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Immunology

Somatic diversification is required to generate the V_κ genes of MOPC 511 and MOPC 167 myeloma proteins

(antibody diversity/recombinant DNA)

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ABSTRACT **The immune response to phosphocholine in BALB/c mice involves one group of heavy chain variable region (V_H) genes and at least three groups of light chain variable region (V_κ) genes, represented by the gene products of the myelomas TEPC 15, MOPC 603, and MOPC 167/MOPC 511. The amino acid sequences of BALB/c myeloma κ chains MOPC 167 and MOPC 511 are known, and they differ by six amino acids. We have isolated several closely related V region genes of immunoglobulin light chains from a mouse sperm DNA phage library, selecting clones that cross-hybridize with a cDNA plasmid probe encoding the light chain of MOPC 167. We identified six strongly hybridizing clones, representing three separate cloning events. We determined the sequence of the coding and immediate flanking regions of three clones, representing the three separate cloning events, and they proved to be identical. This germ-line sequence encoded the amino acid sequence of neither MOPC 167 nor MOPC 511, but required four base pair changes to generate the $V_{\kappa M167}$ cDNA sequence and five base pair changes to generate the $V_{\kappa M511}$ gene. By Southern hybridization experiments, we demonstrated that neither MOPC 511 nor MOPC 167 germ-line genes exist. We conclude that the $V_{\kappa M167}$ and $V_{\kappa M511}$ genes are created somatically.**

An immunoglobulin polypeptide contains two distinct regions, the variable (V) and the constant (C) regions. The variability of amino acid sequences within the V region determines the antigen-binding specificity of the antibody. To explain the origin of antibody diversity, the germline hypothesis proposed that diversity arises from the multiplicity of the V region genes encoded in the germ line, whereas the somatic mutation hypothesis proposed that diversity arises from a few inherited germline genes, which during the life of an individual (ontogeny) undergo somatic variations to create an expanded repertoire of V genes (1–7).

From molecular hybridization (and Southern blotting) data gathered by using subgroup-specific V_κ or V_H probes (H, heavy chain), the number of cross-hybridizing germ-line V genes is estimated to range between 4 and 20 genes per subgroup, with 100 V_κ subgroups (8). However, the question of how much expressed antibody diversity is encoded in the germ-line V genes and how much, if any, is attributable to somatic mechanisms remains unanswered.

Previously, we characterized the organization of the $V_{\kappa M167}$ gene family in germ-line DNA by Southern blotting, and we demonstrated that no major changes in the size of the $V_{\kappa M167}$-related genes occur during embryogenesis, ruling out embryonic episomal insertion of genetic elements (9). In this paper, we describe the isolation of several closely related V_κ gene clones from a λ phage library of mouse sperm DNA by probing with a MOPC 167 (M167) light chain cDNA (p167kRI). Because both $V_{\kappa M167}$ and $V_{\kappa M511}$ genes belong to the $V_\kappa 24$ light chain subgroup, we shall refer to genomic germ-line clones hybridizing to p167kRI as $V_\kappa 24$ genes. We have determined the entire nucleotide sequence of the coding and immediate flanking regions of three highly homologous independent $V_\kappa 24$ clones. These three clones were identical, and their germ-line sequence encoded neither the M167 cDNA sequence nor the amino acid sequence of MOPC 511 (M511) myeloma protein. Because, by Southern blot analysis, we demonstrated that neither the M511 nor the M167 germ-line gene exists, we conclude that the M511 and M167 sequences arise by somatic mechanisms.

MATERIALS AND METHODS

The BALB/c Ga mouse sperm DNA phage library was constructed by *Hae* III/*Alu* I partial digestion of sperm DNA followed by ligation with *Eco*RI linkers and insertion into λ Charon 4A (10). The phage library was screened by plaque hybridization (11) with nick-translated p167kRI (12). The recombinant phages were plaque purified, isolated from overnight confluent plates, and purified by two CsCl step gradients (ref. 13, pp. 70–77). The phage DNA was extracted by the formamide extraction technique (ref. 13, pp. 106–111). The restriction endonucleases were obtained and utilized as directed from New England BioLabs (Beverly, MA) and Bethesda Research Laboratories (Rockville, MD). Horizontal agarose gel electrophoresis and transfer to nitrocellulose or diazobenzyloxymethyl-paper were done as described (ref. 13, pp. 148–161). Cloned mouse DNA fragments were subcloned in pBR322 and the plasmids were grown and isolated as described (ref. 13, pp. 138–141). Nucleotide sequences were determined by the Maxam and Gilbert technique (14). High molecular weight DNA was prepared from sperm according to Joho *et al.* (9) and from liver as described by Nottenburg and Weissman (15).

RESULTS

Isolation and Characterization of $V_\kappa 24$-like Clones. Germline DNA contains several members of the $V_\kappa 24$ family. With *Hae* III-digested DNA, the p167kRI probe detects seven bands at low hybridization stringency (2× NaCl/Cit at 65°C; 1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), two bands of 950 base pairs (bp) and 750 bp at increased stringency (0.3× NaCl/Cit), and only the 950-bp band at 0.1× NaCl/Cit (9).

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Abbreviations: V and C, variable and constant regions of immunoglobulins; H, heavy chain of immunoglobulins; bp, base pair(s); kb, kilobase(s); V_κ gene, V gene of κ light chain; C_κ gene, C gene; $V_\kappa 24$, germline prototype V_κ gene of the immunoglobulin κ subgroup 24; M511 and M167, myelomas MOPC 511 and MOPC 167; $V_{\kappa M511}$ and $V_{\kappa M167}$ designate V_κ genes expressed in the myelomas M511 and M167, respectively; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0 (standard saline citrate).

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We screened 1.7×10^6 recombinant phages with a p167kRI cDNA probe and isolated 15 cross-hybridizing clones. Analysis of all 15 clones by *Hae* III digestion followed by Southern hybridization analysis with p167kRI (Fig. 1) revealed 2 C_κ and 13 V_κ clones. Six of the V_κ clones produced a 950-bp fragment upon *Hae* III digestion. Five clones yielded a 750-bp *Hae* III fragment and two clones showed a 520-bp *Hae* III fragment. When these Southern blots of *Hae* III-digested clones hybridized with p167kRI were washed at 65°C under various salt conditions, the pattern was identical to the pattern seen on whole genomic Southern blots.

The isolated clones were further characterized by restriction enzyme digestion and Southern blot analysis. The inserts ranged between 6 and 18 kilobases (kb). The *Eco*RI digests of the six clones containing 950-bp *Hae* III V_κ fragments demonstrated that the six isolates probably represented only three separate cloning events (Fig. 2). In contrast with *V* genes of H chains (16, 17), each of these clones contained only one detectable $V_{\kappa M167}$-like gene.

Because only the 950-bp *Hae* III band remained after the most stringent washing of Southern filters of *Hae* III-digested sperm DNA probed with $V_{\kappa M167}$, we were interested in determining if the three groups of clones containing 950-bp *Hae* III fragments represented a single gene or three closely related genes. For fine mapping and sequence analysis, we inserted into pBR322 the *Eco*RI/*Bam*HI fragment containing the *V* region from one representative of each of the three groups.

Sequence of the 950-bp *Hae* III Fragment of the V_κ24 Gene. The nucleotide sequences of the coding and immediate flanking regions were determined for each of the three subclones by using the strategy shown in Fig. 2. All sequences were identical in the coding and flanking regions. The V_κ24 family contains two myeloma light chains, M511 and M167, that differ from one another by six amino acids, all of which occur in framework regions (18, 19). The V_κ24 germ-line sequence encoded neither the M167 nor the M511 κ chain. We verified the amino acid sequence of $V_{\kappa M167}$ by determining the sequence of the V region of p167kRI (Fig. 2).

A comparison of our germ-line variable gene sequence and the M167 cDNA sequence demonstrated four base pair differences. Two of these base pair changes resulted in amino acid changes, while two base pair changes were silent. In order to generate the M511 amino acid sequence from this germ-line gene, a minimum of five nucleotide changes would be required. This germ-line gene sequence could also be interpreted to encode a hybrid M167–M511 molecule. The DNA sequence differences among the M167 cDNA, V_κ24, and M511 occur at

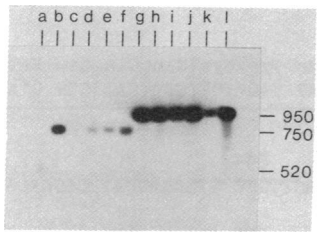

FIG. 1. Identification of the *Hae* III fragments from recombinant phages carrying *V* region genes that cross-hybridize to a M167 cDNA probe, p167kRI. DNA was digested with *Hae* III, electrophoresed through 1.8% agarose, and transferred to nitrocellulose. The DNA on the nitrocellulose was annealed with ^{32}P-labeled p167kRI, then washed with 2× NaCl/Cit at 65°C, and autoradiographed. Lanes: a, Ch V_κ52-1 (exhibits a 520-bp *Hae* III band); b, Ch V_κ75-1; c, Ch V_κ75-3; d, Ch V_κ75-3a; e, Ch V_κ75-3b; f, Ch V_κ75-4 (lanes b–f exhibit 750-bp *Hae* III bands); g, Ch V_κ95-1; h, Ch V_κ95-1a; i, Ch V_κ95-1b; j, Ch V_κ95-2; k, Ch V_κ95-2a; l, Ch V_κ95-2b (lanes g–l exhibit 950-bp *Hae* III bands).

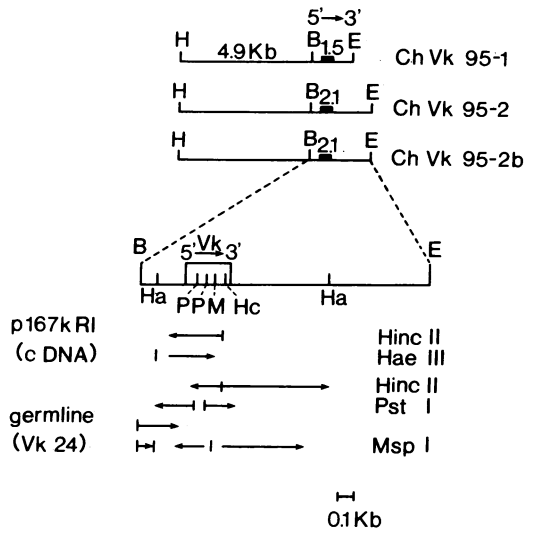

FIG. 2. Partial restriction endonuclease maps of six phage clones, representing three independent cloning events, containing 950-bp *Hae* III fragments of V_κ genes, and the strategy used to determine the *V* region gene sequences of each independent cloning event. The restriction maps were generated by a combination of single and double restriction enzyme digestion, followed by Southern blot hybridization analysis. The boxes represent the V_κ gene coding sequence. The Ch V_κ95-1, Ch V_κ95-2, and Ch V_κ95-2b clones have been isolated three times, two times, and once, respectively. The *Bam*HI/*Eco*RI fragment containing the V_κ coding region was subcloned in pBR322 for DNA sequence analysis. The sequence was determined by generating fragments using *Hae* III, *Hinc*II, *Msp* I, and *Pst* I, as indicated. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae* III; Hc, *Hinc*II; M, *Msp* I; P, *Pst* I.

amino acid residues 11, 39, 41, 45, 49, 55, 68, and 77 (Table 1). At the first four positions where the M167 and M511 sequences differ, the germ-line V_κ24 gene encodes the M167 sequence, and at the remaining two differing positions it encodes the M511 sequence. Thus, conceivably, the $V_{\kappa M511}$ and $V_{\kappa M167}$ genes could arise from this gene either by mutation or by recombination (see *Discussion*).

No Germ-Line M511 Gene Exists. Despite the fact that all the clones containing 950-bp *Hae* III fragments of V_κ genes encoded a single, identical, sequence, a $V_{\kappa M511}$ or a $V_{\kappa M167}$ gene

Table 1. Comparison of DNA sequence and amino acid differences among the M167 cDNA, V_κ24 gene, and M511 protein

Amino acid no.	Germline prototype V_κ24 Amino acid	Codon	M167 cDNA Amino acid	Codon	M511 Amino acid	Codons	Base pair changes
11	Asn	AAT	Asn	AAT	Lys	AA^A_G	1
39	Arg	AGA	Arg	AGA	Gly	GGN	1
41	Gly	GGA	Gly	GGA	Gln	CA^A_G	2
45	Gln	CAG	Gln	CAG	Arg	CGN AG^A_G	1
49	Tyr	TAT	Ser	TCT	Tyr	TAT^T_C	1
55	Ala	GCA	Ala	GCC	Ala	GCN	1
68	Gly	GGA	Arg	AGA	Gly	GGN	1
77	Arg	AGA	Arg	CGA	Arg	CGN AG^A_G	1

The M511 gene's codons are the predicted codons derived from the amino acid sequence (19). The minimal number of base pair changes needed to generate the M167 or M511 amino acid sequence from V_κ24 is indicated. N, any nucleoside.

or both might still exist in the germ line, but may not have been cloned from or been present in our phage library. Because an M511 germ-line gene would have an arginine codon (CGN or AG^{A}_{G}) at amino acid 45 rather than the glutamine (CAG) codon found in the sequenced germ-line gene, we could test for the existence of an M511 germ-line gene by examining whether the *Alu* I site (A-G-C-T) between amino acids 45 and 46 is lost by any germ-line $V_\kappa 24$ genes. When the germ-line $V_\kappa 24$ gene is digested with *Alu* I, fragments of 344 and 198 bp hybridize with p167kRI. Hence, if an M511 germ-line gene exists, a whole genomic Southern blot of *Alu* I-digested sperm DNA hybridized with p167kRI should contain a fragment larger than the $V_\kappa 24$ fragments because the *Alu* I site (box, Fig. 3) is lost. In a Southern blot no larger band was seen although the fragments of the C_κ gene were visible (Fig. 4*A*). Therefore, no M511 gene exits.

No M167 Germ-Line Gene Exists. Similarly, the existence of a germ-line M167 gene was tested by observing that the M167 cDNA had a silent base pair change from the germ-line $V_\kappa 24$ sequence at nucleotide number 570, creating a *Taq* I (T-C-G-A) site (Fig. 3). Therefore, if an M167 germ-line gene existed, a whole genomic Southern blot of *Taq* I-digested DNA, hybridized with p167kRI, and washed under stringent conditions, should demonstrate not only a 2-kb $V_\kappa 24$ germ-line fragment but also a second band. Even though up to 25 μg per lane of *Taq* I-digested DNA was hybridized with p167kRI (Fig. 4*B*), only a single C_κ band and a single V_κ 2-kb band were detected, both without any smaller bands. Hence, no M167 germ-line gene exists.

From the *Alu* I and *Taq* I Southern blot experiments and the frequency of selecting a gene insert containing the 950-bp *Hae* III fragment from our library we suspected that the $V_\kappa 24$ germ-line gene was a single-copy gene. As an independent method of determining whether there were other genes in addition to the cloned germ-line $V_\kappa 24$, we attempted to estimate the germ-line $V_\kappa 24$ copy number per haploid genome relative to the C_κ gene, using *Taq* I, an enzyme for which there are no sites within the V_κ germ-line sequence and the C_κ gene. If another $V_\kappa 24$-like germ-line gene exists in addition to the germ-line sequence we have cloned, then there should be two or more copies of a 2.0-kb *Taq* I band per haploid genome. Alternatively, if only the $V_\kappa 24$ germ-line gene exists, one should detect only one copy per haploid genome. Thus we titrated increasing multiples of 5 μg of *Taq* I-digested DNA per lane by hybridization with p167kRI and washing under stringent conditions (Fig. 4*B*). Several autoradiographs were traced with a microdensitometer to circumvent the problem of nonlinearity in exposure of the film. From this densitometry, incorporating a correction for the degree of *V* (297 bp) versus *C* (527 bp) region homology of the probe, we determined that the normalized intensity ratio of the C_κ gene band to the V_κ gene band was 1.74 ± 0.21. Cognizant of the vagaries affecting the quantitation of band intensities (e.g., gel

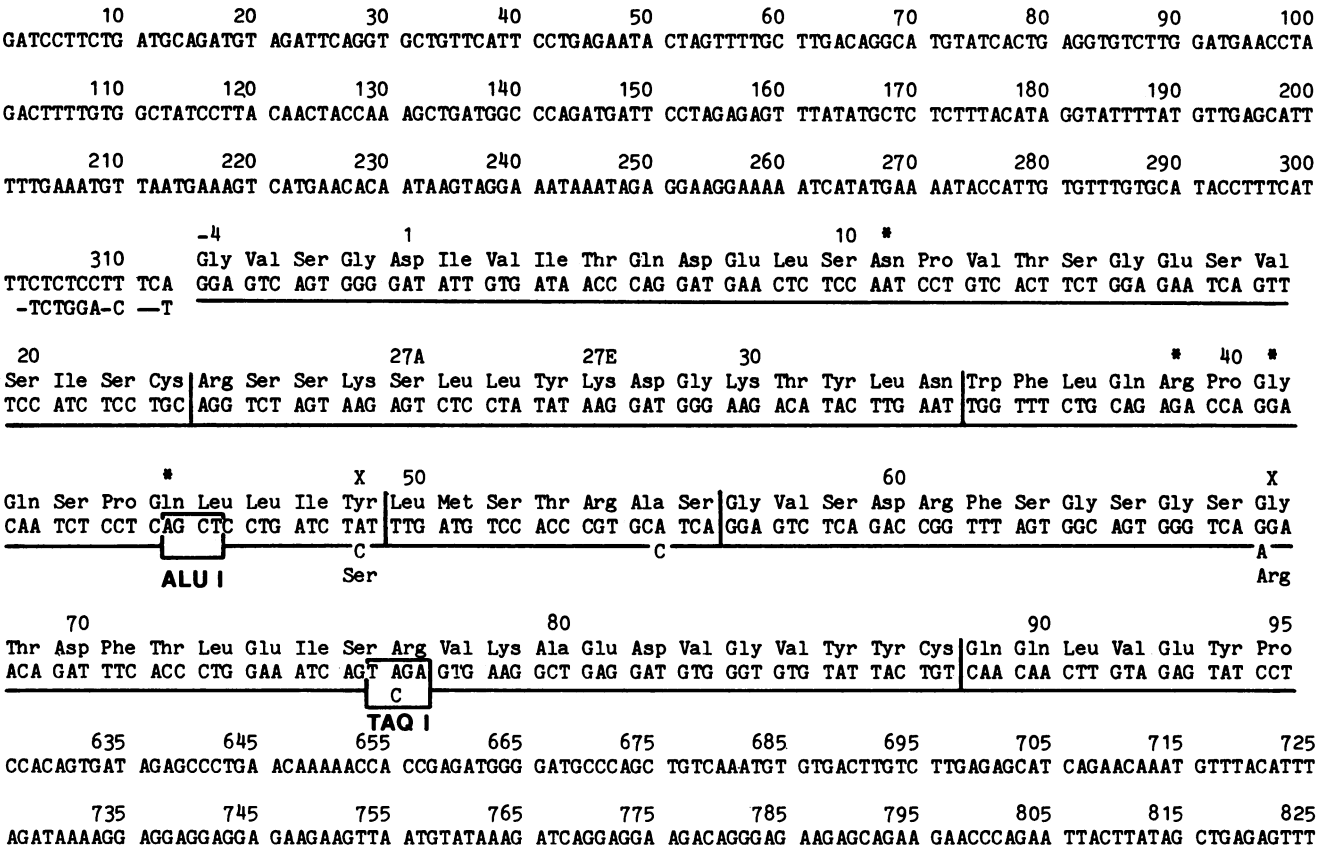

FIG. 3. Comparison of the nucleotide sequence of the germ-line $V_\kappa 24$ sequence to the M167 cDNA sequence, and the M511 and M167 protein sequences. The sequence is 5′ to 3′ with the origin at the *Bam*HI site. The amino acid sequence of the germ-line clone is written above the nucleotide sequence and numbered according to Kabat *et al.* (20). The vertical lines in the coding sequences separate framework and hypervariable regions. The M167 cDNA sequence is beneath the germ-line sequence and the solid line indicates nucleotides identical to the germ-line sequence. Nucleotide differences are indicated by letter, while differences in the sequence causing amino acid changes have the resulting amino acid beneath the sequence. Asterisks indicate amino acid differences between M511 and M167 where the M167 sequence is identical to the $V_\kappa 24$ sequence. Xs indicate amino acid differences between M511 and M167 where the M511 amino acid sequence is identical to the $V_\kappa 24$ sequence. The boxed regions of the sequence locate the *Alu* I site present in the $V_\kappa 24$ gene and the *Taq* I site absent from the $V_\kappa 24$ gene but present in the M167 cDNA sequence.

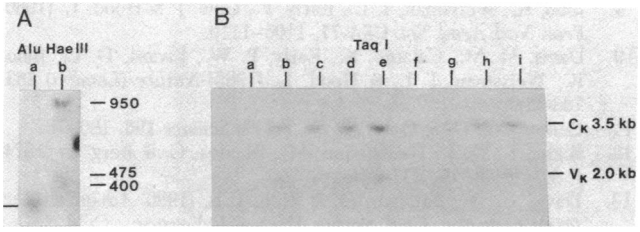

FIG. 4. Hybridization of ^{32}P-labeled p167kRI to a restriction digest of sperm and liver DNA. (*A*) BALB/c Ga sperm DNA was digested with *Alu* I (lane a) and *Hae* III (lane b), electrophoresed through 2.0% agarose, and transferred to nitrocellulose. Fragment lengths are given in bp. (*B*) Lanes a–e contain 5, 10, 15, 20, and 25 μg per lane of *Taq* I-digested BALB/c Ga sperm DNA, respectively. Lanes f–i contain 5, 10, 15, and 20 μg per lane of *Taq* I-digested BALB/c liver DNA. The digested DNA was electrophoresed through 1.5% agarose and transferred to nitrocellulose. The nitrocellulose filters were annealed with ^{32}P-labeled p167kRI, washed with 0.1×NaCl/Cit at 65°C, and autoradiographed.

transfer efficiencies, hybridization efficiencies, probe length homologies, and G+C content), we interpret these results as being consistent with a single germ-line V_κ24 gene.

Therefore, by inference, the M511 and M167 genes are created somatically.

DISCUSSION

The immune response to phosphocholine involves at least three different types of κ light chains represented by the myelomas TEPC15, M603, and M511/M167 (21, 22). The amino acid sequences of M167 and M511 are known, and they differ from each other by six residues. By comparing the M167 cDNA sequence, the M511 and M167 κ amino acid sequences, and the DNA sequence of the most homologous cloned germ-line V region (V_κ24), we examined the relative contributions of germline and somatic mechanisms to the generation of antibody diversity in the M511/M167 (V_κ24) family. The germ-line V_κ24 sequence most homologous to the M167 cDNA sequence encoded the amino acid sequence of neither M167 nor M511. In addition, from preliminary nucleotide sequence analysis, the *V* regions of the germ-line 750- and 520-bp *Hae* III fragments encoded neither the M167 nor the M511 amino acid sequence. From Southern blot analysis, we demonstrated that neither the M511 nor the M167 germ-line gene exists in the BALB/c Ga strain or the BALB/c π strain (from which the M511 and M167 tumors arose), and the most homologous germ-line (V_κ24) gene exists only as a single copy per haploid genome. As a result, we infer that the M511 and M167 V_κ sequences are generated somatically. Recently, E. Selsing and U. Storb (personal communication) have independently analyzed the genomic content of the V_κ24 genes, finding that the $V_{\kappa M511}$ and $V_{\kappa M167}$ genes were absent from both BALB/c J kidney DNA and the nonexpressed, unrearranged V_κ24 gene in the M511 myeloma. Their nucleotide sequence of the V_κ24 germ-line context gene coincides with our nucleotide sequence. Selsing and Storb located the germ-line gene sequence encoding the amino-terminal portion of the M167 leader protein, approximately 350 bp 5′ to the start of the V_κ24 coding sequence.

Somatic diversification mechanisms had previously been proposed in the λ light chain immunoglobulin system from a comparison of DNA molecular hybridization data with the amino acid sequences of several myeloma proteins (23, 24). In short, several proteins appear to be derived from only a few (or one) V_λ genes (25, 26) The DNA sequence of the rearranged V_λ H2020 plasmacytoma gene revealed that the sequence was identical to the λ_1 embryo DNA sequence, except at the two codons encoding residues 25 and 32. Although restriction mapping of the $V_{\lambda 1}$ flanking regions suggested it is present as a single copy per haploid genome (27), a recently duplicated closely related germ-line gene with the same restriction map remains a possiblity. To eliminate the conceivable possiblity of masking more than one V_κ24 gene per haploid genome in this study, we estimated copy number per haploid genome by the restriction fragment analysis described and by titration of restricted DNA with an internal C_κ gene standard. Thus this constitutes even more stringent evidence that somatic diversification of *V* genes can occur, at least in myeloma cells or their precursors.

Analysis of the intensities of the C_κ band relative to the V_κ band in the *Taq* I-digested DNA demonstrated a significantly less intense V_κ band. Although this finding is consistent with a single-copy V_κ24 gene, the quantitation of the C_κ to V_κ genes could resurrect the issue of the C_κ gene copy number. The evidence for a single copy per haploid genome stems from double-digest Southern restriction mapping using one enzyme cutting within the C_κ coding region with a variety of other enzymes with sites in the flanking region (9). However, this method cannot eliminate the possibility that the entire approximately 25-kb region containing the C_κ gene is repeated in the genome. Although a saturation hybridization analysis has detected two C_κ genes (28), we consider it to be unlikely that a region of about 25 kb (carrying C_κ) is exactly repeated. However, formally the possibility exists that a C_κ pseudogene (29) might contribute to the C_κ band intensity. In any case, this evidence supports the assertion of only a single V_κ24 gene.

Recently, Gearhart *et al.* (30) have determined the amino-terminal amino acid sequences of seven V_κ24 BALB/c hybridoma immunoglobulins. Analyzing the first 38 amino acids of the V_κ regions revealed that three of the seven V_κ regions are identical to M167 and the others differ by a single amino acid residue. Two of the four variant V_κ sequences contained differences in the first framework region, while the other two contained differences in the first hypervariable region. Thus, in addition to the M167 and M511 sequences, the four hybridoma V_κ proteins probably also arise from the prototype V_κ24 germ-line gene.

What somatic mechanisms could explain the generation of the M511 or M167 sequence and these other somatic variants from the single-copy prototype V_κ24 germ-line gene? Although a somatic recombination mechanism is compatible with our data, we believe that the hybridoma data make this explanation less plausible. The somatic mutation model of simple base pair changes seems the simplest mechanism accounting for these sequence differences. Unlike the λ light chain system, in which all the base pair changes are preferentially found in the hypervariable regions, the V_κ24 system strongly suggests an enzymatic mechanism introducing mutations randomly along the entire *V* region coding sequence. Although an error-prone DNA polymerase could be responsible for generating these mutations, it could not have the site-specific recognition sequences as proposed by Ben-Sasson (31). The questions remain of (*i*) whether the mutations studied here are limited to the $V_{\kappa M511}$ and $V_{\kappa M167}$ genes without introducing mutations in the C_κ or the flanking regions; and if so, what kind of mechanism can be envisioned for locus-specific mutagenesis; (*ii*) at what stage of embryogenesis or B cell differentiation does this process take place, including its relationship to antigen-driven events; (*iii*) whether germ-line context or just the rearranged *V* genes are somatically mutated; and (*iv*) if such mutations occur on both the expressed and nonexpressed κ chromosomes.

Formally, the possibility exists that diverse segments of different V_κ24-related genes formed the M167 and M511 genes by a process of gene conversion (32), involving closely related 'minigenes' (33, 34) surrounded by gene sequences significantly

different from $V_\kappa 24$. In this context, the six other bands identified on Southern blots with some homology to $V_{\kappa M167}$ under less stringent hybridization conditions may be important.

The mutations characterizing M511 and M167 might have arisen subsequent to (and not prior to) malignant transformation, and therefore reflect some process ongoing in malignant myelomas. The resolution of this possibility would require sequence analysis of isolated genomic genes from normal B cells or plasma cells expressing $V_\kappa 24$-related genes.

Note Added in Proof. Selsing and Storb (35) have recently reported their similar findings.

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