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Mechanisms of antibody diversity: Multiple genes encode structurally related mouse κ variable regions

(amino acid sequence/genetics of antibody diversity/Vκ-21 isotype)

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ABSTRACT **The complete amino acid sequences of the variable regions of three mouse Vκ-21 kappa chains (A22, T111, and CB101) and one partial sequence (B32) have been determined and are compared to four previously reported Vκ-21 variable regions. These eight κ variable region sequences have, with the exception of an amide difference at residue 1, identical amino-terminal 23-residue sequences, all are of the same length, and all have extensive amino acid sequence homology throughout the variable region. When these eight variable regions are grouped by sequence homology, five different groups (Vκ-21A, B, C, D, and E) are present whose members share common sets of amino acids within a group. Three groups of similar homology each contains at least two members (M63 and AB22 in Vκ-21B; M321 and T124 in Vκ-21C; and M70 and B32 in Vκ-21A). The repetition of these five characteristic subgroup sequences in this relatively small sample indicates that these subgroups are isotypes which are controlled by separate germ-line genes. It is unlikely that these sequences could have been randomly somatically generated in different animals from a single germ-line gene (parallel mutation). Although a limited number of comparisons are available, the sequence differences within the Vκ-21A, B, and C isotypes are limited to complementarity-determining regions and may have resulted from somatic mutations. The κ chains comprising the Vκ-21 isotypes offer a unique opportunity to compare the genetic interpretations of the primary amino acid sequence data with the nucleic acid hybridization data.**

The origins of the genetic basis of antibody diversity have been approached through amino acid sequence analyses of immunoglobulin (Ig) variable regions of human, mouse, and rabbit κ (Vκ), λ (Vλ), and heavy chains (VH) (1), and more recently by nucleic acid hybridization (2–4). Amino acid sequences of immunoglobulin variable (V) regions derived from a single inbred strain of mice (e.g., BALB/c) most directly reflect the Ig-producing genetic potential of the individual. In such a system the role of genetic polymorphisms is reduced to a minimum and many of the differences in Ig primary structures can be related to the genetic mechanisms of Ig diversity.

With the availability of several three-dimensional models of Ig V regions that have been determined by x-ray crystallography (5, 6) it has been possible to determine the functional components of the primary sequences of heavy and light chains. The framework segments of the V region sequences determine the conformation of the variable domain and interactions with other domains. Amino acid sequence differences in the framework segments of the V region are thought to be permissive changes that apparently do not alter the ability of the polypeptide to fold into a functional domain (7). The chains fold so that the complementarity-determining regions [also referred to as hypervariable regions (8)] form the antigen-binding site. Wu and Kabat (8) have demonstrated that amino acid sequence variations occur most frequently in these complementarity-determining regions.

A large number of partial amino acid sequences for Igs are now available from the inbred BALB/c strain of mice because of the ease with which plasmacytomas can be induced in this strain (9). The amino-terminal sequences of Vκ and VH exhibit extensive sequence diversity (10–16). V region sequences have been divided into subgroups on the basis of amino acid sequence homology and similarities in V region length. This subgroup diversity has been interpreted to reflect the multiplicity of Vκ and VH genes within the germ line (11, 12).

In order to relate more clearly the Vκ sequences within a subgroup to a genetic mechanism of antibody diversity, the term isotype has been introduced (16). Each different Vκ isotype is presumed to be encoded by at least one unique germ-line gene. A Vκ isotype in the BALB/c mouse has been arbitrarily defined by a characteristic amino-terminal sequence (16). Mouse Vκ sequences that differ from all other mouse Vκ sequences by three amino acids within the amino-terminal 23 residues have been tentatively defined as an isotype. Such sequence differences within the amino-terminal 23-residue sequences are predictive of extensive sequence differences throughout the V regions whose complete sequences have been determined (10, 13, 17). By this criterion, 26 Vκ isotypes (Vκ-1 to Vκ-26) have been distinguished among 63 available sequences (16). It is expected that the criteria used to distinguish isotypes from the primary amino acid sequence data will become more refined as additional complete Vκ sequences become available and enable us to determine what genetic mechanisms are involved in generating antibody diversity.

This involves the sequence analysis of eight V regions that have been assigned to the Vκ-21 isotype (16). In 1973 we (13) compared the sequence diversity in a group of four of these proteins. With the exception of an amide difference at residue 1, the four Vκ-21 amino-terminal sequences (to Cys-23) were identical, all four V regions were of the same length, and all had extensive amino acid sequence homology throughout the variable region. When the four Vκ-21 regions were compared for sequence homology, MOPC 70E (M70) differed from MOPC 63 (M63), MOPC 321 (M321), and TEPC 124 (T124) by 21–22 residues. We concluded (13) that due to the extensive sequence differences between M70 and M321, T124, and M63, this group of Vκ sequences was encoded by at least two germ-line genes. Thus, the Vκ21 isotype was subdivided into Vκ-21A (M70) and Vκ-21B (M321, T124, and M63) (15).

We have since produced anti-isotype antisera that recognize Vκ-21 isotypes (unpublished data) and have identified additional Vκ-21 proteins. In this paper we present the complete sequence of three additional Vκ-21 light chains (A22, T111, and C101) and portions of one other (B32). We will show how these

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Abbreviations: VH, variable region of the heavy chain; Vκ and Vλ, variable regions of the κ and λ light chains, respectively.

additional sequences confirm our original conclusions (13) concerning the multigenic origin of this homologous group of Vκ-21 sequences. Although it may be possible to identify the minimum number of isotypes from differences within the amino-terminal 23-residue sequences, it is not sufficient to identify all potential isotypes. These eight V regions, which would be classified by their amino-terminal 23-residue sequences into a single isotype (Vκ-21), can be further subdivided into five distinct isotypes (Vκ-21A, B, C, D, and E) on the basis of the presence of common sets of amino acids that are shared throughout their V regions. We will also discuss how minimal sequence differences seen between variable regions within a given isotype can be generated.

Nucleic acid hybridization probes for Vκ-21 light chain have been used in studies designed to count the number of Vκ-21 variable region germ-line genes (2–4). These studies have concluded that there are from one to three germ-line genes encoding the Vκ-21 light chain variable regions. The amino acid sequence data presented in this paper suggest that although there is probably not one germ-line gene for each Vκ-21 V region sequence, there are at least five or more germ-line genes encoding Vκ-21 variable regions.

MATERIALS AND METHODS

Plasmacytomas and Vκ-21 Chains: Origins and Identification. The origins of the plasmacytomas and Vκ-21 chains used in the present experiment are given in Table 1. Vκ-21 light chains were identified in a serological screening of BALB/c myeloma tumors with Vκ-21 isotypic antisera (unpublished data). The rabbit antisera were made against purified Bence-Jones proteins or partially reduced and *S*-carboxymethylated light chains from each isotype group (M70, M321, and AB22).

Light Chain Purification. Myeloma immunoglobulins were isolated from ascites of tumor-bearing mice by precipitation in 50% saturated ammonium sulfate at 4° for 18 hr. The precipitates were solubilized and the IgG peak was separated by chromatography on Sephadex G-200 (2 × 150 cm) in 10 mM Tris·HCl/0.14 M NaCl at pH 8.0. Light chains were isolated from partially *S*-carboxymethylated Ig fractions (18) by chromatography on a Sephacryl S-200 (2.5 × 140 cm) column in 6 M urea/ 0.5 M acetic acid. Bence-Jones proteins were isolated, purified, and completely reduced and *S*-carboxymethylated as described (13).

Amino Acid Sequences. One of two possible strategies for sequence determination was chosen based on serological typing results with rabbit antisera against light chains. The two strategies were necessary because of either two methionines (AB22, M63, T111, and CB101) or four methionines (B32) in the light chain sequences. These strategies (Fig. 1) use the isolation of cyanogen bromide and tryptic fragments by Sephadex G-75 gel filtration chromatography (essentially as previously presented) (19). Numbering of light chain residues is according to Gray *et al.* (10).

The sequences of light chains and their fragments were each determined at least twice on a Beckman Model 890C Sequencer with a DMAA program (20). The sequences of light chain fragments were determined on the Beckman Sequencer in the presence of 1 mg of polybrene (Aldrich Chem.) (21) to minimize peptide extraction from the cup. By using polybrene, the complete sequences of fragments of between 5 and 45 residues long could be determined to the last residue. Phenylthiohydantoins were identified on a Waters high-pressure liquid chromatograph with a Dupont Zorbax ODS reverse-phase column using a program modified from Zimmerman *et al.* (22). In addition, an aliquot from each sequencer cycle was back-hydrolyzed (23) and the residue was identified on a Beckman model 121M amino acid analyzer. A radiolabeled control protein (^{14}C-labeled *S*-carboxymethyl pancreatic trypsin inhibitor) (Worthington Biochemicals) was added to each sequencer sample to monitor sequencer chemical efficiency (24).

Table 1. BALB/c Vκ-21 proteins

Tumor	Year found	Inducing agent	m-component	Isotype	Ref.
MOPC 70E	1961	Bayol F	BJ*, IgG	Vκ-21A	10
MOPC 63	1961	Bayol F	BJ	Vκ-21B	13
MOPC 321	1966	Bayol F	BJ	Vκ-21C	13
TEPC 124	1969	Pristane	BJ	Vκ-21C	13
ABPC 20	1973	P-AbV†	IgG_{2b}	?	‡
ABPC 22	1973	P-AbV†	IgM	Vκ-21B	§
TEPC 111	1969	Pristane	IgA	Vκk-21E	§
ABPC 17	1973	P-AbV†	IgA	?	‡
BFPC 32	1969	Bayol F	BJ	Vκ-21A?	§
BFPC 61	1969	Bayol F	BJ	?	‡
TEPC 62	1970	Pristane	BJ	Vκ-21B?	‡
CBPC 101¶	1974	Pristane	IgG_{2a}	Vκ-21D	§

Origins of the BALB/c Vκ-21 myeloma κ light chains. Question marks under the Vκ-21 isotype heading indicate uncertainty as to under which Vκ isotype the light chain is classified.

* BJ = Bence-Jones urinary protein.

† P-AbV = Pristane, Abelson virus.

‡ Unpublished results.

§ This paper.

¶ CBPC 101 is an Ig-congenic mouse of BALB/c background carrying the IgC_H locus of C67BL/Ka. Twenty consecutive backcrosses were made before inbreeding was begun.

RESULTS

The light chain cyanogen bromide fragments from AB22, T111, CB101, and B32 were isolated by gel filtration chromatography essentially as reported (19). Although the cyanogen bromide fragments showed a tendency to aggregate during the isolation, the fragments were recovered in yields of 45% or better. When the sequences of light chains or their fragments were determined, repetitive yields between 93 and 96% were obtained.

The complete V-region sequence of AB22 (Table 2) differs from that of the M63 V region only at position 96, where there is a tyrosine in place of a tryptophan. AB22 and M63 (Vκ-21B) share five characteristic framework amino acids, Asn-1, Val-58, Ala-83, Tyr-87, and Gly-100 (Fig. 2), which distinguish them from the closely related Vκ-21C proteins M321 and T124. Further, AB22 and M63 share three amino acids from the complementarity-determining region, His-34 in L1, Leu-50 in L2, and Asn-91 in L3 (Fig. 2), which differ in M321 and T124. AB22 is therefore designated as the Vκ-21B isotype and is the second complete sequence available in this isotype.

The sequence of T111 differs from those of M63 and A22 (Vκ-21B) by 6–7 residues, from those of M321 and T124 (Vκ-21C) by 6–7 residues, from that of M70 by 22 residues, and from that of C101 by 16 residues. The differences between T111 and M70 or C101 occur almost as frequently in the framework as in the hypervariable regions. Not only is there a higher frequency of framework differences between T111 and the sequences within Vκ-21B or Vκ-21C, but the differences appear to occur in segments. With the exception of the amino terminus, the T111 follows the Vκ-21B sequence through residue 49, the Vκ-21C sequence through residue 83, and (except Ser-91) the Vκ-21B sequence through residue 107. This pattern suggests that T111 may have originated from recombination between Vκ-21B and Vκ-21C genes or a closely related Vκ-21 gene. The fact that there is an NZB myeloma κ chain that is identical to

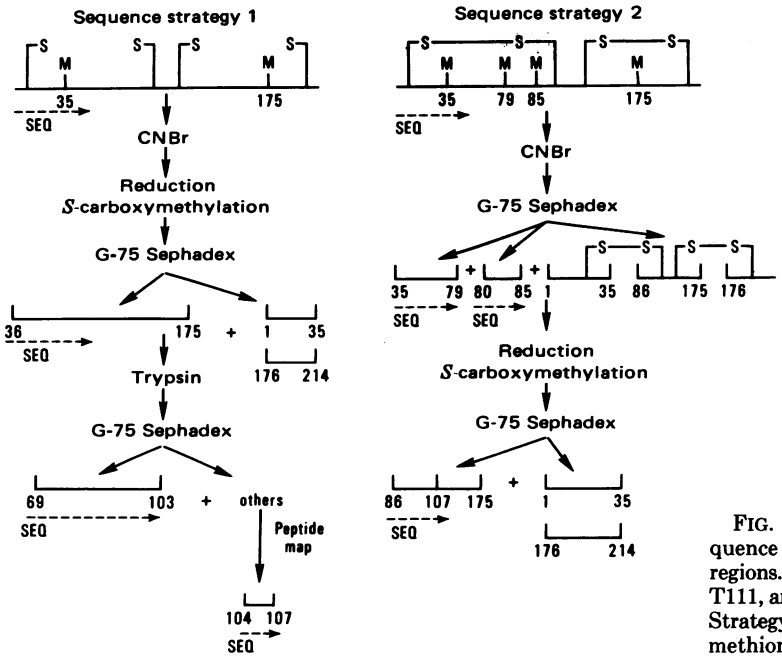

FIG. 1. Strategies used for the amino acid sequence determination of Vκ-21 light chain variable regions. Strategy 1 was used with light chains (A22, T111, and CB101) that contained two methionines. Strategy 2 was used with B32, which contained four methionines.

T111 (M. Weigert, personal communication) suggests that T111 is a distinct isotype from Vκ-21B and C. T111 is designated here as Vκ-21D.

The sequence of CB101 differs from the Vκ-21A, Vκ-21B, and Vκ-21C isotype sequences by 18–19 residues. Approximately half of the differences occur within the hypervariable regions. Many of the framework region differences occur in the segment between L2 and L3 (Fig. 2). CB101 is designated as Vκ-21E isotype.

The incomplete sequence of the B32 protein (Table 2) differs from the sequence of M70 by 3 out of the 88 positions whose sequences have been determined. Two of these substitutions occur within the first hypervariable region and an additional substitution is located in the third hypervariable region. The remaining 19 positions, which have not been identified, are entirely within the framework regions. B32 has been tentatively classified as a Vκ-21A isotype.

The amino acid sequence of M63 between residues 1–53 and 69–107 was repeated during this study. Positions 27, 34, and 87 were found to be different from the originally reported sequence (13).

Table 2. Vκ-21 sequences

		23 a b c d L1 35
21B	A22	N I V L T Q S P A S L A V S L G Q R A T I S C R A S E S V D S Y G N S F M H W Y
	M63	. . . . .
21C	M321	D . . . . . K . . N T . . . . . Q . .
	T124	D . . . . . B W . . . . . Z . .
21D	T111	D . . . . .
21A	M70	D . . . . . Q . . B B S . I . . . N . F
	B32	D . . . . . N . . I . . . N (.) F
21E	C101	D . . . . . K . . Q . . . Y(T) . E(.)Y . N . .

		49 L2 57
	A22	Q Q K P G Q P P K L L I Y L A S N L E S G V P A R F S G S G S R T D F T L T I D
	M63	. . . . . Z . . . . .
	M321	Z Z . . . Z . . . . . R . . . . Z . . I . . . . . B . . . . . B
	T124	Z Z . . . Z . . . . . R . . . . Z . . I . . . . . B . . . . . B
	T111	. . . . . R . . . . . I . . . . . N
	M70	Z Z . . . Z . . . . . A . . . Q G . . . . . G . . . S . N . H
	B32	. . . . . A . (.) . Q G (.) . .
	101	. . N . . . S . . . . . A . . . . . I . . . . . G . . . . . N . H

		88 L3 98
	A22	P V E A D D A A T Y Y C Q Q N N E D P Y T F G G G T K L E I K
	M63	. . . . . W . . . . .
	M321	. . Z . B . V . . . F . Z Z S B Z B . W . . S . . . . .
	T124	. . Z . B B V . . . F . Z Z S B Z A . W . . S . . . . .
	T111	. . . . . V . . . . . (S) . . . . .
	M70	. H Z Z B B T . H . F . Z Z S K . V . W . . . . .
	B32	(H) . E . . T . H . F . . S K . V . . . . .
	101	. . . E E . . . . . (S) . . . . .

Variable region amino acid sequences from Vκ-21 light chains. Sequences are presented in the one-letter amino acid code (25); dots indicate sequence identity with the sequence of A22. Parentheses represent tentatively identified residues. Vertical lines denote boundaries of complementarity-determining hypervariable regions L1, L2, and L3.

DISCUSSION

The pattern of variation that is seen among structurally related Vκ, Vλ, and VH chains has been used to identify whether immunoglobulin sequence diversity is totally encoded within the germ line or is generated somatically from a limited number of germ-line genes (26–31). Mouse Vλ sequences have been shown by Weigert and Riblet (32) to exhibit a minimal amount of variation, limited exclusively to complementarity-determining regions, from a common Vλ sequence. The limited substitution pattern has been interpreted (32, 33) to indicate that Vλ diversity is generated by somatic mutation from one or at most a few germ-line genes. Mouse VH amino-terminal sequences appear to be somewhat less structurally diverse than mouse Vκ (34). It is difficult to assess the total extent of mouse VH diversity because only the amino-terminal 20-residue sequences are available from most VH regions. Also, most of the VH regions examined have been selected on the basis of antigen reactivity, which probably biases the sequence data in favor of structural similarity.

The amino-terminal 23-residue sequences of mouse Vκ are very diverse (11). When these sequences are grouped by sequence homology (sequences in each subgroup differing from sequences in another subgroup by 3 or more residues), 26 different subgroups can be distinguished from the 63 amino-terminal Vκ regions whose sequences have been determined (16). This sequence variation within the amino-terminal 23 residues has been predictive of extensive sequence variation throughout the V region (10, 13, 17). The sequence variation observed between different subgroups is so diverse that each subgroup is thought to be encoded by at least one germ-line gene. We, therefore, refer to each of these 26 different subgroups as iso-

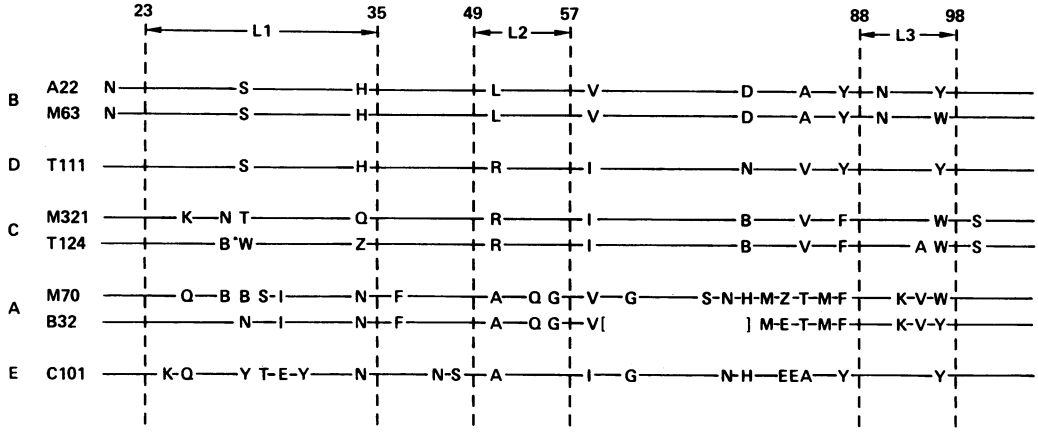

FIG. 2. Diagram of the residues that differ within the Vκ-21 isotype sequences Vertical dashed lines and the numbers above denote framework boundaries. The complementarity-determining residues are 24–34, 50–56, and 89–97 (by the numbering systems in refs. 1 and 8).

types (16). This approach is useful in trying to interpret from the amino acid sequence data the minimum number of germline genes that encode Vκ.

In this paper we compare the amino acid sequences of eight mouse Vκ regions to establish the criteria for defining the genetic control of these closely related V sequences. The sequence of A22 presented in this paper differs from the M63 sequence in only one position in the L3 complementarity segment (third hypervariable region) and shares with M63 the five framework amino acids (positions 1, 58, 83, 87, and 100) that distinguish M63 from M321 and T124 (Fig. 2). In addition, M63 and AB22 share a common set of three amino acids (positions 34, 50, and 91) located in complementarity-determining regions that distinguish A22 and M63 from M321 and T124. Thus, there are now two examples (M321 and T124; AB22 and M63) for which there are multiple amino acid sequences that are repeated in both the framework and complementarity regions. It is unlikely the sequences within both Vκ-21B (A22 and M63) and Vκ-21C (M321 and T124) isotypes could have randomly generated in somatic cells from a common germ-line structural gene (parallel mutation). Vκ-21B differs from Vκ-21C in five framework positions (1, 58, 83, 87, and 100) which would require five independent random mutations that have no apparent selective advantage. Thus, the diversity pattern within the M321, T124, M63, and AB22 sequences is most likely encoded from at least two germ-line genes.

The sequence of T111 appears to follow alternatively the Vκ-21B and Vκ-21C sequences. This suggests that T111 may have originated as a recombinational product of the Vκ-21B and Vκ-21C genes during evolution. An identical Vκ-21 light chain from the NZB mouse strain has been identified (M. Weigert, personal communication). It is unlikely that a T111-like sequence would be generated independently in a different strain of mice unless it was encoded by a germ-line gene distinct from the genes encoding the Vκ-21A, B, C, or E isotypes.

As may be seen in Fig. 2, many of the inter-isotype amino acid sequence differences are located in the framework between 58 and 88. Molecular models from the three-dimensional structure of M603 (7) indicate that most of this primary sequence forms a loop of β structure which is located on the external surface of the domain. A part of this sequence is found on the interface of the domain facing VH. The amino acid side chains of residues 59, 60, 61, 63, 65, 68, 72, 74, 78, and 80 all face externally. Residues 85 and 87 are external and lie in the interface between VH. Residue 87 contacts VH, while residue 85 does not (5). Most of these amino acids then are not involved in maintaining the β pleat formation. These externally located side chains may contribute to the isotypic antigenicity of the Vκ-21 chains and may be responsible for serological specificities that distinguish among Vκ-21 proteins.

The Vκ-21 isotypes represent a significant fraction (8–10%) of normally expressed κ chains in different strains of mice (ref. 35 and unpublished data). These isotypes probably all evolved from a common ancestral gene by a continuing process of gene duplication. The Vκ-21 isotypes have probably been maintained in *Mus musculus* because they contribute to antibodies that react with antigens common in the mouse environment.

The differences within the Vκ-21 isotypes compared in this paper are all located in complementarity-determining regions and hence resemble the pattern of variation described by Weigert and Riblet in Vλ (32). It will be interesting to see if all differences within Vκ-21 isotypes are limited to complementarity-determining regions once a larger sample of sequence comparisons are completed. A genetic mechanism that randomly generates somatic mutation would be expected to produce a high proportion of intra-isotype substitutions (but not necessarily all) within complementarity-determining regions because these residues may undergo strong selection pressure by their ability to produce a reactive active site for antigen binding. However, substitutions outside of the complementarity-determining regions would also be expected to occur as long as these amino acids did not disrupt the tertiary structure of the framework. If additional Vκ sequences are found to group around a number of common sequences (isotypes) and if the substitutions of one light chain are not repeated in the light chains of the same isotype, then this will suggest that these intra-isotype substitutions are generated somatically. Germ-line gene duplication would have to occur very frequently in evolution to produce such a pattern of variation. Although the number of Vκ-21 intra-isotype sequence comparisons is not sufficient to enable us to define the mechanism generating intra-isotype diversity, the sequence comparisons are consistent with a somatic mechanism. The Vκ-21-like myelomas have not been selected for sequence determination on the basis of similar antigen reactivity and, therefore, the substitution pattern should reflect an unbiased sampling of the diversity-generating mechanism.

Seven differences have been found in comparing the proteins within the Vκ-21 family of isotypes, e.g., M321-T124, M70-B32, and AB22-M63. At least three of the seven substitutions (43%) require two base mutations (M63-AB22 1/1, M321-T124 1/3, and M70-B32 1/3 two-base substitutions). The presence of a large fraction of double base mutations would argue against random somatic mutation as the mechanism for generating intra-isotype diversity because random mutation should produce a majority of single base mutations. These double base mutations may be indicative of a special diversity-generating mechanism if they occur frequently in a larger sampling of Vκ-21 sequences. Alternatively, two of the three positions requiring double base mutations occur at residue 96. Determi-

nation of the DNA sequence of mouse Vλ genes (36) has indicated that the Vλ region gene ends near residue 96. Thus, the presence of double base substitutions at this position in Vκ may reflect the changes in the DNA sequence which result as part of the V and C gene-splicing mechanism.

Workers in several laboratories (2–4) have tried to distinguish a germ line from a somatic mutation diversity-generating mechanism by nucleic acid hybridization techniques. The consensus among these workers is that there are no more than three genes coding for Vκ-21 isotype V regions. These studies have recently been criticized (37) for the limits of error with which these predictions are made. There is some uncertainty as to how similar Vκ genes must be in their base sequences in order to cross-hybridize completely. Tonegawa (3) has shown that M321 hybridizes with the same kinetics as that found for globin probes. Yet the Vκ-21-like light chains whose partial sequence we have determined (unpublished data) suggest that there are likely to be more Vκ-21 isotypes than the five which have been presented in this paper. The Vκ-21 isotypes provide an opportunity to compare critically the nucleic acid hybridization data with the genetic interpretations made from primary amino acid sequence data in order to define the extent to which the germ line and somatic mechanisms interact. We would predict that although there probably is not a different germ-line gene encoding each Vκ, the total number of germ-line genes for Vκ-21 variable regions has been underestimated by the nucleic acid hybridization techniques.

The sequence comparisons presented here suggest that Vκ-21 variable regions are encoded by at least five germ-line genes. If each of the other 25 BALB/c Vκ isotypes (most of which have been identified only by their amino-terminal 23-residue sequences) has a pattern of diversity similar to the Vκ-21 light chains, then the number of germ-line genes that encode mouse Vκ may well exceed 125 genes. It is difficult to predict the total number of Vκ germ-line genes from the available Vκ sequence data. Since the Vκ-21 isotypes represent a large fraction (10%) of normal κ chains, it is possible that the Vκ-21 V regions may be represented in the BALB/c genome by more genes than other less frequently occurring Vκ isotypes. There is also no evidence that the number of Vκ isotypes has been saturated. Studies of the amino-terminal sequences of BALB/c myeloma light chains continue to reveal new isotypes (unpublished observations). These studies suggest that there may be more BALB/c isotypes than the 26 that have been published.

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