Mouse immunoglobulin genes: a bacterial plasmid containing the entire coding sequence for a pre-γ2a heavy chain

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ABSTRACT

A DNA sequence complementary to the entire coding part of a mouse γ2a immunoglobulin heavy chain mRNA isolated from a myeloma producing a levan binding protein (UPC 10), has been cloned in the PstI site of pBR 322. Transformants containing sequences complementary to purified γ2a heavy chain mRNA were selected. One transformant, pG2a-10-21, containing a 1750 nucleotide insert, has been characterized by hybrid-arrested translation and purification of γ2a heavy chain mRNA on DNA-DBM cellulose filters. Restriction enzyme analysis and partial sequencing demonstrate that the pG2a-10-21 contains the complete structural sequence for the γ2a heavy chain and predicts the sequence of a 18 amino acid hydrophobic amino terminal extra piece segment.

INTRODUCTION

Antibody molecules are made up of two non identical subunits : light (L) and heavy (H) chains. Numerous studies suggest that virtually any light chain may associate with any heavy chain. Recently a series of experiments have demonstrated that an active gene for κ or λ light chain is formed by somatic recombination between a variable (V) region gene and a J segment close to the constant (C) region gene (1,2). Hybridization saturation experiments (3) and analysis of the structure of Vκ germ-line genes strongly suggest that the large diversity of κ light chains is the result of a site-specific recombination between one of the hundreds germ-line Vκ genes and a J segment without further somatic mutation (4,5).

Similar mechanisms are probably involved in the formation of active genes for heavy chains. Nevertheless a same VH region can be associated with different constant regions (Cμ, Cγ, Cα), (for review, see reference 6). In order to study the problems of the size of the VH genes repertoire and the mechanisms by which a gene segment (VH) can move from one position to another, we have cloned different structural gene sequences corresponding to the main classes of immunoglobulin heavy chains. In this report, we present the analysis of a bacterial DNA recombinant clone encoding the entire V and

C regions of a γ2a heavy chain from a myeloma protein (UPC 10) having a binding ligand specificity for 2-6 levan (7,8).

MATERIALS AND METHODS

1 - Materials

[^{35}S] Methionine, [^{3}H]dCTP, [^{32}P]dCTP and [γ-^{32}P] ATP were obtained from the Radiochemical Center, Amersham, England. All other reagents were obtained from usual commercial sources in the highest grade of purity available.

2 - Tumors

Myeloma used in this study : UPC 10 (γ2a, κ), TEPC 183 (μ, κ), and J 558 (α, λ), derive from NIH Balb/c plasmacytomas and were obtained from P.A. Cazenave.

3 - DNA and enzymes

pBR 322 was obtained from H. Boyer (9) and DNA purified according to Katz et al. (10). Purified reverse transcriptase from avian myeloblastosis virus (AMV) was obtained from J. Beard (Life Sciences Inc., St Petersbourg, USA). E. coli DNA polymerase I (Fraction VII) was purified according to Jovin et al. (11). Terminal deoxynucleotidyl transferase (TdT), T4 polynucleotide kinase and single-strand S1 nuclease were purified as described (12-14). EcoRI, BamHI, HindIII, PstI, AluI and SalI restriction endonucleases were purified according to published procedures (15-16). All other restriction endonucleases were obtained from New England Biolabs.

4 - Heavy chain mRNA purification

Heavy chain mRNAs were purified as described (17). Briefly, frozen tumors were homogenized in 3 M LiCl, 6 M urea. RNA was pelleted and poly(A)-containing RNA was isolated by two successive oligo(dT)-cellulose chromatographies. Heavy chain mRNA was isolated by sucrose gradient centrifugation and/or preparative polyacrylamide gel electrophoresis. The purity at the sucrose gradient stage was about 20-30%, and 50-65% after preparative electrophoresis.

5 - Double-stranded DNA synthesis, hybrid formation and transformation

For cloning experiments, RNA (50 μg/ml) was transcribed into complementary DNA (cDNA) as described (18) with the following modifications : actinomycin D was omitted and incubation was for 30 min at 42°C. After alkaline hydrolysis and gel filtration the ^{3}H cDNA (2.10^{6} cpm/μg) was converted into double-stranded structure by E. coli DNA polymerase I (19).

DNA was extracted with chloroform/isoamylalcohol (24 : 1) and isolated by gel filtration through a G 75 Sephadex column equilibrated in 50 mM NaCl. The excluded fractions were treated with S1 nuclease (18). After incubation, the DNA was extracted, ethanol precipitated and fractionnated on a sucrose gradient (18). Double-stranded DNA (larger than 1200 bp) was elongated with oligo (dC) by TdT for 2 min at 35°C using Co^{2+} as divalent cation (18). pBR 322 digested with PstI was elongated with oligo (dG) by TdT for 15 min at 35°C with Mg^{2+} as divalent cation (18). The reactions were stopped with 20 mM EDTA and 0.2% SDS. Material insoluble at 0-4°C was eliminated by centrifugation and the supernatant was directly used for the annealing reaction using a 1 : 1 molar ratio of (dC) ds-cDNA/(dG) vector DNA (18). Transformation and screening of recombinant clones were carried out as previously described (20). [^{32}P] cDNA used for in situ hybridization was synthesized in the same conditions than [^{3}H] cDNA with the addition of 4 mM sodium pyrophosphate (21).

6 - Characterization of recombinant plasmid by hybrid-arrested translation

Hybrid-arrested translation was performed essentially as described (22), using 1 µg of DNA insert isolated by sucrose gradient centrifugation and 0.4 µg poly(A)-containing RNA. The hybrids were recovered by ethanol precipitation and washed with cold 70% ethanol, and redissolved in water. One half was heated for 2 min at 80°C then cooled on ice. In vitro translation was performed in an mRNA-dependent reticulocyte lysate prepared as described by Pelham and Jackson (23). The incubation conditions were as described (17). Translation products were analysed on 15% SDS-polyacrylamide gels (24), and fluorography was performed as described (25).

7 - Purification of mRNA by hybridization to DNA-DBM filters

Sonicated heat-denatured plasmid DNA was fixed on 1 cm^2 DBM filters prepared just before use as described by Stark and Williams (26). Hybridization of total poly(A)-containing RNA and elution of bound RNA were performed as described (27). RNA was recovered by ethanol precipitation in the presence of 5 µg wheat germ tRNA and translated in vitro.

8 - Restriction mapping and blotting analysis

A detailed restriction map of the DNA insert was established by the method of Smith and Birnstiel (28). Blotting of recombinant DNA was carried out as described by Breathnach et al. (29). 5.10^5 cpm of [^{32}P] cDNA ($1-5.10^7$ cpm/µg) were used for a 150 cm^2 nitrocellulose filter.

9 - DNA sequencing

DNA fragments isolated by polyacrylamide gel electrophoresis were end

labelled with T4 polynucleotide kinase using the γ exchange reaction (30). Sequencing was performed using the partial chemical degradation method of Maxam and Gilbert (31).

10 - Biohazards

Biohazards associated with the experiments described in this publication were examined previously by the French National Committee, and carried out according to the recommandations of this Committee (Le Progrès Scientifique, n° 191, Nov/Dec 1977), under L3-B1 conditions.

RESULTS AND DISCUSSION

1 - Construction and selection of hybrid plasmids containing DNA sequences complementary to heavy chain mRNA from UPC 10 myeloma

Heavy chain mRNA purified as described in Methods was used as a template for AMV DNA polymerase.The yield of the reaction was about 20-30%. Omitting the actinomycin D resulted in a significant increase of the cDNA yield with concomitant increase of S1 nuclease resistance (10-15%). Total cDNA isolated by gel filtration without size selection was converted into double-stranded structure with E. coli DNA polymerase I. In the present case, the yield of conversion of cDNA into double-stranded structure was about 50-60% . From the experience of our laboratory in the cloning of various structural genes corresponding to mRNAs ranging from 600 to 2000 nucleotides, it appears that there is not a general rule concerning the use of AMV DNA polymerase or E. coli DNA polymerase I for the synthesis of the second strand. In some cases AMV DNA polymerase used in the conditions described by Monahan et al. (32) can be as efficient as E. coli DNA polymerase I. After S1 nuclease digestion the DNA molecules were selected on a sucrose gradient and DNA molecules larger than 1200 bp were pooled. Elongation was performed as described in Methods. Stopping the reaction by addition of 20 mM EDTA and 0.2% SDS, and removing material insoluble at 0-4°C permits to use the supernatant directly for the annealing reaction. This prevents lost of material during phenol or chloroform extraction and ethanol precipitation. There is no decrease in the yield of transformation which is about 2.10^3-10^4 transformants/µg of hybrid DNA. Ampicilline sensitive and tetracycline resistant colonies were selected, grown in 50 ml cultures and plasmid DNA was amplified with chloramphenicol. Plasmids were extracted by the cleared lysate procedure (10), RNA digested with pancreatic RNAase and the DNA digested with PstI and EcoRI in order to determine the length of the insert. One plasmid, pG2a-10-21, having a 1750 bp insert, was selected for further

characterization.

2 - Hybridization of the recombinant DNA with [^{32} P] cDNA from different classes of heavy chain mRNAs

Since each myeloma produces only one type of heavy chain (μ, γ, α) and since sequence divergence between the constant part of the heavy chains (33,34) prevents cross hybridization, it can be anticipated that the γ2a recombinant will hybridize only with the cDNA transcribed from the mRNA purified from the UPC 10 tumor. In order to demonstrate that the inserted DNA contains sequences complementary to γ2a heavy chain mRNA, DNA from plasmids pBR 322 and pG2a-10-21 were digested with EcoRI or PstI and electrophoresed on a 2% agarose gel, then DNA was transferred to nitrocellulose filters.

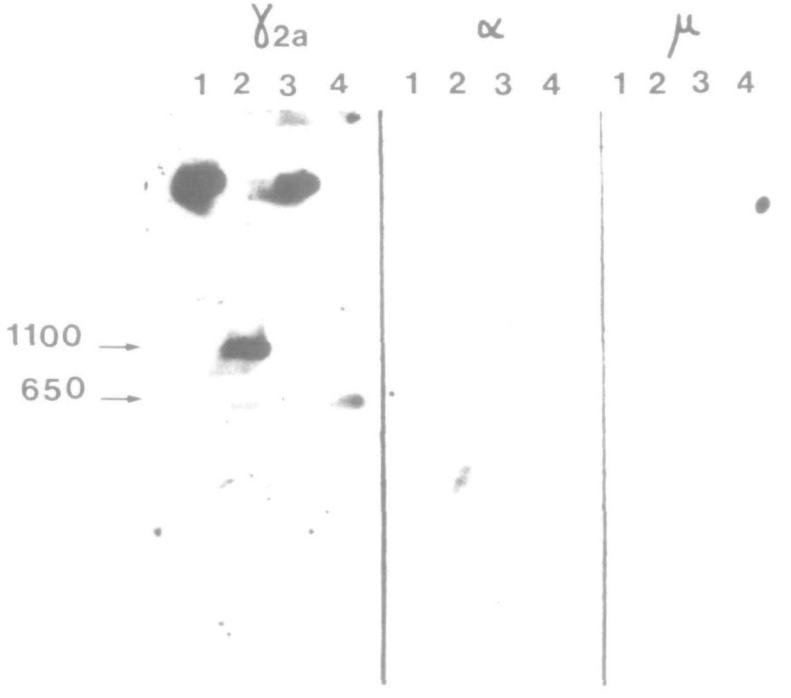

Fig. 1 : Detection of DNA fragments containing sequences complementary to cDNA transcribed from IgG2a mRNA.
pG2a-10-21 and pG2a-10-99 DNA (containing a 1300 bp γ2a insert) were digested with PstI, electrophoresed on a 2% agarose gel and transferred to nitrocellulose filters. The filters were hybridized with [^{32} P] cDNA transcribed from total mRNA isolated from IgG2a (left), IgA (middle) or IgM (right) producing tumors. Lane 1 : undigested pG2a-10-21. Lane 2 : pG2a-10-21 digested with PstI. Lane 3 : undigested pG2a-10-99. Lane 4 : pG2a-10-99 digested with PstI (two 650 bp fragments comigrating).

Three different filters were hybridized with [^{32}P] cDNA (smaller than 1200 nucleotides) transcribed from mRNA prepared from three myelomas producing one of the three main classes of heavy chains : TEPC 183 (μ, κ), UPC 10 (γ2a, κ), and J 558 (α, λ). As shown in Fig.1, the cDNA insert hybridized only with cDNA prepared from tumor producing γ2a heavy chain. Providing that [^{32}P] cDNA are complementary only to the C region of heavy chain, it can be concluded that a differential screening of recombinant clones containing gene sequences expressed only in one type of myeloma can be achieved using unpurified cDNAs.

3 - Hybrid-arrested translation and heavy chain mRNA purification by hybridization to plasmid DNA

The DNA fragment containing the structural gene sequence for γ2a heavy chain was used in the hybrid-arrested translation assay as described

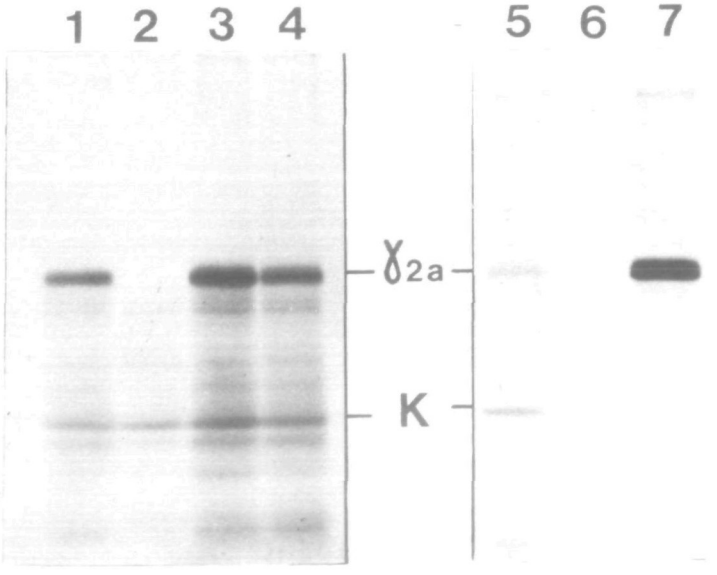

Fig. 2 : Fluorograph from 15% polyacrylamide-SDS slab gels of the in vitro translation products of UPC 10 poly(A)-containing RNA synthesized in an mRNA-dependent rabbit reticulocyte lysate.
Hybrid-arrested translation of γ2a heavy chain mRNA.
Lane 1-4 : translation of poly(A)-containing RNA without treatment (1), after hybridization with the cDNA insert of pG2a-10-21 (2), same as 2 after melting the hybrids (3), after hybridization with pBR 322 (4).
Translation of mRNA hybridizing to pBR 322 (6) and pG2a-10-21 (7) plasmid DNA bound to DBM filters. Lane 5 : translation of RNA not hybridizing to pG2a-10-21 filter.

in Methods. The results shown in Fig.2 indicate that the in vitro translation of the γ2a heavy chain mRNA is completely and selectively abolished when total poly(A)-containing RNA has been hybridized with the DNA insert, then recovered by melting the hybrids. These results were confirmed by the use of the procedure described by Stark and Williams (26). Total plasmid DNA from pG2a-10-21 and pBR 322 was bound to DBM filters and used to select complementary mRNA sequences present in the total poly(A)-containing RNA. Bound RNA was translated in vitro. The results of the analysis of the translation products shown in Fig.2 indicate that γ2a heavy chain mRNA is selectively retained on pG2a-10-21 filters, whereas control filters bearing pBR 322 DNA did not bind γ2a heavy chain mRNA.

4 - Orientation of the insert by blotting analysis

A partial restriction map of pG2a-10-21 was built up by digestions of the DNA with EcoRI, HindIII, BamHI and PstI (Fig.3). In order to orient the insert with respect to transcription, plasmid DNA was digested with BamHI or PstI and the fragments were transferred to nitrocellulose filters. One of them was hybridized with [^{32}P] cDNA having a chain length smaller than 1200 nucleotides corresponding to the constant part of the mRNA (cDNA C). Another blot was hybridized with a full-length transcript of the mRNA

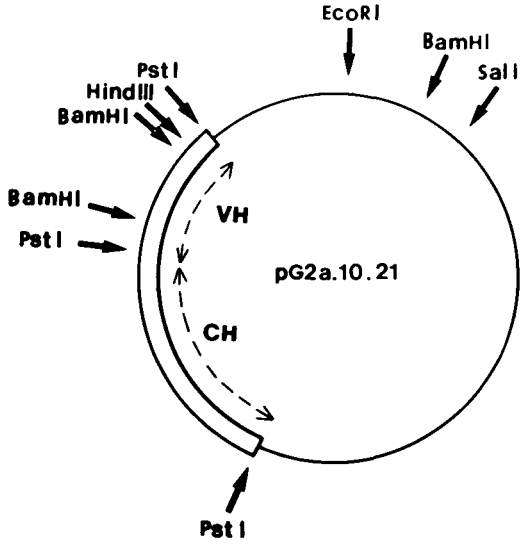

Fig. 3 : A partial restriction map of pG2a-10-21. The map shows the inserted γ2a fragment and its orientation in the pBR 322 plasmid DNA. The approximate cleavage sites for the restriction endonucleases PstI, HindIII, BamHI, EcoRI and SalI are indicated.

(cDNA V+C). The results shown in Fig.4 indicate that the BamHI (440 and 1230 bp) and the PstI (650 bp) fragments hybridized only with the full-length transcripts, and thus contain the VH coding sequence corresponding to the 5' end of the mRNA. This result strongly supports the orientation indicated in Fig.3.

5 - Restriction enzyme mapping and DNA sequencing of pG2a-10-21 with respect to protein sequence

In order to locate more precisely V and C coding regions, a detailed restriction map of the insert was built up as described in Methods. The results shown in Fig.5 were compared to the putative restriction map obtained with a computer program (F. Bregegere, personal communication) from the 447 amino acid sequence of MOPC 173 γ2a heavy chain which has a VH region very similar to that of UPC 10 (35,36). Only five BamHI sites are possibly encoded at nucleotides 46, 409, 481, 1027 and 1207 (starting from the first codon of the V region). As two BamHI sites are actually found separated by 440 bp, the only possible association is 46-481. The HindIII site found 150 bp from the 5' end of the insert and 40 bp from the BamHI site at nu-

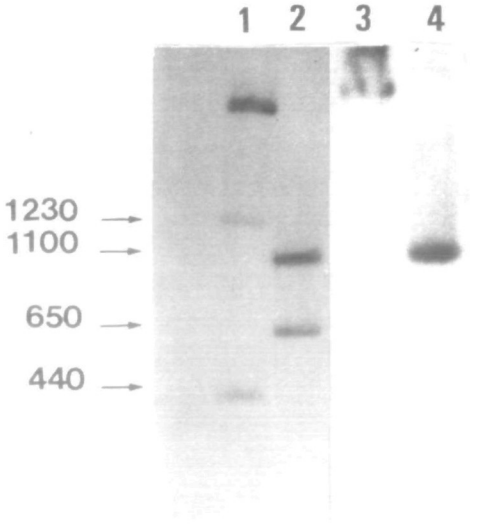

Fig. 4 : Electrophoresis of BamHI and PstI digests of pG2a-10-21 on 2% agarose gels.
BamHI (lanes 1 and 3) and PstI (lanes 2 and 4) digests were electrophoresed and transferred to nitrocellulose filters. One filter was hybridized with full-length [^{32}P] cDNA transcripts of γ2a mRNA (lanes 1 and 2), another one with [^{32}P] cDNA corresponding to the 3' half of γ2a mRNA (lanes 3 and 4).

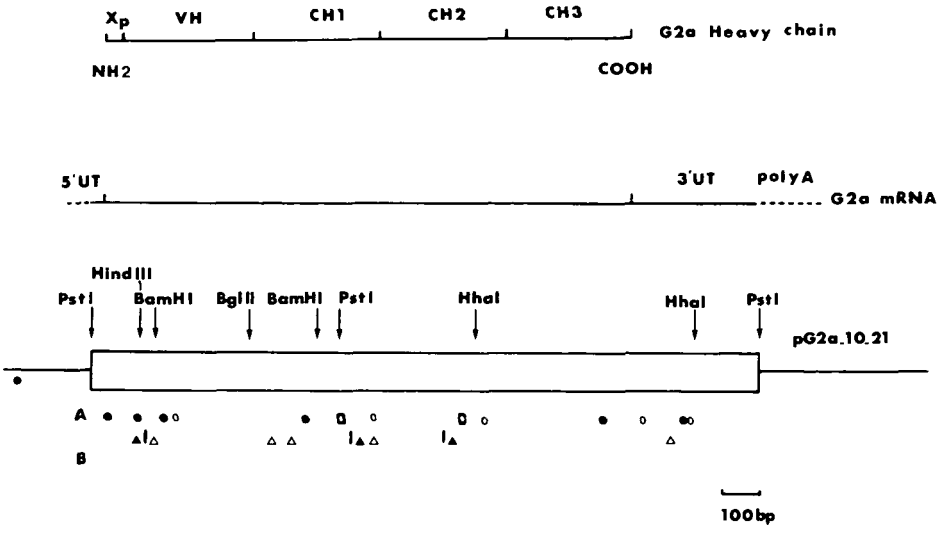

Fig. 5 : Complete restriction enzyme map of pG2a-10-21.
Upper line : γ2a heavy chain protein structure.
Middle line : γ2a heavy chain mRNA structure. The dotted lines represent the segments not present in the insert.
Lower line : restriction sites found in the insert for the indicated enzymes and
A : HinfI (●), HpaII (○), PvuII (▯)
B : TaqI (▲), HaeIII (△), AluI (▮).

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AUG GAU UUU GGG CUG AUU UUU UUU AUU GUU GCU CUU UAA AAA GGG GUC CAG UGU GAG
Met Asp Phe Gly Leu Ile Phe Phe Ile Val Ala Leu Leu Lys Gly Val Gln Cys Glu
-18                                                                  -1   1

    HindIII                                                  BamHI       Glu
    7↓                                                       46↓
     AAG CTT                                                  GGA TCC

GUA AAG CUU CUC GAG UCU GGA GGU GGC CUG GUG CAG CUU GGA GGA UCC CUG AAA CUC UCC UGU
Val Lys Leu Leu Glu Ser Gly Gly Gly Leu Val Gln Leu Gly Gly Ser Leu Lys Leu Ser Cys
                                   10                                20      22
Val Lys Leu Leu Glu Ser Gly Gly Pro Leu Val Gln Leu Gly Gly Ser Leu Lys Leu Ser Cys
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Fig. 6 : Partial nucleotide sequence of γ2a heavy chain mRNA. The nucleotide sequence (upper line) is deduced from the sequence of pG2a-10-21 determined from both HindIII and BamHI sites. The amino acid sequence (middle line) is that deduced from the nucleotide sequence. The amino acid sequence of MOPC 173 γ2a heavy chain is indicated on the lower line.

cleotide 46 fits with a predicted site at nucleotide 7 which corresponds to the third amino acid of the mature protein. The sequence of the region surrounding the HindIII site was determined as described in Methods. The results shown in Fig.6 are in agreement with the protein sequence. It is highly probable that the difference observed at position 10 corresponds the variation between VH genes belonging to the same subgroup (37). The sequence obtained predicts that the γ2a heavy chain is synthesized primarily as a precursor polypeptide : the only AUG initiation codon is found at position -18. The amino terminal extra piece was found quite hydrophobic : 11 hydrophobic residues and 2 neutral residues out of 18. The sequence also demonstrates that the variable segment of UPC 10 γ2a heavy chain belongs to the VHIII subgroup (37). The comparison of the predicted amino acid sequence for the γ2a precursor with that of precursors for λ and κ light chains (38) and MOPC 315 α heavy chain (39) (which belongs to the VHII subgroup) indicated a lack of homology. This strongly suggests that the extra piece is a genetic extension of the variable region. Alltogether, these results indicate that the total coding sequence being 1395 bp, part of the 5' and 3' untranslated regions are also present in the 1750 bp insert.

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