Volume 11 Number 24 1983 Nucleic Acids Research

# Structural analysis of a rabbit immunoglobulin $\kappa 2$ J-C locus reveals multiple deletions

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Received 29 August 1983; Revised and Accepted 14 November 1983

## ABSTRACT

We previously reported that domestic rabbits harbor at least two DNA sequences that hybridize strongly to immunoglobulin κ C region probes in Southern blots. One of these was cloned from a domestic b4 rabbit and identified as the gene for the nominal b4 allotype κ chain which is expressed at high levels. We now have cloned (from a b4 rabbit) the other homologous sequence and find that it encodes a κ chain nearly identical to the κ2 chain of "bas" rabbits, which is not normally expressed at detectable levels in domestic rabbits. Sequence analysis of this κ2 chain reveals a $J_\kappa$-$C_\kappa$ locus with no obvious coding sequence defects that could explain its low expression. However, several base changes in a putative enhancer region as well as deletions (totalling about 1.5 kb) in the J-C intron might be related to low expression. The comparison between these two κ genes raises questions about the selective pressures operating during the evolution of this gene system.

## INTRODUCTION

Studies of rabbit immunoglobulin κ chains have revealed a complexity in this species that is not found in κ chains of human or mouse. Serologic and chemical studies have shown that rabbits express a series of κ chains known as the "b series" allotypes ($b4^v$, b4, b5, b6 and b9 in domestic rabbits) which appear to be inherited as allelic structural genes. Additionally, the observation of allotypes unexpected on the basis of a rabbit's pedigree - "latent allotypes" - has led to more complex models (1). Besides the b series chains, also known as k1, some rabbits express at very low levels another type of κ chain known as κ2, which apparently represents the product of a second isotypic (non-allelic) gene (2). This isotype was first discovered in the "bas" colony of rabbits (3), in which the ability to synthesize a b series κ1 chain (b9) was lost by mutation.

Rabbit κ chain genes are being studied at the molecular level in an attempt to understand the molecular mechanism regulating the variable

expression of these κ immunoglobulins. We recently described (4) the structure of a cloned genomic κ1b4 gene (previously designated b4Nb4) encoding the b4 allotype κ1 chain. Using probes from this gene, we established by Southern blot analysis that domestic rabbits of five b series allotypes harbor at least two sequences homologous to κ1b4: one (which we arbitrarily called the b4B sequences and which includes the κ1b4 gene) demonstrates restriction sites similar to the cloned b4 gene, and another (called the b4A sequences) has a different characteristic restriction map. Here we describe the complete nucleotide sequence of the $J_{\kappa}$-$C_{\kappa}$ b4A locus cloned from a b4 rabbit. The sequence of the C region is nearly identical to that of a κ2 cDNA clone derived from a bas rabbit (5) and apparently represents a functional κ2 gene. Comparison with the κ1b4 gene demonstrates that the κ2 gene has undergone three substantial deletions, two in the J-C intron and one causing the loss of two J regions. The remaining three J regions all appear functional (in contrast to the κ1b4 locus, where all but one of the five J regions appear defective). By Southern blotting we show that in rabbits of the $b4^{v}$, b5, b6 and b9 allotypes the corresponding b4A sequence has similar deleted organization. The deletions and base changes that we observe in the $J_{\kappa}$-$C_{\kappa}$ intron on this κ2 gene may provide an explanation for the low level of expression of κ2, since the J-C intron is believed to contain an enhancer sequence (6,7) and DNAase I hypersensitivity sites (8) that may play a role in κ gene regulation.

While this work was in progress, Heidmann and Rougeon (9) independently reported partial sequence (C region) of a gene apparently identical to that described here and identified it as the κ2 gene on the basis of homology to the partial bas amino acid sequence of Garcia et al. (10).

## MATERIALS AND METHODS:

### Cloning of the EcoRI fragment containing the b4A sequence

High molecular weight liver DNA (11) from a b4 homozygous rabbit (#6123) was digested to completion with EcoRI and size fractionated by preparative electrophoresis. The fractions were analyzed by Southern blotting (12) and hybridization to a $C_{\kappa}$ probe derived from a κ1b4 cDNA clone (13). DNA from a single fraction containing hybridizing fragments (18 kb) was ligated into λJI (14) EcoRI arms. After in vitro packaging (15) of the ligated material, the resulting phage particles ($3 \times 10^5$ pfu) were screened in situ (16) with the κ1b4 C region probe. Positive clones were characterized by restriction mapping of DNA minipreps (17).

## Sequence determination

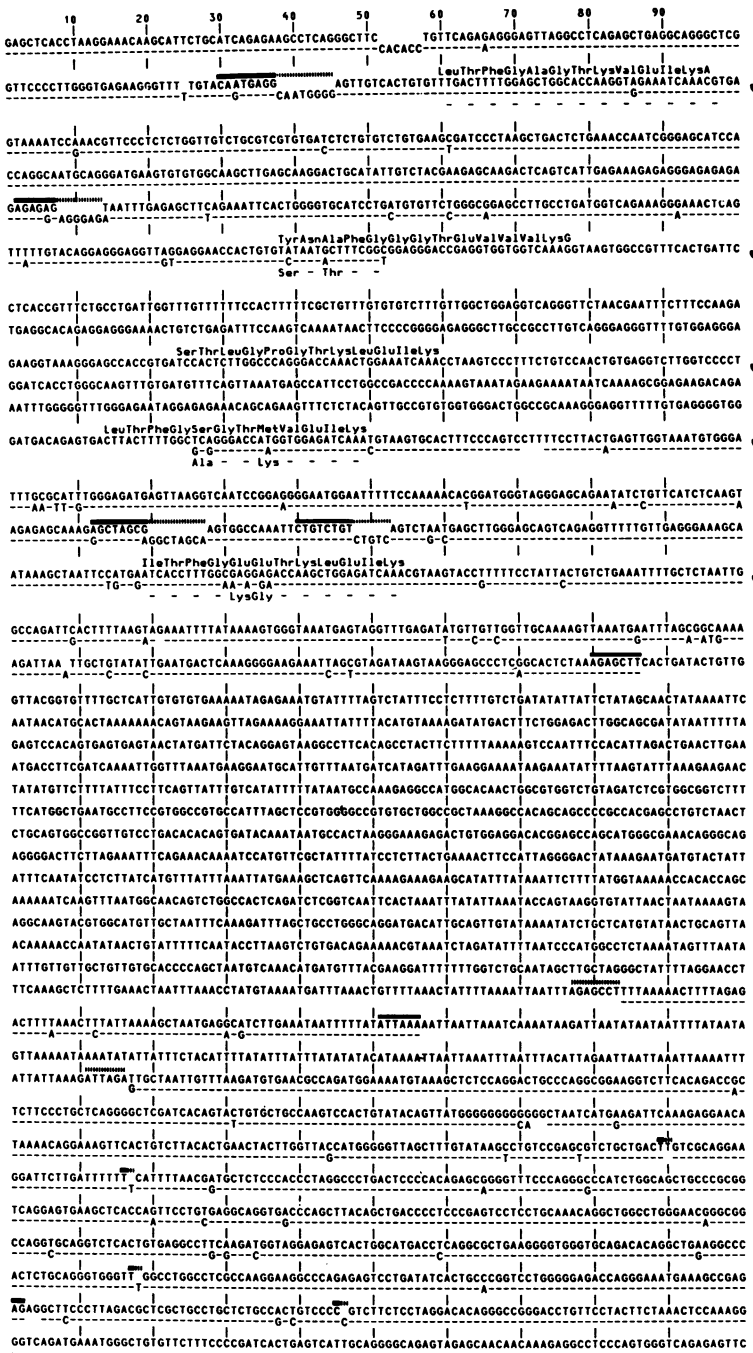
All sequence analysis was done on single stranded DNA derived from fragments subcloned in the M13 phages mp8, mp9, mp10 or mp11 (18). The sequence was determined by the Sanger dideoxynucleotide terminators methods (19) using the M13 sequencing kit from Bethesda Research Laboratories. The Korn-Queen program (20) was used for sequence analysis.

## RESULTS

### Clone isolation

We have previously shown that DNA from a b4 rabbit contains, in addition to the nominal b4 κ gene (κ1b4), a second sequence (b4A) that is strongly homologous to κ1b4 C region probes; this sequence is located on an 18 kb EcoRI fragment (4). To isolate this b4A sequence for characterization, agarose gel-purified 18 kb fragments from a complete EcoRI digest of b4 rabbit DNA were ligated into λJ1 phage arms and packaged. Resultant phages were screened with a κ1b4 C probe, yielding 19 clones that all contained the same 18 kb EcoRI fragment.


Figure 1. Restriction map and sequencing strategies for the entire κ1 and κ2 J-C loci. Restriction maps of the two loci were derived from DNA restriction digests, Southern blots and sequence analysis. Each arrow represents the origin, the direction and the extent of nucleotide sequence of individual M13 subclones determined according to the Sanger dideoxynucleotide terminators procedure. Bold rectangles correspond to the location of the J and C coding regions.


J1
J2
J3
J4
J5

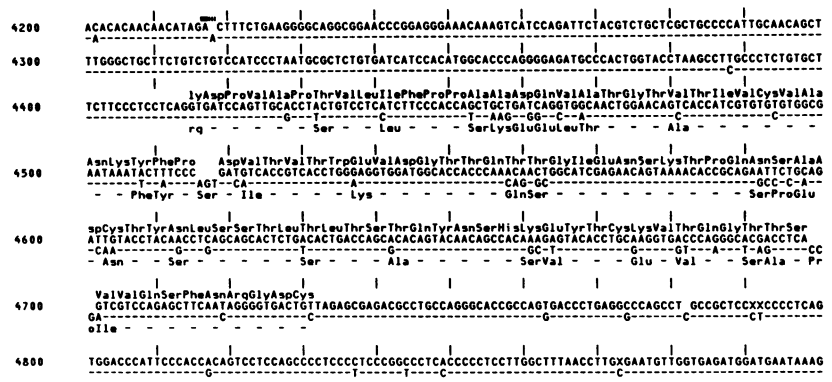


Figure 2. Nucleotide sequence of the κ1 and κ2 genes from a b4 rabbit. The two sequences are presented with the κ1 sequence above, κ2 below. The κ2 sequence is represented by a hyphen when it is identical to κ1 and by the appropriate nucleotides where discrepancies occur. Gaps have been introduced in both genes to maximize homology. The κ1 amino acid translation is given above the κ1 gene sequence; the κ2 amino acid translation is given below the gene only for residues different from those of κ1. In the κ1 translation the Gly at the J-C junction is encoded in part by J2 ("G" of Gly above position 577) and in part by the C region ("ly" of Gly above positions 4415-4416). Similar convention represents the Arg at the κ2 J-C junction. The short direct repeats possibly involved in deletion mechanism are highlighted at the borders of the deleted areas: a continuous line represents the repeat deleted in one of the sequences. Portions of the κ1b4 sequence have been published previously (4, 7).

## Identification of the b4A gene by sequence analysis

Southern blots of restriction digests of the cloned 18 kb EcoRI b4A fragment indicated that sequences homologous to the κ1b4 $J_K$ and $C_K$ probes are contained in a 3.1 kb SstI fragment. This fragment was submitted to nucleotide sequence analysis and, to facilitate comparison with the κ1b4 gene, previous analysis of the latter was extended to complete the J-C intron sequence. The sequencing strategies are presented in Fig. 1 and the resulting sequences are shown in Fig. 2. In Fig. 2 the entire sequence of the nominal b4 locus (κ1b4) is taken as reference, with its amino acid translation indicated above. The sequence of the homologous region from the b4A clone is presented on the line below; dashes indicate nucleotide identity between the two sequences. Similarly, the translated residues of the b4A sequence are indicated (below the nucleotide sequence) only where they depart from the κ1b4 sequence.

This comparison reveals in the b4A locus a sequence that (i) is homologous to the κ1b4 C coding region, (ii) translates to a κ-like protein sequence with no termination codons, and (iii) possesses the identical mRNA

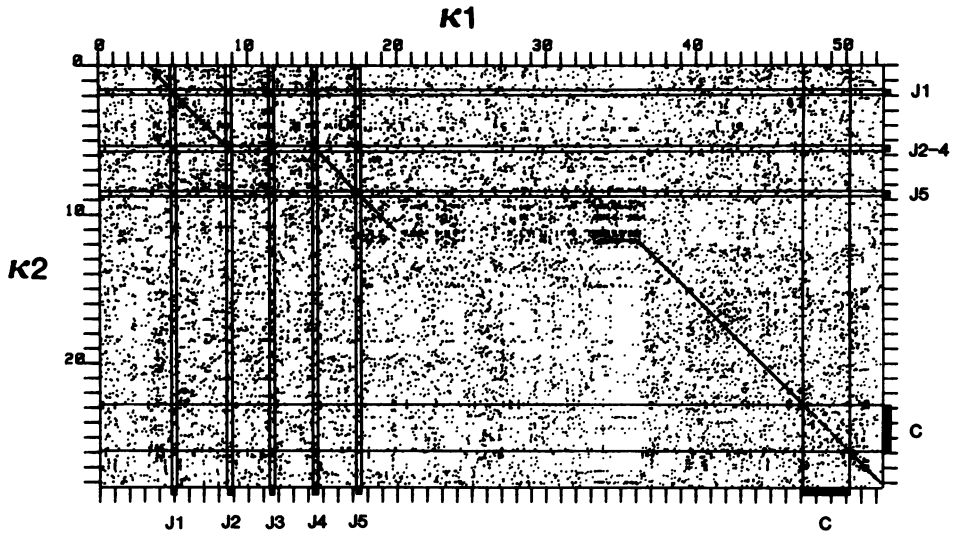



Figure 3. Dot matrix nucleotide sequence comparison of the κ1 and κ2 genes. The computer program used for the comparison has been previously described (29). The κ1b4 sequence is represented on the horizontal axis with κ2b4 on the vertical. Each dot represents a stretch of 5 bp at the indicated position that is identical between the two sequences; a segment of homologous sequence appears as a 45° diagonal line. One division on the axes corresponds to 100 bp. The coding regions are indicated by heavy rectangles on the axes and by reference lines across the display. The three major deletions of κ2 are clearly shown as displacements of the main homology diagonal.

splice acceptor site ("AG..." at position 4413 in Fig. 2) and mRNA polyadenylation signal (AATAAA at position 4894) found in κ1b4; it thus appears to be a potentially functional κ gene. In fact the b4A sequence matches, with a single substitution, the nucleotide sequence of a cDNA clone encoding the bas κ2 immunoglobulin chain (5) and yields a translation consistent with the partial amino acid sequence of a bas κ2 chain determined by Garcia et al. (10). We therefore identify it as the "κ2b4" gene to indicate that it encodes the κ2 isotype and was derived from a rabbit secreting the b4 allotype. If, as current evidence suggests, the κ2 gene is closely linked to the b series κ gene, then the genes for the two isotypes may be inherited as haplotypes, with a particular κ1 and κ2 pair generally found together on the same chromosome. As discussed below, we currently identify the κ2 gene with the Southern blot bands previously designated "b4A" and the κ1 gene (nominal b series gene) with the "b4B" bands.

Our κ2b4 sequence is identical to that reported earlier by Heidmann and

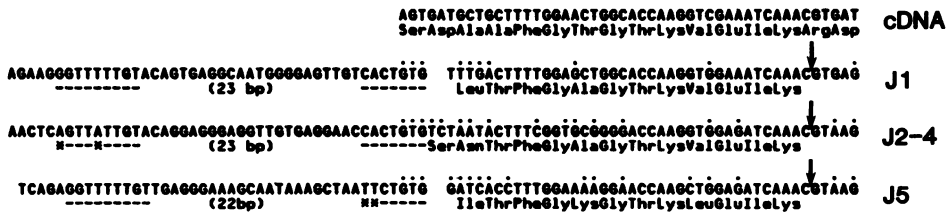



Figure 4. The germline J sequences of the κ2 locus are potentially functional. The three J sequences found in the germline κ2 locus are presented aligned together and with the corresponding region of a bas κ2 cDNA (5). The flanking sequences include the heptamer and nonamer elements thought critical for V-J recombination; these are underlined and the spacing between them indicated. Deviations from the consensus sequence for these signals are indicated as asterisks. The mRNA splice sites are indicated by arrows. Dots above each germline J region represent the nucleotides that depart from the cDNA sequence within the presumed J-derived region of the cDNA clone (codons 97-108); J1 with only one substitution apparently represents the J region used in the cDNA.

Rougeon (9), which they identified as representing κ2 on the basis of similarity to the partial amino acid sequence of Garcia et al. (10).

Deletions in the κ2 gene include part of the J locus

Comparison between the κ1b4 and κ2b4 nucleotide sequences 5' to the C region reveals several large deletions in the κ2b4 gene. This is well illustrated by the dot matrix display of Fig. 3. In this figure the κ1b4 sequence is represented on the horizontal axis, with κ2b4 on the vertical. The three deletions are clearly shown as displacements of the homology diagnoals. Two of the deletions (position 1687-3083 and 3158-3317 of Fig. 2) together elminate about 1.5 kb in the $J_K$-$C_K$ intron. In the J region cluster, another deletion (position 553-1125 of Fig. 2) that apparently resulted from an unequal recombination between J2 and J4 has eliminated the entire DNA segment between these two J regions, including J3; the remaining parts of the J2 and J4 joined together constitute a complete J region. The J1 and J5 regions of κ1b4 have homologous counterparts in the κ2b4 cluster. To highlight the homology with the κ1b4 gene, the J regions of the κ2b4 locus have been designated J1, J2-4 and J5. As in the homologous κ1b4 sequences, these three J regions terminate at their 3' end by the "GT.." mRNA donor splice site and are flanked on their 5' side by sequences homologous to the heptamer and nonamer elements involved in V-J recombination (Fig. 4). In the κ1b4 locus, the J1 and J5 regions may be defective (4) - J1 because of the abnormal length (15 bp) of the spacer between the nonamer and heptamer signal

elements (functional spacers are either 11-12 or 22-23 bp) and J5 because its heptamer sequence departs from the consensus sequence at 4 out of 7 nucleotides. In contrast, the homologous J sequences of the κ2b4 gene do not bear these defects (Fig. 4) and so all three J regions of this gene may be functional.

The J1 nucleotide sequence differs from that of the Bernstein et al. cDNA clone (5) by one substitution (a silent G/C mutation in codon 104, Fig. 4), whereas both J2 and J5 differ from the cDNA by six substitutions (resulting in respectively one and two amino acid replacements); this cDNA thus most likely represents a transcript including J1 and C from a κ2b9 gene that is strongly homologous, though perhaps not identical, to κ2b4.

The b4A κ sequences in $b4^v$, b5, b6 and b9 DNA have deletions similar to those in the κ2b4 gene.

Previous studies (4) have indicated that domestic rabbits of all b series allotypes harbor sequences (the b4A group) with restriction maps grossly identical to that of the κ2b4 gene described here. The deletions observed in the cloned κ2b4 locus prompted us to ask whether the b4A sequences in other rabbits have the same deleted organization, as would be expected from their similar restriction maps. To answer this question, several probes were derived from the κ1b4 gene (map at bottom of Fig. 5) and were hybridized to blots of DNA from the different allotypes. If the organization of the b4A and b4B sequences is the same in each allotype, we would expect the X1 and X2 probes to detect only the b4B sequences in all the DNAs, whereas the J2, IVS and C probes should detect both the b4A and b4B sequences. Indeed, Fig. 5 shows that in each allotype the 3.1 kb SstI band, which in b4 DNA represents the κ2 sequence, is detected by neither the X1 or X2 probe, whereas it is revealed by the C region probe. Other experiments demonstrated that the J2 and IVS probes detected both the b4A and b4B sequences [(4) and unpublished experiments]. From these experiments we conclude that in $b4^v$, b4, b5, b6 and b9 allotypes, the b4A sequences have a similar deleted organization.

## DISCUSSION

### Identification of rabbit κ gene classes

We have cloned from a homozygous domestic rabbit a $J_\kappa$-$C_\kappa$ germline gene with a C region sequence nearly identical to that of a κ2 cDNA clone derived from a bas rabbit. The bas chain has been identified as a second κ isotype (κ2) because it can be detected serologically in some wild rabbits that are heterozygous for b series (κ1) synthesis (2). However, κ2 expression has not

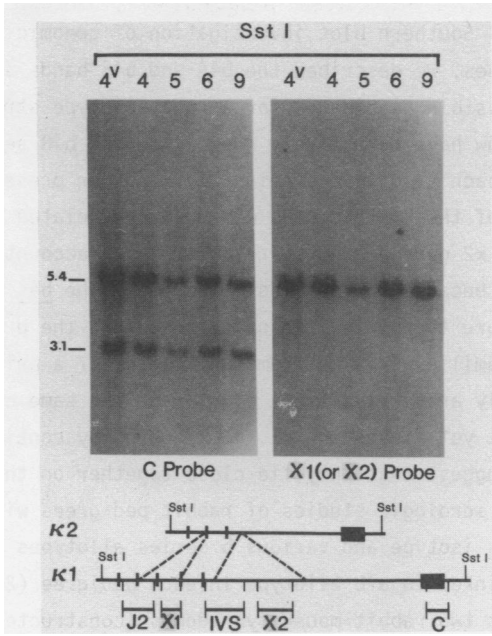



Figure 5. Rabbits of the b4$^{V}$, b5, b6 and b9 pedigrees each contain sequences with a deleted organization similar to that of the κ2b4 gene. SstI-digested DNA samples from homozygous rabbits of each allotypic strain were electrophoresed in parallel in an agarose gel and transferred to a nitrocellulose filter. The blots were then hybridized to the X1, X2 or C probes (as indicated under each panel), which were constructed from the κ1 gene as indicated in the map at the bottom of the figure. The other probes shown in the map (J2 and IVS) were used in similar experiments employing different restriction enzymes (data not shown). The blots probed with X1 and X2 appeared identical except for a faint trace of the 3.1 kb band detected in all DNAs by the X1 probe, presumably because of a small segment (about 28 bp) of J region sequence which was present in this probe. The probes were as follows: J2 - 575 bp Sau3A fragment; X1 - 228 bp Sau3A to AvaII; IVS - 568 bp Sau3A fragment: X2 - 490 bp PstI fragment; C - 342 bp PvuII to AvaII.

been reported in domestic rabbits, so the discovery of a κ2 gene with no apparent defects in a domestic b4 rabbit is of interest. Do all rabbits possess a κ2 gene even though they may not express it in amounts detectable by the usual anti-bas serologic assays? It is notable that we have detected Southern blot bands with properties similar to κ2b4 in DNA from domestic rabbits of all the major b series allotypes. We suggest that these bands (which we previously designated b4A sequences) may represent κ2 genes in these rabbits because (i) they have restriction patterns for EcoRI and SstI identical to that of κ2b4 and (ii) they show evidence of at least the largest two of the deletions that we find in κ2b4 (Fig. 5).

In our original Southern blot investigation of genomic DNA from rabbits of different allotypes, we described the b4A and b4B bands and noted that they represented possible candidates for latent allotype structural genes (4). However, we now have tentatively identified the b4B sequences as the nominal κ1 gene in each allotypic strain (21), and our present paper supports the identification of the b4A bands as κ2 sequences related to the bas phenotype. The κ2 gene products are unlikely to account for latent allotype expression because the original studies on the bas κ chain emphasized its failure to react with antisera against the b series allotypes.

In most gene families arising from duplication of a primordial gene, the members of the family are arrayed in a cluster on the same chromosome. Although we have not yet linked the κ1 and κ2 genes by contiguous clones, two lines of evidence suggest that they lie close together on the same chromosome. First, serologic studies of rabbit pedigrees with antisera specific for the bas isotype and various b series allotypes indicate that the $bas^+$ phenotype is linked to a b allotype in each pedigree (2). A second line of evidence concerns two rabbit-mouse hybridomas (constructed in our laboratory)
that secrete rabbit κ chains but have no detectable rabbit chromosome on karyotype analysis (22). Since the rabbit κ chain genes in these hybridomas are presumably located on a small fragment of rabbit chromosome translocated onto a mouse chromosome, it is significant that both hybridomas demonstrate Southern blot bands representing κ1 and κ2 genes (13, and unpublished results); these genes thus must lie near enough to each other to be found on the same small chromosome fragment. Close linkage of κ1 and κ2 would justify our view of κ1-κ2 haplotypes in which a given κ1 gene would generally be found in association with a particular κ2 gene.

Molecular evolution of the rabbit κ genes

Comparision of the κ1b4 and κ2b4 loci reveals several results of the evolutionary history of these genes. Numerous "segmental mutations" (i.e. insertions or deletions) of varying sizes are found throughout the sequenced regions of the two genes. Theoretically, segmental mutations observed in a comparison between two genes could have occurred by an insertion in one sequence or a deletion in the other. In the present case, availability of the human κ gene sequence over much of the relevant region allows the conclusion that the three major segmental mutations in this locus, and many of the smaller ones, are in fact deletions rather than insertions. This follows from the presence in the human gene of sequences homologous to many

of those segments deleted in the rabbit κ1 or κ2; it is clearly implausible that such homology could have occurred as a result of independent homologous insertions in both the human and rabbit genomes. (Corresponding comparisons of the rabbit sequences with the mouse κ locus instead of the human are less informative because of the lower overall homology between rabbit and mouse intron sequences.) Most of the deletions occurred by the eliminations of DNA between short duplicated sequences, with one copy of the repeat remaining after the deletion (see horizontal bars in Fig. 2 that mark these repeats). Similar repeats have frequently been seen associated with deletions in prokaryote and eukaryote DNA and have been explained by a mechanism involving "slipped mispairing" during DNA replication (25,26,27).

In contrast, the large deletion that eliminated J3 and the two introns between J2 and J4 may have occurred by an unequal crossover event between the homologous J2 and J4, since the ends of this deletion are probably within the region of homology represented by the J coding sequence. The exact crossover point cannot be ascertained, but the 5' part of the J2-4 in κ2 most closely resembles J2 while the 3' part resembles J4. A "slipped mispairing" involving J2 and J4 as repeats cannot, however, be ruled out for this deletion.

It seems worth noting that of the segmental mutations that occurred after the κ1-κ2 divergence, all three large ones (159, 572 and 1397 bp) appear to be deletions in κ2 while 9 of the 13 smaller sequential mutations (1 to 8 bp) are nominally deletions in κ1. If this difference is significant it could reflect some asymmetry in the mechanism of the mutation events, e.g. some fateful cellular abnormality (in the germline of a rabbit ancestor) that produced the three large deletions in the κ2 locus at one time; however, this idea is clearly pure speculation.

The base substitutions evident between the κ1 and κ2 genes present a striking puzzle: significantly more substitutions are seen within the coding regions than in the non-coding regions. The percent nucleotide discrepancy between the two genes in the C coding regions is 17% while it is only 3% in the 110 bp immediately 5' of the C region and 4% in the 3' UT. A similar clustering of mutations in coding vs non-coding regions was observed by Sheppard and Gutman (28) in a comparison of rat κ alleles, whereas in most other comparisons between homologous genes, coding sequences are more highly conserved than flanking regions. A second unusual feature observed in the comparison of κ1 vs κ2 and (more strikingly) in the comparison between the two rat alleles is the low frequency of silent mutations. Between the κ1 and

κ2 C-coding regions, 54 nucleotide changes in 40 codons have resulted in 31 amino acid changes, while between the rat κ alleles, 12 base substitutions determine 11 amino acid differences. In contrast, most comparisons between homologous genes show higher frequency of silent than replacement changes. These observations suggest that natural selection has favored either sequence conservation at the nucleic acid level (perhaps on the basis of secondary structure or binding properties) or diversification of the C region amino acid sequences (conceivably as adaptation to alterations in the heavy chain structure). Since it is not known how long ago the κ1 and κ2 sequences diverged from their common ancestor, it is unclear whether the non-coding sequences have been unusually strongly conserved or the coding sequences unusually vigorously diversified.

Low expression of κ2b4

In rabbits expressing b series allotypes, the level of expression of the κ2 gene is normally very low and often undetectable. Indeed the rabbit whose DNA was the source of our κ2b4 clone expressed no detectable κ2 in serum (J. Roland, personal communication). As we have seen, no obvious defects in the J or C region genes can account for the decreased κ2b4 expression. However, recent studies suggest that other elements in the $J_K$-$C_K$ locus might be necessary for maximal gene expression. Two sites of DNAase I hypersensitivity, possibly reflecting active chromatin structure, have been described in murine myeloma cell lines expression κ light chains (8). One of these sites, located about .7 kb 5' of the C region gene, is in the approximate position of an enhancer sequence which has been hypothesized in the $J_K$-$C_K$ intron (6). We have identified by nucleotide sequence analysis of the mouse, human and rabbit $J_K$-$C_K$ introns a segment of DNA highly conserved among the three species (which we called KICR, for kappa intron conserved region) and we have proposed that this segment may correspond to the enhancer element of the $J_K$-$C_K$ intron (7). Comparison of the nominal b4 κ1 gene with the κ2 gene in this region (position 3631 to 3756 in Fig. 2) reveals only five point mutations. One of these changes (A/G at position 3665) occurs at a position which is conserved within the three species and which falls in a portion of the KICR homologous to proposed viral enhancer elements. The second DNAase I hypersensitivity site (8) was positioned at about 1.7 kb 5' of the C region gene in the murine $J_K$-$C_K$ intron. Comparison of the rabbit κ1b4 gene with the murine gene shows that in the rabbit the homologous position would map in a region that was deleted in the κ2 gene.

Clearly, present evidence is insufficient to attribute the low levels of

κ2 chains observed in rabbit sera to regulatory defects of κ2 gene transcription, since other explanations remain possible. These include: (i) defects in the κ2 V region genes (i.e., a κ2-specific set of V regions with few active genes); (ii) problems of mRNA processing, transport or stability; or (iii) instability or poor function of the κ2 protein. Nevertheless, the substitutions we find in the κ2 KICR or the deletions in the $J_{\kappa}$-$C_{\kappa}$ intron may affect κ2 gene expression; functional studies with contructs from the J-C intron should reveal the importance of these features.

ACKNOWLEDGEMENTS

We would like to thank K. Bernstein and R. Mage for their data on the bas cDNA sequence, Dorothy Trinh for expert technical assistance, John Evans and Jacob Maizel for providing the dot matrix display and Virginia Shaw for her expert and patient editorial assistance.

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