Immunoglobulin diversity: analysis of the germ-line V_H gene repertoire of the murine anti-GAT response

Claudine Schiff, Michèle Milili and Michel Fougereau

Centre d'Immunologie INSERM-CNRS de Marseille-Luminy Case 906, 13288 Marseille Cedex 9, France

Received 29 March 1983; Revised and Accepted 27 May 1983

SUMMARY

A cDNA clone was constructed from a mRNA encoding an anti-GAT (Glu^{60} Ala^{30A} Tyr^{10}) BALB/c monoclonal antibody heavy chain. Its sequence, covering codons -5 to 162 and therefore encompassing the complete V-D-J region, was determined. Surprisingly, the sequence of the V_H gene-encoded region was almost identical with that of the BALB/c V_H anti-NP (4-hydroxy-3-nitrophenyl) acetyl V_H region, suggesting that the same V_H germ-line might be used to encode two heavy chains contributing to antibodies of discrete specificities. A specific V_H probe was derived and annealed to Eco RI and Bgl II restriction fragments of liver (unrearranged) DNA extracted from the BALB/c, DBA/2 and C57BL/6 mouse strains that differ in their H chain allotypes. Under stringent conditions, only a few bands were identified by Southern blotting. The different patterns observed suggest that the V_H anti-GAT repertoire differs between these strains eventhough their various anti-GAT antibodies express the same public idiotypic specificities.

INTRODUCTION

Immunoglobulins are symmetrical molecules composed of heavy and light chains that both contribute to the structure of the antibody combining site through interactions of their variable (V_H and V_L) domains (1). Immunoglobulin germ-line genes need to be rearranged before expression : two genes are used for the V_L domain (V_L and J_L), three genes for the V_H domain (V_H, D_H, and J_H), in addition to a leader (L) segment gene located 5' to the V_H and V_L genes (2-6). Furthermore, somatic events affecting both chains (7, 8) amplify antibody diversity. The relative proportion of the germ-line and somatic origin of this type of diversity is still unknown. Determination of the overall number of V germ-line genes is required. Various approaches have been used, such as Southern blots of genomic DNA restriction fragments to a series of cDNA V probes (9, 10) and the isolation of V germ-line genes from mouse genomic libraries (8, 11, 12), especially in connection with the expression of public idiotypes considered to be germ-line markers of both of the V_H and V_L domains.

The random synthetic terpolymer poly Glu^{60} Ala^{30} Tyr^{10} (GAT), elicits in various strains of mice anti-GAT antibodies that express the public idiotypic specificities termed CGAT (13) or pGAT (14). These specificities require both the H and L chains of the idiotype in order to be expressed (15). Analyses of the NH_2-terminal amino acid sequences (residues 1-43) of 16 H-L pairs isolated from monoclonal anti-GAT antibodies (16) suggest that the V_H and the V_L regions of the pGAT idiotype are each encoded by a very small number of germ-line genes. More recently, we reported (17) on the complete sequence of four BALB/c V_H regions, using mRNA nucleotide sequencing, as determined by a modification (18) of the chain terminator method (19). Two anti-GAT heavy chains (G5 Bb 2.2. and G8 Ca 1.7), which were derived from monoclonal BALB/c antibodies expressing identical public and private idiotypic specificities, had completely identical sequences, encompassing the entire V-D-J region. This sequence could therefore be considered rather close to that of the V_H germ-line gene, and thus the G8 Ca 1.7 hybridoma provided a good material to derive a V_H-"GAT" cDNA clone. In this paper, we report the isolation and nucleotide sequence of such a clone, that was used to identify the number and size of cross-hybridizing germ-line restriction DNA fragments prepared from three different strains of mice.

MATERIALS AND METHODS

Enzymes

Avian myeloblastosis virus (AMV) reverse transcriptase was obtained from Dr. J. Beard (Life Science Inc. St Petersbourg, USA). DNA polymerase I Klenow's fragment and S1 nuclease were purchased from Boehringer, terminal transferase from P.L. Biochemicals and T4 ligase and various restriction enzymes from Bethesda Research Laboratories.

Hybridoma production

Hybridoma G8 Ca 1.7 ($\gamma 1, \kappa$) was derived from a fusion between spleen cells of a BALB/c mouse (Charles Rivers, France) previously hyperimmunized with GAT and the myeloma cell line X 63.Ag8.653 (20), as reported earlier (21). Hybridoma cells were serially transplanted subcutaneously in BALB/c mice, and the solid tumors harvested were frozen in liquid nitrogen and stored at -70°C before use.

Heavy chain mRNA preparation

Total RNA was extracted from frozen tumors by the LiCl-Urea method (22). The Poly-A^+ RNA containing fraction was eluted from oligo-dT-cellulose (T7,

Collaborative Research) and enriched for H-chain mRNA by sucrose gradient (5-20%) ultracentrifugation as assayed by cell-free translation.

Construction of the cDNA library

cDNA was synthesized (23) from 10 µg of poly A^+-RNA enriched in H-chain mRNA under conditions allowing production of "full-length" transcripts (24-26). After treatment with S1 nuclease and Sepharose 4B gel filtration, cDNA fragments larger than 800 bp were inserted at the Pst I site of pBR 322 using the dC-dG tailing procedure (27) and a 1:1 (vector : cDNA) molar ratio. E. Coli transformation and screening of recombinant clones were performed (28) initially with a cDNA probe derived from the pG 1.6 γ1 cDNA clone (29). The size of the inserts was estimated by gel electrophoresis of Pst I digests of purified recombinant DNA (30).

DNA sequencing

Sequencing was performed (31) using either terminal transferase mediated addition of ^{32}P-cordycepin (Amersham) (32) or 3' addition of ^{32}P-dGTP with Klenow's fragment (33).

Preparation of genomic DNA

Mouse DNA was extracted (34) from adult liver.

Southern transfer analysis

Ten µg of DNA restriction fragments were separated by gel electrophoresis, transferred to nitrocellulose (35) and hybridized with the V_H cDNA "GAT" ^{32}P-labelled probe (probe c, Fig. 1). The probe was ligated to itself with T4 ligase (36) and nick translated to a specific activity of 4-6 x 10^8 cpm/µg (37). Hybridizations under stringent conditions were performed at 65°C in 1X SSC and DH + solution (10 x Denhardt's solution, 0.1% SDS and 50 µg/ml Salmon sperm DNA) followed by washings in 1X SSC DH + solution and 0.1X SSC (9). For non stringent conditions, the same temperature was used but hybridization and washing buffer was 6X SSC.

Biohazards

Experiments were carried out in agreement with the recommendations of the ad hoc French National Committee under the L2-B1 classification.

RESULTS

Isolation of a "GAT" V_H cDNA clone

From 5 g of anti-GAT hybridoma G8 Ca 1.7 frozen solid tumor, about 20 mg of total RNA was extracted, fractionated into 400 µg of poly-A^+-RNA and further subfractionated into 40 µg of H-chain mRNA-containing poly-A^+ RNA. The synthesis of cDNA initiated with 10 µg of H-chain enriched mRNA yielded

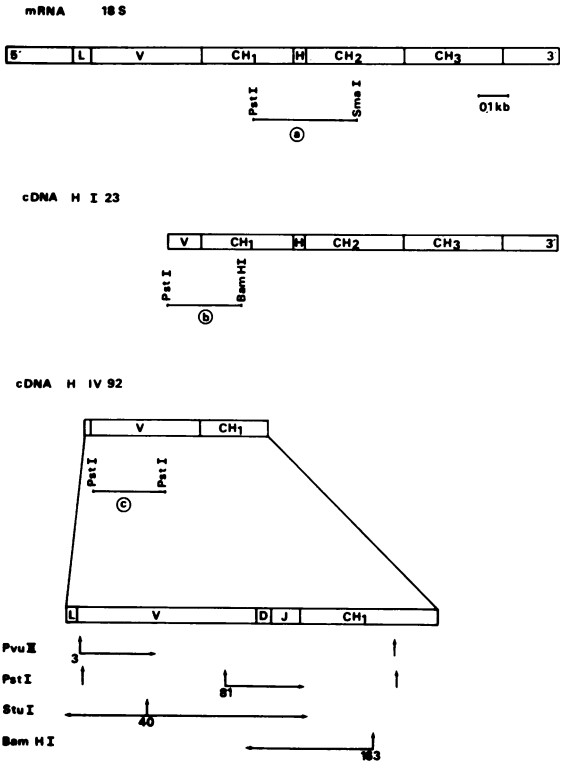

Figure 1 : Characterization of the anti-GAT V_H cDNA clones. Probe a was derived from a C_H cDNA clone (29). Probe b was derived from our cDNA H I23 clone as indicated. Probe c was a Pst I fragment from our H IV 92 cDNA clone. The restriction map and sequence strategy of IV 92 clone are indicated.

3.8 µg of $cDNA_{ss}$ and 1.4 µg of $cDNA_{ds}$. Upon treatment with S1 nuclease, 0.9 µg of $cDNA_{ds}$ was isolated of which 3 ng was inserted into pBR 322. When screened with a Pst I/Sma I digest of pG 1.6 cDNA (29) that contained the second half of C_H1, the hinge region and the first half of C_H2 of the γ1 chain (codons 178 to 288, probe a, Fig. 1) 39 of the 1459 recombinant clones obtained gave a positive signal. Among those clones having the largest inserts, one clone termed I 23 and consisting of a 1200 bp insert, was further characterized by restriction mapping. This clone contained the 3'untranslated region, the complete constant region, and the 3' end of the V region. Nucleotide sequencing indicated that it started at codon 81 at its 5'-terminus. Clone I 23 was used to prepare a second probe by cleavage with

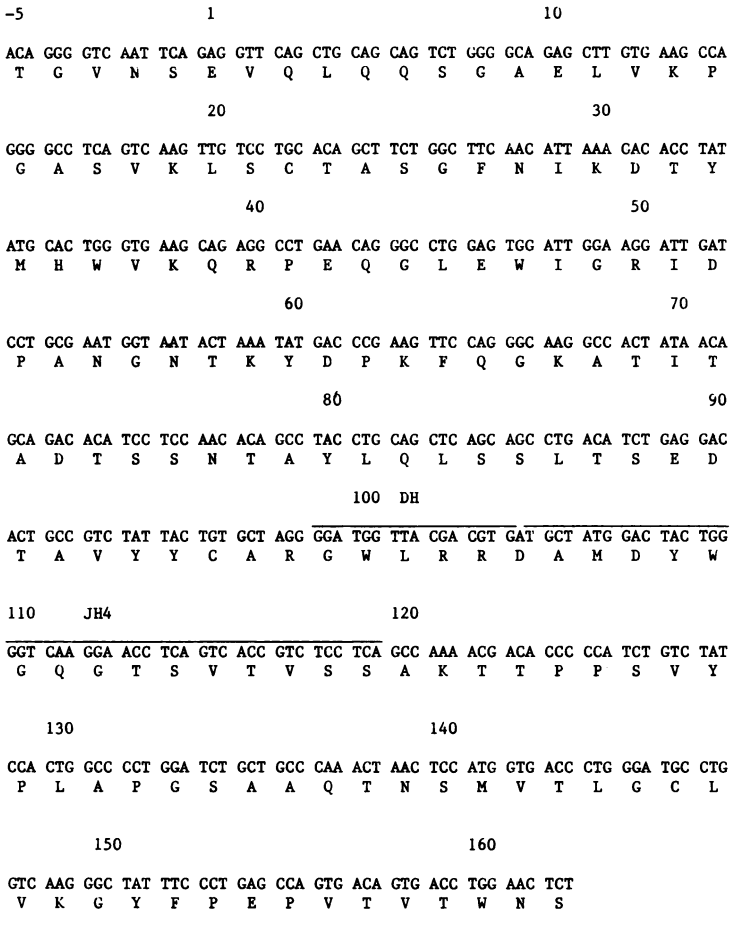

Figure 2 : Nucleotide sequence of the anti-GAT H IV 92 cDNA, and the deduced corresponding amino acid sequence (given in the one-letter code).

Pst I and Bam H I. This 250 bp long probe was shown to contain the end of the V region and the beginning of the C_H1 region (probe b, Fig. 1). The 12 clones assayed to be positive with this probe were included in the 39 clones previously identified with the pG 1.6 probe. One clone termed IV 92, that gave a weak signal with the pG 1.6 probe a (Fig. 1) and a strong signal with probe b (Fig. 1) was further shown to contain a 600 bp long insert consisting of the entire V region, including the 3' end of the leader segment and the 5' end of the constant region (Fig. 1).

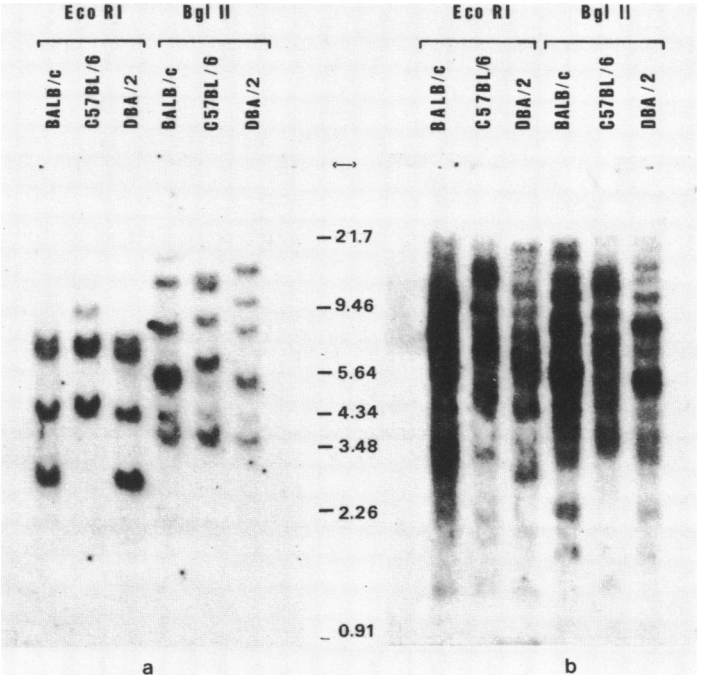

Figure 3 : Southern blots of liver DNA Eco RI and Bgl II fragments using stringent (a) and non stringent (b) conditions as revealed with probe c. Exposure times were 8 and 1 days, respectively. Size references : Eco R I and Hind III digest of λ phage.

Nucleotide sequence of the G8 Ca 1.7 V_H IV 92 clone

The sequence determined according to the strategy given in Fig.1 is in complete agreement with the NH_2-terminal amino acid sequence (residues 1-43) reported for the corresponding heavy chain (16), and is presented in Fig. 2. This figure indicates that the insert of the IV 92 cDNA clone begins 15 nucleotides 5' to the NH_2-terminal glutamic acid codon. This sequence also agrees with that reported for the corresponding mRNA (17), as determined by a modification (18) of the dideoxy method (19).

DNA hybridization patterns

DNA extracted from BALB/c, C57Bl/6 and DBA/2 adult liver was consistently larger than 50 kb. Eco R I and Bgl II fragments of such DNA were hybridized with probe c derived by Pst I cleavage of the IV 92 cDNA clone (Fig. 1) and which covered most of the V_H region (codons 4 to 81). Under low stringency conditions, 15 to 25 bands were observed, whereas at high stringency a limited number of bands (3 to 7) were evident (Fig.3 and Table

Table 1 : Size characteristics of the various restriction fragments depicted in figure 3 (stringent conditions only).

	Eco R I (Kb)				Bgl II (Kb)						
BALB/c	7.5	7.0	4.3	3.0	20	12	8.5	5.5	4.2	3.9	
C57Bl/6	9.4	7.2	4.5		13	11.5	9	6.5	5.3	4.2	3.9
DBA/2	7.5	7.0	4.3	3.0	15	10.5	8.5	5.5	4.2	3.9	

1). BALB/c and DBA/2 possess identical Eco R I bands but differ in some of their Bgl II bands. C57Bl/6 banding patterns strain differed from those of the other two strains ; the Eco R I fragments yielded only three main bands and the Bgl II pattern overlapped only partially with that of the BALB/c and DBA/2 fragments (Table 1).

DISCUSSION

Different approaches have been used to estimate the number of germ-line genes that encode the V_H and the V_L regions of immunoglobulins (38-41). Isolation of V genes has been reported in several antibody systems well defined by their idiotypic markers. We have found that BALB/c and DBA/2 anti-GAT monoclonal antibodies use a restricted repertoire of V_H and V_L chains likely controlled by a structurally closely related subset of a very few germ-line genes (16, 17).

We describe here the isolation of an anti-GAT V_H cDNA clone that covers codons -5 to 193, the sequence of which was determined until to codon 162 inclusive, thus encompassing the V-D-J regions. It should be stressed that this cDNA, although synthesized from the 3' poly-A end of the corresponding mRNA, has most of the constant region deleted. It was first identified with probe a (Fig. 1) because of the overlap of the 3' terminal 60 nucleotides.

When compared with other V_H sequences, that of G8 Ca 1.7 clearly belongs to the V_H II subgroup, as defined by Kabat et al. (42) and which contains a wide collection of heavy chains, including BALB/c anti-dextran (43), and C57BL/6 anti-(4-hydroxy-3-nitrophenyl) acetyl (NP) antibodies (44). Despite the homology observed, it seems unlikely that these different V_H segments be encoded by the same germ-line gene. Surprisingly, a very high homology was observed between the V_H G8 Ca 1.7 (anti-GAT) sequence, and that recently reported for the V_H region of a BALB/c anti-NP antibody (45),

```
          -5                  1                                  10
G8Cal.7 ACA GGG GTC AAT TCA GAG GTT CAG CTG CAG CAG TCT GGG GCA GAG CTT GTG

17.2.25 ___________________________________________________________________

        Lys                        20
        AAG CCA GGG GCC TCA GTC AAG TTG TCC TGC ACA GCT TCT GGC TTC AAC ATT

        _G_____________________________________G_____________________________
        Arg

        30                                    40
        AAA GAC ACC TAT ATG CAC TGG GTG AAG CAG AGG CCT GAA CAG GGC CTG GAG

        ___________________________________________________________________

                  50                                    60
        TGG ATT GGA AGG ATT GAT CCT GCG AAT GGT AAT ACT AAA TAT GAC CCG AAG

        ___________________________________________________________________

                             70                       Ser            80
        TTC CAG GGC AAG GCC ACT ATA ACA GCA GAC ACA TCC TCC AAC ACA GCC TAC

        ___________________________________________A_____________________
                                                   Thr

                                          90
        CGT CAG CTC AGC AGC CTG ACA TCT GAG GAC ACT GCC GTC TAT TAC TGT GCT AG

        ___________________________________________________________________
```

Figure 4 : Comparison of anti-GAT (G8 Ca 1.7, this paper) and anti-NP^a (45) V_H nucleotide and amino acid sequences.

expressing the NP^a idiotypic marker (46). Comparison of the two sequences is given in Figure 4. Only three nucleotide differences were identified, one silent (codon 23), and two leading to an amino acid replacement (Lys$\leftrightarrow$Arg at position 13 and Ser$\leftrightarrow$ Thr at position 76). The same J_H4 gene was used in both cases, but the D segments were different. Very close V_H structures may therefore be used by antibody molecules of discrete specificities. Evidently, the contribution of different D gene products appears of decisive importance in dictating the actual specificity, together with the structure of the complementary light chains, which, in the present case, are of the κ and λ type, for the anti-GAT and anti-NP, respectively. From the genetic stand point, the main issue remains the overall number of germ-line genes which encode for the various segments of immunoglobulins. It would therefore be of interest to investigate whether both the anti-GAT and the anti-NP V_H regions are indeed derived from the same germ-line gene.

Using probe c, covering codons 4-81 and stringent conditions, simple

hybridization patterns were observed with Eco R I and Bgl II restriction germ-line DNA fragments (Fig. 3a). These observations confirm that anti-GAT antibodies expressing the pGAT or CGAT public idiotypic specificities (13, 14) indeed use a very small number of germ-line V_H genes. Furthermore, it cannot be concluded from this approach whether all the bands actually represent an anti-GAT V_H gene. For instance, some bands might reflect cross-reactive or pseudo-genes, which would lower the actual number of germ-line genes. It was also observed in Figure 3a that hybridization patterns differed at least partially whenever different strains were compared. In fact cross-hybridization observed with fragments derived from the C57BL/6 strain may not reflect the actual genes used in this strain to synthesize anti-GAT antibodies, since, at least in one case analyzed (Rocca-Serra et al. submitted), the V_H structure was only 75% homologous to that described for the BALB/c strain.

Because of the conditions of high stringency that were used, only genes having a very similar sequence should cross-hybridize. As the four anti-GAT V_H BALB/c sequences recently reported (17) share greater than 95% nucleotide homology, they clearly should cross-hybridize if they represent discrete germ-line genes. Similarly because of the extremely high level of homology observed between the two BALB/c anti-GAT and anti-NP V_H sequences, it seems very clear that either V_H cDNA probe will lead to the same hybridization pattern. At this point, it cannot be decided whether one or the other of the anti-GAT or anti-NP V_H nucleotide sequence actually represents the direct expression of a unique germ-line gene. If this were the case, the most likely candidate would rather be the anti-GAT sequence since two monoclonal antibodies, derived from separate fusions, expressed identical V_H regions (17). Clearly, the final answer will rely on the isolation and characterization of the various cross-hybridizing genes derived from a genomic library.

ACKNOWLEDGEMENTS

We are much indebted to Dr. F. Rougeon and his collaborators for invaluable help and advice, to Dr. Honjo for providing us with the pG 1.6 cDNA clone, to Dr. J. Thèze for his kind gift of the G8 Ca 1.7 hybridoma cells, and to Dr. Klaus Rajewsky for helpful discussions.

*To whom reprint requests should be sent

REFERENCES

1. Amzel,L.M., Poljak,R., Saul,F., Varga,J. and Richards,F. (1974) Proc. Natl. Acad. Sci. USA 71, 1427-1431.
2. Brack,C., Hirama,M., Lenhard-Schuller,R. and Tonegawa,S. (1978) Cell 15, 1-14.
3. Sakano,H., Hüppi,K., Heinrich,G. and Tonegawa,S. (1979) Nature 280, 288-294.
4. Seidman,J.G., Max,E.E. and Leder,P. (1979) Nature 280, 370-375.
5. Early,P., Huang,H., Davis,M., Calame,K. and Hood,L. (1980) Cell 19, 981-992.
6. Kurosawa,Y. and Tonegawa,S. (1982) J. Exp. Med. 155, 201-218.
7. Gearhart,P.J., Johnson,N.D., Doulas,R. and Hood,L. (1981) Nature 291, 29-34.
8. Bothwell,A.L.M., Paskind,M., Reth,M., Imanishi-Kari,T., Rajewsky,K. and Baltimore,D. (1981) Cell 24, 625-637.
9. Bentley,D.L. and Rabbitts,T.H. (1981) Cell 24, 613-623.
10. Adams,J.M., Kemp,D.J., Bernard,O., Gough,N., Webb,E., Tyler,B., Gerondakis,S. and Cory,S. (1981) Immunological Rev. 59, 5-31.
11. Crews,S., Griffin,J., Huang,H., Calame,K. and Hood,L. (1981) Cell 25, 59-66.
12. Givol,D., Zakut,R., Effron,K., Rechavi,G., Ram,D. and Cohen,J.B. (1981) Nature 292, 426-430.
13. Ju,S.T., Kipps,T.J., Thèze,J., Benacerraf,B. and Dorf,M.E. (1978) J. Immunol. 121, 1034-1039.
14. Thèze,J. and Moreau,J.L. (1978) Ann. Immunol. (Institut Pasteur) 129c, 721-726.
15. Sommé,G., Rocca-Serra,J., Leclercq,L., Moreau,J.-L., Mazié,J.-C., Moinier,D., Fougereau,M. and Thèze, J. (1982) Molec. Immunol. 19, 1011-1019.
16. Ruf,J., Tonnelle,C., Rocca-Serra,J., Moinier,D., Pierres,M., Ju,S.-T., Dorf,M., Thèze,J. and Fougereau,M. (1983) Proc. Natl. Acad. Sci. USA, in press.
17. Rocca-Serra,J., Matthes,H.W., Kaartinen,M., Milstein,C., Thèze,J. and Fougereau,M. (1983) EMBO J. in press.
18. Hamlyn,P.H., Brownlee,G.G., Cheng,C.C., Gait,M.J. and Milstein,C. (1978) Cell 15, 1067-1075.
19. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
20. Kearney,J.F., Radbruch,A., Liesegang,B. and Rajewsky,K. (1979) J. Immunol. 123, 1548-1550.
21. Leclercq,L., Mazié,J.-C., Sommé,G. and Thèze,J. (1982) Molec. Immunol. 19, 1001-1009.
22. Auffray,C. and Rougeon,F. (1980) Eur. J. Biochem. 107, 303-314.
23. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) Molecular Cloning, pp. 230-240, Cold Spring Harbor Laboratory.
24. Efstratiadis,A., Kafatos,F.C., Maxam,A.M. and Maniatis,T. (1976) Cell 7, 279-288.
25. Buell,G.N., Wickens,M.P., Payvar,F. and Schimke,R.T. (1978) J. Biol. Chem. 253, 2471-2482.
26. Retzel,E.F., Collet,M.S. and Faras,A.J. (1980) Biochemistry 19, 513-517.
27. Rougeon,F. and Mach,B. (1977) J. Biol. Chem. 252, 2209-2217.
28. Roskam,W.G. and Rougeon,F. (1979) Nucl. Acids Res. 7, 305-320.
29. Obata,M., Yamawaki-Kataoka,Y., Takahashi,N., Kataoka,T.,Shimizu,A., Mano,Y., Seidman,J.G., Peterlin,B.M., Leder,P. and Honjo,T.(1980) Gene 9, 87-97.
30. Birnboim,H.C. and Doly,J. (1979) Nucl. Acids Res. 7, 1513-1523.

31. Maxam,A.M. and Gilbert,W. (1980) Methods in Enzymology, K. Moldave and L. Grossman, Eds., 65, pp. 499-560, Academic Press, New York.
32. Roychoudhury,R., Jay,E. and Wu,R. (1976) Nucl. Acids Res. 3, 101-115.
33. Drouin,J. (1980) J. Mol. Biol. 140, 15-34.
34. Gross-Bellard,M., Oudet,P., Chambon,P. (1973) Eur. J. Biochem. 36, 32-38.
35. Southern,E.M. (1975) J. Mol. Biol. 98, 503-517.
36. Heidmann,O. and Rougeon,F. (1982) Cell 28, 507-513.
37. Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) J. Mol. Biol. 113, 237-251.
38. Ben-Neriah,Y., Cohen,J.B., Rechavi,G., Zakut,R. and Givol.D. (1981) Eur. J. Immunol. 11, 1017-1022.
39. Siekevitz,M., Gefter,M., Brodeur,P., Riblet,R. and Marshak-Rothstein,A. (1982) Eur. J. Immunol. 12, 1023-1032.
40. Cory,S., Kemp,D.J., Bernard,O., Gough,N., Gerondakis,S., Tyler,B., Webb,E. and Adams,J.M. (1981) in Immunoglobulin Idiotypes, Janeway,C., Sercarz,E.E and Wigzell,H. Eds., pp. 49-58.
41. Rabbitts,T.H., Matthyssens,G., Hamlyn,P.H. (1980) Nature 284, 238-243.
42. Kabat,E.A., Wu,T.T. and Bilofsky,H. (1979) NIH Publication n° 80 2008.
43. Schilling,J., Clevinger,B., Davie,J.M. and Hood,L. (1980) Nature 283, 35-40.
44. Bothwell,A.L.M., Paskind,M., Reth,M., Imanishi-Kari,T., Rajewsky,K. and Baltimore,D. (1981) Cell 24, 625-637.
45. Loh,D.Y., Bothwell,A.L.M., White-Scharf,M.E., Imanishi-Kari,T. and Baltimore,D. (1983) Cell 33, 85-93.
46. White-Scharf,M.E. and Imanishi-Kari,T. (1981) Eur. J. Immunol. 11, 897-904.