Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9253–9256, October 1991
Medical Sciences

Wegener autoantigen and myeloblastin are encoded by a single mRNA

(proteinase 3/serine protease/leukemia)

CATHERINE LABBAYE, PHILIPPE MUSETTE, AND YVON E. CAYRE*

Department of Physiology and Cellular Biophysics and Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032

Communicated by Lloyd J. Old, July 25, 1991 (received for review February 11, 1991)

ABSTRACT **Myeloblastin is a serine protease that has been identified in the human leukemia cell line HL-60. Down-regulation of this protease can inhibit proliferation and induce differentiation of promyelocyte-like human leukemic cells. Proteinase 3, a serine protease of human neutrophils, has been identified as the Wegener autoantigen. A high level of homology between myeloblastin and proteinase 3 has suggested that they may be a single serine protease. We have recently completed the 5′-terminal nucleotide sequence of proteinase 3 and shown that its mRNA was also expressed in HL-60 cells and in cells from patients with acute myeloid leukemia. Here we demonstrate that myeloblastin and proteinase 3 are encoded by a single mRNA.**

Myeloblastin (MBN) is a serine protease involved in the control of growth and differentiation of human leukemic cells (1). This protease is expressed in the human acute promyelocytic leukemia cell line HL-60 where it is down-regulated during induced differentiation (1). Another serine protease, proteinase 3 (PR-3) (2), originally described in human neutrophils, has been identified as the autoantigen of Wegener granulomatosis, a systemic vasculitis complicated by myelomonocyte proliferation (3). The revised incomplete PR-3 sequence (4) suggested that this serine protease is identical to azurophil granule protein 7 (4, 5), a serine protease of azurophil granules. The sequence of PR-3 is also identical to the sequence of the neutrophil protease p29 (4, 6, 7). Down-regulation of MBN in immature leukemic cells has been shown to inhibit proliferation and induce differentiation of immature human leukemic cells (1). By considering the high level of homology between the partial revised sequence of PR-3 and MBN (1, 4, 7), it is tempting to speculate that, if MBN and PR-3 were identical, then the Wegener granulomatosis could be an autoimmune disorder resulting in the inhibition of MBN activity and enhanced granulocytic differentiation. This type of hypothesis has been proposed by other investigators (4). However, despite recent studies suggesting that the Wegener autoantigen is similar to MBN (4, 7), the identity of the two proteins has remained controversial. This was due to the fact that only partial nucleotide sequences were available (4, 8) and that the predicted amino acid sequence of PR-3 differed from the sequence of the purified protein or its fragments (8). Campanelli *et al.* (8) have suggested that the sequence discrepancies may arise from ambiguities in amino acid sequencing, that PR-3 may be polymorphic, that an additional gene(s) is linked in tandem to PR-3, or that PR-3 and MBN are the result of alternative splicing. Using HL-60 cells, we have achieved anchored-PCR amplification (9), cloning, and sequencing of the missing 5′-terminal nucleotide sequence of PR-3 (10). This sequence, extending 5′ upstream of the MBN sequence (1), contained a putative initiation codon surrounded by a ribosomal binding site consensus sequence (CCC ACC ATG GCT) (10, 11) that was absent from the incomplete 5′ sequence of PR-3 (8). The predicted N-terminal sequence of PR-3 suggested that this sequence may act as a signal peptide (10, 12). We have shown (10) that PR-3 mRNA is expressed in HL-60 cells and in cells from patients with acute leukemia of myeloid origin. The goal of the present study has been to clarify whether MBN and PR-3 are a unique protease and to eliminate the possibility that MBN and PR-3 are the result of an alternative splicing encoding for two enzymes (13). Here we demonstrate that a single mRNA encodes for the Wegener autoantigen and MBN.†

MATERIALS AND METHODS

Cells and Culture Conditions. Cells were grown in RPMI 1640 medium with 10% (vol/vol) fetal bovine serum and 2 mM L-glutamine. 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] was dissolved in 95% ethanol and used at a final concentration of 0.6 μM. The human cell lines used were the myeloblastic KG-1, the myelomonoblastic ML-3, the monocytoid U937, and K562 cells, which are early myeloid blasts and/or erythroblasts (for review, see ref. 14). 1F10, 1F10-PMA, and 1F10-D3 cells were as described (15).

RNA Isolation and Northern Blot Analysis. Total RNA was prepared as described (16). Poly(A)$^+$ mRNA was isolated on an oligo(dT)-cellulose column (Collaborative Research). For Northern blots, RNA preparations were denatured and processed as described (17). All RNA blots contained 10 μg of total RNA per lane or 2 μg of poly(A)$^+$ mRNA. The filters were hybridized to a 5′-end-labeled oligodeoxynucleotide probe (2×10^6 cpm/ml) corresponding to positions 1–19 of the PR-3 5′-terminal sequence as described (10) or to a MBN nick-translated cDNA probe corresponding to positions 260–630 of the MBN sequence (1) (Fig. 1). Ethidium bromide-stained 28S and 18S rRNAs were used as assessment of RNA quantities in gel prior to transfer (1). When indicated, a β_2-microglobulin nick-translated cDNA probe (18) was used for assessment of RNA quantities in each lane.

S1 Nuclease Assay with Double-Stranded DNA Probe. A 235-bp PCR-amplified cDNA was obtained by a standard PCR-amplification protocol (19), using HL-60 mRNA and two primers, the 19-mer sense 5′-ATGGCTCACCGGCCCCCA-3′ (10) and the 18-mer antisense 5′-CTGGGGTATGTCCCGCAG-3′ (1) sequences from positions 217 to 234 in Fig. 1. This double-stranded 235-bp PCR fragment was sequenced (Applied Biosystems) (Fig. 1) and used for mapping mRNAs from HL-60 cells with S1 nuclease as described

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MBN, myeloblastin; PR-3, proteinase 3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M75154).

```
-48
 GAT TGG CTA TAA GAG GAG CTT GAT CGT GGG TGC ACC CTG GAC CCC ACC

 1                                  18
ATG GCT CAC CGG CCC CCC AGC CCT GCC CTG GCG TCC GTG CTG CTG GCC

TTG CTG CTG AGC GGT GCT GCC CGA GCT GCG GAG ATC GTG GGC GGG CAC

                                        124
GAG GCG CAG CCA CAC TCC CGG CCC TAC ATG GCC TCC CTG CAG ATG CGG

       150
GGG AAC CCG GGC AGC CAC TTC TGC GGA GGC ACC TTG ATC CAC CCC AGC

                                   217                  234
TTC GTG CTG ACG GCC GCG CAC TGC CTG CGG GAC ATA CCC CAG CGC CTG

GTG AAC GTG GTG CTC GGA GCC CAC AAC GTG CGG ACG CAG GAG CCC ACC

CAG CAG CAC TTC TCG GTG GCT CAG GTG TTT CTG AAC AAC TAC GAC GCG

                                                        382
GAG AAC AAA CTG AAC GAC ATT CTC CTC ATC CAG CTG AGC AGC CCA GCC

                               753
AAC CTC ..... GTG GAG GCC AAG GGC CGC CCC TGA
```

FIG. 1. Nucleotide sequence of PR-3/MBN indicating the position of the various primers and probes used in our experiments. The positions of primers and of the MBN probe are indicated with underlined bold letters and numbered above the sequence. The box from positions 1 to 234 indicates the 235-base-pair (bp) PCR fragment sequence. The ATG at position 124 corresponds to position 1 of the MBN sequence (1). The sequence from positions 382 to 753 represents the MBN cDNA probe corresponding to positions 260 to 630 of the MBN sequence. Dots correspond to positions 270 to 625 of the MBN sequence.

(20). After S1 nuclease assay, the samples were electrophoresed through an alkaline agarose gel and blotted for hybridization with the nick-translated 235-bp PCR-amplified cDNA probe.

Southern Blot Analysis. The 235-bp PCR-amplified and the MBN (1) cDNA probes were nick-translated and hybridized to Southern blots of HL-60 genomic DNA prepared as described (21) and digested with *Pst* I, *Pvu* II, and *Hin*dIII restriction enzymes. Samples were electrophoresed through a 0.8% agarose gel and blotted onto a Nytran membrane (22).

Primer-Extension Assay. Primer extension was conducted as described (20) by using 3 μg of poly(A)$^+$ mRNA and two antisense primers, a 19-mer 5'-end oligodeoxynucleotide from the PR-3 sequence (10), 5'-TGGGGGGCCGGTGAGC-CAT-3', and a 27-mer antisense oligodeoxynucleotide, 5'-GTTCCCCCGCATCTGCAGGGAGGCCAT-3', from positions 1 to 26 of the initial MBN sequence (1) corresponding to positions 124–150 of the PR-3/MBN sequence (Fig. 1). Reverse transcription was conducted using avian myeloblastosis virus reverse transcriptase (Molecular Genetic Resources, Tampa, FL), and the samples were electrophoresed through a denaturing 8% polyacrylamide gel. Yeast tRNA (10 μg) was used as negative control.

RESULTS

PR3 mRNA Expression and Regulation in Various Human Leukemic Cell Lines. We have analyzed the expression and the regulation of PR-3 mRNA in HL-60 and 1F10 cells. A synthetic oligodeoxynucleotide probe corresponding to the first 19 nucleotides downstream from the putative initiation codon of PR-3 (10) was hybridized to Northern blots of RNAs from HL-60 cells. As shown in Fig. 2, these cells expressed an ≈1.3-kilobase PR-3 mRNA that was down-regulated after treatment by 1,25-$(OH)_2D_3$. By using the same PR-oligodeoxynucleotide probe, PR-3 mRNA was also found in 1F10 and 1F10-D3 cells that have been shown to contain MBN (1); as with MBN (1), PR-3 mRNA was not detected in 1F10-PMA cells (Fig. 3). PR-3 mRNA was present in both U937 and ML-3 human leukemic cells (Fig. 4); however, it was not detected in KG-1 and K562, two cell lines found to be negative for MBN mRNA (1). Thus our results show that expression and regulation of PR-3 mRNA and MBN mRNA in various leukemic cell lines were similar (1).

S1 Nuclease Mapping of HL-60 RNAs. We have used a PCR-amplification protocol (19) to obtain a 235-bp cDNA starting at position 1 of the PR-3 sequence (10) and encompassing part of the sequence for MBN from positions 1 to 112 (1). After verification of its specificity by Northern blot hybridization to HL-60 RNA (Fig. 5*A*), this double-stranded 235-bp PCR fragment was sequenced (Fig. 1) and used for mapping HL-60 total cellular RNAs with S1 nuclease. As shown in Fig. 5*B*, S1 nuclease generated a single protected RNA fragment of 235 bp. The same experiment was conducted with the same results using HL-60 poly(A)$^+$ RNA (data not shown). This result demonstrated the existence of a single PR-3/MBN mRNA in HL-60 cells.

Identification of PR-3/MBN mRNA Cap Site. Primer extension was conducted to localize the PR-3/MBN cap site. By using a primer corresponding to positions 1–26 of the MBN sequence (1), a cap site was found at approximately position −140 upstream of the primer (Fig. 6*A*). This position corresponds to approximately position −17 upstream of the putative PR-3 initiation codon (10). By using another primer

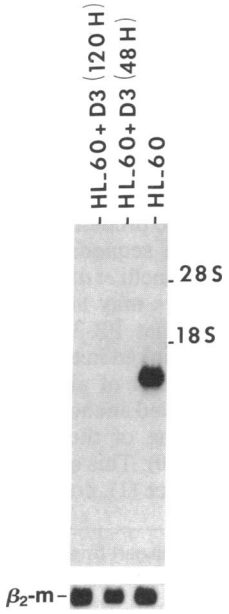

FIG. 2. (*Upper*) Autoradiogram of PR-3 mRNA in HL-60 cells. Poly(A)$^+$ RNA was prepared from HL-60 cells either untreated or treated with 1,25-$(OH)_2D_3$ for 120 or 48 hr. (*Lower*) Same blot hybridized with a β_2-microglobulin (β_2-m) nick-translated cDNA probe for assessment of RNA quantities in each lane. The 28S and 18S rRNAs are indicated as size markers.

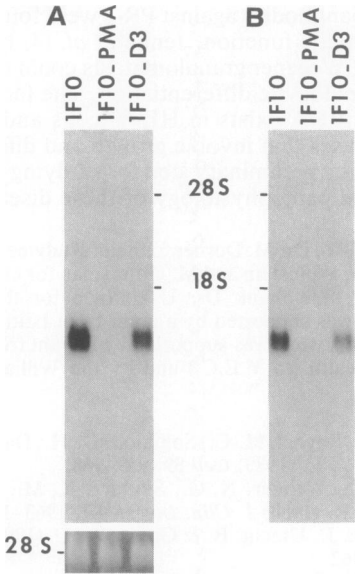

FIG. 3. Autoradiograms of 1F10-cell total RNA with MBN and PR-3 probes. (*A*) Autoradiogram of 1F10 cell RNA with the MBN cDNA probe. (*B*) Same blot after washing and hybridization with the PR-3 oligodeoxynucleotide probe. The 28S and 18S rRNAs are indicated as size markers. In *A*, an ethidium bromide-stained 28S rRNA from the gel prior to transfer is shown at the bottom to assess RNA quantities in each lane.

from positions 1 to 19 of the PR-3 sequence (10), a cap site was detected at approximately position −20 upstream of the putative PR-3 initiation codon (Fig. 6*B*). These results suggest that the cap site of PR-3/MBN is located around positions −17 to −20 of the putative initiation codon (10).

PR-3/MBN mRNA Is Encoded by a Unique Gene. To determine whether PR-3/MBN is encoded by a unique gene, we used both the MBN cDNA probe (1) (corresponding to positions 260–630 of the MBN sequence) and the above-described 235-bp PCR-amplified cDNA probe that corresponds to positions 1–234 of the PR-3 5′-terminal nucleotide sequence, as indicated above, to hybridize to Southern blots of genomic HL-60 DNA. Because the two probes did not

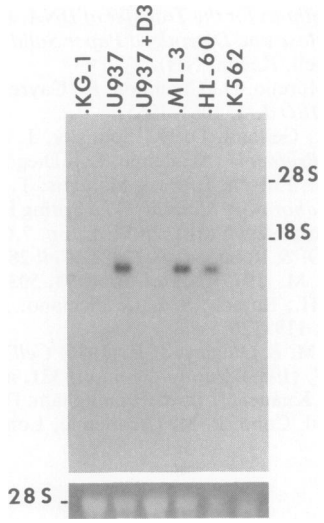

FIG. 4. (*Upper*) Autoradiogram of PR-3 mRNA in various human cell lines. RNAs were from the following cell lines: K562, HL-60, ML-3, U937 before and after treatment with 1,25-$(OH)_2D_3$, and KG1. (*Lower*) Ethidium bromide-stained 28S rRNA to assess RNA quantities in each lane. The 28S and 18S rRNAs are indicated as size markers.

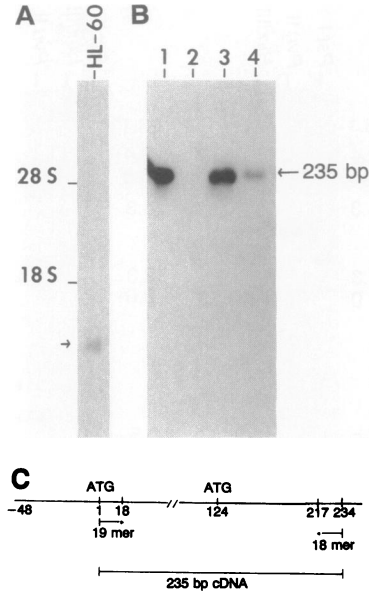

FIG. 5. S1 nuclease mapping of HL-60 RNAs. (*A*) Control hybridization of the 235-bp PCR-amplified cDNA fragment to HL-60 total RNA. Arrow indicates the position of PR-3 mRNA. The 28S and 18S rRNAs are indicated as size markers. (*B*) S1 nuclease mapping of HL-60 RNA. Lanes: 1, 235-bp PCR-amplified cDNA fragment; 2, control digestion of the 235-bp fragments by S1 nuclease; 3 and 4, S1 nuclease-protected fragment from two annealing conditions at 42°C and 60°C, respectively. (*C*) Schematic diagram showing the sites of the primers used for generating the 235-bp cDNA fragment.

encompass the same sequences of the gene, their pattern of hybridization was different. However, for each probe, a single band was detected after *Pst* I and *Hin*dIII digestion (Fig. 7). Each probe also hybridized to a major band after *Pvu* II digestion. Therefore, these data are consistent with a unique gene encoding PR-3/MBN mRNA.

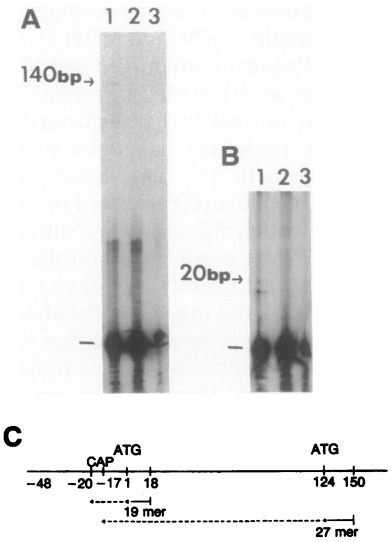

FIG. 6. Primer-extension assay of HL-60 poly(A)$^+$ RNA. (*A*) Primer extension using the 27-mer oligodeoxynucleotide MBN primer. Lanes: 1, HL-60 RNA; 2, yeast tRNA control; 3, primer alone. Arrow indicates the position of the cap site at ≈140 bp upstream of the primer. (*B*) Primer extension using the 19-mer 5′-end PR-3 oligodeoxynucleotide primer. Lanes: 1–3, as indicated above. Arrow indicates the position of the cap site at ≈20 bp upstream of the primer. In *A* and *B*, the tick marks indicate the top of each primer. (*C*) Schematic diagram showing the sites of the primers and their extension.

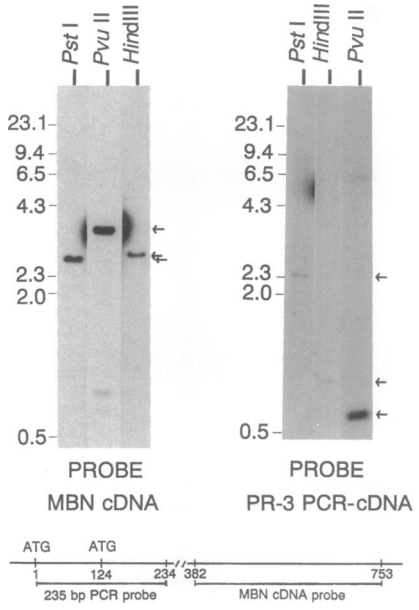

FIG. 7. (*Upper*) Southern blot analysis of HL-60 cells genomic DNA. Restriction enzymes used are indicated above each lane. The two probes used for hybridization are indicated below the autoradiograms. Size markers are as indicated in kb (kilobases). (*Lower*) Schematic diagram showing the positions of the probes.

DISCUSSION

We have demonstrated that MBN, initially identified in human leukemic cells (1), and the Wegener autoantigen are encoded by a single mRNA corresponding to a single gene. Our 5'-terminal PR-3 sequence (10) contained a putative initiation codon surrounded by a ribosomal binding site consensus sequence (11). This codon preceded a sequence that is likely to encode a complete signal peptide, a classical feature of serine proteases (12). Though only partial PR-3 amino acid (5, 7) and cDNA sequences (4, 8) were available, several authors have observed sequence similarities for PR-3, azurophil granule protein 7, p29, and MBN (4, 7, 8) and have suggested that the Wegener autoantigen and MBN are either highly homologous or identical (4, 7). Protease inhibitors can affect the growth of normal and transformed cells; subsequently, a role for proteases has been proposed in the regulation of cell growth (23, 24). Serine proteases are involved in a series of cell-surface-related proteolytic events that are required for cell proliferation and differentiation (for review, see ref. 25). These events are controlled by inhibitors of serine proteases (26). Recently, we have identified MBN as a serine protease involved in the control of cell growth and differentiation of human promyelocyte-like leukemic cells (1). The Wegener granulomatosis is complicated by myelomonocytic proliferation. Though the cause and pathogenesis of the Wegener granulomatosis remain unknown, circulating autoantibodies directed against a unique antigen in cytoplasmic granules of human neutrophils and monocytes have been regarded as a possible pathogenetic factor in this disease (4). Since down-regulation of MBN resulted in differentiation and growth arrest of human leukemic cells (1) and most autoantibodies against PR-3 were found to interfere with its enzymatic function, Jenne *et al.* (4) have proposed that fulminant Wegener granulomatosis could be the result of enhanced granulocytic differentiation. The fact that a single PR-3/MBN mRNA exists in HL-60 cells and is associated with two diseases that involve growth and differentiation of myeloid cells is a preliminary step for studying the role of this protease in the pathophysiology of these diseases.

We are grateful to Dr. M. Dorner for helpful advice and discussions and to Drs. I. B. Weinstein and M. Gottesman for critical reading of the manuscript. We thank Dr. Uskokovic for the gift of 1,25-$(OH)_2D_3$. C.L. was supported by a grant from Istituto Superiore di Sanita, Italy. This work was supported by a grant from the National Institutes of Health (to Y.E.C.) and by the William J. Matheson Foundation.

1. Bories, D., Raynal, M.-C., Solomon, D. H., Darzynkiewicz, Z. & Cayre, Y. E. (1989) *Cell* **59**, 959–968.
2. Kao, R. C., Wehner, N. G., Skubitz, K. M., Gray, B. H. & Hoidal, J. R. (1988) *J. Clin. Invest.* **82**, 1963–1968.
3. Lüdemann, J., Utecht, B. & Gross, W. L. (1990) *J. Exp. Med.* **171**, 357–362.
4. Jenne, D. E., Tschopp, J., Lüdemann, J., Utecht, B. & Gross, W. L. (1990) *Nature (London)* **346**, 520.
5. Wilde, C. G., Snable, J. L., Griffith, J. E. & Scott, R. W. (1990) *J. Biol. Chem.* **265**, 2038–2041.
6. Niles, J. L., McCluskey, R. T., Ahmad, M. F. & Arnaout, N. A. (1989) *Blood* **74**, 1888–1893.
7. Gupta, S. K., Niles, J. L., McCluskey, R. T. & Arnaout, M. A. (1990) *Blood* **76**, 2162.
8. Campanelli, D., Melchior, M., Fu, Y., Nakata, M., Shuman, M., Nathan, C. & Gabay, J. E. (1990) *J. Exp. Med.* **172**, 1709–1715.
9. Loh, E. Y., Elliott, J. F., Cwirla, S., Lanier, L. L. & Davis, M. M. (1989) *Science* **243**, 217–220.
10. Musette, P., Casanova, J. L., Labbaye, C., Dorner, M. H., Kourilsky, P. & Cayre, Y. E. (1991) *Blood* **77**, 1398–1399.
11. Kozak, M. (1984) *Nature (London)* **308**, 241–246.
12. von Heijne, G. (1985) *J. Mol. Biol.* **184**, 99–105.
13. Rao, N. V., Wehner, N. G., Marshall, B. C., Gray, W. R., Gray, B. H. & Hoidal, J. R. (1991) *J. Biol. Chem.* **266**, 9540–9548.
14. Koeffler, M. P. (1983) *Blood* **62**, 709–721.
15. Cayre, Y., Raynal, M.-C., Darzynkiewicz, Z. & Dorner, M. H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7619–7623.
16. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
17. Wahl, G. M., Ong, E., Meinkoth, J., Franco, R. & Barinaga, M. (1981) *Methods for the Transfer of DNA, RNA and Proteins to Nitrocellulose and Diazotized Paper Solid Supports* (Schleicher & Schuell, Keene, NH).
18. Daniel, F., Morello, D., Chambon, P., Cayre, Y. & Kourilsky, P. (1983) *EMBO J.* **2**, 1061–1065.
19. Innis, M. A., Gelfand, D. H., Sminsky, J. J. & White, T. J. (1990) *PCR Protocols* (Academic, San Diego), pp. 3–28.
20. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, New York), 2nd Ed., pp. 7.62–7.65.
21. Heidmann, O. & Rougeon, F. (1982) *Cell* **28**, 507–513.
22. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
23. Gibson, W. H., Burack, S. L. & Picciano, A. (1984) *J. Cell. Physiol.* **119**, 119–126.
24. Sullivan, L. M. & Quigley, J. P. (1986) *Cell* **45**, 905–915.
25. Carrel, R. W. (1988) *Nature (London)* **331**, 478–479.
26. Baker, J. B., Knauer, J. D. & Cunningham, D. D. (1986) in *The Receptors*, ed. Conn, P. M. (Academic, London), Vol. 3, pp. 153–172.