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κ-chain allotypes and isotypes in the rabbit: cDNA sequences of clones encoding *b9* suggest an evolutionary pathway and possible role of the interdomain disulfide bond in quantitative allotype expression

(immunoglobulins/κ light chain genes/variable regions/joining regions/constant regions)

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ABSTRACT **The constant regions of rabbit κ light chains are unusual because the sequences of the allotypic forms can differ more from each other than do some variable regions with which they associate. We report the nucleic acid sequence of a full-length cDNA clone of *b9* allotype and show comparisons to available sequences of the rabbit κ allotypes *b4*, *b5*, and *bas-N4*. Our analyses suggest that the primordial rabbit κ gene encoded a *bas*-like sequence. They also reveal a surprising difference in the position of the variable region cysteine that forms the interdomain disulfide bond that is unique to most rabbit κ chains. One *b9* cDNA sequence lacks the usual cysteine-80 and instead encodes cysteine-108, which in three-dimensional models appears capable of forming the interdomain disulfide bond with cysteine-171 in the constant region. A partial sequence of a second *b9* clone encodes both cysteine-80 and cysteine-108; the translation product of this clone could have a free reactive sulfhydryl group that might lead to an unstable nonfunctional Ig molecule. The fact that pre-B cells with *b9* κ chains do not differentiate and expand into productive Ig-producing cells with frequencies comparable to the other allotypes may be explained if a substantial proportion of the gene products have a free sulfhydryl group. Our sequence results suggest that in cells differentiating to produce κ light chains of *b9* allotype the number and location of the cysteines influence immunoglobulin expression.**

We have undertaken cDNA cloning and sequence analysis to define the κ genetic region (*b* locus) and to study the regulated expression of κ light chains in the rabbit. There are four common rabbit κ allotypes: *b4*, *b5*, *b6*, and *b9* (1–3). Recently, a Basilea strain of rabbit has been developed that expresses low amounts of *bas* κ light chains and exhibits compensatory production of λ light chains (4). Experiments from four laboratories indicate that *bas* is an isotypic form of rabbit κ (*K2*) (5–10). These and earlier observations of latent (unexpected) allotype expression have led to proposals that rabbit κ allotypes are in fact isotypes whose expression is regulated in allelic fashion (11–14). Regulated expression of κ allotypes is also evident in heterozygous rabbits where serum levels of the κ allotypes follow a "pecking order" (*b4* > *b5* ≥ *b6* > *b9* > *bas*) (15). The pecking order appears to be established after the stage when DNA rearrangements necessary for κ gene expression have occurred because equal numbers of pre-B cells expressing *b9* and (e.g.) *b5* are found in heterozygous rabbits (16). We report the sequence of a full-length *b9* cDNA clone and compare both the nucleic acid and deduced amino acid sequences with sequences of *b4* (17), *b5* (18), and *bas-N4* (5, 10). We will refer to this *bas* gene sequence and its potential protein product as *bas-N4* because it was isolated from the DNA of a rabbit with nominal allotype *b4*. Our results have given us new insights into the evolutionary relationships of the constant region κ (C_κ) allotypes. An unexpected finding is the difference in the location of the κ variable region (V_κ) cysteine that forms the interdomain disulfide bond that is unique to most rabbit κ light chains. In addition, our sequence analysis has revealed that some *b9* mRNAs encode light chains with a free cysteine; the translation product of such mRNA may not readily form functional Ig molecules. Our data raise the possibility that the number and location of cysteines in the V_κ and κ joining region (J_κ) influence differentiation and clonal expansion of B cells, resulting in low expression of the *b9* κ light chains compared to the other "allelic" *b* allotypes. Recent sequence analysis of a Basilea κ light chain cDNA in our laboratory also suggests a possible relationship of protein structure to limited expression (19).

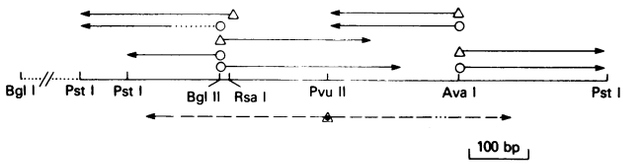

FIG. 1. Strategy of sequence analysis of clones pκb9-17D9 (solid lines) and pκb9-9G12 (dashed lines). Arrows and lines indicate direction and length of sequence obtained from sites 3′ labeled using the large fragment of DNA polymerase I and [α-^{32}P]dNTP (○) to fill in, 3′ labeled with [^{32}P]ddATP (●), or labeled 5′ using [γ-^{32}P]ATP and polynucleotide kinase (△). Dots indicate portions of fragments that were not resequenced.

MATERIALS AND METHODS

A cDNA library was constructed from splenic mRNA isolated from a *Trypanosoma equiperdum*-infected rabbit (CW247-4) of *b9* κ light chain allotype (20). Clones were identified by colony hybridization, screening with nick-translated *Pst* I fragment of pκb5-F2, a cDNA clone encoding *V* and *C* regions of *b5* allotype (18). Eleven positive clones were chosen at random and two containing large inserts (pκb9-17D9 and pκb9-9G12) were selected for sequence analysis by the method of Maxam and Gilbert (21). The strategy for sequence analysis of pκb9-17D9 is shown in Fig. 1. Confirmatory partial sequence of pκb9-9G12 was obtained on both strands from the *Pvu* II site allowing us to read through the *Ava* I site in the *C* region.

Sequence comparisons were made by using the National Biomedical Research Foundation's database and computer programs. After sequences were aligned to maximize homologies, the matrix of mutation differences was used to estimate the structure of a rootless evolutionary tree (22) and

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Abbreviations: V_κ, J_κ, and C_κ, variable, joining, and constant regions of κ light chain; bp, base pair(s); CDR, complementarity-determining region; FR, framework.

from additional information a proposed rooted tree was constructed (see *Discussion*).

RESULTS

Fig. 2*a* shows the nucleic acid and deduced amino acid sequence of pκb9-17D9. The V_κ sequence was compared to that of our *b5* cDNA clone pκb5-F2 (18) and to a homologous *b4* protein sequence 311 (24) (Fig. 2*b*). A gap has been inserted in CDR1 to allow for alignment with the 311 protein sequence. The deduced protein sequence of a second *b9* cDNA clone (pκb9-9G12) and the 311 light chain have a CDR1 of similar length. The leader and first two framework regions of the two cDNA sequences are highly conserved. In contrast, FR3 contains a region of variability (positions 77–81) where five amino acid differences result from four one-base changes and a single two-base change. The cysteine (TGT) at position 80 in pκb5-F2 is replaced with an arginine (CGT) in pκb9-17D9. However, pκb9-9G12 encodes cysteine at this position and a partial *b9* protein sequence (4153-1) was reported with a cysteine at position 80 (29, 30). The cysteines at positions 23 and 88 remain invariant.

Although a portion of pκb9-17D9 J_κ, *b5* J_κ, and *b4* J_κ (*J2*) (25) have considerable homology, the 3′ portions are very different (Fig. 2*c*). A codon for cysteine occurs in pκb9-17D9 at position 108; that is the site of the J_κ–C_κ RNA splice. The nucleic acid sequence of the second *b9* cDNA clone, pκb9-9G12, differs from the J_κ of pκb9-17D9 at 14 of 39 positions (36%) but also encodes a cysteine at position 108. There have been no published *b9* protein sequences that include the J_κ.

The nucleic acid and deduced amino acid sequence comparisons of the rabbit C_κ are shown in Fig. 3. Table 1 lists the percent homologies of the different allotypes at the nucleic acid and protein levels. We used the sequence of the *bas-N4*

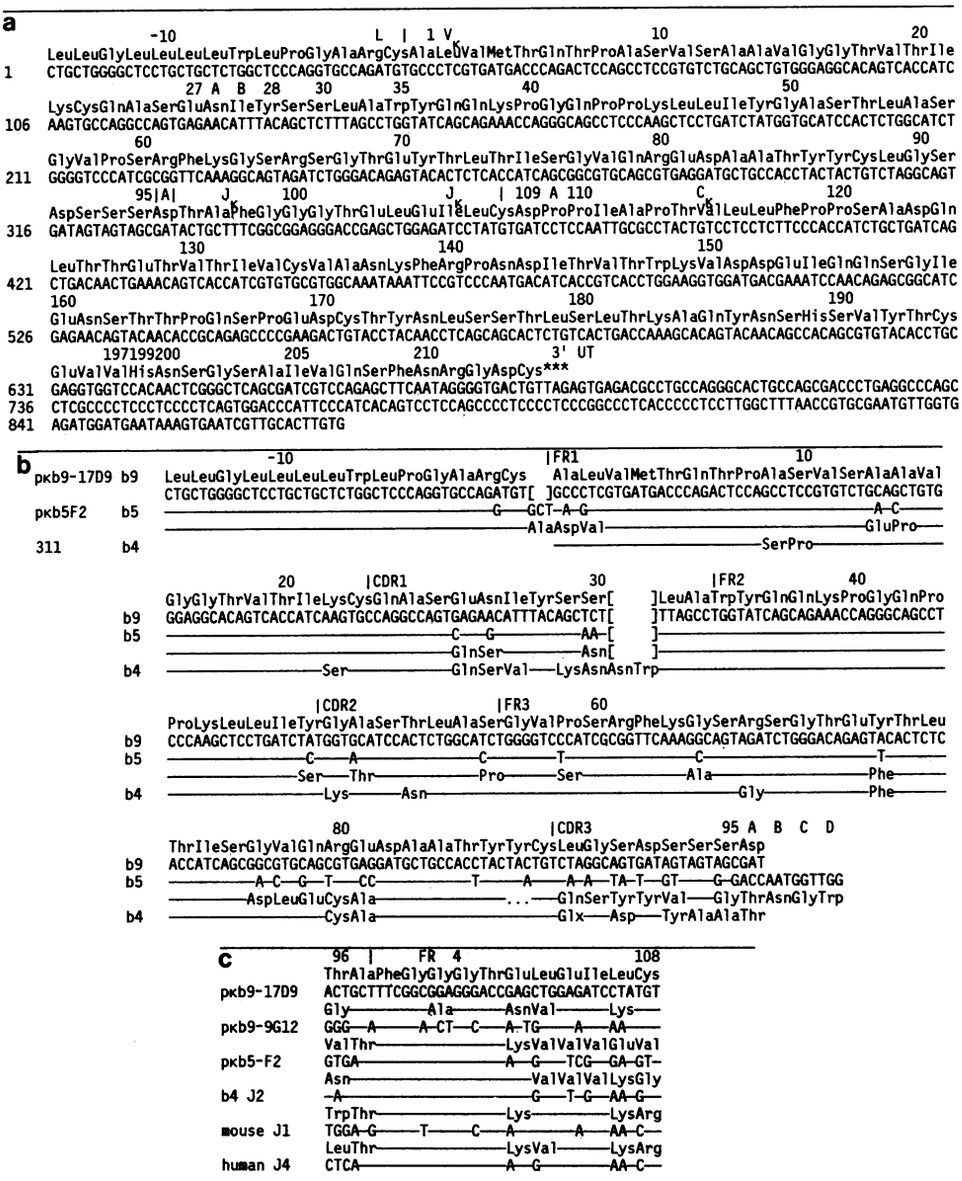


FIG. 2. (*a*) The complete DNA and deduced amino acid sequences of cDNA clone pκb9-17D9. The bases are numbered along the left margin and amino acids are numbered above by using the standard system of Kabat *et al.* (23). We have designated leader (L), V_κ, J_κ, C_κ, and 3′ untranslated (UT) regions. (*b*) Comparison of the DNA and deduced amino acid sequences of pκb9-17D9 in the leader and *V* region with a comparable *b5* cDNA sequence (pκb5-F2) (18) and a light chain protein of *b4* allotype (311) (24). The . . . at position 87 of the *b5* amino acid sequence reflects the TAA stop codon found in this cDNA. We have designated framework (FR) and complementarity-determining (CDR) regions. (*c*) Comparison of the DNA and deduced amino acid sequence of the *J* region of pκb9-17D9 with a second *b9* cDNA sequence (pκb9-9G12), a *b5* cDNA sequence (pκb5-F2), the expressed *J2* of *b4* genomic DNA (25), and genomic mouse *J1* (26) and human *J4* (27, 28) genes.

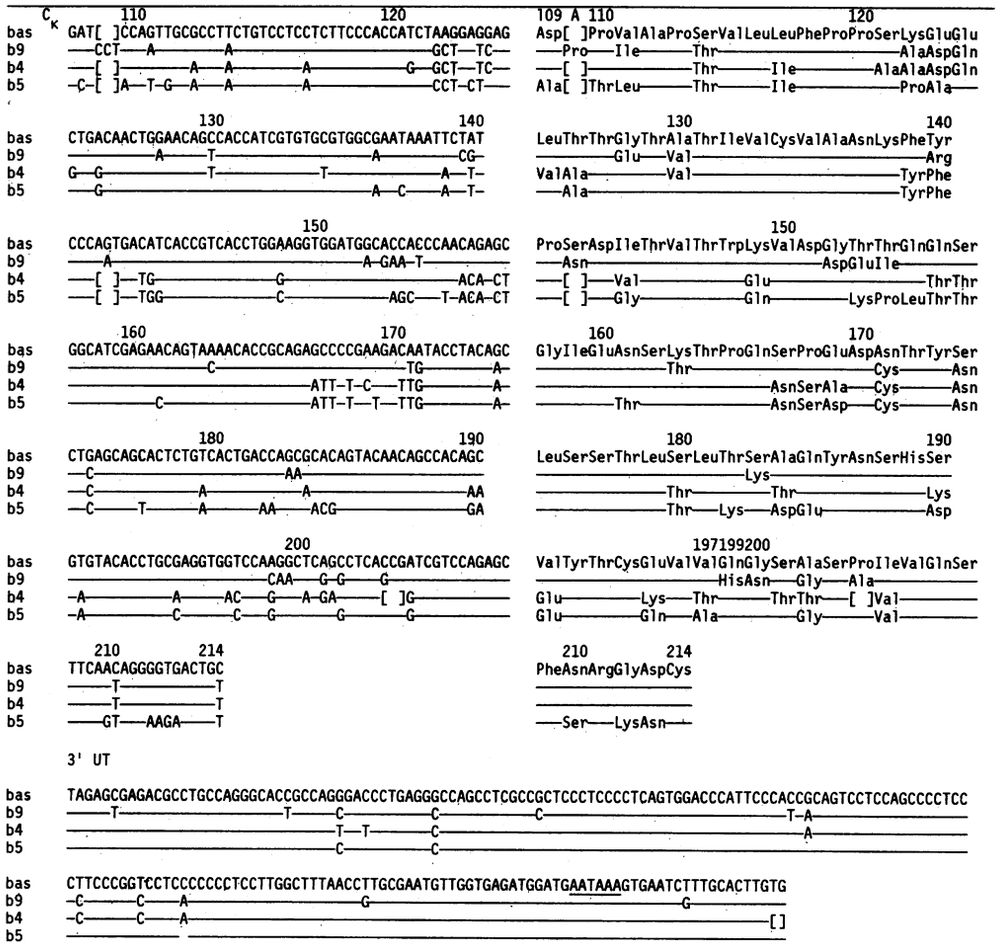


FIG. 3. Comparisons of the DNA and deduced amino acid sequences of rabbit κ light chains of *bas-N4* (5, 10), *b9* (pκb9-17D9), *b4* (17), and *b5* (pκb5-F2) (18) types. Across the bottom, the DNA sequences of the 3′ untranslated (UT) regions of each type are shown and compared. The polyadenylylation signal sequence is underlined. Lines signify identity to the first (*bas-N4*) sequence.

as our prototype because a pattern emerged that suggests an evolutionary pathway from a duplicated ancestral *bas*-like gene (see *Discussion*). Whereas the intradomain disulfide bonds are conserved among the different species, only Lagomorph κ chains have evolved a third disulfide bond linking the V_κ and C_κ domains. The *b9* sequence encodes a cysteine (TGT) at position 171 as do the *b4* and *b5* sequences; however, the *bas-N4* C_κ sequence (5, 10) encodes an asparagine (AAT) instead. Of the 315 nucleotides represented in the *bas* sequence, 229 positions are conserved among all of the allotypes. Only 14 positions differ in each of the allotypes, 10 involving identical substitutions. At 22 additional sites *b4* and *b5* have identical substitutions, whereas *b4* and *b9* share 4 and *b5* and *b9* only 3 additional identical substitutions. Of these sequences, *b9* is most similar to *bas* (80% amino acid, 89% nucleic acid homologies), followed by *b4* and then *b5* (Table 1). When the *b9*, *b4*, and *b5* *C* region sequences are compared with *bas-N4*, 78–80% of all of the codons with differences have replacement changes. In addition, changes in the first, second, and third positions of the codons occur with similar frequencies.

Table 1. Comparisons of DNA and amino acid sequences of rabbit light chain *C* regions of different allotypes

	C_k, % amino acid homology			
	b4	*b5*	*b9*	*bas*
b4		73	68	70
b5	86 (96)		59	65
b9	83 (96)	79 (94)		80
bas	84 (96)	80 (98)	89 (94)	
	C_k, % nucleic acid homology (3′ UT)			

Numbers shown in parentheses are the % homologies of the nucleic acid sequences of the 3′ untranslated (UT) regions.

There are length differences in the C_κ of the different allotypes. The *b9* contains an extra codon at the start of the C_κ. The placement of the gaps to maximize homologies of the four sequences can vary, depending upon the alignment of the nucleotides or amino acids. Light chains of *b4* and *b5* allotype lack an additional codon at position 142 in the alignment shown; *b4* also lacks a codon at position 204. A gap has been inserted at position 198 in each rabbit sequence according to the numbering system of Kabat *et al.* (23). The cysteines at positions 134 and 194 that form the intradomain disulfide bond remain constant between the allotypes, as does cysteine-214, which forms the interchain disulfide bond. Interspersed within the conserved areas of *C* region sequence are highly variable regions, which can potentially contribute to the allotypic determinants.

The sequence of the 187 nucleotides of the 3′ untranslated region of pκb9-17D9 is highly homologous to other published 3′ untranslated regions of *b4*, *b5*, and *bas* (96%, 94%, and 94% homology, respectively) (Fig. 3, Table 1). *bas* and *b5* sequences have one gap each to maximize homologies.

DISCUSSION

In this paper we report the sequence of a cDNA clone encoding rabbit κ light chain of *b9* allotype (pκb9-17D9). The de-

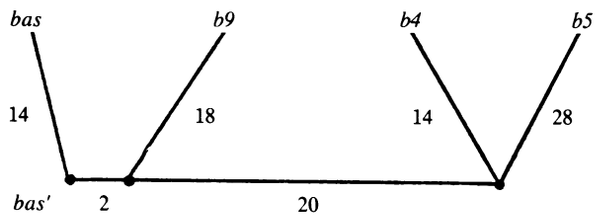
duced amino acid sequence of the *b9* cDNA clone is very similar to the published *b9* C_κ protein sequence (31). The amide differences at positions 128 and 142 are most likely due to protein sequence analysis errors, as are the missing valines at positions 191 and 197. The complete C_κ nucleic acid sequence reported here has been confirmed in the second *b9* cDNA clone pκb9-9G12. The Ala(Gly,Ser) reported by Farnsworth *et al.* (31) at positions 202–204 was most likely incorrect, as both *b9* cDNA clones encoded Gly-Ser-Ala at these positions. Some *b9* V_κs contain an allotype-correlated glutamic acid at position 16 (32); however, pκb9-17D9 encodes the alternative glycine.

We have compared both the nucleic acid and deduced amino acid sequences of pκb9-17D9 to those previously obtained for *b4* (17), *b5* (18), and *bas-N4* (5, 10). Whereas the V_κs of the *b5* and *b9* sequences shown differ by 12% of their nucleotides, the C_κs of *b5* and *b9* differ by 21%, representing a difference of 41% in amino acid sequence. In contrast to the C_κ diversity, the sequences of the 3′ untranslated regions are highly conserved among the different types (94–98% homology). The significance of this sequence conservation has been discussed previously with regard to the possible function of this region (5, 33, 34). The wide divergence in C_κ sequences suggests that there may be some selective pressure favoring replacement changes.

Our data support the existence of separate J_κs for the different allotypes. In the two *b9* clones subjected to sequence analysis so far, two different J_κs have been found. A unique expressed J_κ has been found to date in *b5* proteins and cDNAs (18, 35) that differs from the five genomic J_κ genes associated with *b4* C_κ (25) and the three associated with *bas-N4* (10). It is evident in comparing the rabbit CDR3 and J_κ that there are large length differences. Whether this is due to the different lengths of the coding regions of V_κ or J_κ (or both), to different splice sites between the two regions, or to an additional diversity-generating mechanism (36, 37) is not known.

The availability of the C_κ nucleic acid sequences for four of the rabbit κ allotypes has enabled us to search for possible evolutionary relationships between them. These analyses reveal a high degree of sequence conservation between *bas-N4* and each of the other allotypes. Because only 14 of the 315 *bas-N4* positions differ in each of the allotypes—at all other positions, at least one allotype has the same nucleotide as *bas*—we propose that the primordial Lagomorph κ gene had a *bas*-like sequence. The following observations lend support to this premise: (*i*) In general, the *bas-N4* sequence is more homologous to the C_κ of mouse (66%) and rat (67%) than are the other κ allotypes (61–63%). (*ii*) *bas-N4* κ, as well as human, mouse, and rat, lack the cysteine at position 171 that forms the interdomain disulfide bond that is unique to rabbit κ. (*iii*) *bas-N4* has been shown to be an isotypic form of rabbit κ, although all rabbits may not express this common gene.

We used the difference matrix method to derive a rootless evolutionary tree that is independent of evolutionary rates (22). We then derived the following rooted evolutionary tree that shows a proposed ancestral *bas* gene (*bas′*) that differs from modern *bas* at the 14 positions that are also different in *b9*, *b4*, and *b5*:



Although the rootless form is a unique solution consistent with the mutational differences, the proposed rooted tree is not the only solution (for example, an ancestral 4′ is tenable). However, because *bas* is an isotype, duplication of the primordial *bas* gene could have allowed the rapid accumulation of mutations in the evolving *b9*, *b4*, and *b5* (and *b6*) genes. The gain of cysteine-171 (AAT-TGT) in the *b* allotypes could have occurred after duplication of the primordial *bas* but before the evolution of the separate allotypes. The 8 additional identical substitutions in *b9*, *b5*, and *b4* could also represent mutations that occurred early after duplication; alternatively, they could represent mutations in the modern *bas* compared to the primordial sequence. The occurrence of 22 additional identical substitutions in the *b4* and *b5* sequences suggests that these sequences diverged after the *b9* divergence. However, these sequences may not have evolved strictly through classical evolutionary pathways; rather, they may have undergone accelerated diversification by mechanisms akin to gene conversion (38, 39) that appear to have rapidly generated differences between members of other gene families (40, 41).

The *b9* sequence reported here lacks the normal cysteine-80 and instead has a cysteine at position 108. We have examined the three-dimensional structure of the homologous mouse κ protein MOPC 603 (42) to locate the α-carbons involved in the disulfide bonds. Analysis of the refined model

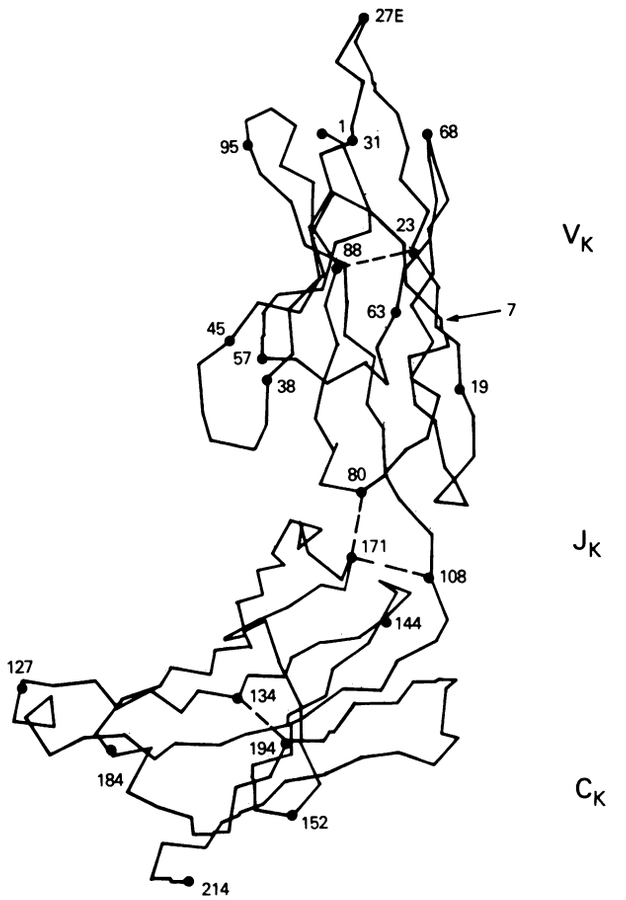


FIG. 4. The α-carbon backbone of the κ light chain of the Fab fragment of mouse myeloma protein MOPC 603 deduced from its x-ray crystallographic structure (ref. 42; Y. Satow, G. H. Cohen, and D. R. Davies, personal communication) is shown with some key amino acid positions numbered according to the standard nomenclature of Kabat *et al.* (23). Dashed lines connect the α-carbons of positions 80 and 171 and of positions 108 and 171. These appear to be alternative locations for disulfide bonds in different rabbit κ light chains. The α-carbons of the cysteines that form the invariant intradomain disulfide bonds are also connected by dashed lines.

at 2.7 Å resolution for MOPC 603 (Y. Satow, G. H. Cohen, and D. R. Davies, personal communication) shows that cysteine-80 and cysteine-108 are both in sufficient proximity to cysteine-171 to form the interdomain disulfide bond (Fig. 4). In this model, the cysteine-80 to cysteine-171 and cysteine-108 to cysteine-171 α-carbon distances are $\approx$7.3 Å and $\approx$7.0 Å, respectively, while cysteine-80 to cysteine-108 are separated by $\approx$10 Å. The α-carbons of the cysteines that form the V (positions 23–88) and C region (positions 134–194) intradomain disulfide bonds are $\approx$6.5 Å and $\approx$6.6 Å apart.

Two populations of κ light chains (K_A and K_B) have been described on the basis of their chromatographic separation following oxidative sulfitolysis. Most *b4*, *b5*, and *b6* κ chains are of K_B type, whereas most *b9* κ chains are of K_A type (43). It was suggested that the difference in the susceptibility to oxidative sulfitolysis of *b9* K_A and K_B light chains resulted from a difference in position of the interdomain disulfide bond (44). A bond between cysteine-80 and cysteine-171 may indeed be less readily oxidized and thus form a more stable globular light chain. Because most V_κs from *b4*, *b5*, and *b6* rabbits have cysteine-80, it may have resulted from a selectively favored mutation that was expanded or spread by gene conversion-like events (38, 39) through many pre-existing genes. In *b9* rabbits, the majority of expressed light chains are of the K_A type (43, 44)—that is, we believe, the population bearing the cysteine-108 to cysteine-171 disulfide bond. Their expression appears to depend upon available V_κs that lack cysteine-80 or conceivably undergo somatic mutations (e.g., TGT-CGT). Likewise, the expression of the minor K_B population is dependent upon both available V_κ genes encoding cysteine-80 and J_κ genes lacking cysteine-108, either in the genome or lost via somatic mutations.

A second *b9* clone (pκb9-9G12) encodes both cysteine-80 and cysteine-108. If, as is likely, this second clone is representative of a significant proportion of the *b9* mRNA, a light chain translation product would be synthesized with an extra free cysteine. If the cysteine that forms the more thermodynamically stable molecule bonds with cysteine-171, the remaining cysteine with its free sulfhydryl group may make this molecule unstable or reactive (45). It is well documented that a pecking order exists in *b* allotype expression (*b4* > *b5* $\geq$ *b6* > *b9*). However, in pre-B cells of heterozygous rabbits, equal numbers of cells expressing *b5* or *b9* light chains are found, suggesting that selection does not occur at the level of DNA rearrangement, but rather that during differentiation of the pre-B and early B cell, maturation of cells secreting *b5* is favored (16). Could it be that the free sulfhydryl group in the populations of *b9* molecules with both cysteine-80 and cysteine-108 is the source of the selective disadvantage due to molecular instability or reactivity of the free sulfhydryl group? We think that our studies have not only revealed an evolutionary pathway of development of the rabbit κ allotypes but also offer a plausible explanation for the relatively low expression of *b9* κ light chains based upon their structure.

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