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Molecular cloning of rabbit γ heavy chain mRNA

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ABSTRACT

A cDNA library of rabbit spleen mRNA was screened for immunoglobulin heavy chain sequences. In this paper we report the nucleotide sequence of two cDNA clones containing part of the constant region of the rabbit γ heavy chain mRNA. The sequence encodes part of the CH2 domain (amino acids 268 to 340), the entire CH3 domain (amino acids 341 to 447) and the 3' untranslated region. This nucleotide sequence has been compared to the corresponding sequences of mouse γ1, γ2a and γ2b genes. The homologies between rabbit γ chain gene sequence and each of the mouse γ chain gene sequences are of the same magnitude order. This comparison shows that the CH2 domains are more homologous to each other than CH3 domains or 3' untranslated sequences. The presence of species specific nucleotide positions suggests that mouse γ chain genes could have evolved from a common ancestor shortly after mouse-rabbit species separation. Genomic blot analysis of rabbit liver DNA with the rabbit Cγ probes shows a limited number of related sequences, with little restriction site polymorphism between individual rabbits.

INTRODUCTION

Immunoglobulins are composed of two light chains and two heavy chains, each of which has a variable N-terminal sequence (V region) and a constant COOH-terminal sequence (C region). Extensive studies of the mouse immunoglobulin genes have shown that the V and C regions are encoded by separate genetic segments on the chromosome, which are joined during the B lymphocyte differentiation (1-3). In case of heavy chain gene, a complete V_H gene is generated by the joining of V_H, D and J_H gene segments (4-6). This active V_H gene may be sequentially associated with two or more different C_H genes which determine the immunoglobulin class (IgM, IgD, IgG, IgA or IgE) (7, 8).

Rabbit immunoglobulin heavy chains pose two specific questions, relative to the organization and expression of V and C genes. First, the rabbit and other Leporidae are unique in having antigenic markers present in the variable region of heavy chains.

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The existence of stable allelic markers present on multiple V_H chains raises questions about the organization of rabbit V_H genes (for review, see references 9 and 10).

The second question concerns the number of rabbit Cγ genes. Many mammalian species, such as man and mouse, are known to express four different IgG subclasses (11) corresponding to different amino acid sequences of the Cγ region. No similar division in γ subclasses have been reported for rabbit IgG. Comparison of rabbit γ chain amino acid sequences shows very little amino acid variations. Partial amino acid sequence on a purified tryptic peptide have shown some further amino acid substitutions and have been proposed to be subclass-related (12). However, no clear correlation between amino acid sequences and specific effector functions has been made for the rabbit Cγ region. DNA analysis should permit us to determine if the rabbit genome contains multiple different Cγ genes.

We have already described the construction of a cDNA library starting from hyperimmunized rabbit spleen mRNAs (13). We report here the characterization and the partial nucleotide sequence of a rabbit Cγ mRNA. This sequence has been compared to the corresponding nucleotide sequences of mouse γ1, γ2a and γ2b heavy chain mRNAs (14, 15, 16). We then used the Cγ sequence as a probe, in order to determine by the Southern filter hybridization technique the number of Cγ genes in the rabbit genome.

MATERIALS AND METHODS

1 - Chemicals and enzymes

(^{35}S)-Methionine (1000 Ci/mmole), (α - ^{32}P)dXTP (400 Ci/mmole) and (γ - ^{32}P)ATP were purchased from the Radiochemical Centre Amersham (England). 3'-(α - ^{32}P)dATP (Cordycepin Triphosphate) was obtained from New England Nuclear (Boston, Massachusetts, U.S.A.).

T4 Polynucleotide kinase, terminal deoxynucleotidyl transferase, PstI and EcoRI restriction endonucleases were purified according to published procedures (17-19). T4 DNA ligase, BstNI and PvuII restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Purified reverse transcriptase from avian myeloblastosis virus was obtained from J. Beard (Life Sciences, Inc., S^t Petersburg, FL.), Escherichia coli DNA polymerase I and DNase I from Boehringer (Mannheim, FRG).

2 - Screening of rabbit spleen cDNA library

Poly(A)-containing RNAs were purified from rabbit spleen and rabbit liver up to the sucrose gradient, immunoglobulin RNAs were identified by in

vitro translation, immunoprecipitation and SDS-polyacrylamide gel electrophoresis, as previously described (20). Synthesis of cDNA probes and screening of recombinant clones were performed in conditions previously described (21). Supercoiled DNAs were purified according to Katz et al. (22). The sized plasmid inserts were determined by the rapid alkali method (23).

3 - DNA sequencing

Nucleotide sequence was determined using the partial chemical degradation method of Maxam and Gilbert (24), in conditions previously described (14). Five bases reactions were used (G, G+A, T+C, C, A > C). The products were analysed on 20%, 8% and 6% 0.35 mm thick urea-polyacrylamide gels according to Sanger and Coulson (25).

4 - Southern blot experiments

Preparation of rabbit liver DNAs, restriction endonucleases digestions, agarose gel electrophoresis, transfer to nitrocellulose and hybridization conditions have been reported elsewhere (26).

5 - Construction of the probes

60 µg of plasmid pRγ73 were digested with BstNI and PvuII restriction endonucleases. CH2 and CH3 specific fragments (respectively 97 and 360 bp) were isolated by polyacrylamide gel electrophoresis and electroelution in a dialyzing flange. 1.5 µg of CH2 fragment and 5 µg of CH3 fragment were independently ligated to themselves as reported elsewhere (26). These fragments were nick-translated to high specific activity according to Rigby et al. (27).

RESULTS AND DISCUSSION

1 - Characterization of Cγ recombinant plasmids

We have previously reported the construction of a cDNA library with mRNA isolated from one hyperimmunized rabbit spleen (13). This library was screened for Cγ sequences by differential *in situ* hybridizations with two kinds of probes. Spleen mRNA was enriched in 16S mRNA by a 5-20% sucrose gradient. Each fraction was tested in an *in vitro* translation system and products were immunoprecipitated with anti-a2 antibodies. Fractions containing heavy chain mRNA were then pooled and used to synthetize ^{32}P-cDNA probe. Replicate filters of the colonies were hybridized independently with the γ-rich ^{32}P-cDNA and with ^{32}P-cDNA made from total liver mRNA. Clones giving a very strong signal with the γ-rich probe and no signal with the liver probe were further analyzed by rapid alkali procedure in order to determine the size insert.

Three of them, with inserts ranging from 450 to 1200 bp, were digested with PstI restriction endonuclease and limited nucleotide sequence

was carried out using the partial degradation method of Maxam and Gilbert. Two clones (pRγ49 and pRγ73) contained inserts coding for part of the rabbit Cγ region.

2 - Nucleotide sequence of rabbit Cγ mRNA

Complete nucleotide sequence determination of plasmids pRγ49 and pRγ73 was performed according to the method of Maxam and Gilbert (24) after labeling the restriction sites as shown in Figure 1. The derived amino-acid sequence was compared to the known protein sequence (28-30). The two clones overlie part of the CH2 nucleotide sequence, from amino acid 268 to 340, the entire CH3 sequence from position 341 to 447 and the 3' untranslated nucleotide sequence. When compared to the known protein sequence, there are 8 D/N or E/Q changes. Three uncertain amino acids are determined (at positions 274, 280 and 281) and two amino acids are different (E/G at position 380 and W/Y at position 404). There is an additional Lysine at the carboxyterminal end, which is processed by a post-translational mechanism, as it is reported for the mouse γ chains (34, 15, 16). Furthermore, the CH3 sequence ends with GGTAAA. This sequence is a splice site for the transmembrane immunoglobulin gene segment (31-33). The alanine at position 309 specifies the allotype 15

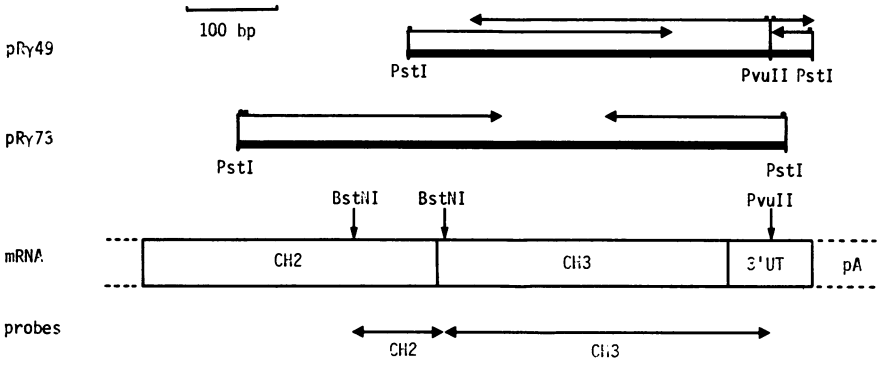

Fig. 1 : Strategy for nucleotide sequencing and hybridization probes.

The plasmid inserts (pRγ49 and pRγ73) and the restriction sites used for sequencing are indicated in the upper part of the figure. The direction and extent of nucleotide reading are represented by horizontal arrows. Filled squares on arrows specify fragments labeled at 3' ends, empty circles indicate fragments labeled at 5' ends.

The different segments of the mRNA are shown in the middle part of the figure : CH2, CH3 = regions coding for the second and third domains of the protein ; 3'UT = 3' untranslated region ; pA = polyadenylic acid.

The fragments used as probes in genomic blot analysis are indicated in the lower part of the figure. CH2-probe and CH3-probe are respectively, BstNI-BstNI and BstNI-PvuII restriction fragments of plasmid pRγ73.

of the e series (30). The 3' untranslated region contains 91 nucleotides and starts with UGA as terminal codon of the coding sequence. Since there are only two A residues in the pRγ49 clone, it is not sure that the entire 3' untranslated sequence is present.

3 - Comparison of the coding sequence of rabbit and mouse Cγ mRNAs

The nucleotide sequence of rabbit γ chain has been compared to the mouse γ1, γ2a and γ2b sequences (14, 15, 16). As shown is Figures 2A and 2B, the coding sequences of mouse and rabbit γ chains can be aligned over 540 nucleotides without introducing any gaps. The percentage of homology between each region of mouse and rabbit γ chains are given in Table 1. At the nucleotide level, the rabbit γ chain sequence appears slightly more homologous to the mouse γ1 domains (76% for CH2 and 69% for CH3). The CH2 domain appears more conserved between species than the CH3, both at the amino acid and nucleotide level. When compared to the mouse γ1 chain, the CH2 domain contains relatively short sequences of high homology (96% between nucleotides 874 and 898, 97% between nucleotides 983 and 1014). 96% of homology is found between nucleotides 898 and 924, when compared to the mouse γ2b. In any case , some nucleotide sequences appear highly conserved between the four γ chains (nucleotides 115 to 163 and 181 to 213). In the CH3 domain, which exhibits greater divergence, the mismatches are scattered throughout the nucleotide sequence . No obvious long region of sequence homology can be found between the four γ chains, except for the last twenty nucleotides of the coding region including the carboxy-terminal Glys-Lys codons.

The comparison of the nucleotide coding sequences between rabbit and mouse γ chains shows that, in the three mouse γ chains, numerous nucleotides are species specific. These nucleotides are indicated under the sequences in Figures 2A and 2B. Furthermore, Table 1 shows that the percent homology between rabbit and mouse γ chains is very similar for each mouse γ subclass. This supports the hypothesis that mouse γ chain genes duplicated after species divergence. However, since the distance between rabbit and mouse γ chains is very similar to the distance between γ1 and γ2a or γ1 and γ2b in the coding region, the divergence of mouse γ1 and precursor-γ2 genes could have occured relatively shortly after speciation.

4 - Comparison of 3' untranslated regions of mouse and rabbit γ mRNA

The 91 nucleotides of the 3' untranslated region of rabbit γ chain present in clone pRγ49 have been compared to the corresponding mouse sequences. Figure 2C shows the alignment of the four sequences. Gaps have been introduced in order to maximize the percent homology. The cloned 3' untranslated rabbit

Figure 2A :

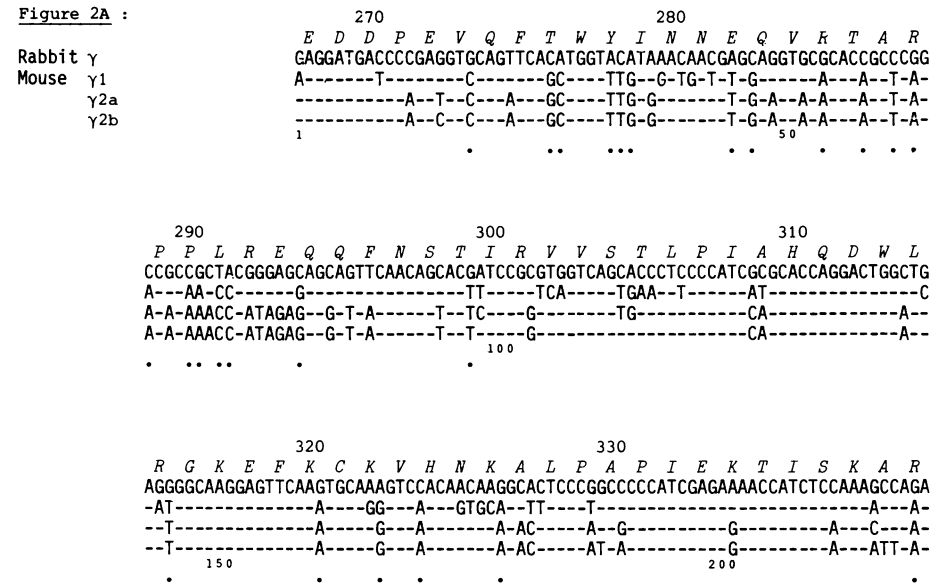

Figure 2B :

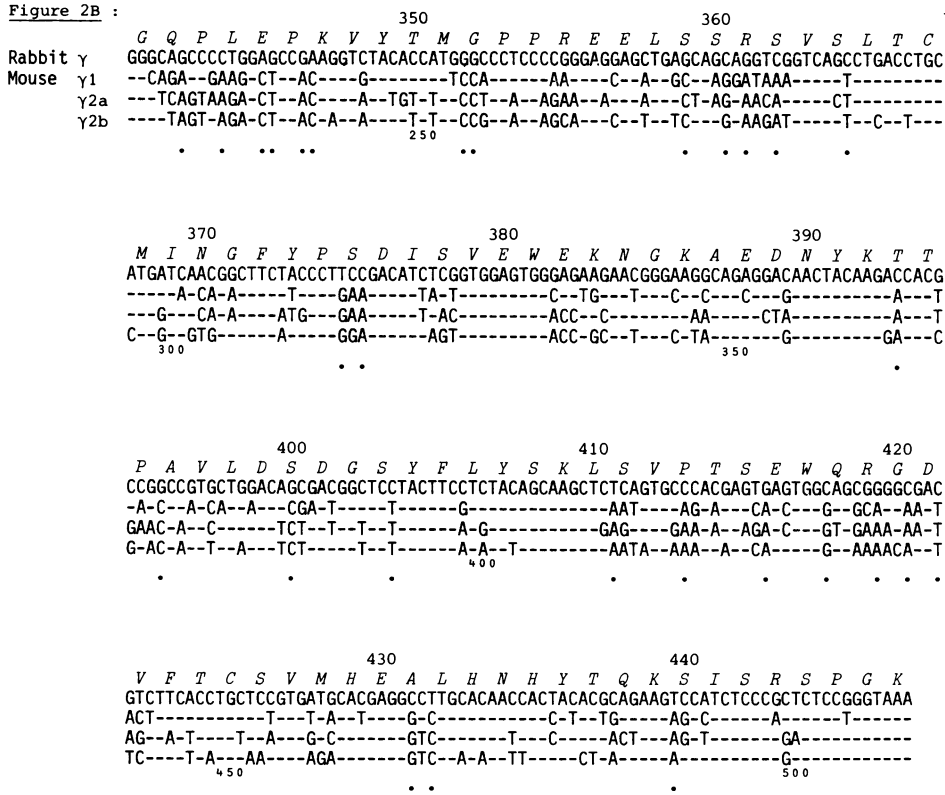

Figure 2C : *Ter*

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                TGAGCGCTGTG CCGGCG AGCTGCCCCTCTCCC   TCCCCCCCACGCCGCAG
Rabbit γ        ---T-C-A---T--TTG- ---    -----GG    ---TA-  -G-A-T-T-ACA
Mouse  γ1       -----T-A-CA --CA-AA-A-- -T-AGG---AAAGAGA-A-----A-T --T
       γ2a      -----T-A-CA --CA-AA---- -T-AGG---TAAGAGA-A-TGG-A-- --T
       γ2b                                                 550

                 CT  GTGCACCCCGCAC   ACAAATAAAGCACCCAGCTCTGCCCTGAA
                C--ACC-C------T-C-TGT-T----------------A-----T--GGAA
                 --CCA---TT---TTGT   -T----------------AA----TG-G-CCATGTAA
                 A-CCA----T---TTGT   -T----------T-----AAA---TG-T-CCATGTAA
                                              600
```

Fig. 2 : Partial nucleotide sequence of the constant and 3' untranslated regions of the rabbit Cγ mRNA and comparison with the mouse Cγ1, Cγ2a and Cγ2b mRNAs.

The nucleotide sequences have been divided into three parts : A = CH2 ; B = CH3 ; C = 3'UT region.

Only the nucleotides which differ from rabbit γ heavy chain are mentionned for mouse γ heavy chains. Homologous positions are indicated by dashes. Gaps have been introduced in the 3' untranslated region in order to maximize the homology. The sequence AATAAA in this region is underlined.

In the upper lines, the amino-acid sequence predicted from the rabbit nucleotide sequence is shown in italic letters by the one letter code (37). Numbering of amino-acids is according to the mouse γ2a heavy chain (15).

sequence is the most homologous, in length and nucleotide sequence, to the mouse γ1 3' untranslated region. However, since it is not sure if the entire 3'UT region is present in clone pRγ49, the percentage of homology between rabbit and mouse γ2a or γ2b regions has been calculated on the total length including gaps, but excluding the last 6 nucleotides (CCATGT) of γ2a and γ2b sequences. The alignments show a 16 nucleotide long region of high homology between the four γ sequences (only one substitution at nucleotide position 601 between rabbit and mouse γ2b sequences). This homologous region is also observed in the case of rabbit and mouse κ mRNA (13) and contains the pentanucleotide AATAAA specific of eukaryotic mRNAs (35).

5 - Genomic blot analysis

In order to carry out γ-gene enumeration in the rabbit genome, DNA of three individual rabbits were analyzed according to the Southern blot hybridization technique (36). DNAs were digested with restriction endonucleases which do not cut in the nucleotide sequence shown in Figure 2. Since the CH2 domain of γ heavy chains appears to be the more conservated between and intra species, we analyzed EcoRI-digested DNAs with a nick-translated CH2-probe, constructed as described in Materials and Methods. This probe contains 87 nucleotides of the CH2 coding sequence and 10 nucleotides of the CH3 coding sequence, between two BstNI sites (Figure 1). Nitrocellulose filter was washed

Table 1 : Comparison of amino-acid and nucleotide sequences in different segments between rabbit and mouse γ heavy chains

	CH2	CH3	3'UT
Rγ/Mγ1	64 / 76	56 / 69	61
Rγ/Mγ2a	64 / 75	55 / 64	59
Rγ/Mγ2b	66 / 75	60 / 65	56
Mγ1/Mγ2a	69 / 78	62 / 71	71
Mγ1/Mγ2b	67 / 77	54 / 67	71
Mγ2a/Mγ2b	95 / 97	61 / 74	89

The sequences were aligned in order to maximize the percent homology (amino-acid or nucleotide identities x 100/ total number of amino-acids or nucleotides compared including gaps). For the γ-CH2 domains, only the amino-acids from position 268 to 340 and the corresponding nucleotides were compared.

The upper part of the squares indicated the amino-acid percent homology, the lower part the nucleotide percent homology.

R = Rabbit ; M = Mouse.

under high stringency conditions, ie. 0.1 x SSC at 65°C for 30 mn, in order to detect only highly homologous sequences. The autoradiogram is shown in Figure 3A. Rabbits 1 and 3 exhibit two EcoRI restriction fragments of 11 and 12.5 kb hybridizing with the CH2-probe. In the rabbit 2, the CH2-probe reveals only one EcoRI fragment of 12.5 kb, whose intensity appears to be approximately twice those of the two others rabbit's bands. Since the same amount of digested

DNA was layed on the agarose gel, the duplicity of the bands in two rabbits could correspond to restriction site polymorphism on parental chromosomes, rather than to duplicate genes. Rabbit DNAs were then hybridized with a CH3-probe containing 311 nucleotides of CH3 coding sequences and 49 nucleotides of 3' untranslated region. The autoradiograms of EcoRI and PstI digested DNAs, hybridized with the CH3-probe, are shown in Figure 3B. The PstI restriction pattern shows two bands of about 3.5 kb for rabbits 1 and 3, and only one more intense band of 3.5 kb for rabbit 2. On the other hand, the three rabbit EcoRI-digested DNAs, hybridized with the CH3-probe, exhibit

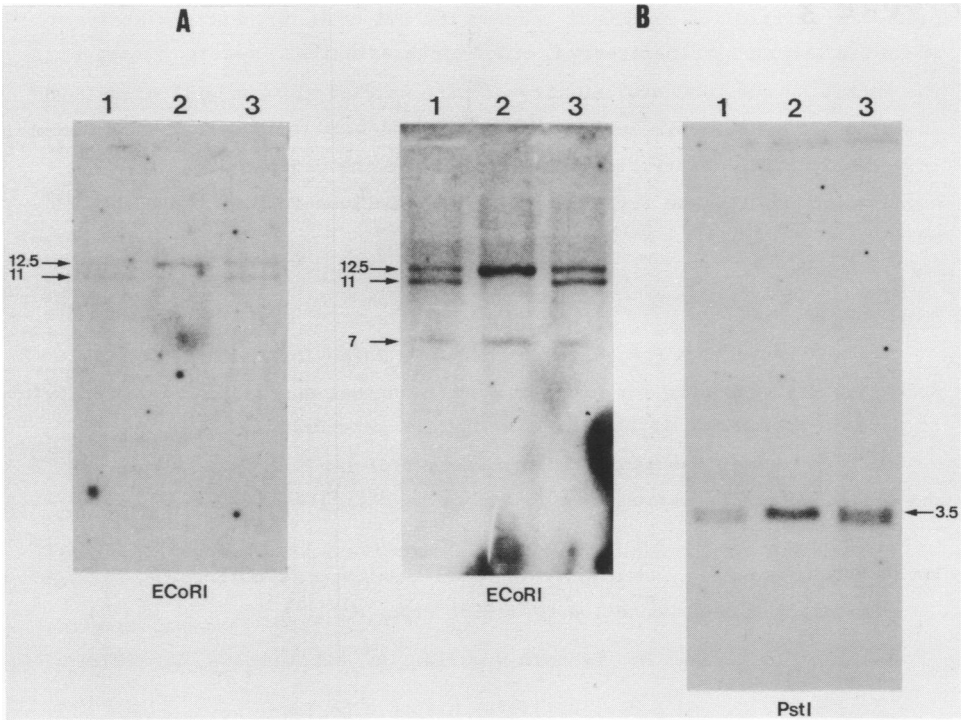

Fig. 3 : Southern hybridization pattern of γ related sequences in individual rabbit DNAs.

25 µg of three individual rabbit DNAs (1, 2 and 3) digested by ECoRI or PstI restriction endonucleases were electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose filters and hybridized with rabbit C -probe. Filters were washed under high stringency conditions (0.1xSSC, 65°C, 30 mn).

Fig. 3A : DNAs were hybridized with a γ-CH2 probe.

Fig. 3B : DNAs were hybridized with a γ-CH3 probe.

Fragments size were estimated from a ECoRI digest of λ bacteriophage DNA run in a parallel channel.

in addition to the 11 and 12.5 kb EcoRI fragments faint band of 7 kb. Since no other protein data are available for rabbit γ chain, we can not determine at this stage to what corresponds this homologous sequence. The absence of an additional faint band in PstI-digested DNA hybridized with the CH3-probe could correspond to the localization of this homologous region on a PstI fragment smaller than 0.5 kb and not detectable by genomic blot analysis. Thus, it appears by genomic blot experiments, that there is a limited number of sequences related to the cloned Cγ mRNA, in the rabbit genome. Under similar conditions (0.2 x SSC, 65°C, 30 mn), Cory et al. have identified with a Cγ2a probe three individual restriction fragments bearing the Cγ2a, Cγ2b and Cγ3 genes in the mouse genome (8). However, they could only detect with a Cγ1-probe the restriction fragments bearing the Cγ1 gene (8). Further analyses at the DNA level are now necessary in order to characterize Cγ related sequences in the rabbit genome. The availability of pure specific probes will allow such investigations. Furthermore, the Cγ probe is being used to isolate V_H-cDNA recombinant in a rabbit cDNA library, in order to study at the genomic level the organization of V_H gene segments and their relationship with the allotypes of the a series.

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