Bethesda, MD 20892, USA

+ A Howard Hughes Medical Scholar

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

contain mRNA and proteins for at least two distinct members of the $G_{i\alpha}$ genus.

2. MATERIALS AND METHODS

The human promyelocytic leukemia cell line HL60 was maintained as reported [14]. HL60 were induced to differentiate to granulocytoid cells with 750 mM dibutyryl cyclic AMP (Sigma) for 1–3 days. Alternatively, cells were differentiated with 1.25% dimethylsulfoxide (DMSO) for 5 days. Plasma membranes were prepared by homogenization and sucrose gradient centrifugation as described in [14]. Poly(A)$^+$ RNA was selected from total cellular RNA by 2 passages through an oligo(dT) cellulose column [17].

Synthetic 48mer oligodeoxyribonucleotides were synthesized [18] which correspond to a divergent area of the coding region of cDNA sequences from human $G_{i\alpha\text{-}1}$, rat $G_{i\alpha\text{-}2}$, rat $G_{i\alpha\text{-}3}$, and rat $G_{o\alpha}$ (table 1). A 48mer complementary to the bases encoding amino acids 379–394 of rat $G_{s\alpha}$ [5′-GAGCAGCT-CGTATTGGCGAAGATGCATGCGCTGGAT-GATGTCACGGCA-3′] (Reed, R. and Jones, D., personal communication) was also constructed. Probes were 5′-end labelled with [α-^{32}P]dATP by the terminal deoxynucleotidyl transferase method [18]. A c-myc cDNA probe [20] was labelled with [α-^{32}P]dCTP by nick translation [19].

Poly(A)$^+$ RNA (5 μg/lane) was separated on a 2.2 M formaldehyde, 1.2% agarose gel and transferred to nylon membranes. Hybridization was performed with ^{32}P-labelled probes at 37°C as described [18]. Specific transcripts were identified by autoradiography after extensive washing of the membranes at 55°C in 150 mM NaCl wash buffer. Calf liver ribosomal RNA was used as chain length markers.

AS6 is a rabbit antiserum raised against the synthetic decapeptide, KENLKDCGLF (carboxy-terminus of bovine transducin-α) as previously described [14]. AS6 binds to purified bovine transducin-α, to purified $G_{i\alpha}$ from bovine brain and neutrophils, but not to purified bovine brain G_o or G_s [14]. LE3 is a rabbit antiserum raised against the synthetic decapeptide, LERIAQSDYI (amino acid 160–169 predicted by $G_{i\alpha\text{-}2}$ cDNA clones, and divergent from $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}3}$ clones) as described in [16] (table 1). This antiserum recognizes a 40 kDa pertussis toxin substrate from human neutrophils but does not bind to the 41 kDa pertussis toxin substrate from bovine brain, or to purified α-subunits of transducin, G_o or G_s [16].

Purified HL60 membranes were subjected to SDS-PAGE and transferred to nitrocellulose. AS6 or LE3 binding to blots was detected with an indirect immunoperoxidase method using preimmune serum and/or decapeptide competition as controls [14,16].

3. RESULTS AND DISCUSSION

Comparison of $G_{i\alpha}$ cDNA sequences from different species reveals marked conservation and indicates common subtypes (table 1): 94% homology in the case of human and bovine $G_{i\alpha\text{-}1}$; 87–90% homology in the case of human, rat and murine $G_{i\alpha\text{-}2}$. Moreover, the predicted amino acid sequences of $G_{i\alpha\text{-}1}$ are identical across species and those of $G_{i\alpha\text{-}2}$ diverge at only 3–8 positions. The amino acid sequences predicted from rat and human $G_{i\alpha\text{-}3}$ cDNAs diverge at a single residue ([8] and Reed, R. and Jones, D., personal communication).

Comparison of predicted amino acid sequences from different $G_{i\alpha}$ subtypes shows approx. 88% homology between $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}2}$, 86% homology between $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$, and 94.5% homology between $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}3}$ with the majority of divergence accruing from differences in the region from amino acid positions 82–142. The synthetic 48mer oligonucleotides indicated in the methods were derived from regions which contained $\leq$50% homology between each of the $G_{i\alpha}$ subtypes and $\leq$43% for the $G_{i\alpha}$ group compared to $G_{o\alpha}$ and $G_{s\alpha}$ at the nucleotide level.

The Northern blots in fig.1 indicate that both uninduced and induced HL60 cells contain mRNA transcripts specific for $G_{s\alpha}$ (1.9 kb) and for the $G_{i\alpha\text{-}2}$ (2.0 kb) and $G_{i\alpha\text{-}3}$ (2.6 kb) subtypes, but not for $G_{o\alpha}$ or for the $G_{i\alpha\text{-}1}$ subtype. The sizes of these transcripts are similar to those previously reported for $G_{s\alpha}$ [3], $G_{i\alpha\text{-}2}$ [7], and $G_{i\alpha\text{-}3}$ [9]. In all cases where transcript is detected in the uninduced cells the abundance of the specific G_α transcript increases with maturation, but that of c-myc decreases in accord with previous observations [20].

To determine whether evidence of multiple $G_{i\alpha}$ subtypes in HL60 cells could be detected at the

Table 1

Divergent region of G_α cDNAs[a]

		Amino acid nos	Corresponding nucleotide sequence
Human	$G_{i\alpha-1}$	108-123	TTTGTGCTAGCTGGAGCTGCTAAGAA---GGCTTTATGACTGCAGAACTT
Human	$G_{i\alpha-2}$	108-124	····CA··GT·CT·CA·C··C··G··GCAA···G·GC·CC···AT··C··G
Rat	$G_{i\alpha-2}$	108-124	··C·CA··GT·CT·T·····C··G··GCAA···A·GC·TC·G·A···C··G
Rat	$G_{i\alpha-3}$	108-123	A·A·GTTAT·TATT·TT·····G···GGA·T·A·G·CTT·A·A------A
Rat	$G_{o\alpha}$	108-124	·G··ACG·G·TGA·TCGCATG··G··CACT·AACCAT·CT·····G
Human	$G_{i\alpha-1}$	124-139	GCTGGAGTTATAAAGAGATTGTGGAAAGATAGTGGTGTACAAGCCTGT
Human	$G_{i\alpha-2}$	125-140	T·C··C··C··CCG···GC·C···GCT··C·A·····G··G·····C
Rat	$G_{i\alpha-2}$	125-140	T·G··C··C··CCG···GC·C···GCT··CCA·····G·····C
Rat	$G_{i\alpha-3}$	124-139	··A··C··G··T··AC·T··A···CG···G·C··G··G··G··A··C
Rat	$G_{i\alpha-}$	125-140	CT·TCT·CC··G·T·C··C·C···GGC··CTCG··GA·C··G·AG··C

Predicted amino acid sequences[b]

		Amino acid nos	Amino acid sequence	Amino acid nos.	Amino acid sequence
Bovine	$T_{d\alpha}$	341-350	KENLKDCGLF (AS6)	155-164	···LVTPG·V
Human	$G_{i\alpha-1}$	345-354	·N·····	159-168	·D····PN··
Human	$G_{i\alpha-2}$	346-355	·N·····	160-169	LERIAQSDYI (LE3)
Human	$G_{i\alpha-3}$	345-354	·N···E···Y	159-168	·D··S··N··
Rat	$G_{o\alpha}$	346-355	AN··RG···Y	160-169	·D··GAA··Q

[a] Nucleotide sequence alignment of predicted amino acid residues 108–140, in frame for the indicated G protein α-subunit cDNAs. The dashes indicate a single amino acid gap in the coding region placed to allow maximal homology. The dots indicate positions that are identical to the comparison sequence of human $G_{i\alpha-1}$. The synthetic oligonucleotide probes used in fig.1 are the inverse complement of the underlined regions

[b] Comparative alignment of amino acid residues from 2 regions of the predicted primary structure of the indicated G protein α-subunit cDNAs. Rabbit antisera indicated in the parentheses were raised against synthetic decapeptides corresponding to the underlined sequences. The dots indicate amino acids that are identical to the comparison sequences (underlined) in the corresponding region of different G protein α-subunits

protein level, we performed immunoblots of plasma membrane proteins with the antisera AS6 and LE3 described in section 2 (fig.2). AS6 recognizes a single broad band spanning the 40–41 kDa range in plasma membranes from both uninduced HL60 cells and dibutyryl cAMP-induced HL60 cells. LE3 also recognizes a single broad band in both uninduced and induced HL60 cells, but reactivity is limited more narrowly to about 40 kDa. This difference in AS6 and LE3 is apparent even when the amount of membrane loaded on the gel or the dilution of either antibody is varied (not shown). The intensity of Western blot labelling by both AS6 and LE3 per μg of HL60 membrane protein increases with induction, yet the distinction between the staining pattern of these antibodies is maintained.

In separate experiments DMSO was used as the inducing agent with HL60 cells in order to examine the effects of an induction regimen other than dibutyryl cAMP. AS6 and LE3 showed the same pattern of reactivity in HL60 cells induced with DMSO as that shown wity dibutyryl cAMP induction in fig.2. In addition there was a clear increase in the amount of immunoreactive protein over 5 days of induction with DMSO (shown for LE3 in fig.3).

These data indicate that mRNA for two subtypes of the $G_{i\alpha}$ genus of G proteins identified to date by cDNA cloning, $G_{i\alpha-2}$ and $G_{i\alpha-3}$, are

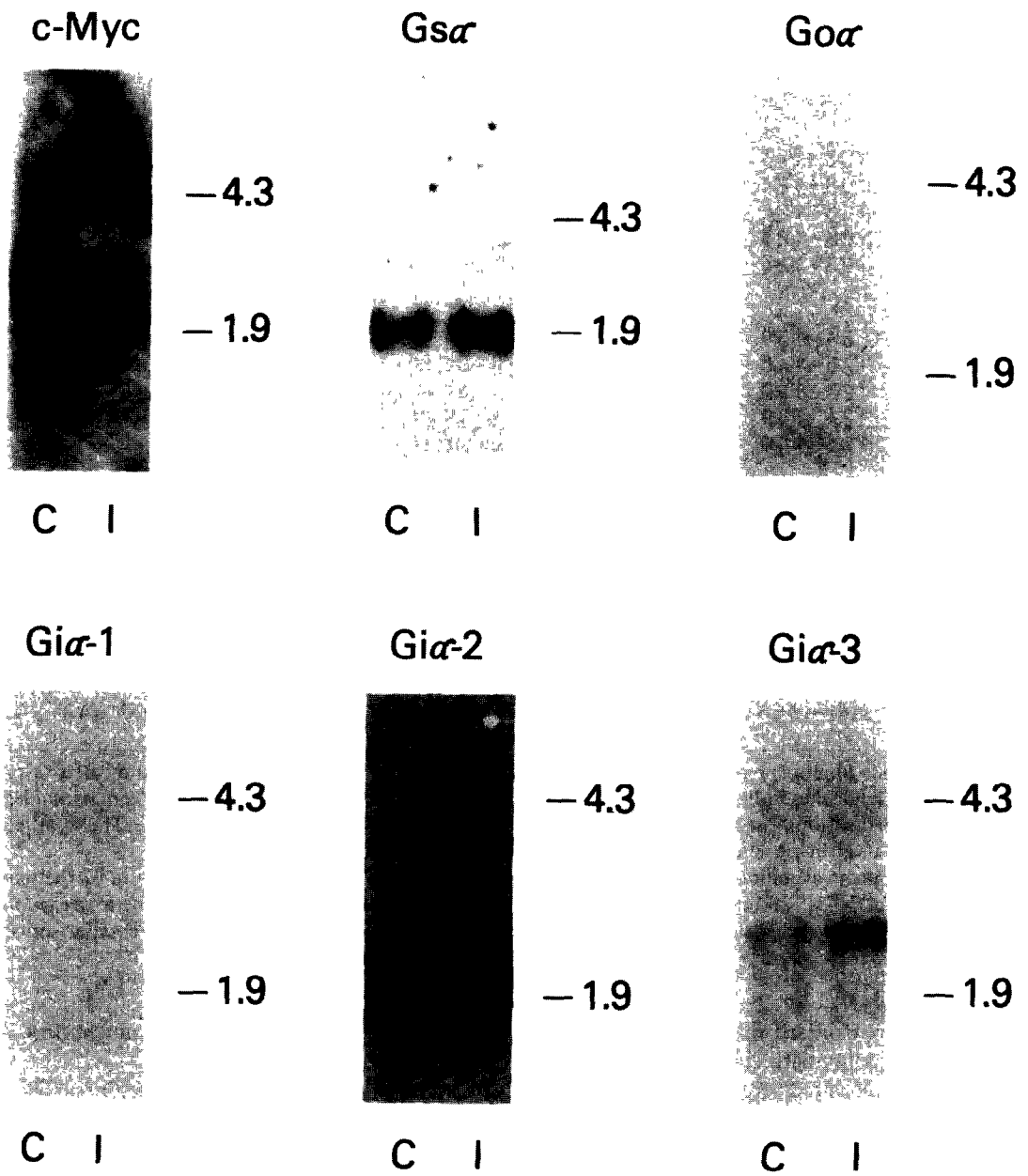

Fig.1. Northern blot analysis. For each panel, poly(A)$^+$ enriched RNA (5 μg/lane) from control (lane C) and induced (lane I) HL60 cells was subjected to electrophoresis, blotted, and hybridized with the probes indicated at the top of each panel (see section 2 and table 1). The dash marks on the right of each panel indicate the chain lengths of the RNA molecular mass markers in kilobases.

transcribed in HL60 cells. At the protein level there is also evidence for more than one $G_{i\alpha}$ subtype as indicated by the immunoblots generated with AS6 and LE3 antisera. Based on the derivation of the LE3 antiserum, we suggest that one of the $G_{i\alpha}$ peptides present is encoded by $G_{i\alpha\text{-}2}$ and is 40 kDa in molecular mass. AS6 is known to bind strongly to both transducin-α and the predominant brain form

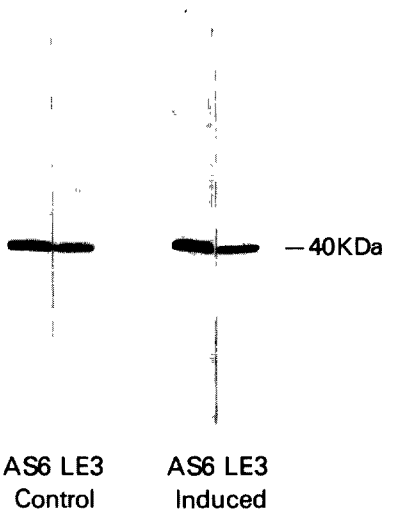

Fig.2. Western blot analysis. For each panel, plasma membrane proteins (100 μg/lane) from control and dibutyryl cAMP-induced HL60 cells were subjected to SDS-polyacrylamide (10%) gel electrophoresis, blotted, and then incubated with rabbit antisera AS6 (1:250 dilution), or antisera LE3 (1:100 dilution) followed by goat anti-rabbit IgG-peroxidase as described in section 2. The molecular mass of the lower portion of the immunoreactive peroxidase positive band is indicated at the right of the panels.

of $G_{i\alpha}$, indicating that it is likely to bind to all of the $G_{i\alpha}$ subtypes, though this remains to be proven. In HL60 cell membranes there is AS6 immunoreactive material with a molecular mass slightly higher than that recognized by LE3. While the identity of this material may be a protein encoded by $G_{i\alpha\text{-}3}$, it could be another, as yet unidentified, $G_{i\alpha}$ subtype.

Although more than one $G_{i\alpha}$ subtype has been identified in Northern blots of brain mRNA, this material is a tissue with multiple cell types. The use of a human cloned tissue culture line, HL60, demonstrates that both $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$ transcripts are being produced in the same cell type. Myeloid cells contain both phospholipase C-coupled and adenylate cyclase-coupled receptor activation systems, and both systems are linked to $G_{i\alpha}$ functions based on pertussis toxin sensitivity [21–23]. It is possible to speculate that at least two $G_{i\alpha}$ subtypes with different effector system specificities are required. Preliminary studies suggest that the formyl peptide chemotactic receptor, a phospholipase

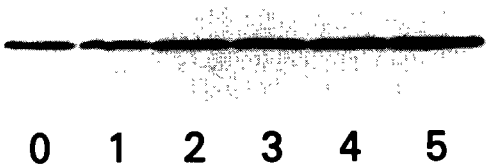

Fig.3. Western blot analysis. Plasma membrane proteins (100 μg/lane) from uninduced (lane 0), and day 1 to 5 (lanes 1–5) DMSO induced HL60 cells were electrophoresed and analyzed with antiserum LE3 as described for fig.2. As in fig.2 the LE3 immunoreactive band is 40 kDa.

C-coupled receptor, interacts with a 40 kDa pertussis toxin-sensitive substrate [24]. Our studies would suggest that the 40 kDa $G_{i\alpha}$ in HL60 cells is $G_{i\alpha\text{-}2}$, though our data cannot exclude the possibility that there is more than one 40 kDa $G_{i\alpha}$ subtype in these cells.

It is of note that both $G_{i\alpha\text{-}1}$ and $G_{o\alpha}$ transcripts were not detected in HL60 cells. $G_{o\alpha}$ transcripts have been detected in brain and heart (review [2]). The significance of its absence from HL60 cells is not known, but indicates that tissue specificity exists for G_o expression. The absence of $G_{i\alpha\text{-}1}$ transcript from HL60 cells supports the concept of tissue specificity for $G_{i\alpha}$ subtypes as well.

ACKNOWLEDGEMENTS

We wish to thank Dr C. Unson of Rockefeller University, New York and Dr G. Milligan of Glasgow University, Scotland for their assistance in the production of the synthetic peptides and peptide antisera, and we thank Dr D. Jones and Dr R. Reed of Johns Hopkins University, Baltimore for unpublished information on cDNA sequences and mRNA size for rat $G_{i\alpha}$ subunits.

REFERENCES

[1] Gilman, A.G. (1984) Cell 36, 577–579.
[2] Spiegel, A. (1987) Mol. Cell. Endocrinol. 49, 1–16.
[3] Bray, P., Carter, A., Simons, C., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. and Nirenberg, M. (1986) Proc. Natl. Acad. Sci. USA 83, 8893–8897.
[4] Bray, P., Carter, A., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. and Nirenberg, M. (1987) Proc. Natl. Acad. Sci. USA 84, in press.

[5] Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K. and Kaziro, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 3776–3780.
[6] Lerea, C.L., Somers, D.E., Hurley, J.B., Klock, I.B. and Bunt-Milam, A.H. (1986) Science 234, 77–80.
[7] Didsbury, J.R., Ho, Y. and Snyderman, R. (1987) FEBS Lett. 211, 160–164.
[8] Suki, W.N., Abramowitz, J., Codina, J. and Birnbaumer, L. (1987) FEBS Lett., in press.
[9] Didsbury, J.R. and Snyderman, R. (1987) Clin. Res. 35, 656A.
[10] Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Hirose, T., Inayama, S. and Numa, S. (1986) FEBS Lett. 195, 220–224.
[11] Sullivan, K.A., Liao, Y., Alborzi, A., Beiderman, B., Chang, F., Masters, S.B., Levinson, A.D. and Bourne, H.R. (1986) Proc. Natl. Acad. Sci. USA 83, 6687–6691.
[12] Gierschik, P., Milligan, G., Pines, M., Codina, J., Klee, W. and Spiegel, A. (1986) Proc. Natl. Acad. Sci. USA 83, 2258–2262.
[13] Gierschik, P., Falloon, J., Milligan, G., Pines, M., Gallin, J.I. and Spiegel, A. (1986) J. Biol. Chem. 261, 8058–8062.
[14] Falloon, J., Malech, H.L., Milligan, G., Unson, C., Kahn, R., Goldsmith, P. and Spiegel, A. (1986) FEBS Lett. 209, 352–356.
[15] Oinuma, M., Katada, T. and Ui, M. (1987) J. Biol. Chem. 262, 8347–8353.
[16] Goldsmith, P., Gierschik, P., Milligan, G., Unson, C., Vinitsky, R., Malech, H. and Spiegel, A. (1987) J. Biol. Chem., in press.
[17] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[18] Brann, M., Collins, R. and Spiegel, A. (1987) submitted.
[19] Maniatis, T., Fritsch, E.F. and Sambrook, M. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[20] Slungaard, A., Confer, D.L. and Schubach, W.H. (1987) J. Clin. Invest. 79, 1542–1547.
[21] Smith, C.D., Lane, B.D., Kusaka, I., Verghese, M.W. and Snyderman, R. (1985) J. Biol. Chem. 260, 5875–5878.
[22] Smith, C.D., Cox, C.C. and Snyderman, R. (1986) Science 232, 97–100.
[23] Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M. and Takai, Y. (1986) J. Biol. Chem. 261, 11558–11562.
[24] Polakis, P. and Snyderman, R. (1987) Clin. Res. 35, 487A.