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# Partial Amino Acid Sequence of a Rabbit Immunoglobulin Light Chain of Allotype b5†

Hammadi Ayadi, Sophie Dutka, Pierre Paroutaud, and A. Donny Strosberg*

ABSTRACT: The amino acid sequence of a rabbit immunoglobulin light chain of allotype b5 has been nearly completed. A comparison of its structure with that of light chains of allotypes b4, b6, and b9 confirms that the constant regions of these various κ chains differ by 20–35%. The substitutions are clustered in parts of the second half of the chain, and the b5 form bears more resemblance to the b6 chain than to any other, in good agreement with previous serological data. The analysis of the variable region reveals the existence of certain allotype-associated residues which have also been reported in other b5 chains, but not in proteins of the other allotypes. An examination of the rabbit light chain sequences between positions 96 and 107 suggests that this portion of the chain may be encoded separately by a joining "J" DNA segment, as has been described previously for murine and human immunoglobulins. In the rabbit, however, these $J_\kappa$ regions appear to differ from one allotype to another. Together with the extensive variations of the constant regions, these data suggest that the rabbit κ gene organization more closely resembles the murine λ system (four different $C_\lambda$ genes each flanked by its J segment) than the murine κ system (a single $C_\kappa$ gene).

Serological studies have documented the existence of four major domestic rabbit κ light chain allotypes: b4, b5, b6, (Oudin, 1960), and b9 (Dubiski & Muller, 1967). Breeding studies suggest that these allotypes are the products of codominant allelic structural genes.

Amino acid sequence studies have revealed amino acid substitutions in the light chain variable region ($V_L$)[1] which seem to correlate with the allotypes: statistical differences in the expression of particular N-terminal sequences in light chains of different allotypes (Waterfield et al., 1973) have been confirmed by the analysis of individual chains, mostly of allotype b9 (Fraser et al., 1978).

Sequence studies of the light chain constant region ($C_L$) indicate between 20 and 35% differences between chains of allotypes b4, b6, and b9 (Zeeuws & Strosberg, 1975; Farnsworth et al., 1976; Emorine et al., 1979). A small number of substitutions appear to distinguish constant regions of the same allotype (Sogn & Kindt, 1976; Strosberg et al., 1974; Emorine et al., 1979). Data for the b5 allotype have been

† From the Laboratory of Molecular Immunology, Institut de Recherche en Biologie Moleculaire, CNRS–Université Paris VII, 75251 Paris Cedex 05, France. *Received March 8, 1982.* This work was supported by grants from the Centre National de la Recherche Scientifique (Bases Structurales de l'allotypie LP 000601) and from the Délégation Générale de la Recherche Scientifique et Technique (Immunologie des Allogreffes 79.70504).

[1] Abbreviations: $V_L$, variable region of light chain; $C_L$, constant region of light chain; HPLC, high-pressure liquid chromatography; DTT, dithiothreitol; IAA, iodoacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PITC, phenyl isothiocyanate; TFA, trifluoroacetic acid; DABITC, (dimethylamino)azobenzene isothiocyanate; Gdn·HCl, guanidine hydrochloride; TPCK, tosylphenylalanyl chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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obtained only recently (Chersi et al., 1980; Emorine et al., 1979) and suggest that b5 and b6 are more similar to each other than to either b4 or b9, in good agreement with earlier predictions from the serology (Landucci-Tosi et al., 1975).

In the present work, we wish to extend these previous findings and to report variations among b5 chains of various origins. We discuss the implications of our findings of variable region allotype association and constant region polymorphism in view of the recent findings of the molecular biology of immunoglobulin genes.

## Experimental Procedures

### *Materials*

TPCK-treated trypsin was obtained from Worthington. V8 (*Staphylococcus aureus*) protease was obtained from Miles Laboratories.

### *Methods*

*Isolation of IgG.* Immunoglobulins of restricted heterogeneity with κ light chain b5 allotype were prepared from rabbits hyperimmunized by three weekly injections of strain III pneumococcal vaccine (Chen et al., 1973); serum was analyzed by cellulose acetate microzone electrophoresis (Beckman cell, Model R-200).

IgG was purified by ion-exchange chromatography on DE52 (Whatman) in 0.0175 M phosphate buffer, pH 6.3. Homogeneity of the IgG was verified by isoelectrofocusing on polyacrylamide gels in 6 M urea.

*Preparation of κ Light Chains.* Twenty milligrams of lyophilized IgG was dissolved in 20 mL of 0.5 M Tris-HCl, pH 8.2, containing 7 M guanidine hydrochloride and 30 mg of DTT and incubated at 37 °C for 3 h. The fully reduced Ig was then radioalkylated at 0 °C by addition of 50 μCi of [$^{14}$C]IAA for 10 min. Alkylation was completed by the addition of 114 mg of IAA and incubation continued for an additional 20 min. This solution was dialyzed against 5 M guanidine and fractionated on Sephadex G-100 in 5 M guanidine hydrochloride to obtain light chains. The purity of light chains was verified by $NaDodSO_4$–polyacrylamide gel electrophoresis.

*Enzymatic Digestion. (A) Tryptic Digestion.* Tryptic digestion was performed on 20 mg of κ light chain in 1% $NH_4HCO_3$ by adding TPCK-treated trypsin dissolved in $10^{-5}$ M HCl at a ratio of 1% (w/w). After 3 h the digest was resolved on a Sephadex G-25 superfine column equilibrated in 1% $NH_4HCO_3$.

*(B) Staphylococcus aureus Protease Digestion.* κ light chains were digested at 37 °C for 18 h in 50 mM $NH_4HCO_3$ (pH 7.8). The ratio of enzyme to substrate was 1% (w/w). The digest was resolved as the tryptic digestion.

*Peptide Separation.* Peptides were further purified by gel filtration on Bio-Gel P6 and P10 equilibrated with 1 M $NH_4HCO_3$ and/or by high-pressure liquid chromatography (HPLC) on a $C_{18}$ μBondapak (Waters Associates) column using a linear gradient of acetonitrile for elution.

Homogeneity of the peptides was verified by two-dimensional electrophoresis/chromatography on microcrystalline cellulose sheets and by N-terminal determination using the manual Edman degradation technique.

*Sequence Determination.* Manual sequencing and automated sequencing were used in this work. In the sequenator the following systems were employed: 0.33 M quadrol, 5% PITC, conversion with TFA. The phenylthiohydantoin amino acid derivatives were identified by HPLC (Zeeuws & Strosberg, 1978). In the manual sequence, we used the procedure previously described by Chang et al. (1978) using DABITC, (*N,N*-dimethylamino)azobenzene 4′-isothiocyanate. Each step of the degradation of radioactive peptides was counted in an Intertechnique liquid scintillation counter.

## Results

Rabbit 2699, homozygous for allotype b5, responded to hyperimmunization with *Pneumococcus* strain III by the production of large amounts of antibodies of restricted heterogeneity. IgG was prepared on DEAE-cellulose, and the purity was analyzed by electrofocusing. The light and heavy chains from fully reduced and alkylated immunoglobulins were separated by gel filtration on Sephadex G-100 in 5 M Gdn·HCl. The purity of the b5 light chains was verified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$). The N-terminal sequence of the whole light chain was determined by automated Edman degradation and the first 54 amino acids residues were identified (Figure 1); a limited heterogeneity was revealed at a number of positions. For determination of the rest of the sequence, light chains were digested respectively with trypsin and V8 protease, and peptides were separated and purified by gel filtration followed by high-pressure liquid chromatography.

*Analysis of Tryptic Peptides.* Tryptic peptides from the digestion of 20 mg of light chains were separated on Sephadex G-25SF. Four fractions were obtained: T1 to T4 (Figure 2). Fraction T1 was further resolved on P6 and P10 Bio-Gel: three peptides were isolated, and their origin in the intact light chain was determined from their composition and the homology of their sequence with that of previously sequenced rabbit chains. Peptide T1a (positions 64–82) was sequenced up to position 73; its composition indicated the presence of two radioalkylated cysteines. Peptide T1b (positions 23–39) was sequenced automatically; the first residue of this peptide was a radioactive sulfocarboxymethylcysteine, and the 13th residue was a tryptophan residue, corresponding to tryptophan-35, characteristic of all rabbit κ light chains. Peptide T1-5 (positions 104–130) corresponded to the end of the J region and to the beginning of the C region. The radioactivity of the 31st step confirmed the presence of the radioalkylated cysteine at position 134 in the C region. This peptide was sequenced manually until position 111 and automatically until position 130. Peptide T3 was entirely sequenced automatically and positioned between positions 163–182. The radioalkylated cysteine at position 170 which is characteristic of rabbit $C_\kappa$ light chains and joins C to V through the cysteine at position 80 was identified both by radioactivity at this position and by HPLC. Fraction T4 was further resolved on HPLC. The fact that T4 was the last fraction eluted from the Sephadex G-25 superfine column suggested that T4 was composed of small peptides which could be separated and purified by HPLC. Peptides T4-2 (positions 209–211), T4-3 (positions 40–45), and T4-5 (positions 97–103) were isolated and entirely sequenced both manually and automatically. Peptide T4-2 contained the C-terminal radioalkylated cysteine (position 211) which links the light to the heavy chain. It was identified by radioactivity. T4-7 (positions 46–63) was sequenced manually and automatically. At position 50, we identified a serine residue as found in b6 chains instead of arginine as usually seen in b4 or glycine as in b9 chains. Peptide T4-8 (positions 139–162) was isolated and entirely sequenced automatically.

*Analysis of V8 Proteolytic Peptides.* The V8 proteolytic digest was resolved on HPLC, and three important peptides were obtained (Figure 3): E6, E7, and E11. Peptide E6 (positions 160–190) was isolated and entirely sequenced. We confirmed the presence of lysine at position 162 and the cysteine at position 170. Peptide E7 (positions 191–211) was

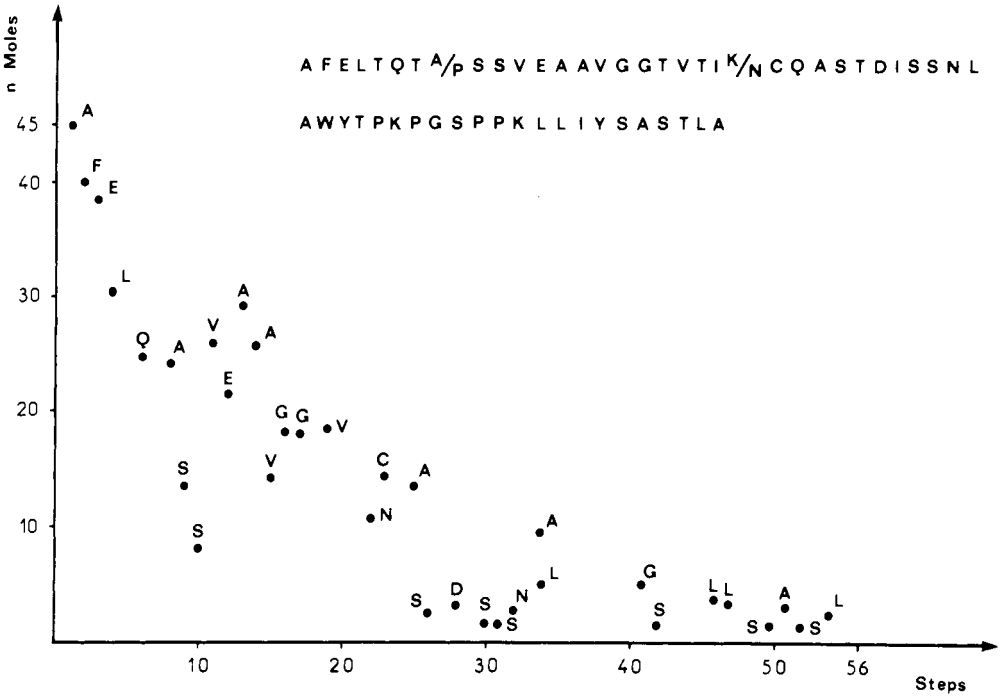


FIGURE 1: Automated Edman degradation of fully reduced and carboxymethylated light chain L2699. Two alternative residues have been found at positions 8 and 22. Repetitive yields at every step are presented for all amino acids except threonine, proline, and lysine.

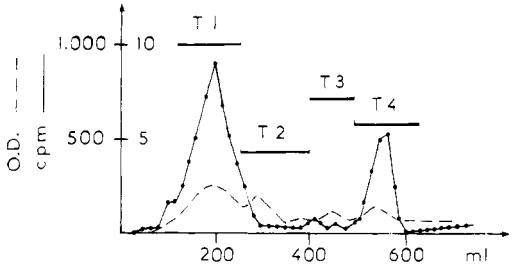


FIGURE 2: Gel filtration patterns of the tryptic digest of b5 light chains on a Sephadex G-25 column (1.5 × 190 cm). The flow rate was 15 mL/h, and the buffer was 1% $NH_4HCO_3$.

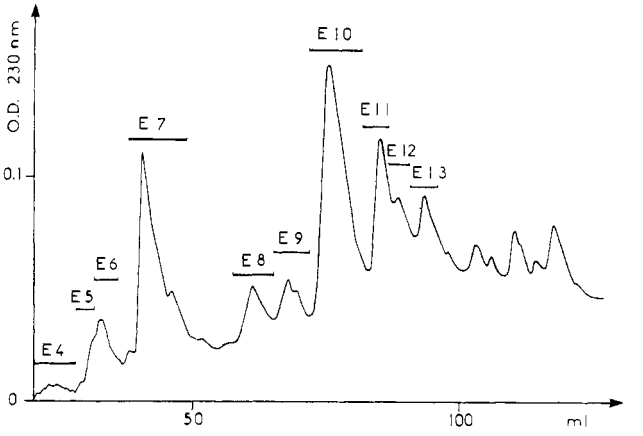


FIGURE 3: Purification of the V8 protease digest of b5 light chain on HPLC. Elution was done as follows: solvent A is $H_2O$/(20 mM) $CH_3COONH_4$. Solvent B is acetonitrile. Elution was obtained with a gradient 0% B to 60% B in 1 h. The flow rate is 1 mL/min.

sequenced manually and automatically. Cysteine-193 was identified and confirmed by the presence of radioactivity at this step. Peptide E10 (positions 108–140) was sequenced, confirming the presence of a glutamic acid residue at position 107. This finding confirms the differences at this region between b5 and b4 as was predicted from peptide T1-5. The radioalkylated cysteine at position 134 was verified.

## Discussion

*Differences between b5 and the Other Allotypes.* The comparison between b5 light chain (Figure 4) sequenced in this work and those of previously sequenced chains of allotypes b4, b6, and b9 permits the following conclusions: (1) Extensive similarities exist in the amino-terminal variable region and the framework between b4, b6, b9, and b5 (Figure 5). (2) The percentage of amino acid substitutions between the b4 and b5 J regions, the only two allotypes for which the complete sequence is known, is 18% (Figure 6). Indeed, among the 11 amino acid residues of the J regions, 2 are different. (3) The percentage of amino acid substitutions between the b4, b6, b9, and b5 constant regions differs by 20–35%; however, the variations are most frequent in certain regions: from 120 to 130, from 148 to 156, from 180 to 184, and from 194 to 200 (Figure 7).

*Differences among b5 Chains.* The comparison of the results reported here with those reported previously for another b5 light chain, obtained after allotypic suppression of a b4/b5 rabbit (Chersi et al., 1980), suggests that variations may exist among the constant regions of b5 chains, as has been previously shown for b4 (Strosberg et al., 1974; Sogn & Kindt, 1978) and for b6 (Emorine et al., 1979). Among the six differences between the b5 constant region described by Chersi and that of light chain 2699, two (leucine for valine at position 195 and proline for alanine at position 196) could be technical, since they were deduced from amino acid compositions in Chersi et al. report. Three differences (alanine for proline at position 113, proline for serine at position 122, and threonine for alanine at position 129) are more intriguing since they occur in the region in which substitutions were observed between b4 and b4 Var (serine in place of alanine at position 121 and leucine in place of glutamine at position 124). The remaining difference, glutamine for glutamic acid, is more usual.

*Three-Dimensional Localization of Differences between the Various Allotypes.* A visual examination of the three-dimensional model of human Fab New (Saul et al., 1978) in-

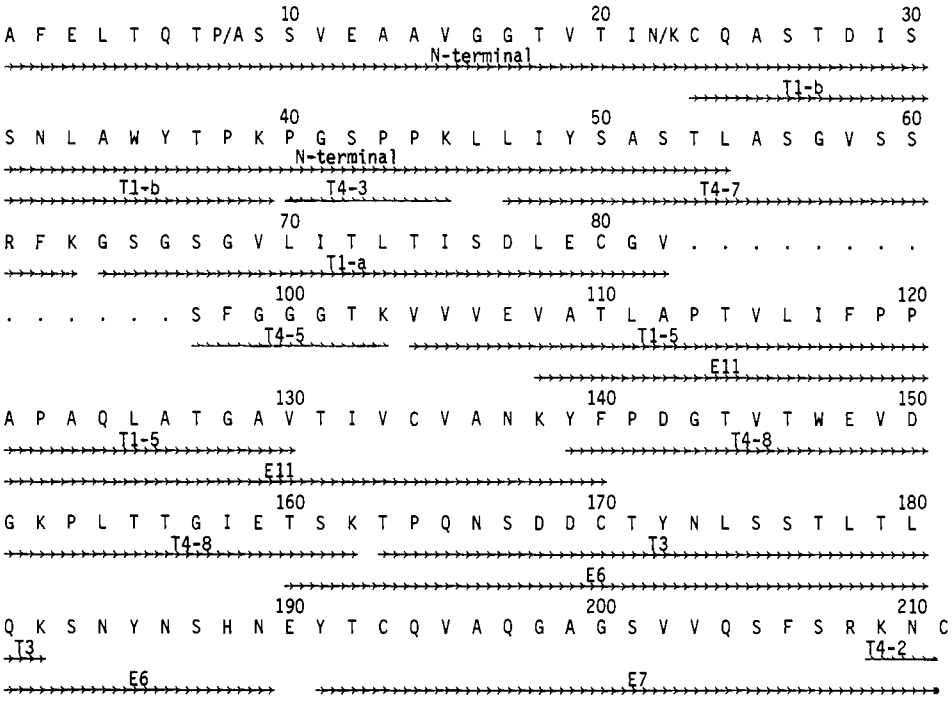


FIGURE 4: Amino acid sequence of 2699 b5 κ light chain. TPCK-trypsin peptides are designated T, and V8 protease peptides are designated E. Residues underlined by half-arrows have been sequenced manually. Residues underlined by full arrows have been sequenced automatically.

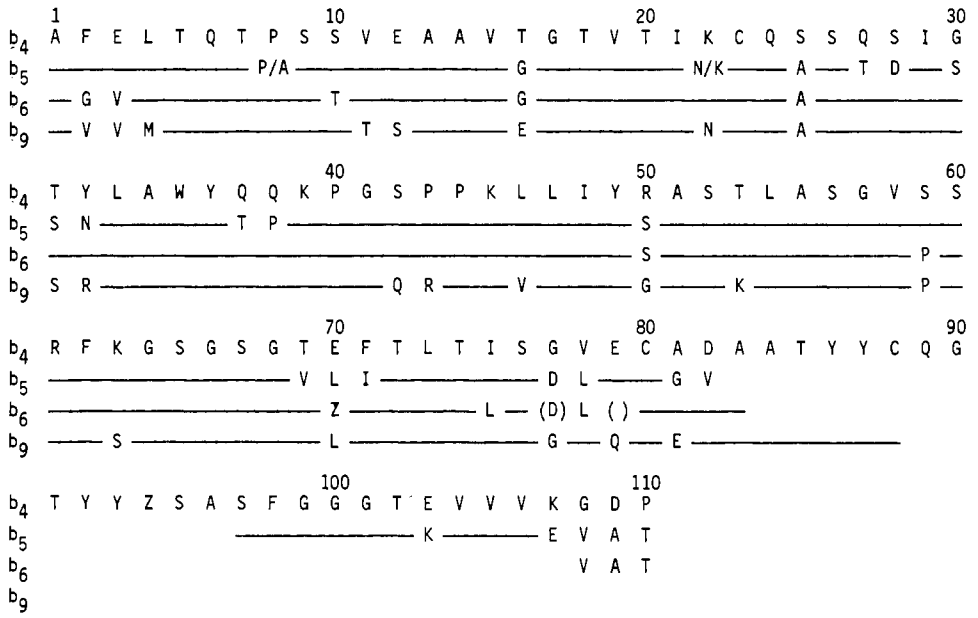


FIGURE 5: Variable region from 2699 b5 κ light chain compared to the same positions of chains bearing the various group b allotypes. b4, b6, and b9 are respectively from Van Hoegaerden & Strosberg (1978), Fraser et al. (1978), and Emorine et al. (1979).

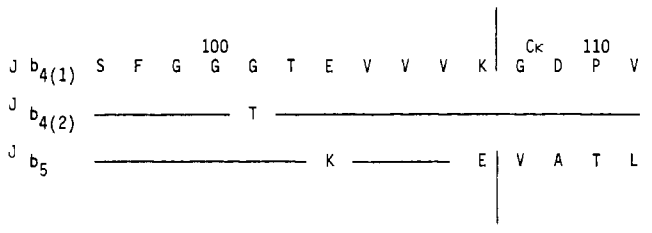


FIGURE 6: Comparison between b5 and b4 J segments. b4 (1) and b4 (2) are respectively from Van Hoegaerden & Strosberg (1978) and Braun et al. (1976). b5 is this work.

dicates that most of the amino acid substitutions observed upon comparison of the rabbit light chains of the different allotypes do correspond to positions which are on the surface of the light chain. Some of these residues are in contact with the heavy chain: this is the case for positions 117, 121, 133, 159, and 188. Others such as the lysine at position 107 are accessible not only to solvent molecules but also to large proteins such as proteolytic enzymes (trypsin) or anti-allotype antibodies.

To render the comparison between the human λ and the rabbit κ chains more significant, we had to introduce a number of gaps. Thus we aligned residue 107 from rabbit κ light chains with position 109 of λ New and continued without any shift up to position 147, where the tryptophan residue of the λ New position 150 was aligned with position 147 of rabbit κ light chain. The residues at positions 191, 210, 211, and 214 of the λ New had to be deleted to maximize the homology between the chains. The cysteine at position 213 was positioned at position 211 (the C-terminal cysteine residue for the rabbit

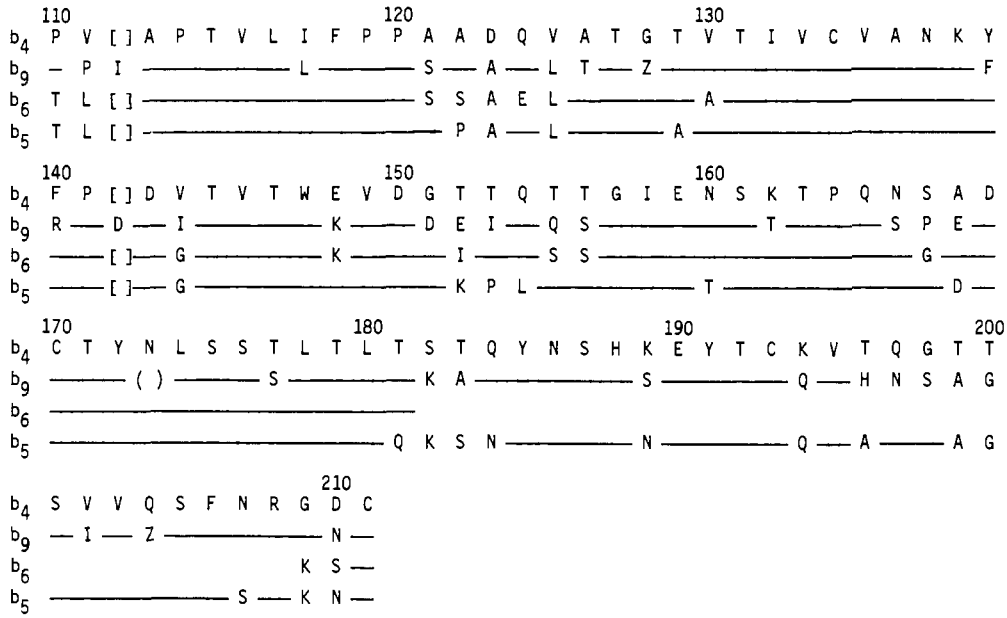


FIGURE 7: Constant region from the light chain 2699 (b5) compared to the homologous position of the various group b allotypes. b4, b6, and b9 are respectively from Van Hoegaerden & Strosberg (1978), Emorine et al. (1979), and Farnsworth et al. (1976).

κ light chain). Three gaps had thus to be introduced at positions 210, 190, and 151.

The four segments of the $C_\kappa$ chains which concentrate most of the substitutions are all exposed on the surface, as might be expected, since most of the residues at these positions are polar. It is likely that they participate in the structure of the b allotypic determinants. In addition, some residues which are not included in these parts of the C region are also at the exterior of the molecule. This is the case for position 107 occupied by a lysine (b4, b6, b9) or glutamic acid residues (b5) and position 110 occupied by a proline (b4, b9) or a threonine residue (b5 and b6). These two positions correspond to the end of the J region and are accessible to proteolytic enzymes. It is likely that they also participate in the antigenicity of the b allotype. Position 167 is occupied by a serine (b4 and b5), proline (b9), or glycine residue (b6); alanine (b4 and b6), glutamic acid (b9), and aspartic acid (b5) at position 168 are also located at the external surface of the molecule and are likely to participate in the antigenicity of the b allotype. Some residues from the C region are in close contact with the first constant region of the heavy chain (CH1); these residues are mostly hydrophobic such as the phenylalanine residue at position 119 and the valine residue at position 133. The valine residue at position 135 and the cysteine residue at position 170 are located near the V region. The latter forms a disulfide bond with cysteine-80. The two positions are in close proximity in both the human Mcg (Schiffer et al., 1973) and New chains. The leucine residue at position 154 is located at the interior of the molecule and constitutes together with the isoleucine residue at position 158 and the tryptophan residue at position 147 a hydrophobic "box" likely to stabilize this part of the C region.

Finally, it is worth indicating that positions 153 and 191, both apparently involved in allotypic determinants of the rabbit κ chains, are also associated with the human κ chain Km markers; Km(1) corresponds to valine (153) and leucine (191), Km(2) to alanine (153) and leucine (191), and Km(3) to alanine (153) and valine (191). The isotypic $Oz^+$ and $Oz^-$ variants of the human λ chains have respectively lysine and arginine at position 190. Thus, these positions are undoubtedly crucial in the antigenicity of both rabbit and human light chains.

This paper presents for the first time the complete J and C region sequences of a b5 κ light chain. Peptides were positioned in the whole light chain sequence by extensive homology with known proteins. The structure of this sequenced b5 light chain corresponds to the general structure of a rabbit κ light chain.

The comparison of the V region sequence of this chain with other b allotype V regions confirms the presence of hypervariable and framework regions. The framework displays residues common to all κ light chains such as cysteines at positions 23, 80, and 89 and tryptophan at position 35, but other residues are associated with a given allotype. Indeed, framework substitutions appear more frequently in some allotypes than in others such as the glutamic acid residue at position 16 and the leucine residue at position 70 in many but not all the b9 chains (Thunberg et al., 1973; Fraser et al., 1978). Mage et al. (1973) described the presence of alanine at position 8 for b5 light chain which was also found in this work. The data suggest that some $V_\kappa$ regions are associated preferentially with $C_\kappa$ regions of a given allotype, as was suggested earlier by Waterfield et al. (1973) and more recently by Fraser et al. (1978) and Emorine et al. (1979).

Sequence studies of a great number of mouse κ light chains suggested that the 96–107 region is encoded separately in the DNA (Weigert et al., 1978). Nucleotide sequence studies have confirmed this hypothesis and have shown that a single C gene is joined to a set of V genes through four different J segments (Sakano et al., 1979). A comparison of all available sequences of rabbit κ light chains suggests that a similar J portion may be defined between positions 97 and 107. The sequence of the J region of the b5 allotype is different from the region of the b4 allotype at two positions (Figure 6). Only one b4 protein was found to differ at a single position where a threonine replaced a glycine residue: this replacement could have arisen from somatic mutation or could reflect the existence of a second germ-line b4 $J_\kappa$ gene segment. These observations suggest that for every $C_\kappa$ allotype there is one J region or a set of different J regions. The existence of allotype-associated residues supports this hypothesis.

The rabbit κ light chain constant regions vary from one allotype to another by 20–35%. For a given specificity, variants have been described. The b4 Var differs from b4 by two

residues (serine for alanine at position 121 and leucine for glutamine at position 124). Genetic data show that b4 is an allele of b4 Var (Sogn & Kindt, 1978). Several different residues have also been observed in the b4 light chain sequence at position 172 (Asn, Leu, and Val) (Appella et al., 1973; Chen et al., 1975; Van Hoegaerden & Strosberg, 1978). Variations between two chains of allotype b6 have been reported by Strosberg & Janssens (1976) and Emorine et al. (1979); the comparison between the b5 chain described here and the b5 chain reported by Chersi et al. (1980) shows six substitutions in these C regions, at least three of which are likely to correspond to polymorphic variations. In the present work, no sequence heterogeneity was found for the C region.

The DNA hybridization studies with a b4 probe (Heidmann & Rougeon, 1982) show that individual rabbits, whatever the allotype, contain at least two DNA regions closely related to the b4 structural C gene as well as several DNA sequences less strongly related to this gene. It appears that restriction patterns vary from one allotype to another. The nucleotide sequence of each hybridization band has not yet been determined. However, the hybridization data and the observation of the expression of latent allotypes (Strosberg, 1977) can be interpreted as indications that some or all rabbits have the genetic information for more than one b allotype.

Recent DNA studies (Gough et al., 1979) favor a single murine $C_\kappa$ gene in BALB/c mice. The murine $\lambda$ system is different: four $C_\lambda$ isotypic regions have been identified. Two gene groups have been identified, one for $C_{\lambda 2}$, $C_{\lambda 3}$ and another for $C_{\lambda 1}$, $C_{\lambda 4}$; these two groups are separated from each other and are separate V and J gene segments. In the rabbit $\kappa$ light chain system, there may be a similar gene organization since the rabbit $C_\kappa$ sequences also differ about 30% as do the murine $C_\lambda$, and there appear, from the limited data, to be allotype associated residues in both $V_\kappa$ and $J_\kappa$.

While the amino acid sequences strongly suggest that all the $C_\kappa$ genes arose from a common ancestor gene by duplication, the b5 and b6 alleles clearly diverged at a later stage since they are more similar to each other than to either b4 or b9. This similarity is observed in the V and the C regions, suggesting a synchronous evolution or the sharing of V regions between the b5 and b6 chains.

Acknowledgments

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