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Clusters of point mutations are found exclusively around rearranged antibody variable genes

(somatic mutation/nucleotide substitutions/error-prone repair)

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ABSTRACT **We have examined the nucleotide sequences of a series of murine antibody genes derived from one κ light chain gene in order to gain insight into the mechanism that specifically mutates variable genes. Six rearranged V_{K167} genes from hybridoma and myeloma cells were cloned from bacteriophage λ libraries. The sequences were compared to the germ-line sequence of the V_{K167} gene, the J_K genes, and the C_K gene to identify sites of mutation. Four of six rearranged genes had extensive mutation which occurred exclusively in a 1-kilobase region of DNA centered around the V-J gene. No mutations were found at more distant sites in the intervening sequence or in the constant gene. The frequency of mutation was approximately 0.5% (32 mutations per 6,749 base pairs). Mutations were mostly due to nucleotide substitutions with no preference for transitions or transversions. The location of mutations around each gene indicates that they occur in clusters at random sites. The observation of mutations in the intervening sequence downstream from the J_{K5} gene rules out models for the mechanism of mutagenesis that rely solely on gene conversion or recombination. The distribution and high frequency of mutations are most easily explained by a mechanism of error-prone repair that occurs during several cycles of cell division.**

The enormous diversity of antibodies produced by an individual is generated by various mechanisms that take advantage of the unique DNA rearrangements that occur during the development of antibody-secreting lymphocytes (B cells). For example, considerable diversity in the variable regions of κ light chains results from multiple germ-line genes encoding variable (V_K) and joining (J_K) regions (1–3), random pairing of different V_K and J_K gene segments (4), and variability at the site of ligation of V_K and J_K gene segments (3).

Nevertheless, there is an even greater amount of diversity in antibodies that cannot be explained by the rearrangement of gene segments. Recent experiments show that the number of germ-line genes is far less than the number of antibody proteins, providing strong evidence that variable genes undergo somatic diversification. The data are particularly convincing in cases in which multiple antibodies have been derived from one gene. For example, one variable gene for λ light chains codes for 8 different variable regions (5–7), and one variable gene for heavy chains codes for 10 unique variable regions that bind phosphorylcholine (8, 9). In each case, the variants are clearly derived from a unique prototype sequence by the accumulation of somatic mutations.

In order to gain insight into the mechanism producing the high rate of mutation in variable genes, we have determined the sequences of genomic clones from several different myeloma and hybridoma cells that expressed a rearranged V_K gene derived from a unique germ-line gene. The use of genomic clones rather than cDNA clones has allowed us to determine the boundaries of mutation in the sequences flanking the variable gene. In addition, genomic cloning allows determination of sequences within the intervening sequence at a distance from the variable gene and also around the constant gene. Our results show that 32 point mutations from four rearranged genes are distributed exclusively in the region surrounding and including the rearranged V-J gene, confirming previous reports on mutation in V_H and V_K genes (10, 11). Furthermore, we show that nucleotide substitutions occur in clusters located at random sites around the rearranged gene.

MATERIALS AND METHODS

Preparation of DNA for Partial Libraries. Hybridoma cell lines HPCG9, HPCG10, HPCG13, and HPCG22, derived from BALB/cJ mice purchased from The Jackson Laboratory were maintained in tissue culture. Myeloma cell lines M511 and M167, obtained from M. Potter (National Institutes of Health), were maintained as ascites in BALB/cJ mice. DNA was prepared according to Bogenhagen and Clayton (12). Using the Southern blot hybridization method, we identified a 4.7-kilobase (kb) *Bam*HI fragment that contained the rearranged V_{K167}, J_{K5}, and C_K genes and a 7.0-kb *Hin*dIII fragment that contained the V_{K167} and J_{K5} genes. All of the rearranged V_{K167} genes were found to be joined to J_{K5}.

For partial genomic libraries, 50 μg of DNA was digested with *Bam*HI or *Hin*dIII and fractionated by agarose gel electrophoresis. The region of the gel containing *Bam*HI fragments in the size range 4.0–5.5 kb or *Hin*dIII fragments in the size range 6.2–7.8 kb was excised and the DNA was recovered by electroelution. Charon 28 viral DNA (13) was digested with *Bam*HI or *Hin*dIII. *Bam*HI right and left arms were prepared by centrifuging the digested DNA in a 5–20% sucrose gradient in the Beckman SW 41 rotor at 24,000 rpm for 12 hr at 24°C. The appropriate fractions were pooled, dialyzed against 10 mM Tris·HCl, pH 7.6/1 mM EDTA, and centrifuged in an equilibrium CsCl gradient in a Beckman 50 Ti rotor at 36,000 rpm for 40 hr. The inclusion of this buoyant CsCl gradient was found to improve the efficiency of ligation. *Hin*dIII right and left arms were used directly after enzyme digestion.

λ Phage Libraries. Two micrograms of gel-purified lymphocyte DNA fragments was ligated to 4 μg of *Bam*HI or *Hin*dIII Charon 28 arms by using 5 units of T4 DNA ligase in a 20-μl reaction mixture and incubation at 4°C overnight. The ligated DNA was packaged into virus particles (14), and the library was

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Abbreviation: kb, kilobase(s).

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plated on *Escherichia coli* strain C600. The yields were approximately 1×10^5 plaques per μg of ligated DNA with *Bam*HI arms and 6×10^6 plaques per μg with *Hin*dIII arms. The libraries were simultaneously screened with two DNA probes containing the V_{K167} and C_K genes. A cDNA copy of the rearranged V_{K167} gene was obtained from L. Hood (California Institute of Technology) (15) and modified to exclude the *J* gene segment. A 2.7-kb *Bgl* II fragment containing the C_K gene was furnished by J. Seidman (National Institutes of Health) (3). DNA fragments were labeled with ^{32}P by nick-translation, and the libraries were screened according to the procedures of Benton and Davis (16). Approximately one to five viral plaques per μg of ligated DNA contained both the rearranged V_{K167} and C_K genes as determined by autoradiography.

DNA Sequence Analysis. The method of Maxam and Gilbert (17) was used to analyze the C_K genes. The 4.7-kb *Bam*HI fragment from a Charon 28 clone containing the rearranged V_{K167} and C_K genes was subcloned into plasmid pBR322. DNA was digested with *Hpa* I and labeled with ^{32}P (18). The method of Sanger *et al.* (19) was used to analyze the V_K gene and its flanking sequences. DNA from Charon 28 clones or pBR322 subclones was digested with various restriction enzymes, and fragments were ligated to the appropriate restriction sites in M13mp8 and -mp9 viral vectors to allow preparation of single-stranded DNA for chain terminator sequence determination.

RESULTS AND DISCUSSION

Mutations Occur in a 1-kb Region Centered Around the *V-J* Gene. The protein sequences of light chains from several hybridomas and myelomas that bind phosphorylcholine indicated that they were similar to a prototype sequence from the M167 myeloma (9). A germ-line gene, V_{K167}, encoding the prototype sequence was subsequently identified and its sequence was determined by Selsing and Storb (20) and Gershenfeld *et al.* (21). The light chains from myelomas M167 and M511 and hybridomas HPCG10 and HPCG13 had amino acid substitutions from the sequence encoded by the germ-line gene, suggesting that they had undergone mutation. To determine the extent of mutation in rearranged V_{K167} genes and their flanking sequences, we cloned and analyzed the genes from several cell lines. The sequence determination strategy was to analyze the *V-J* gene and its flanking sequences, a portion of the intervening sequence, and a portion of the *C* gene and its flanking regions. The sequences were then compared to the germ-line sequences of the V_{K167} gene (20, 21), the J_{K5} gene (22, 23), and the C_K gene (22) to identify sites of mutation. The consensus sequence of the nonmutated, rearranged *V-J* gene is shown in Fig. 1.

The results in Table 1 and Fig. 2 show that the number of mutations in the rearranged *V-J* genes varied from none in HPCG9 and HPCG22 to 5 in M511, 6 in HPCG10, 10 in HPCG13, and 11 in M167. Four amino acid substitutions identified by protein sequence analysis were confirmed by nucleotide sequence, and five were not confirmed. All of the mutations are due to single nucleotide substitutions with the exception of a deletion of three bases in M511 at position 1,280–1,282. There was no specificity for any particular nucleotide to mutate, and no preference for transitions (purine to purine, or pyrimidine to pyrimidine) as opposed to transversions (purine to pyrimidine, or pyrimidine to purine).

We found a mutation frequency of 0.5% occurring exclusively in the vicinity of the rearranged *V-J* gene. Mutations extended approximately 400 nucleotides into the intervening se-

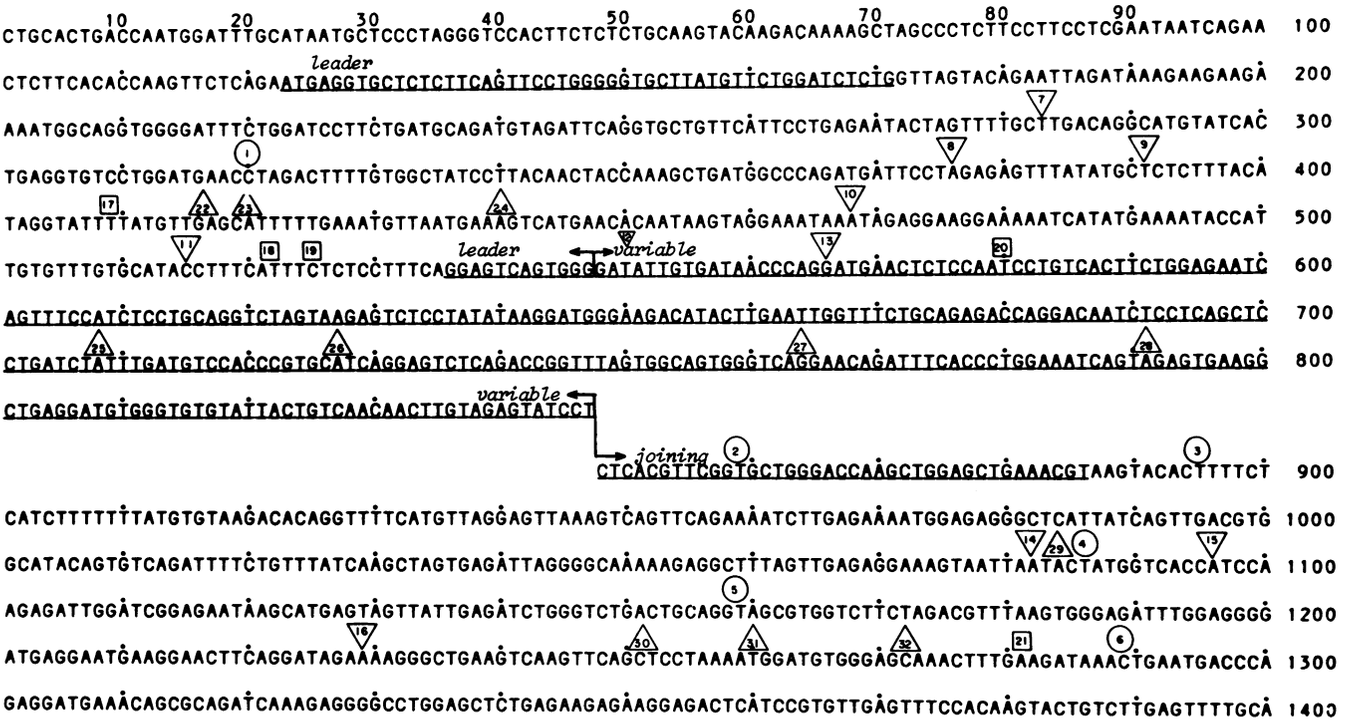

FIG. 1. Sequence of the rearranged V_{K167}-J_{K5} gene. The consensus sequence was obtained from the sequences of six genes: two germ-line genes (HPCG9 and HPCG22) and four mutant genes at bases where there was no mutation. All six genes had the identical sequence except for sites of mutation. This sequence differs from the V_{K167} sequence (reported in ref. 20) at positions 2 and 207 and from the intervening sequences (reported in ref. 22) at positions 1,113 and 1,334 and (reported in ref. 23) at positions 914, 938, 1,028, and 1,029. The amino acid-encoding region is underlined. The nucleotide sequence at the *V-J* junction (845–847) codes for proline in the six examples in this report, but the codon varies due to imprecise joining of *V* and *J* gene segments. The proline codon is CCG in every case except that in HPCG13 it is CCT. The location of the mutations numbered in Table 1 are shown by the respective number and a symbol: ○, HPCG10; ▽, HPCG13; □, M511; and △, M167.

Table 1. Nucleotide substitutions in V_{K167} caused by somatic mutation

HPCG10	HPCG13	M511	M167
1. 320, C→G	7. 283, T→G	17. 409, T→C	22. 417, A→G
2. 859, T→C	8. 376, A→T	18. 522, T→A	23. 420, A→G
3. 895, T→C	9. 391, T→C	19. 525, C→T	24. 440, A→G
4. 1,086, T→A	10. 467, A→G	20. 580, T→G*	25. 708, A→C*
5. 1,159, T→G	11. 515, C→A	21. 1,280–1,282▽	26. 727, A→C
6. 1,289, C→T	12. 550, T→C		27. 764, G→A*
	13. 566, G→A*		28. 791, A→C
	14. 1,082, A→T		29. 1,085, C→T
	15. 1,096, A→C		30. 1,251, C→T
	16. 1,229, A→G		31. 1,260, T→G
			32. 1,272, C→T

Numbers correspond to the position of the nucleotide in Fig. 1. The nucleotide sequences correct the following errors in protein sequence from Gearhart *et al.* (9): HPCG9, amino acid 33 is aspartic acid, not serine; and HPCG10, amino acid 34 is glycine, not glutamine. The predicted protein sequence of the rearranged *V* gene from the M511 plasmacytoma differs from the protein sequence in ref. 24 at amino acids 39, 41, and 45. A total of 17 transition and 14 transversion mutations were identified. ▽, Deletion.

* Substitution changes the amino acid from that encoded in the germ-line sequence.

quences surrounding the *V* gene but were not found in a region of the intervening sequence >1 kb downstream from the *V-J* gene or in the *C* gene. Other investigators also have not detected mutations in *C* genes (7, 10, 11). However, the distribution of mutation may vary in different genes; Kim *et al.* (10) reported substitutions occurring 2.3 kb away from a rearranged V_H gene. Because our sequence analysis was limited to the region shown in Fig. 2, we cannot rule out the occurrence of mutations at a slightly greater distance from the *V-J* gene.

The localized occurrence of mutations rules out the possibility that substitutions are due to any of several artifacts. First, the mutations were not the result of genetic polymorphism in an inbred strain of mice. Three hybridomas—HPCG9, HPCG10, and HPCG13—with different nucleotide sequences (Fig. 2) were derived from one mouse (9). Only two different genes would be expected if a mouse had a maternal and paternal chromosome each containing a different V_{K167} gene. Also, the distribution of variant nucleotides would be expected to be random, and not concentrated around the *V-J* gene, if there were genetic drift. Second, mutations were not due to artifacts of cloning and sequence analysis of the genes because they were only found around the *V-J* gene, not around the *C* gene. Both the *V-J* and *C* genes were isolated and cloned on the same restriction fragment, and mutations would be scattered if they were due to cloning artifacts. Furthermore, the nucleotide changes confirmed several of the substitutions found by protein sequence analysis. Third, mutations were not due to random substitutions generated during the passage of cells in animals or tissue culture because substitutions were found only around the *V-J* gene. If mutations occurred *in vitro*, they must have been generated specifically in this region. This is possible because Cook *et al.* (25) have shown that amino acid substitutions can occur *in vitro* in the V_H region of antibodies from a myeloma cell line.

The distribution of mutations may be influenced by selection for or against variations in the protein sequence. Cells expressing variant antibodies with higher affinity for antigen than the germ-line-encoded antibody will be preferentially selected by antigen and expanded clonally. Selection operates at the level of the intact antibody protein—i.e., both the heavy and light chains are selected together. It is interesting to note that all of the κ light chain genes with mutations are paired with heavy chains containing extensive mutation (9, 10). For example, in the M167 plasmacytoma, the V_H gene has a mutation frequency of 3.8% and the V_K gene has a mutation frequency of 1%. Thus, one mechanism may act on both chromosomes containing rearranged heavy and light chain genes in a B cell. We also note that both HPCG10 and HPCG13 variant antibodies have a severalfold higher affinity for the diazophenylphosphorylcholine antigen than does the HPCG9 antibody which has the germ-line sequence (26). Thus, somatic mutation can produce antibodies with higher affinity for antigen than their germ-line-encoded counterparts.

Although selection may contribute to the mutation frequency, it is unlikely to account for all of the genetically silent mutations observed here. In the coding region of the *V-J* gene, only four of eight nucleotide changes caused amino acid substitutions (Table 1). None of the several mutations observed in HPCG10 resulted in a change in the amino acid sequence. Furthermore, mutations occurred as frequently in the flanking regions (0.4–0.6%) as in the *V-J* coding region (0.4%) (Fig. 2), which suggests that there is no strong selection for mutations to accumulate in the coding region. The rate of mutation must be very high to produce so many unselected mutations.

Site-Specific Mechanism for Error-Prone Replication or Repair May Cause Mutation. Several models have been proposed to account for the somatic diversification of antibody genes. The data presented here suggest a site-specific mechanism of

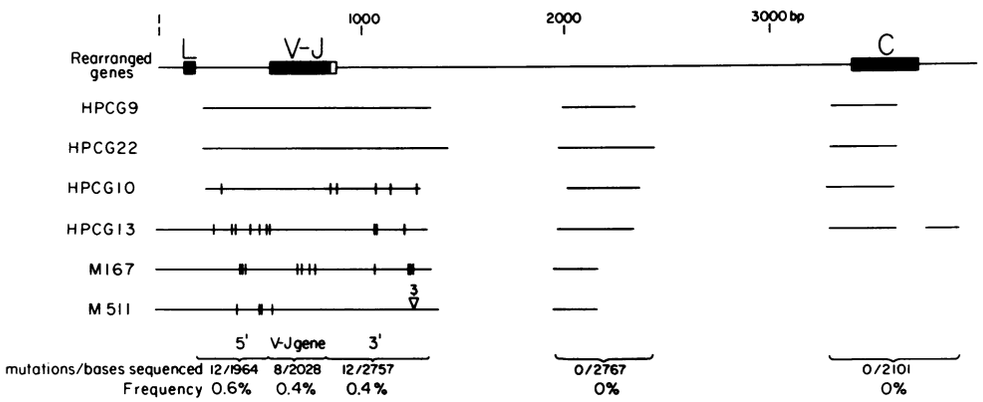

FIG. 2. Mutations are localized to the *V-J* gene. The horizontal lines for hybridoma and myeloma genes indicate identity to the germ-line sequence shown at the top. Vertical lines, single nucleotide substitutions; ▽, deletion of three bases; open box next to *V* gene segment, *J* gene segment. HPCG9 and HPCG10 are IgG3 proteins; HPCG22 and HPCG13 are IgG1 proteins; and M167 and M511 are IgA proteins. HPCG9, HPCG10, and HPCG13 were derived from one mouse.

error-prone DNA replication or repair. Other mechanisms can be ruled out by the following evidence, although they may contribute to diversity in other systems.

(*i*) Gene recombination postulates that two related genes recombine to produce a hybrid gene (1, 27). The entire nucleotide sequence of the five J_K genes and their flanking sequences is known (22). In the four examples in Fig. 2, none of the mutations in *J* genes or their 3′ flanking sequences can be derived from recombination between other *J* segments.

(*ii*) Gene conversion proposes that one gene acquires the base changes characteristic of another related gene (28). In the data presented here, the occurrence of mutations in the *J* gene or its 3′ flanking sequence similarly could not be explained by gene conversion between related *J* gene segments.

(*iii*) Some potential base-specific mutagenic mechanisms are inconsistent with our results. For example, deamination of 5-methylcytosine to thymine (29) or deamination of adenine to hypoxanthine (30) would produce specific transition mutations only and could not account for the transversion mutations observed (Table 1).

(*iv*) Terminal deoxynucleotidyltransferase will add bases, preferably guanine and cytosine, to a 3′ hydroxyl end regardless of template specificity (31). This mechanism would also predict insertions in a sequence. In the V_{K167} somatic genes, there was no preference for bases to mutate to guanine or cytosine and no evidence for insertions.

(*v*) During the ligation of *V* and *J* gene segments, the ends of DNA molecules may be damaged and errors may be introduced during repair (20). This would predict that all of the mutations occur during the discrete time interval of *V-J* joining. During general DNA repair, a gap is produced by an excision enzyme and then filled in by a repair polymerase, which may be error-prone (32). The largest patch sizes observed during repair which could contain mutations were ≈100 base pairs long, which is inconsistent with a 1-kb region of mutation in *V* genes (Fig. 2). Also, if mutations were fixed shortly after *V-J* joining, they would be found frequently in the first antibodies to be expressed—IgM molecules. A B cell first produces IgM molecules and then switches to produce IgG and IgA molecules. However, protein sequence data (9) revealed that the frequency of mutation in the heavy and light chains from IgM antibodies was significantly lower than that in the IgG and IgA antibodies. Mutations may accumulate in IgG- and IgA-producing cells because they divide more than do IgM-producing cells.

The key feature of somatic mutation is the local occurrence of mutations within and surrounding the rearranged *V-J* genes. Point mutations may be introduced by an error-prone repair process that is initiated by a nicking enzyme that is specific for the nucleotide sequence or the chromatin structure of the rearranged *V-J* gene (33). The overall frequency of mutation of 0.5% is extraordinary in view of the normally high fidelity of DNA replication. The amount of nucleotide sequence data available for other gene systems is insufficient to permit calculation of a similar frequency of mutation. However, it has been estimated that the frequency of mutation in nonimmunoglobulin genes in lymphocyte cell lines may be $<10^{-6}$ per gene locus (34). No known DNA polymerase introduces errors at the high frequency observed in *V* genes, although DNA polymerase β is relatively error-prone (35). Therefore, mutations may accumulate during cell division due to many cycles of error-prone replication or repair.

Mutations Occur in Clusters. The location of substitutions in each gene in Fig. 2 suggests that they occur in random clusters in both the coding and flanking sequences. We have performed a statistical analysis of the data to determine whether the clustering of mutations is significant. This analysis is formally similar to the analysis of DNA–protein binding sites on a one-dimensional matrix as formulated by McGhee and Von Hippel (36). To avoid biasing the data in favor of clustering by including genes that do not show mutation, we considered only the genes that exhibit mutation.

If mutations occur in clusters, the observed spacing between adjacent mutant residues (or gaps between mutations) will be shorter than expected from a random model. In the four examples of genes containing mutations, we observed 32 mutations in 4,480 residues, giving a mutation frequency, *M* of 0.7%. Thus, we would expect on the average 1 mutation per 140 nucleotides. However, the observed spacing between adjacent mutations is considerably shorter than expected from the average mutation frequency. We have constructed a probabilistic model of the distribution of gap sizes expected from a random occurrence of mutations at $M = 0.007$. Given a mutation at one residue, the probability of a second mutation occurring after a gap of exactly *g* residues is $P = M(1 - M)^{g-1}$. Thus, the probability that a second mutation occurs within *S* nucleotides of another mutation (i.e., gap size $g \leq S$) is given by $\Sigma_{g=1}^{S} M(1 - M)^{g-1}$. The distribution of gap sizes anticipated by this model is compared with the observed distribution of gap sizes in Fig. 3. Clearly, we find substantially more small gaps, reflecting clusters of mutations, than expected from the random model. The median gap size observed was less than 40 nucleotides, which is significantly smaller than the expected median of 100.

We can envision at least two mechanisms that would result in a clustering of mutations. First, a series of mutations may be produced in one short, concerted passage of an error-prone polymerase. Alternatively, the existence of a first mutation before DNA replication might serve to reactivate an error-prone process leading to a second, nearby mutation. In both cases, it is predicted that a cluster of several mutations is made during a brief time interval.

In order to study the mechanism of somatic mutation, it is important to determine when it happens during B-cell development. Several lines of evidence indicate that mutations accumulate with time after the joining of *V* and *J* gene segments. First, IgG and IgA antibodies contain more mutations than do IgM molecules (9). Second, the high frequency of mutations

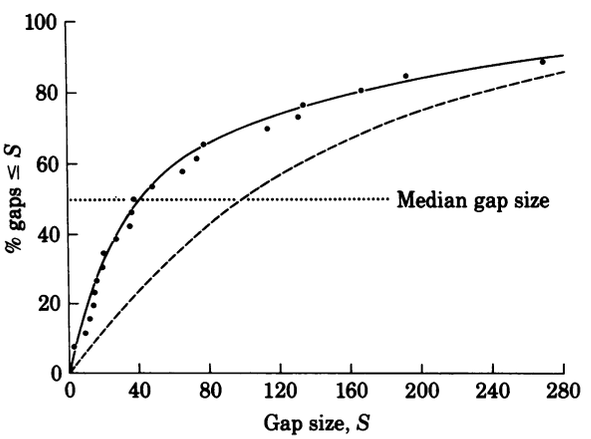

FIG. 3. Mutations occur in clusters. The fraction of gaps smaller than or equal to *S* is plotted as a function of the gap size, *S*. The dashed curve shows the distribution of gap sizes expected for a random occurrence of mutations at a frequency of 0.7%. The solid curve, drawn through the observed data points, indicates that a disproportionate number of small gaps was observed. The horizontal dotted line represents the median gap size (ordinate = 50%).

argues that they accumulate during several cycles of error-prone repair. Third, the random location of clusters within a gene suggests that each cluster may be initiated by a separate repair event.

Somatic mutation appears to be a common mechanism to increase diversity in antibodies because two variant genes, HPCG10 and HPCG13, were derived from one mouse. However, mutation is not obligatory; HPCG9 and HPCG22 have the germ-line sequence. Thus, an animal could initially respond to antigen with its germ-line repertoire of antibodies. After antigen stimulation and repeated cell division, mutational enzymes may act on the rearranged *V-J* gene and introduce base substitutions. Separate lines of B cells undergo mutations in random sites around the *V-J* gene, as evidenced by the nonoverlapping substitutions in HPCG10 and HPCG13. Within a cell line, it is expected that clonal daughter cells will share mutations. The elucidation of this unusual mechanism that produces extensive, localized, base substitutions around the rearranged variable gene will require additional studies of a more dynamic character.

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