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Immunology

Nucleotide sequence of constant and 3′ untranslated regions of a κ immunoglobulin light chain mRNA of a homozygous *b4* rabbit

(hyperimmunization/spleen/*in vitro* translation/gene cloning)

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ABSTRACT **A homozygous *a2/a2* and *b4/b4* rabbit has been hyperimmunized with *Micrococcus lysodeikticus*. Poly(A)-containing RNA has been isolated from the spleen and translated *in vitro*, and translation products have been analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Double-stranded cDNA has been synthesized from poly(A)-containing RNA template and inserted in the *Pst* I endonuclease site of plasmid pBR322 by using the oligo(dC)·oligo(dG) tailing procedure. Tetracycline-resistant ampicillin-sensitive clones containing cDNA complementary to a κ light chain mRNA have been selected by differential screening and their ability to hybridize to a spleen mRNA having the same size as a mouse κ light chain mRNA. Two clones, pRk-15 and pRk-32, have been selected to determine the nucleotide sequence of the constant and 3′ untranslated regions of κ light chain mRNA, by the Maxam and Gilbert partial degradation method. Comparison of homologous regions of mouse κ chain mRNA and *b4* rabbit κ chain mRNA reveals 61% homology in the constant region and 59% homology in the 3′ untranslated region.**

Serological studies of rabbit immunoglobulins have revealed an important genetic polymorphism called allotypy (1, 2). Structures responsible for allotypic specificities have been located on constant (C) and variable (V) regions of heavy chains and on the C region of light (L) chains (for a review, see ref. 3). Genetic studies on the inheritance of allotypes have shown that these polymorphic forms segregate in a Mendelian fashion, implying that they are encoded by allelic structural genes (4).

Four allotypic forms of the domestic rabbit κ chain have been characterized (5, 6). They are designated by the symbols *b4*, *b5*, *b6*, and *b9*. The sequences of the C_κ region of *b4* and *b9* chains exhibit a surprising divergence; they differ by 35% of their amino acid sequences, and nine two-base substitutions and three gaps are found (7, 8). Furthermore, serological analysis has shown that one heterozygous *b4/b5* rabbit can express three allotypes of the *b* series (*b4*, *b5*, *b6*) (9). The extensive divergence of allelic forms of rabbit κ chains and the occurrence of latent allotypes have led to the hypothesis that *b* allotypes in the rabbit are encoded by multiple closely linked genes regulated by a polymorphic control mechanism (8, 10).

Studying, at the DNA level, the gene segments coding for the rabbit *b* allotypes presents two main difficulties: (*i*) there is no rabbit myeloma cell line; (*ii*) the extensive divergence between rabbit and mouse C sequence (44% homology at the amino acid level) makes uncertain the possibility of screening a rabbit cDNA library with a cloned mouse C_κ probe.

Starting from hyperimmunized rabbit spleen, we have constructed cDNA clones complementary to a rabbit κ chain mRNA. The sequence of the constant and 3′ untranslated regions of a rabbit *b4* κ chain mRNA has been determined and compared to corresponding mouse sequences.

MATERIALS AND METHODS

Enzymes and Chemicals. Terminal deoxynucleotidyl transferase, phage T4 polynucleotide kinase, and restriction endonuclease *Pst* I were purified according to published procedures (11–13). Purified reverse transcriptase RNA-dependent DNA polymerase from avian myeloblastosis virus was obtained from J. Beard (Life Sciences, Inc., St. Petersburg, FL). *Escherichia coli* DNA polymerase I was obtained from Boehringer Mannheim. Other restriction endonucleases (*Ava* II, *Pvu* II) and T4 DNA ligase were purchased from New England BioLabs.

[^{35}S]Methionine (1000 Ci/mmol), α-^{32}P-labeled dCTP, dATP, dGTP, and dTTP (each at 410 Ci/mmol), and [γ-^{32}P]ATP were purchased from the Radiochemical Centre (Amersham, England) (1 Ci = 3.7×10^{10} becquerels). 3′-[α-^{32}P]dATP (cordycepin triphosphate) was obtained from New England Nuclear.

Bacteria and Plasmids. The bacterial strain used for cloning experiments was *E. coli* C600 (r_k^-, m_k^-, *recBC*). The plasmid pBR322 (obtained from H. Boyer) was prepared according to Katz *et al.* (14).

Animals and Antisera. Rabbits (Bouscat Giant) were injected intravenously three times a week with 5 mg of *Micrococcus lysodeikticus* for 12 weeks.

Antiserum against *a2* allotype was prepared as described (15).

mRNA Purification. Total RNAs were extracted from rabbit spleen or rabbit liver, poly(A)-containing RNAs were purified up to the sucrose gradient step, and immunoglobulin mRNAs were identified by *in vitro* translation, immunoprecipitation, and $NaDodSO_4$/polyacrylamide gel electrophoresis, as described (16).

Construction of Hybrid Plasmids and Differential Screening. Synthesis of double-stranded cDNA, construction of hybrid plasmids by oligo(dC)·oligo(dG) tailing in the *Pst* I site of pBR322, transformation of *E. coli* C600, and screening of recombinant clones were performed as described (17).

RNA Blotting Experiments. Poly(A)-containing RNA treated with glyoxal (18) was electrophoresed on 1.5% agarose gels and transferred to diazobenzyloxymethyl (DBM)-cellulose paper, according to Alwine *et al.* (19). Prehybridization, hybridization, and washing conditions were as described (19).

Plasmid DNAs were labeled by nick-translation with *E. coli* DNA polymerase I (20) and [α-^{32}P]dCTP and [α-^{32}P]dTTP at 100 cpm/pg.

DNA Sequence Analysis. The nucleotide sequence of the cDNA inserts was determined by using the partial chemical

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Abbreviations: C, constant region of immunoglobulin chains; L chain, light chain of immunoglobulin; DBM, diazobenzyloxymethyl.

degradation method of Maxam and Gilbert (21) under conditions previously described (22), using thin (0.35 mm) polyacrylamide gels (23) and with one modification: strand separation was performed in 50% (vol/vol) di-methyl sulfoxide) instead of 30% to maximize the denaturation of strands.

RESULTS

***In Vitro* Translation of Rabbit Spleen Poly(A)-Containing RNA.** Poly(A)-containing RNA was isolated from the spleen of a homozygous *a2/a2, b4/b4* rabbit that had been hyperimmunized with *M. lysodeikticus*. It was translated *in vitro* in a mRNA-dependent reticulocyte lysate, and ^{35}S-labeled proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The results in Fig. 1 show that the translation pattern of proteins synthesized in response to rabbit spleen mRNAs resembles that obtained in response to mRNAs from mouse myeloma cells producing a monoclonal IgG, built of γ2a and κ chains. Translation products were immunoprecipitated by the anti-a2 serum. The results (Fig. 1, lane 4) reveal that the 50,000-dalton protein is the precursor of the secreted rabbit γ chain. Translation products having roughly the same size as the precursor of the mouse κ chain have been identified as rabbit κ chain by subsequent cloning experiments.

Identification of Bacterial Clones Containing Rabbit κ Chain cDNA Sequences. κ light chain material seems to be one of the main components of proteins synthesized in the *in vitro* system. The results of RNA blot hybridizations presented in Fig. 2 *Left* show that there is no cross-hybridization between rabbit spleen poly(A)-containing RNA and mouse C_κ probe, under conditions in which rabbit and mouse β-globin sequences cross-hybridize very well (data not shown). To overcome the difficulties made by the limiting amounts of biological material and the very high sequence divergence between mouse and rabbit C region sequences, the following strategy has been used to pick cDNA clones encoding the C region. A cDNA library was constructed from total poly(A)-containing RNA. We synthesized [^{32}P]cDNA probes from 12S rabbit liver RNA and from 12S spleen RNA. Duplicates of the colonies were screened with the two probes, and we selected clones hybridizing only with cDNA complementary to spleen mRNA. Because it can be supposed that rabbit L chain mRNA has the same size as the mouse L chain mRNA, spleen-specific cDNA clones were nick-translated and used in RNA blot hybridization experiments to select those hybridizing to a spleen mRNA of about 1200 nucleotides. Because of the lack of homology between rabbit and mouse C_κ sequences, we mixed mouse C_κ and rabbit cDNA probes. Starting from 50 μg of spleen mRNA, we obtained 2 μg of double-stranded DNA. The efficiency of transformation was 1.2×10^5/μg of vector. Two out of six clones exhibiting the pattern shown in Fig. 2 *Right* were selected for DNA sequence determination.

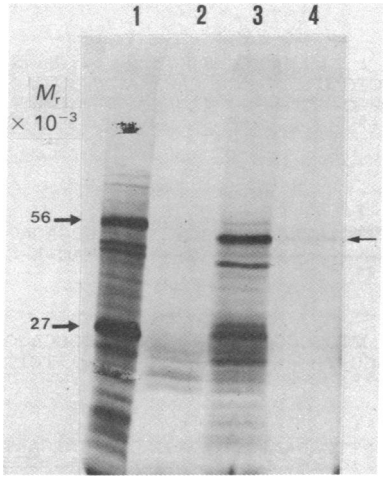

FIG. 1. *In vitro* translation of poly(A)-containing RNA extracted from mouse myeloma cells and rabbit spleen. The translation products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The samples contained in the gel slots were: lane 1, complete assay system plus poly(A)-containing RNA from mouse myeloma cells MOPC173, synthesizing a γ2a and κ IgG; lane 2, complete assay system with no exogenous RNA; lane 3, complete assay system plus poly(A)-containing RNA from homozygous (*a2/a2, b4/b4*) rabbit spleen; lane 4, rabbit spleen translation products immunoprecipitated by anti-a2 antibodies. The molecular weights of the mouse immunoglobulin γ heavy chain (56,000) and κ L chain (27,000) are indicated on the left by arrows.

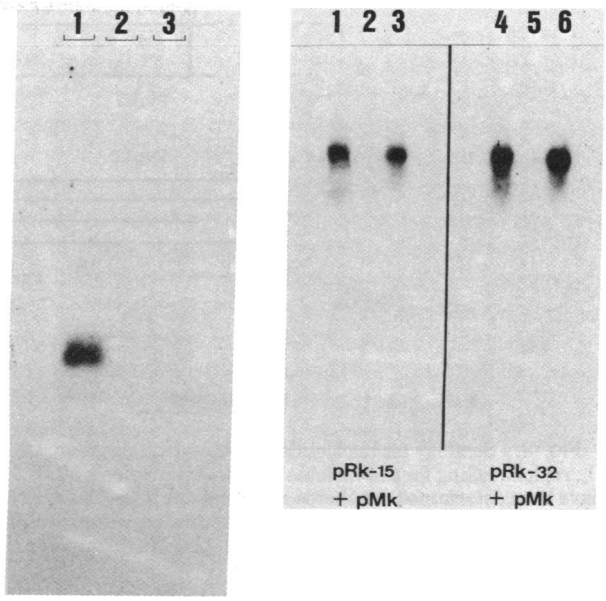

FIG. 2. (*Left*) Absence of cross-hybridization between rabbit and mouse κ immunoglobulin nucleotide sequences. Poly(A)-containing RNAs from rabbit spleen and from mouse myeloma cells were fractionated on a 1.5% agarose gel, transferred to DBM-cellulose paper, and hybridized with a mouse C_κ probe. Hybridization and washing conditions were 30% (vol/vol) formamide/5× standard saline/citrate (1× standard saline citrate = 0.15 M NaCl/0.015 M sodium citrate) at 34°C. Lane 1, 0.5 μg of poly(A)-containing RNA from mouse myeloma cells MOPC173; lane 2, 0.5 μg of poly(A)-RNA from a *a2/a2, b4/b5* rabbit spleen; lane 3, 0.5 μg of poly(A)-RNA from a *a2/a3, b4/b4* rabbit spleen.

(*Right*) Identification of the cloned C_κ fragments by hybridization to rabbit spleen mRNAs. In these experiments, 0.5 μg of each poly(A)-containing RNA was electrophoresed on a 1.5% agarose gel and hybridized under the standard conditions with two nick-translated probes: the mouse C_κ plasmid and one of the selected rabbit plasmids. Lanes 1 and 4, poly(A)-RNA from a *a2/a2, b4/b4* rabbit spleen; lanes 2 and 5, poly(A)-RNA from a *a2/a2, b4/b4* rabbit liver; lanes 3 and 6, poly(A)-RNA from mouse myeloma cells MOPC173; lanes 1–3, hybridization with the rabbit plasmid pRk-15 and the mouse C_κ plasmid (pMk); lanes 4–6, hybridization with the rabbit plasmid pRk-32 and the mouse C_κ plasmid (pMk).

Nucleotide Sequence of the C and 3′ Untranslated Region of a Rabbit C_κ L Chain mRNA. Two cDNA clones (pRk-32 and pRk-15) were used to determine the sequence of mRNA encoding the C and 3′ untranslated regions. The strategy for DNA sequence analysis is shown in Fig. 3 and the results are shown in Fig. 4. The amino acid sequence derived from the nucleotide sequence identifies the cloned gene segments as a light chain gene of *b4* allotype. The first amino acid of the C region corresponds to the amino-acid position 108 (7). The DNA sequence predicts the sequence of a 104 amino acid protein that corre-

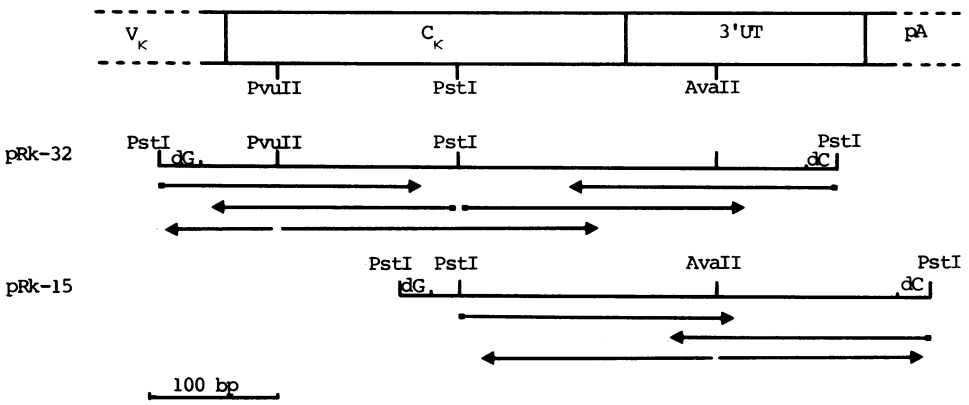

FIG. 3. Strategy for nucleotide sequence determination. On the upper part are indicated the different segments of the rabbit κ mRNA: V_κ and C_κ, regions coding for the variable and constant regions; 3'UT, 3' untranslated region; pA, polyadenylate. The portions whose nucleotide sequences have been determined are shown as continuous lines. The restriction sites used for sequence analysis are in the upper part of the pRk-15 and pRk-32 inserts. The direction and extent of nucleotide reading are indicated by horizontal arrows in the lower part of the inserts. Filled squares on arrows specify fragments labeled at 3' ends. All other fragments were labeled at their 5' ends. bp, Base pairs.

sponds practically to the sequence of the C_κ *b4* allotype determined by Chen *et al.* (7) with one exception, asparagine/aspartic acid at the position 164, and that is identical to the one reported by van Hoegaerden and Strosberg (26). The 3' untranslated region is 182 nucleotides long (208 in mouse, 199 in human) and contains the sequence A-A-T-A-A-A, 17 nucleotides before the poly(A) addition site (27). The sequence of pRk-32 contains part of a J (joining) piece (amino acid 101 to 107). Results on the J_κ gene segments will be reported later.

Sequence Comparison of Mouse and Rabbit C Region Gene. The alignment maximizing the homology between mouse and rabbit C_κ chain gene is presented in Fig. 4. The two sequences exhibit a high degree of divergence in coding sequence: the overall homology is only 61% at the nucleotide level, whereas

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Residue   101                                 110                                 120                                 130
Amino acid T  E  V  V  V  K  G  D  P  V  A  P  T  V  L  I  F  P  P  A  A  D  Q  V  A  T  G  T  V  T
Rabbit    ACCGAGGTGGTGGTCAAAGGTGATCCAGTTGCACCTACTGTCCTCATCTTCCCACCAGCTGCTGATCAGGTGGCAACTGGAACAGTCACC
Mouse                                 -C-GAT-C------A-----ATC-------------T-CAG---G---T-AA--T-----GGT-C-T-A
                                      1         10        20        30        40        50        60

                                    140                                 150
                  I  V  C  V  A  N  K  V  F  P  (  )  D  V  T  V  T  W  E  V  D  G  T  T  Q  T  T  G  I  E  N
                  ATCGTGTGTGTGGCGAATAAATACTTTCCC(  )GATGTCACCGTCACCTGGGAGGTGGATGGCACCACCCAAACAACTGGCATCGAGAAC
                  G-------CT-CTT---C--C-T--AC---AAA--CA---AT----AG---A--A-T-------GTGAA-G-CA--A----G--CT----
                  70        80        90        100       110       120       130       140       150

                  160                                 170                                 180
                  S  K  T  P  Q  N  S  A  D  C  T  V  N  L  S  S  T  L  T  L  T  S  T  Q  V  N  S  H  K  E
                  AGTAAAACACCGCAGAATTCTGCAGATTGTACCTACAACCTCAGCAGCACTCTGACACTGACCAGCACACAGTACAACAGCCACAAAGAG
                  ---TGG--TGAT---G-CAGCAA---CA-C-------G-A-G--------C--C--GT------AGGACG----TG-AC-A--T--CAGC
                  160       170       180       190       200       210       220       230       240

                  190                                 200                                 210
                  V  T  C  K  V  T  Q  G  T  (  )  T  S  V  (  )  V  Q  S  F  N  R  G  D  C  Ter
                  TACACCTGCAAGGTGACCCAGGGCACG(  )ACCTCAGTC(  )GTCCAGAGCTTCAATAGGGGTGACTGTTAGAGCGAGACG(  )CCTGCCAG
                  --T-----TG---CC--T--CAAG--ATCA--T---CC-ATT---A----------C---AA---G--------AC-A-G-T----AG-C
                  250       260       270       280       290       300       310       320       330

                  GGCACCGCCAGTGATCCTGAGG(  )CCCAGCCTCGCCGCT(                  )CCCTCCCCTCAGTGGACCCATTCCCACCACAGTCCTCC
                  -C----A----CTCC--A-CTCCAT--T-T-T--C-TT--AAGGTCTTGGAGG-T-----A--AGC----T-CCA-TGTTG-G--G----
                  340       350       360       370       380       390       400       410       420

                  AGCC(  )CCTCCCCTCC(  )CGGC(  )CCT(  )CACC(  )CCC(  )TCCTTGGCTTTAACCTTGCGAATGTTGGT(  )GAGATGGATGAATAAAGTGAATC
                  -AA-CT-------A--TC-TT-T---C-T--T---TT-----------T-T-A---T---A--T-CA--A-ATAT-C----------G--
                  430       440       450       460       470       480       490       500       510

                  TTTGCACTTGAAA...
                  -----------
                  520       530
```

FIG. 4. Comparison of the C and 3' untranslated sequences of rabbit and mouse κ immunoglobulins. Only the nucleotides that differ from rabbit κ L chain are mentioned for mouse κ L chain. The homologous positions are indicated by dashes. In the upper lines, the amino acid sequence predicted from the rabbit nucleotide sequence is indicated by the one-letter code (24). Ter, chain termination. Numbering of amino acids is according to Chen *et al.* (7) and numbering of nucleotides starts with position 1 at the first position of the constant region (amino acid 108). The mouse κ L chain sequence is from ref. 25. The parentheses indicate the deletions required to maximize the homology. The sequence A-A-T-A-A-A in the 3' untranslated region is underlined.

Table 1. Comparison of nucleotide sequences of rabbit and mouse κ cDNA and rabbit and mouse β-globin cDNA

Segment	A + T / G + C' %	Nucleotides compared (R/M)	Sequence comparison: Gaps	Transitions	Transversions	Total	Similarity index, %
C_κ coding sequence	0.89 (R) 1.06 (M)	309/318	9	55	60	124 (39%)	61
κ 3'UT region	0.64 (R) 0.99 (M)	185/211	26	25	35	86 (41%)	59
β-Globin coding sequence	0.84 (R) 0.75 (M)	441/441	0	49	35	84 (19%)	81
β-Globin 3'UT region	1.5 (R) 1.7 (M)	95/134	41	16	7	64 (47%)	53

R, rabbit; M, mouse; 3'UT, 3' untranslated region. The β-globin data are from ref. 28; the κ mouse sequences are from ref. 25. Gaps are introduced into the compared sequences, as shown in Fig. 4, to maximize the homology. Values in parentheses correspond to percent based on "adjusted sequence" length, which is obtained by adding the number of base pairs and the number of nucleotides in the gaps. The similarity index is 100% − % [(gaps + transitions + transversions)/("adjusted sequence" length)].

there is 81% homology between the coding sequence of mouse and rabbit β-globin (Table 1). The coding sequences differ by three deletions: positions 141, 198, and 202. Of 106 codons compared, 30 are identical. There are 19 silent mutations involving a single base pair change; serine at position 166 is encoded by TCT in the rabbit and by AGC in the mouse. Fifty-three codons differ, 21 resulting from one-base substitutions, 24 from two-base substitutions, and 8 from three-base substitutions.

A sequence of 12 amino acids within a 13 amino acid segment conserved between human and mouse C_κ has been characterized by Hieter *et al.* (25). The human C_κ sequence contains a repeat sequence C-A-G-C-A centered at each of five serine codons in this region and differ from the mouse sequence only by a silent change (25). The analysis of the corresponding region of C_κ rabbit sequence (segment 163–175) does not reveal a special conservation of this sequence: the homology between mouse and rabbit segment is only 67% at the nucleotide level (12 substitutions in 12 codons); there are only three serines instead of five, one using TCT instead of AGC. It can also be noticed that three short regions appear more conserved at the nucleotide level than the whole coding block: 84% homology in the segment 110–119; 79% homology in the segment 170–180; 81% in the segment 203–210 preceding the untranslated region. The same termination codon, TAG, is found in the mouse, human, and rabbit κ chain mRNA.

Sequence Comparison of Rabbit and Mouse 3' Untranslated Region. Fig. 4 shows the alignment of the 3' untranslated regions of rabbit and mouse κ chain mRNA. The overall homology in the 3' untranslated region is slightly lower than in the coding block: 59% versus 61% (Table 1). Alignment shows that there is a large deletion of 12 nucleotides and nine smaller deletions in the rabbit untranslated segment, resulting in deletion of 26 bases. An obvious region of homology of 23 nucleotides, including the hexanucleotide A-A-T-A-A-A, is found preceding the poly(A) addition site. The same degree of homology is found with the corresponding segment of human κ chain mRNA. However, the significance of these homologies does not appear clear, in view of the greater divergence of the corresponding segments in the globin gene family (29).

DISCUSSION

The results reported in the present paper show that the spleen of a hyperimmunized animal can provide an alternative to myeloma cells for synthesizing immunoglobulin cDNA probes. Starting from total poly(A)-containing RNA isolated from a rabbit spleen, C_κ cDNA clones have been isolated. Two aspects of the strategy used to isolate C_κ clones merit further comment, because of their general applicability. Because cloned mouse C_κ probes cannot be used to screen a rabbit library, we have searched for recombinants harboring rabbit C_κ sequences among those exhibiting two properties: (*i*) the ability to hybridize with a [^{32}P]cDNA transcribed from rabbit spleen 12S mRNA but not with a [^{32}P]cDNA complementary to rabbit liver 12S mRNA; (*ii*) the ability to hybridize with a rabbit spleen mRNA having the same size as the mouse κ chain mRNA.

Generally, alleles at a single genetic locus differ only by one or two amino acid positions. Thus, the mouse and human C_κ chain genes that are contained at a single genetic locus show only a few allelic variations (30–33). This situation resembles that observed with human hemoglobin variants. Most of them differ only by one residue. In contrast, closely linked genes resulting from duplication from a common ancestor exhibit more sequence divergence than allelic forms of the gene. This is, for example, the case of the two BALB/c mouse nonallelic β-globin genes. The two β-globin polypeptides differ at 6 of 146 amino acid positions, suggesting that duplication of these genes took place 15–30 million years ago (34).

Serological and structural studies of *b* allotypes have revealed a complex pattern of heterogeneity. Sogn and Kindt have described a $b4^{var}$, which differs from the *b4* sequence by two residues (35). Breeding studies suggest that *b4* and $b4^{var}$ could correspond to alternative forms of a *b4* chain gene at a single genetic locus. Nevertheless, an allotypic specificity of the *b* series appears in fact to be a family of variants of this specificity always found together in a rabbit serum (4, 36). Moreover, three different residues have been characterized at position 172 in homogenous *b4* chains (37–39). Sequence heterogeneity has been reported also in the case of *b6* and *b5* C region (40–42). These observations suggest that a rabbit could have duplicated C_κ genes corresponding to one of the allotypic specificity of the *b* series.

A second pattern of variation is found when the sequences of the different polymorphic forms of the κ L chain are compared. The C regions of *b4* and *b9* L chains differ in 35% of the amino acid positions. Recently, Chersi *et al.* have reported the partial sequence of the C region of a *b5* L chain, which is 77% homologous to *b4* L chain (42). This extensive divergence of rabbit C_κ allotypes, which contrasts with the fact that human and mouse C_κ sequences exhibit few allelic variations, had led to the hypothesis that the rabbit chromosome contains at least four C_κ genes whose expression is regulated by a polymorphic control mechanism (8, 10). The observation that a single rabbit can express three *b* allotypes is consistent with this model. The

availability of pure rabbit C_κ probes should allow examination of the relationship between gene organization and expression of complex allotypes.

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