

DNA Breaks in Hypermutating Immunoglobulin Genes: Evidence for a Break-and-Repair Pathway of Somatic Hypermutation

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ABSTRACT

To test the hypothesis that immunoglobulin gene hypermutation *in vivo* employs a pathway in which DNA breaks are introduced and subsequently repaired to produce mutations, we have used a PCR-based assay to detect and identify single-strand DNA breaks in $\lambda 1$ genes of actively hypermutating primary murine germinal center B cells. We find that there is a two- to threefold excess of breaks in $\lambda 1$ genes of hypermutating B cells, relative to nonhypermutating B cells, and that 1.3% of germinal center B cells contain breaks in the $\lambda 1$ gene that are associated with hypermutation. Breaks were found in both top and bottom DNA strands and were localized to the region of $\lambda 1$ that actively hypermutates, but duplex breaks accounted for only a subset of breaks identified. Almost half of the breaks in hypermutating B cells occurred at hotspots, sites at which two or more independent breaks were identified. Breaksite hotspots were associated with characteristic sequence motifs: a pyrimidine-rich motif, either RCTYT or CCYC; and RGYW, a sequence motif associated with hypermutation hotspots. The sequence motifs identified at breaksite hotspots should inform the design of substrates for characterization of activities that participate in the hypermutation pathway.

SOMATIC hypermutation is a highly regulated process that specifically targets immunoglobulin genes for very high levels of mutation. The great majority of the mutations are single base substitutions, with more transitions than transversions. The mutation rate is about one mutation per kilobase pair per generation, which is 10^6 -fold higher than the typical mutation rate in mammalian somatic cells. In mice and human, hypermutation occurs after challenge with antigen and is coupled with selection for high-affinity B cell clones. Following mutation and selection, a B cell can produce antibody with greatly increased affinity for antigen. Hypermutation thus increases the efficiency of the humoral immune response.

Extensive study of hypermutation has produced some understanding of the biological requirements for this process (reviewed by KLEIN *et al.* 1998; KONG *et al.* 1998a; NEUBERGER *et al.* 1998; STORB *et al.* 1998; WINTER and GEARHART 1998). Transcriptional activation is necessary but not sufficient to target an immunoglobulin gene for hypermutation, and hypermutated sequences are restricted to the region 1–2 kb downstream of the promoter. Hypermutation occurs in specialized physiologi-

cal microenvironments called germinal centers (KELSOE 1996). A huge database of hypermutated sequences has been compiled, and comparison of these sequences has revealed common hotspots for hypermutation. The sequence motif RGYW ($R = A$ or G ; $Y = C$ or T ; $W = A$ or T) is frequently found at hypermutated sites (ROGOZIN and KOLCHANOV 1992; BETZ *et al.* 1993; JOLLY *et al.* 1996; ROGOZIN *et al.* 1996). Reporter genes (β -globin, *gpt*, and *neo*) can be targeted for hypermutation when they are substituted for immunoglobulin variable regions in transgenic constructs, and the RGYW motif is also a hotspot for mutation in these reporter genes (YÉLAMOS *et al.* 1995).

Hypermutation alters immunoglobulin loci in vertebrates from nurse sharks to mammals, but the regulation and products of hypermutation differ from species to species. In chicken (REYNAUD *et al.* 1987; THOMPSON and NEIMAN 1987), rabbit (BECKER and KNIGHT 1990; WEINSTEIN *et al.* 1994; MAGE 1998), pig (BUTLER *et al.* 1996), and cattle (PARNG *et al.* 1995, 1996), hypermutation occurs prior to encounter with antigen to diversify the preimmune repertoire, and most mutations are templated. In sheep, hypermutation also occurs prior to encounter with antigen, but mutations are untemplated (REYNAUD *et al.* 1991). In mouse (BETZ *et al.* 1993; WAGNER *et al.* 1995; JOLLY *et al.* 1996; KONG *et al.* 1998b), human (DORNER *et al.* 1998; DUNN-WALTERS *et al.* 1998; VARADE *et al.* 1998; FOSTER *et al.* 1999), and the distantly related nurse shark, a cold-blooded vertebrate (DIAZ *et al.* 1998, 1999), hypermutation occurs after antigen stimulation and most mutations are untemplated. In an

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attempt to reconcile the apparent paradox of closely related organisms using distinct mechanisms to achieve hypermutation, we have proposed that hypermutation in all organisms occurs by a break-and-repair pathway (MAIZELS 1995; KONG *et al.* 1998a; HARRIS *et al.* 1999). First, a nick or double-strand break (DSB) disrupts the DNA duplex, and then repair by *either* gene conversion or unfaithful copying would produce either templated or untemplated mutations. This model generates a testable hypothesis: that DNA lesions related to hypermutation can be found in immunoglobulin genes of primary hypermutating B cells.

One line of evidence that breaks occur as part of the immunoglobulin gene hypermutation mechanism in humans and mice comes from the identification of deletion and insertion mutations produced as a by-product of hypermutation *in vivo* (GOOSSENS *et al.* 1998; WILSON *et al.* 1998a,b). These sorts of mutations are likely to result from inaccurate repair of DNA breaks. Another line of evidence comes from analysis of immunoglobulin loci in lymphoid malignancies. The involvement of DNA breaks in many B cell lymphomas is well documented, and while some of these breaks appear to result from aberrant V(D)J recombination or switch recombination, others appear to result from breaks induced during hypermutation and implicate aberrant hypermutation events as contributing to some B cell malignancies (GOOSSENS *et al.* 1998; KUPPERS *et al.* 1999).

Direct evidence for the involvement of DNA breaks in hypermutation first came from analysis of a human B cell lymphoma line, Ramos, which carries out constitutive immunoglobulin gene hypermutation in cell culture, although at a level approximately fivefold below that of primary germinal center B cells (SALE and NEUBERGER 1998). SALE and NEUBERGER (1998) found an increase in the level of insertion and deletion mutations in the κ light chain gene V region in Ramos cells, as well as untemplated nucleotide insertion in transfectants expressing terminal deoxynucleotidyl transferase. Recently, two groups used ligation-mediated PCR to identify DSBs in immunoglobulin genes in B cells carrying out hypermutation. PAPAVALIOLIS and SCHATZ (2000) identified DSBs in the immunoglobulin V regions of the Ramos cell line, and by analysis of a reporter transgene confirmed that breaks could also be identified in hypermutating B cells *in vivo*. BROSS *et al.* (2000) also identified DSBs in a "knock-in" mouse model, generated by targeting a modified immunoglobulin heavy chain gene to the heavy chain locus. Both groups showed that the appearance of breaks is dependent upon transcriptional activation, as would be predicted by the dependence of hypermutation on proximal enhancer elements.

We have assayed for the presence of breaks in murine immunoglobulin $\lambda 1$ light chain genes using a PCR-based assay designed to identify single-strand breaks. We find that the level of single-strand DNA breaks is

two- to threefold higher in hypermutating B cells than in nonhypermutating B cells isolated from the same mouse. We measured breaks in both the endogenous $\lambda 1$ gene of wild-type C57BL/6 mice and in a $\lambda 1$ transgene that hypermutates actively (KONG *et al.* 1998b) and find similar levels and patterns of breaks in the endogenous gene and the transgene. Sequence analysis shows that a significant fraction of the breaks in hypermutating B cells are clustered at distinct hotspots, which contain one of two pyrimidine-rich consensus motifs, RCTYT or CCYC. The hypermutation hotspot sequence motif, RGYW, was also found at or near most of the breaksite hotspots. Our observations support the hypothesis that immunoglobulin gene hypermutation depends upon a break-and-repair pathway. They also provide direct evidence for DNA breaks at an endogenous immunoglobulin locus *in vivo*. The consensus sequence motifs at breaksite hotspots may define the specificity of one or more activities essential to the mechanism of immunoglobulin gene hypermutation.

MATERIALS AND METHODS

Isolation of PNA^{hi} and PNA^{lo} B cells and DNA preparation:

The endogenous $\lambda 1$ gene was analyzed in wild-type C57BL/6 mice. A rearranged $\lambda 1$ -E λ_{24} transgene, which undergoes active hypermutation, was analyzed in the LZ15-90 transgenic line (KONG *et al.* 1998b). Mice were immunized intraperitoneally with 100 μ g alum-precipitated NP-CGG and 2×10^9 *Bacillus pertussis*. Secondary immunization was with 10 μ g NP-CGG in saline (PEAKMAN and MAIZELS 1998). Single cell suspensions were prepared from spleen, lymph nodes, and Peyer's patches. Cells were stained with PNA-FITC and B220-RPE as previously described (KONG *et al.* 1998b) and sorted using a FACSVantage cell sorter (Beckton Dickinson, San Jose, CA).

Identification of breaksites: We developed a method for rapid identification of breaksites in small numbers of mammalian cells, referred to as breaksite batch mapping (KONG and MAIZELS 2001). This method considerably increases the speed, throughput, and convenience of breaksite identification by direct sequence analysis of ligation-mediated PCR (LM-PCR) products in batch, with no gel purification or cloning steps. This method also includes two minor modifications that enhance the sensitivity and specificity of standard LM-PCR protocols: use of a biotinylated primer in the extension step and use of nested PCR primers in the amplification step. This method, which is described in detail elsewhere (KONG and MAIZELS 2001), was used for identification of breaksites in murine $\lambda 1$ genes. Briefly, genomic DNA was prepared from sorted B cells, carefully quantitated, and a small amount of DNA (2000 cell-equivalents) was denatured and annealed to a biotinylated primer specific for the region of interest. The primer was extended with Sequenase (United States Biochemical, Cleveland) to produce duplex DNA extension products that terminated at the site of the break with a blunt end. (A minority of Sequenase extension products may not be blunt but instead contain a nontemplated 3' A, resulting in a modest underestimation of total breaks.) After ligation to a duplex linker containing one blunt and one staggered end (generated by annealing oligonucleotides LL3 and LP2), biotinylated extension products were isolated on streptavidin-magnetic beads (Dyna, Great Neck, NY). The recovered extension products were diluted and aliquoted so that subsequent amplification

reactions contained 250 cell-equivalents for analysis of the endogenous $\lambda 1$ gene and 50 cell-equivalents for analysis of the $\lambda 1$ transgene, which is present at six to eight copies/cell (KONG *et al.* 1998b). DNAs were amplified by nested PCR using primer pairs specific to the region of interest or the linker. PCR products were analyzed by Southern blotting or sequence analysis. In some cases, LM-PCR products were separated by gel electrophoresis and sequenced after subcloning. In most cases, the final PCR product mixtures were sequenced directly on an ABI 3700 sequencer, and the breakpoints were identified according to the position of the linker sequence on the sequencing profiles.

The duplex linker was generated by annealing oligonucleotides LL3 (CGAGTTCAGTCCGTAGACCATGGAGATCTGAATTC) and LP2 (GAATTCAGATCTCC). Linker-specific primers for nested PCR were LL2 (GTAGACCATGGAGATCTGAATTC) and LL4 (CGAGTTCAGTCCGTAGAC). For identification of breaks in the endogenous $\lambda 1$ gene top strand, extension was carried out using the biotinylated primer LRB (BBBBATGCTCTTGCTGTCAGG, B = biotin), and $\lambda 1$ -specific primers for nested PCR were LR2 (GTAGAAATCAGTGATCGTAC) and LR3 (ACAGGGTGACTGATGGCGAAG). Extension and amplification primers for the endogenous $\lambda 1$ gene bottom strand were LFB2 (BBBBGATAGTGGGTGTTTATG), wLF2 (ACTCTGGATAAGCCTGAAC), and wLF3 (GATGATTAATGCCCTGAGCTC). For the $\lambda 1$ transgene, top strand-specific primers were LRB3 (BBBBGAATGTTCTGTGCTCTC), LR4d (CTGTGTCTCTCTATGAC), and LR4e (GTTTTCTTCTCCATGAGATAGC); and bottom strand-specific primers were LFB (BBBBCATGAGATCACTGTTCTC), LF2 (CAGTACGGAGCACACAG), and LF3c2 (GCAACAATGCGCATCTTGCTC). LR3a (CATCTTTCAGTAGCAATCCTGG), LR5a (TATTCCTCACAAAGTTCACCAGC), LF3c3 (GATTTGCTACTGATGACTGG), and wLF3a (CAGTGTAGTAGATTTCCATGAC) were end-labeled and used as probes for hybridization following extension and amplification with the LRB, LRB3, LFB, and LFB2 primer sets, respectively. Primers used for sequencing were LR3a, LR3b (CCTTGCCATTGACCTCCAAATAC), LR4a (TATGCCTTCTGGGTACAAG), LR4c (CTAGGAGCCCAGCTTCCAAAC), LR4d, LR4e, LR5a, LR5b (GAGATTA GACATGAAAGGCTACAG), LR6 (TGGTTGCTGTACCATAGAG), and LR7 (TGAGTCACAACAGCCTG) for top strand breaks; and LF3c3, wLF3a, LF4 (CAATGCCATCTTGCTCTC), LF5a (ACCGAGCTCCAGGTGTTCC), LF5b (GAACCAAAGTACTGTCC), LF7 (TCTCATGGAGAAGGAAAACC), LF8 (GAGAAAAAGTTCAAGCGAGTG), and LF9 (CCAGGATTGCTACTGAAAG) for bottom strand breaks.

RESULTS

DNA breaks in $\lambda 1$ light chain genes: Somatic hypermutation occurs in sequestered microenvironments, called germinal centers (reviewed by PRZYLEPA *et al.* 1998). Germinal centers are dynamic structures that form in secondary lymphoid tissues like the spleen, lymph nodes, and Peyer's patches. In the germinal center, B cells make intimate contact with antigen, proliferate actively, and undergo selection for production of high-affinity antibodies. To ask if breaks are present in $\lambda 1$ genes of hypermutating primary B cells, DNA was prepared from hypermutating germinal center B cells (PNA^{hi} B220⁺) and nongerminal center B cells (PNA^{lo} B220⁺), which had been sorted by FACS from secondary lymphoid tissues of individual immunized mice. Break

sites were detected using $\lambda 1$ -specific primers, as described in MATERIALS AND METHODS. Break sites were analyzed not only in the endogenous $\lambda 1$ gene of wild-type C57BL/6 mice but also in a $\lambda 1$ transgene that had been shown to undergo active transcription and hypermutation (KONG *et al.* 1998b).

Figure 1 shows examples of one set of amplification reactions, in which breaks in DNA preparations from spleen, lymph nodes, or Peyer's patches of a single C57BL/6 mouse were amplified, resolved by gel electrophoresis, blotted, and hybridized with a $\lambda 1$ -specific oligonucleotide probe. In each of the tissues analyzed, essentially every band visible by ethidium bromide staining hybridized with the probe (Figure 1A), verifying the specificity of the PCR-based assay. Specificity of other primer sets was comparable (data not shown). As illustrated by the examples in Figure 1, there were more distinct product bands produced upon amplification of samples from germinal center B cells than nongerminal center B cells, indicative of more DNA breaks in this cell type. This was the case for samples from spleen (Figure 1A), lymph node (Figure 1B), and Peyer's patches (Figure 1C).

Table 1 summarizes experiments that compared the number of single-strand DNA breaks in the top (non-transcribed) strand of the $\lambda 1$ gene in germinal center and nongerminal center B cells from five individual mice, three wild type (mice 1–3), and two transgenics (mice 4 and 5). The ratio of single-strand breaks in germinal center and nongerminal center B cells ranged from 2.3 to 3.3 and was similar in wild-type and transgenic mice. The absolute number of breaks was higher in the transgenic mice because the $\lambda 1$ transgene is present at six to eight copies per cell (KONG *et al.* 1998b). The average level of top strand breaks per 100 cells in the single copy endogenous $\lambda 1$ genes of the wild-type mice was 1.0 in the germinal center B cells, and 0.35 in the nongerminal center B cells. Thus, in the $\lambda 1$ genes of primary germinal center B cells there was an excess of 0.65 single-strand breaks on the top strand per 100 cells. Similar results were obtained when DNA break frequencies on the bottom (transcribed) strand were analyzed (data not shown). We therefore estimate that ~1.3% of primary germinal center B cells contain breaks associated with hypermutation in either strand of the $\lambda 1$ genes.

Breaksite hotspots in $\lambda 1$ genes from germinal center B cells: The precise sites of DNA breaks were determined by sequencing LM-PCR products. Sequences of 183 breaks were determined, 84 from the endogenous $\lambda 1$ genes and 99 from the $\lambda 1$ transgenes. Breaksites were identified in DNA from both germinal center (PNA^{hi}) and nongerminal center (PNA^{lo}) B cells. The breaksites are mapped in Figure 2. Strikingly, in the samples from the germinal center B cells, there were numerous examples of multiple independent breaks at single sites. In contrast, there was only one example of a site with more

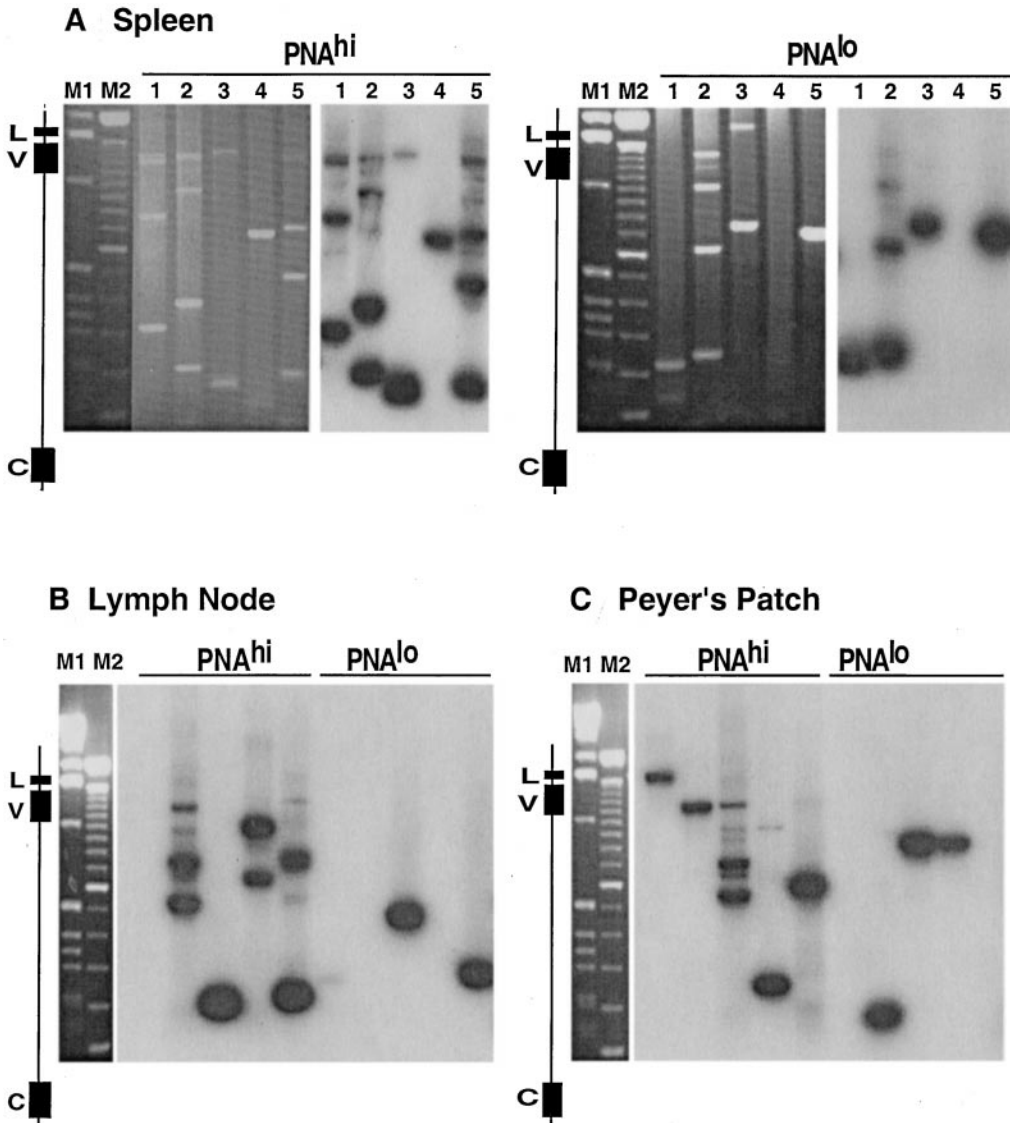


FIGURE 1.—Identification of DNA breaks in germinal center (PNA^{hi}) and nongerminal center (PNA^{lo}) primary B cells. DNA was analyzed in B cells that originated from three different lymphoid tissues: (A) spleen; (B) lymph node; and (C) Peyer's patches. Breaks were identified as described in MATERIALS AND METHODS, using the primer set LRB, LR2, and LR3, which amplifies breaks in the top (non-transcribed) DNA strand, and template DNA from germinal center (PNA^{hi}) and nongerminal center (PNA^{lo}) B cells from a C57BL/6 mouse. The DNA products were separated by agarose gel electrophoresis and hybridized with ³²P-labeled oligonucleotide LR3a, complementary to the J λ 1-C λ 1 intron. The ethidium bromide-stained gel is shown at left (M1, 1-kb ladder; M2, 100-bp ladder), and the corresponding Southern blot at right. A diagram of the rearranged λ 1 gene at the left of A–C provides an approximate map of where breaks occurred. L, leader; V, variable region; C, constant region.

than one break in the samples from nongerminal center B cells, and there were a total of only 2 breaks at this site.

Table 2 compiles these data, showing the number of breaks and breaksites in DNA samples from germinal center and nongerminal center B cells. Of the total of 133 breaks identified in germinal center B cells, 55 (or 41%) were at sites of 2 or more breaks. This contrasts with the results from nongerminal center B cells, where only 2 of 50 breaks (4%) mapped to the same site. If breaks occurred randomly, for example, during DNA preparation, some small fraction of repeated hits would be expected in the dataset, and this fraction would increase with the number of breaks analyzed. Nonetheless, the difference in the number of breaksites identified in the PNA^{hi} and PNA^{lo} cell samples is not sufficient to explain the large difference in multiple hits at individual breaksites. Instead, these results suggest that germinal center B cells carry breaks at specific sites in the λ 1 gene.

Identical breaksite hotspots in the endogenous and transgenic λ 1 genes: Multiple independent breaks were identified at nine sites in the endogenous λ 1 gene, and nine sites in the λ 1 transgene. The sequences of these sites and the number of breaks identified at each site are shown in Figure 3. For simplicity, we refer to sites at which multiple independent breaks were identified as breaksite hotspots. Two top strand and two bottom strand breaksite hotspots were identical in the endogenous λ 1 gene and the transgene and are marked with asterisks in Figure 3, at positions 236 (bottom strand) and 377 (top strand). The breaksite hotspot at position 236 is in the V λ 1 region, 33 bp upstream of CDR3. Nine breaks at this site were independently identified, 7 in samples from wild-type mice and 2 in samples from the transgenic line. The breaksite hotspot at position 377 is in the J λ 1-C λ 1 intron. Ten breaks at this site were independently identified in the top strand, 5 in samples from wild-type mice, and 5 in samples from the transgenic line. Three breaks were also identified in the

TABLE 1
Top strand DNA beaks/100 cells in $\lambda 1$ genes of
germinal center (PNA^{hi}) and nongerminal
center (PNA^{lo}) primary B cells

	Mouse				
	1	2	3	4	5
PNA ^{hi}	0.80	1.2	1.0	9.0	8.5
PNA ^{lo}	0.35	0.40	0.30	4.0	3.0
PNA ^{hi} /PNA ^{lo}	2.3	3.0	3.3	2.3	2.8

Mice 1–3 were wild-type C57BL/6 mice, and mice 4 and 5 were LZ15-90 transgenics carrying six to eight copies per cell of the $\lambda 1$ -E λ_{2-4} transgene. All mice were immunized with NP-CCG. B cells analyzed derived from the spleen in all cases except mouse 3, where they derived from Peyer's patches. Mice 1, 4, and 5 were immunized once and killed 12–14 days later. Mouse 2 was immunized twice, and mouse 3 was immunized three times; these two mice were killed 3–4 days after the last immunization.

bottom strand at the same position. As discussed below, identification of breaks at the same site in top and bottom strands is consistent with the possibility that at least some of the breaks are DSBs. In addition, 3 top strand breaks were identified one nucleotide downstream (po-

sition 378), for a total of 16 breaks clustered at positions 377–378. A cluster of breaks was also observed in the bottom strand (positions 256–257) ~12 bp upstream of CDR3. These breaksite hotspot clusters are indicated by braces in Figure 3.

The breaks in the endogenous $\lambda 1$ gene and the $\lambda 1$ transgene were amplified with different primers. Identification of identical breaksite hotspots in these samples therefore supports the premise that these breaks reflect specific cleavage *in vivo* and are not artifacts of PCR amplification. The fact that identical breaksite hotspots were found in samples from both wild-type C57BL/6 mice and $\lambda 1$ transgenics also eliminates chromosomal location or details of gene organization as possible contributors in determining breaksites.

A duplex breaksite hotspot: Breaksite hotspots are mapped in Figure 4A. As the map shows, the most frequently targeted breaksite hotspots identified in this dataset map at the 3' end of the V $\lambda 1$ region or 5' end of the J $\lambda 1$ -C $\lambda 1$ intron. This region of the $\lambda 1$ gene is highly mutated *in vivo* (GONZÁLEZ-FERNÁNDEZ *et al.* 1994; AZUMA 1998). One breaksite hotspot (position 236) maps just upstream of CDR3. Another (position 269) is within CDR3 and just 1 bp upstream of a hypermutation hotspot at Ala89, characterized by frequent

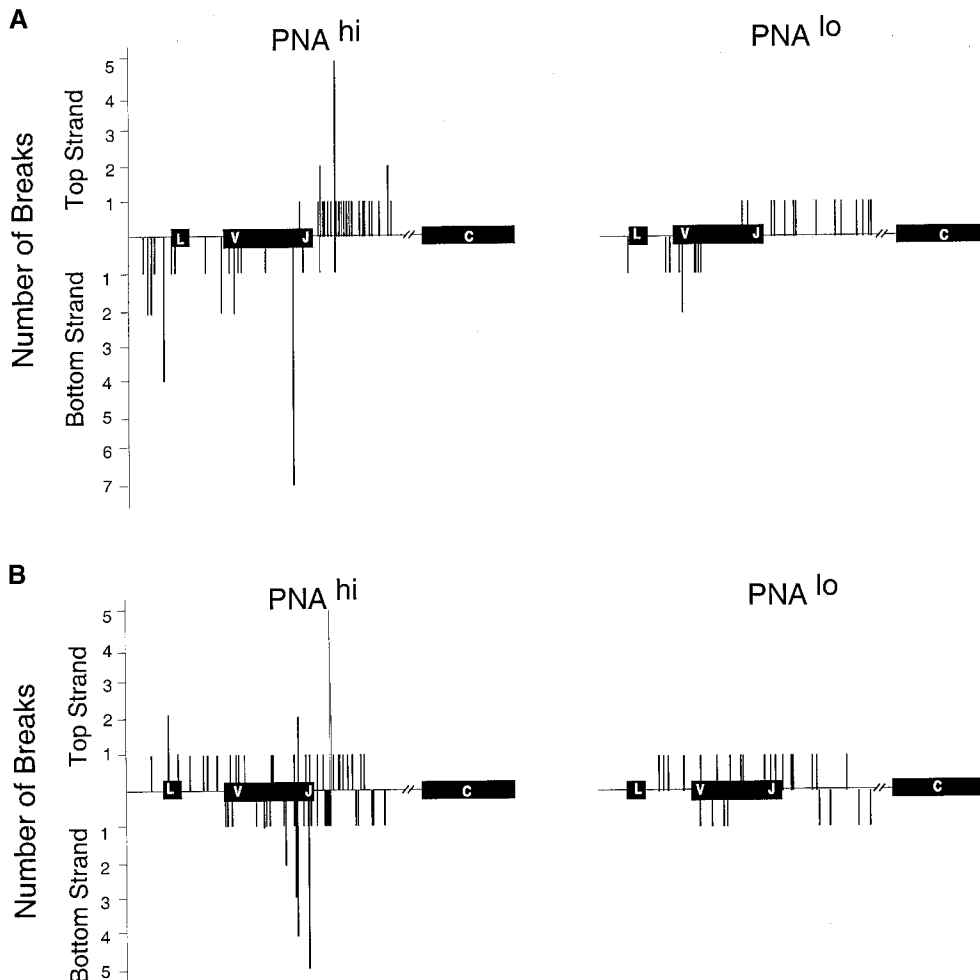


FIGURE 2.—Sites of DNA breaks in $\lambda 1$ genes in germinal center (PNA^{hi}) and nongerminal center (PNA^{lo}) primary B cells. The positions of breaksites were determined by sequencing LM-PCR products. Separate maps are shown for breaks identified in PNA^{hi} and PNA^{lo} cells from (A) the endogenous $\lambda 1$ gene in wild-type C57BL/6 mice and (B) the $\lambda 1$ transgene in the LZ15-90 line. Breaks on the top (non-transcribed) strand are shown above the line, and breaks on the bottom (transcribed) strand below the line. L, leader region; VJ, variable region; C, constant region.

TABLE 2
Breaksites in germinal center (PNA^{hi}) and
nongerminal center (PNA^{lo}) B cells

	PNA ^{hi}			PNA ^{lo}		
	WT ^a	TG ^b	Total	WT ^a	TG ^b	Total
Top strand	29	37	66	14	18	32
Bottom strand	31	36	67	10	8	18
Total breaks	60	73	133	24	26	50
Breaks at sites						
with >1 break	28	27	55	2	0	2
% breaks at sites						
with >1 break	47	37	41	9	0	4

^a WT, endogenous $\lambda 1$ gene in wild-type C57BL/6 mice.

^b TG, $\lambda 1$ transgene in the LZ15-90 line.

mutation of the G in the GCT triplet (GONZÁLEZ-FERNÁNDEZ *et al.* 1994; JOLLY *et al.* 1996). The most heavily targeted hotspot in this collection maps in the intron (position 377–378), just downstream of the 3' end of the $\lambda 1$ region. This breaksite in the $\lambda 1$ -C $\lambda 1$ intron is ~30 bp upstream of a region of the intron that hypermutates very actively (GONZÁLEZ-FERNÁNDEZ *et al.* 1994). Breaks at this site occurred in both top and bottom strands (see Figures 3 and 4A) and in samples from both wild-type mice and transgenics.

Recurrent sequence motifs at breaksite hotspots: *RGYW* has been identified as a consensus site at which hypermutation frequently occurs (ROGOZIN and KOLCHANOV 1992; BETZ *et al.* 1993; JOLLY *et al.* 1996). Almost all breaksites contain at least one match to either the *RGYW* motif or to its reverse complement, *WRCY* (Figure 3). Matches to the *WRCY* motif are in most cases at the breaksite.

All of the breaksite hotspots also contain one of two pyrimidine-rich motifs (Figure 3). An *RCTY*T consensus motif occurs at or just downstream of all breaksite hotspots on the top strand and at hotspots upstream of the leader on the bottom strand. This motif is in many cases preceded by a T, which fuses the *WRCY* hotspot motif (as *TRCT*) and the *RCTY*T motif to generate an expanded consensus motif, *TRCTY*T. A *CCYC* consensus motif occurs at or near hotspots in the variable region and the $\lambda 1$ -C $\lambda 1$ intron on the bottom strand. The same motifs are found in samples from both transgenic and wild-type germinal center B cells.

The potential for the DNA sequences spanning the breaksite hotspots to form secondary structures was analyzed by using the Mfold program (<http://mfold.wustl.edu/folder/dna/form1.cgi>). While many breaksite hotspots had structural potential, this analysis did not identify structural motifs consistently associated with cleavage sites. Nonetheless, because predictions of secondary structure are sensitive to parameters such as sequence length and temperature, this does not rule out the possi-

		Number of breaks at site	Position
Top Strand			
AGCTGTATCA	<u>TGCTCTTCTT</u>	2	-197
TATATTTCTG	<u>TGCTCTATGG</u>	2	269
TAGGTGAGTG	<u>ACTCCTTCCT</u>	2	335
GACTTGAGGT	<u>GCTTTTGTGTT</u> †	10* (5, 5)	377
ACTTGAGGTGC	<u>TTTTTGTGTT</u>	3	378
AAATGCAGCA	<u>CTTCAATAAG</u>	2	577
Bottom Strand			
ATGCAAATTA	<u>CTTTCATTTA</u>	2	-258
CTAGTAATGC	<u>AAATFACITTT</u>	2	-252
CCAGGTCTAT	<u>TCTTTTCAAT</u>	4	-171
ATGAGAAATC	<u>CAGTCATCAG</u>	2	-32
GGGAAATGGC	<u>CCCTGCAAAC</u>	2	-10
AGTCTGTCCG	<u>CCTGTGATGG</u>	9* (7, 2)	236
AAATATATTTG	<u>CCTCATCCTC</u>	3	256
GAAATATATTT	<u>GCCTCATCCT</u>	4	257
CAGTTTGTGTT	<u>CCTCCACCGA</u>	3	308
AACAAAAAGC	<u>ACCTCAAGTC</u> †	3	377

Consensus sequences: RCTYT, CCYC, *RGYW*, *WRCY*

FIGURE 3.—Sequences of breaksite hotspots. Sequences flanking all sites at which multiple breaks were identified are shown. Breaks are indicated by gaps. The number of times each breaksite was independently identified is shown in the second column, and the third column shows the position of the break, using the numbering system of GONZÁLEZ-FERNÁNDEZ *et al.* (1994), in which the first nucleotide of the V region is number 1 and the last nucleotide in the J region is position 328. Breaksites identified on both top and bottom strands are indicated by double daggers (‡). Breaksites identified in samples from both wild-type and transgenic mice are indicated by asterisks (*), and numbers identified in wild-type and transgenics, respectively, are shown in parentheses. In two cases, breaks were separated by only a single base. These clustered breaksites are indicated by brackets, and the breaksite sequences are aligned. Pyrimidine-rich consensus sequence motifs *RCTY*T and *CCYC* are underlined. Matches to the hypermutation hotspot *RGYW* and its reverse complement, *WRCY*, are in lightface type.

bility that DNA must become transiently structured for cleavage.

Breaks localize to the region of the $\lambda 1$ gene that hypermutates: To ask if the breaks in primary PNA^{hi} B cells reflected generalized degradation of genomic DNA rather than a feature of the hypermutation mechanism, we wanted to compare breaks in regions of DNA that mutate at very different rates. We chose for comparison two regions of the $\lambda 1$ -C $\lambda 1$ intron. The upstream region of this intron hypermutates actively and includes a hotspot that hypermutates at a level comparable to the complementarity-determining regions (CDRs) within the V segments; and the downstream region mutates at a much lower level (GONZÁLEZ-FERNÁNDEZ *et al.* 1994; AZUMA 1998). The $\lambda 1$ -C $\lambda 1$ region has a further advan-

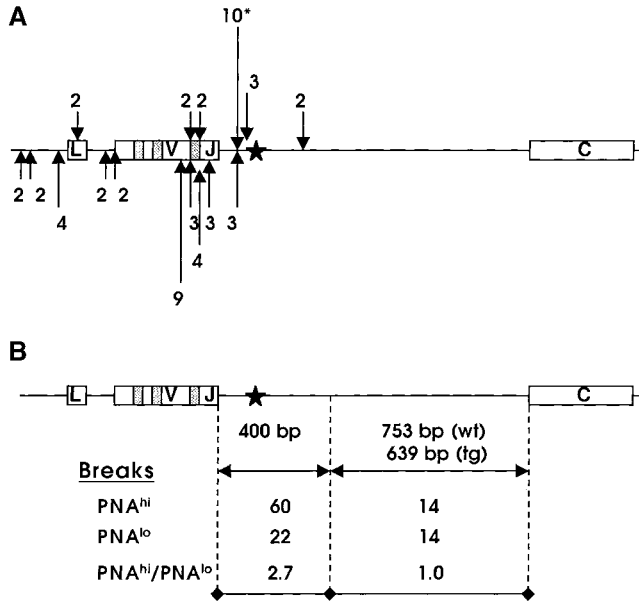


FIGURE 4.—Breaksites correlate with somatic hypermutation. (A) Breaksite hotspots mapped in the $\lambda 1$ gene. The murine $\lambda 1$ locus is shown. Arrows denote positions of breaksite hotspots listed in Figure 3. Numbers above arrows denote the number of breaks at each position among the 133 breaksites identified from PNA^{hi} cells. (B) Breaks localize to the region of the J $\lambda 1$ -C $\lambda 1$ intron that hypermutates. The numbers and ratios of breaks in two intervals within the intron are compared in germinal center (PNA^{hi}) and nongerminal center (PNA^{lo}) B cells. The upstream interval is 400 bp in length, undergoes active hypermutation, and includes a hypermutation hotspot (GONZÁLEZ-FERNÁNDEZ *et al.* 1994) that is denoted by a star. The downstream interval is 753 bp long in wild-type mice and 639 bp in the transgenics and mutates at much lower levels.

tage for this analysis, namely, that it does not encode protein and is not subject to clonal selection. Moreover, by comparing hypermutation of different zones within the same gene, rather than two different genes, the comparison could be made independently of differences in levels of transcription. We separately analyzed breaks in two regions within the J $\lambda 1$ -C $\lambda 1$ intron: a 400-bp upstream interval, with its 5' border at the J $\lambda 1$ boundary, and a downstream interval containing the remainder of the intron, with the 3' boundary at C $\lambda 1$. The downstream interval is 753 bp in the wild-type mice and 639 bp in the transgenics. This difference is due to a 114-bp deletion introduced into the intron during transgene construction (HENGSTSCHLÄGER *et al.* 1994).

Figure 4B shows that in DNA from germinal center B cells there were 60 breaks in the upstream interval and 14 breaks in the downstream interval and that in nongerminal center B cells there were 22 breaks in the upstream interval and 14 breaks in the downstream interval. The ratio of breaks in germinal center (PNA^{hi}) to nongerminal center (PNA^{lo}) cells is therefore 2.7 in the upstream interval and 1.0 in the downstream interval. Because the same PCR primers and protocols were used to amplify DNA from both cell populations, the

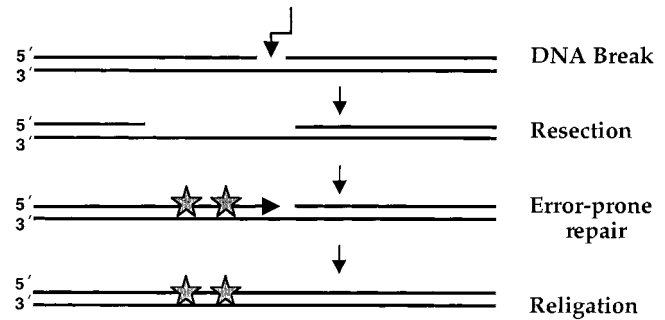


FIGURE 5.—DNA break-and-repair model for somatic hypermutation. A single-strand break introduced into the DNA is shown (top line). The broken strand is resected by a 3'-5' exonuclease (second line); mutations (stars) are introduced by an error-prone polymerase (third line); and the DNA is religated (last line). Errors introduced into one DNA strand can be fixed by subsequent DNA replication or by mismatch repair. A similar model can also be drawn for initiation of hypermutation by a double-strand break. This polarity for resection is shown to emphasize how a 5' end may persist during both resection and repair, but our data do not preclude resection by a nuclease of opposite polarity or strand displacement by a repair polymerase.

data are very unlikely to reflect particulars of the PCR analysis. We conclude that the excess of breaks in germinal center B cells is confined to regions of DNA that actively hypermutate. This provides further evidence that the breaks are associated with the hypermutation mechanism.

DISCUSSION

We have identified single-strand breaks in $\lambda 1$ genes of actively hypermutating primary germinal center B cells. These breaks appear to be intermediates in the hypermutation pathway, for the following reasons: (1) There are more breaks in $\lambda 1$ genes from germinal center B cells than from nongerminal center B cells; (2) a significant fraction (41%) of the breaks in germinal center B cells occur at breaksite hotspots, sites at which two or more independent breaks were identified; (3) characteristic motifs could be identified at breaksite hotspots, including the RGYW motif (or its reverse complement, WRCY), which is associated with hotspots for somatic hypermutation; and (4) breaks are concentrated in DNA regions that hypermutate.

Figure 5 presents a simple model for how repair of a DNA break could produce mutations. In the first step, DNA undergoes cleavage, shown here as producing a single-strand break. In the second step, DNA is resected; next, error-prone repair introduces mutations; and in the last step the duplex is religated. The figure shows resection occurring by a 3' \rightarrow 5' exonuclease to dramatize the fact that the 5' end of a DNA break may persist unaltered between the initial cleavage event and the final religation step. In contrast, the 3' end of the DNA

break is effectively a moving target, which may be attacked by an exonuclease during resection, and which will necessarily travel in the 5' → 3' direction during repair by DNA polymerase. The model in Figure 5 is similar in outline to the model originally proposed by BRENNER and MILSTEIN (1966) to explain the origin of antibody variability. The pathway outlined in Figure 5 could readily accommodate initial cleavage to produce a double-strand break, resection by a nuclease with opposite polarity, or strand displacement that accompanies repair synthesis. Cleavage followed by strand transfer rather than error-prone repair could result in templated mutation, as previously proposed by our laboratory (MAIZELS 1995; KONG *et al.* 1998a; HARRIS *et al.* 1999).

The LM-PCR assay we used ligates an identifying linker to a free 5' end. The assay thus reports both single-strand breaks and DSBs. If 5' → 3' exonucleolytic degradation accompanies either repair or strand displacement, the dynamic changes in the position of the 5' end will also be identified as nicks at distinct sites; but the assay will not register 3' → 5' exonucleolytic digestion or extension of the 3' end by a repair polymerase. Thus, some of the breaksites we have identified in V λ 1 regions of primary germinal center B cells may represent initial sites of DNA cleavage, and others may represent sites at which resection or repair is ongoing. Several of the breaksite hotspots were clustered (Figure 3). Clustering is consistent with a mechanism in which initial cleavage is imprecise or in which limited processing of the breaksite occurs prior to linker ligation.

Other laboratories identified DSBs in immunoglobulin genes in hypermutating B cells (BROSS *et al.* 2000; PAPAVALIOLIOU and SCHATZ 2000). Because the assay we used detects breaks on both strands, DSBs would be expected to comprise a subset of the breaks we identified. At least one breaksite, at positions 377–378, almost certainly reflects cleavage of the DNA duplex, because breaks were identified on both top and bottom strands at this site (see Figures 3 and 4A). This is the most predominant breaksite identified in the λ 1 gene: breaks at this site account for 12% of the total breaks in germinal center B cells and 29% of the breaks at hotspots. Breaks were found at this site in both the endogenous λ 1 gene and the transgene. This site is only 30 bp upstream from the region of the J λ 1-C λ 1 intron that hypermutates very actively (positions 406–431; GONZÁLEZ-FERNÁNDEZ *et al.* 1994), and it is possible that initial cleavage at 377–378 is followed by repair that preferentially alters the sequence at this downstream region. Other breaksite hotspots we identified may also correspond to sites of duplex cleavage, but the fact that we did not detect breaks on both strands at some of the most frequently cleaved sites makes it unlikely that all breaks are DSBs. We estimate that 1.3% of primary germinal center B cells contain breaks in the λ 1 regions associated with hypermutation (Table 1). DSBs were estimated to be

present in the V_H region of ~4% of Ramos cells (PAPAVALIOLIOU and SCHATZ 2000). The higher fraction of Ramos cells with breaks may reflect longer persistence of breaks in cultured cells, either because breaks are repaired less rapidly in this cell line or because cultured cells cycle less rapidly than primary germinal center B cells.

Several lines of evidence suggest that there may be two or more components to the hypermutation apparatus (MILSTEIN *et al.* 1998; RADA *et al.* 1998; SALE and NEUBERGER 1998; EHRENSTEIN and NEUBERGER 1999; SPENCER *et al.* 1999; COWELL and KEPLER 2000). It is possible that one distinguishing feature of these two components is creation or persistence of single-strand breaks and DSBs. The possibility that one component of this process involves single-strand breaks is consistent with considerable evidence pointing to the involvement of mismatch repair in somatic hypermutation (CASALHO *et al.* 1998; FREY *et al.* 1998; KIM and STORB 1998; PHUNG *et al.* 1998, 1999; RADA *et al.* 1998; WINTER *et al.* 1998; KONG and MAIZELS 1999; VORA *et al.* 1999; WIESENDANGER *et al.* 2000) and with the absence of a requirement for DNA protein kinase (BEMARK *et al.* 2000) in hypermutation. The presence of single-strand breaks is also consistent with observations of BROSS *et al.* (2000), who identified DSBs in heavy chain genes in murine germinal center B cells. This group found that the DNA end at the promoter-proximal side of breaks was more readily ligated to blunt duplex linkers than the promoter-distal end, leading them to suggest that a single-strand lesion may be processed to produce a blunt end.

Specific sequence motifs could be identified at or near most breaksite hotspots. Matches to the hypermutation consensus, RGYW, and either of two pyrimidine-rich consensus motifs were also evident at hotspots. The RCTYT consensus motif was found at top strand breaksite hotspots and at bottom strand hotspots upstream of the beginning of the leader intron. The CCYC motif was found at bottom strand breaksite hotspots within and downstream of the V region. The sites at which multiple independent breaks were identified in the V λ 2 region of the human B cell line Ramos (PAPAVALIOLIOU and SCHATZ 2000) also contain matches to either the RCTYT or the CCYC motif. Although our data do not bear directly on the question of whether transcription plays a role in production of DNA breaks, the observation that different sequence motifs predominated at breaksite hotspots on the transcribed and nontranscribed strands is intriguing in light of the possibility that the hypermutation apparatus differentiates between the transcribed and nontranscribed DNA strands.

The consensus motifs at hotspots are especially compelling evidence in support of the notion that the breaks reflect some aspect of the hypermutation mechanism. The breaksite hotspot sequence motifs identified in our experiments may represent sites of endonuclease cleavage or sites at which the exonucleases or polymerases pause during DNA repair. If the breaksite hotspot motifs

do define the specificity of one or more activities essential to the hypermutation mechanism, then it should be possible to identify these enzymes and establish their functions.

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