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Rearrangement and Selection in the Developing Vκ Repertoire of the Mouse: An Analysis of the Usage of Two Vκ Gene Segments

Elizabeth A. Whitcomb and Peter H. Brodeur

Detailed analysis of the rearrangement and expression of two mouse Vκ genes has been used to examine B cell repertoire development. The Vκ1-A gene is used by a large proportion (9.6%) of splenic B cells in the adult primary repertoire, whereas the Vκ22 gene is used at a much lower frequency (0.16%). Consistent with these results, quantitative PCR (Q-PCR) assays revealed that the number of splenic B cells with rearranged Vκ1-A genes is much greater than the number with rearranged Vκ22 genes. Q-PCR was also performed on both normal bone marrow pre-B cells and transformed pre-B cells induced to rearrange their κ loci at high frequency. In contrast to splenic B cell rearrangements, the numbers of Vκ1-A and Vκ22 rearrangements in pre-B cells differ by only two- or threefold, suggesting that the intrinsic rearrangement frequencies of these two Vκ genes are not significantly different. Further evidence of disproportionate selection was obtained by comparing the percentages of productive rearrangements amplified from genomic splenic DNA. Sequence analysis showed 84% (37 of 44) of the Vκ1-A rearrangements but only 57% (29 of 51) of the Vκ22 rearrangements to be in-frame. Together these results suggest that B cells expressing Vκ1-A-encoded light chains are preferentially selected either in the periphery or in the transition from pre-B to B cell. Sequence data also reveal a surprisingly restricted diversity of VJ junctions, apparently due to biases introduced by the rearrangement mechanism.

repertoire development, as will any limitations on the association of particular heavy and light chains.

Although the potential for multiple mechanisms to participate in shaping the primary B cell repertoire is well documented, the relative influence of these mechanisms and the stage at which they operate are still largely unknown. In the present study we examine the rearrangement of two \( \kappa \) gene segments. We have determined that the \( \kappa 1 \)-A gene is preferentially used in the adult repertoire and have compared this over-represented \( \kappa \) gene segment with the \( \kappa 22 \) gene, which is represented at a much lower frequency. Consideration of rearrangement frequencies at several stages of B cell development, productive/nonproductive rearrangement ratios, and analysis of VJ junctions from splenic B cells and bone marrow pre-B cells demonstrate that selection is more influential than rearrangement frequency in preferential \( \kappa 1 \)-A gene utilization. Our data also support the possibility that sequence-based biases of the rearrangement mechanism have led to the selection of germline \( \kappa \) and J\( \kappa \) sequences that preferentially form particular, presumably beneficial, CDR3 sequences.

**Materials and Methods**

**Mice**

Female BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Tufts University School of Medicine (Boston, MA) animal facility.

**\( \kappa \) cDNA phage libraries**

Details of the construction of the \( \kappa \) cDNA libraries have been published (22). Briefly, cDNAs were synthesized using poly(A)\(^+\) RNA isolated from BALB/cByJ spleen cells following 3-day culture with 50 \( \mu \)g/ml LPS. Each library was prepared using spleen cells pooled from four or five 11- to 15-wk-old female mice. First-strand synthesis was conducted using an oligonucleotide primer specific for the \( \kappa \) constant region (Ce). Double-stranded cDNA was prepared and treated with EcoRI methylase, ligated to EcoRI linkers, and size fractionated (600–650 bp) by electrophoresis through 5\% polyacrylamide. These CDNA's were cloned into \( \lambda \)gt10. Two libraries, libraries 1 and 2, have been amplified once. The original (nonamplified) libraries contained 240,000 (library 1) and 700,000 (library 2) \( \kappa \)C sequences. Libraries 1 and 2, have been amplified once. The original (nonamplified) libraries have been published (27). Details of the construction of the V\( \kappa 1 \) cDNA libraries have been published.

**Preparation of bone marrow cells**

Bone marrow cells were harvested from tibias and femurs of 2- to 5-mo-old mice by flushing with Dulbecco’s PBS (5\% FCS; HyClone, Logan, UT). Erythrocytes were resuspended in Dulbecco’s PBS (10\% FCS; HyClone, Logan, UT). Erythrocytes were resuspended in Dulbecco’s PBS (10\% FCS) and maintained in the Tufts University School of Medicine (Bar Harbor, ME) and maintained in the Tufts University School of Medicine.

**Preparation of spleen cells**

Splenocyte IgD\(^+\) cells were prepared from individual 3- to 5-mo-old mice by panning with the IgG fraction of polyclonal sheep anti-mouse IgD (The Binding Site, San Diego, CA). Spleen cell suspensions were prepared in Dulbecco’s PBS (10\% FCS; HyClone, Logan, UT). Erythrocytes were removed by lysis with 17 mM Tris-Cl and 140 mM ammonium chloride (pH 7.2), and white blood cells were resuspended in Dulbecco’s PBS (5\% FCS). Cells (3 \times 10\(^7\)) were gently overlaid onto 100-mm plates previously coated with 0.75 mg/ml anti-IgD in 10 ml 50 mM Tris-Cl, pH 8.5. Cells were allowed to adhere for 1 h at 4°C with occasional swirling. Adherent cells were removed by washing with Dulbecco’s PBS (5\% FCS) with the aid of a rubber policeman. Cell purity was determined by flow cytometry following staining with FITC-coupled goat anti-mouse IgM (Fisher Scientific, Pittsburgh, PA) and biotin-conjugated anti-mouse Thy1.2 (Becton Dickinson, Mountain View, CA). The second step reagent for biotin-conjugated Abs was either FITC-conjugated streptavidin (Sigma, St. Louis, MO) or phycocerythrin-conjugated streptavidin (PharMingen, San Diego, CA). Spleen preparations were at least 95\% IgM\(^+\) and were <1\% Thy1.2\(^-\).

**Preparation of DNA for PCR**

For all V\( \kappa 1 \) rearrangements from bone marrow, the initial amplification of 30 cycles was performed with a 5\' \( \kappa \)-specific primer (V\( \kappa 1\)-5' or V\( \kappa 22\)-5') and the J\( \kappa 5 \) primer. A sample (5\%–10\%) of the resulting product was used to further amplify selected regions with an internal \( \kappa \)V-specific primer (V\( \kappa 1\)-3' or V\( \kappa 22\)-3') and the J\( \kappa 5 \) primer. Amplification of V\( \kappa 22 \) rearrangements from spleen used the same two-stage, nested primer protocol (amplification using the V\( \kappa 22\)-1-3' primer followed by amplification using the V\( \kappa 22\)-3'/J\( \kappa 5 \) pair). Amplification of splenic V\( \kappa 1 \)-A rearrangements required only one set of 30 cycles using a V\( \kappa 1\)-A-specific primer (V\( \kappa 1\)-5' or V\( \kappa 1\)-3') and the J\( \kappa 5 \) primer. Following amplification, the 5' overhangs of the PCR products were filled in by treatment with T4 DNA polymerase (Perkin-Elmer, Branchburg, NJ) as previously described (27). The resulting product was subjected to a further 30 cycles of nested amplification with an internal V\( \kappa \)-specific primer (V\( \kappa 1\)-3' or V\( \kappa 22\)-3') and the J\( \kappa 5 \) primer. A sample (5\%–10\%) of the resulting product was used to further amplify selected regions with an internal \( \kappa \)V-specific primer (V\( \kappa 1\)-3' or V\( \kappa 22\)-3') and the J\( \kappa 5 \) primer. Amplification of V\( \kappa 22 \) rearrangements from spleen used the same two-stage, nested primer protocol (amplification using the V\( \kappa 22\)-1-3' primer followed by amplification using the V\( \kappa 22\)-3'/J\( \kappa 5 \) pair). Amplification of splenic V\( \kappa 1 \)-A rearrangements required only one set of 30 cycles using a V\( \kappa 1\)-A-specific primer (V\( \kappa 1\)-5' or V\( \kappa 1\)-3') and the J\( \kappa 5 \) primer. Following amplification, the 5' overhangs of the PCR products were filled in by treatment with T4 DNA polymerase I (Life Technologies, Gaithersburg, MD) for 15 min at 37°C. The products corresponding to J\( \kappa 5 \) rearrangements were purified by agarose gel electrophoresis and ligated into SmaI-digested pGem-Z (Promega, Madison, WI) as previously described (27).

**Q-PCR competitive constructs**

Q-PCR competitors were made by amplifying the 5' and 3' ends of full-length V to J\( \kappa 5 \) rearrangements for both the V\( \kappa 1\)-A and V\( \kappa 22 \) genes using the following primers (EcoRI sequences are underlined). A 137-bp portion of the 5' end of the V\( \kappa 1\)-A gene was amplified using the primers: 5'-GAATTCGAGACTGCTGGCTTC-3' (forward, codons 38 – 44) and the V\( \kappa 1\)-5' reverse primer, described above (codons 3 – 9). A 117-bp portion of the 5' end of the V\( \kappa 22 \) gene was amplified using 5'-GAATTCGACTGCTGGCTTC-3' (forward, codons 46 – 52) and the V\( \kappa 22\)-5'.
reverse primer described above (codons 18–25). The 3’ end of both rearrangements consisted of the identical 53 bp of Jκ5-coding and 3’-flanking sequence amplified with the Jκ5/intron primer (forward, see above) and 5’-GAATTCGCTCACGTTCGGTGCTG-3’ (reverse, codons 95–101 of Vκ-Jκ5 rearrangement). The purified products were digested with EcoRI and ligated overnight, and ligated product was reamplified with the external primers. The resultant products were purified and cloned as described for PCR products. The full-length amplified product is approximately 300 bp, while the competitors are approximately 200 bp. The Vκ1-A and Vκ22 competitor constructs are denoted Vκ1-ΔA and Vκ22Δ, respectively.

**Q-PCR assay**

Dilutions of PstII (Life Technologies)-linearized competitor plasmids were added to otherwise identical PCR reactions. Vκ1-A assays used Jκ5/intron (forward) and Vκ1-5’ (reverse) primer pairs. Vκ22 assays used Jκ5/intron (forward) and Vκ22-5’ (reverse) primers. The reactions were resolved on an agarose gel, transferred to nitrocellulose and hybridized with an oligonucleotide probe specific for either Vκ1-A or Vκ22 (see below). Hybridization was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and copy numbers were calculated as described by Piatak et al. (28). Copy numbers were determined by plotting log(signal of target/signal of competitor) on the y-axis against log(copy number of competitor) on the x-axis. In our assay, the target refers to endogenous rearrangements of either Vκ1-A or Vκ22 to the Jκ segment. Competitor refers to either Vκ1-ΔA or Vκ22Δ, described above. The genomic rearrangement copy number was calculated from the y-intercept of the plotted values, the point at which the signals of the competitor and the target are equivalent.

**Southern blots**

High m.w. DNA was digested to completion and separated by electrophoresis through 0.8 to 1% agarose gels. PCR products were separated through 2% agarose gels. DNA was transferred to BA-S85 nitrocellulose (Schleicher and Schuell, Keene, NH) by capillary transfer. Hybridization with restriction fragment probes was performed as described previously (29). Hybridization with oligonucleotide probes was conducted in 5 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate), 2 × Denhardt’s solution (0.4% each of Ficoll-400, polyvinylpyrrolidone-360, and BSA), 0.5% SDS, and 100 μg/ml sonicated salmon sperm DNA. Unless otherwise indicated, all Southern blot reagents were obtained from Sigma. The Vκ1-A specific oligonucleotide used for Q-PCR hybridization was 5’-GT CAGAGCCCTGTACACAG-3’. The Vκ22-specific oligonucleotide used for Q-PCR hybridization was 5’-CAAGCAAACACAAGGTGCAC-3’. Both Vκ1-A and Vκ22 oligonucleotide hybridization probes were used at 60°C.

**Cloning the germline Vκ22 gene**

Approximately 200 ng of size-fractionated (4 kbp range) EcoRI fragments from BALB/c liver DNA were ligated with 1 μg of λgt10 EcoRI arms (Promega) and packaged in vitro using Packagene extracts (Promega). Recombinant phage containing the germline Vκ22 gene were identified by plaque lift hybridization using a Vκ22 family probe as described previously (22).

**Sequencing**

Double-stranded sequencing of purified plasmid preparations was performed with the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) according to the manufacturer’s protocols.

**Results**

Previous analyses of both Vκ1 and Vκ utilization suggested that individual Vκ genes are not equally represented in the naive repertoire. To gain insight into the mechanism(s) responsible for the unequal usage of Vκ genes, we compared the rearrangement of two individual Vκ genes, Vκ1-A and Vκ22. These two functional Vκ gene segments were chosen for comprehensive study because, as detailed below, they are used at remarkably different frequencies in the adult primary Ab repertoire. The Vκ gene utilization studies described below involve the analysis of two Vκ cDNA phage libraries, both prepared using RNA isolated from LPS-stimulated BALB/c spleen cells. This molecular cloning approach to repertoire analysis has been described in detail (22, 30) (see Materials and Methods).

| Table I. Utilization of Vκ1-A and Vκ22 gene segments in adult spleen cells |
|-----------------------------|-----------------------------|-----------------------------|
| Vκ cDNA Library | Library 1 | Library 2 | Total |
| Vκ1-Α/Cκ | 569/7,100 | 1303/12,451 | 1,872/19,551 |
| (8.0%) | (10.5%) | (9.6%) |
| Vκ22/Cκ | 39/26,243 | 20/9,932 | 59/36,175 |
| (0.15%) | (0.20%) | (0.16%) |

Utilization was determined based on the frequency of Vκ1-A and Vκ22 gene sequences within Vκ cDNA phage libraries. Each of two independent phage libraries were prepared using RNA from spleen cells stimulated with LPS in vitro for 3 days. Each library was constructed from spleen cells pooled from five BALB/c mice. Values were determined by hybridization of plaque lifts with either a Vκ1-A-specific oligonucleotide probe or a Vκ22-specific fragment probe. The number of Cκ positive phage was determined using a Cκ probe.

The construction and characterization of Vκ library 1 and Vκ library 2 are described in Kalled and Broder (22). Seventy percent of the phage in library 1 and Cκ+, and 37% of the phage in library 2 are Cκ+.

Number of Vκ1-A† cDNAs / number of Cκ+ cDNAs screened.

Number of Vκ22‡ cDNAs / number of Cκ+ cDNAs screened.

Summation of data for libraries 1 and 2.

**Utilization of the Vκ1-A gene**

The Vκ1 family consists of three functional genes (Vκ1-A, Vκ1-B, and Vκ1-C) in the BALB/c germ line (31). We and others have shown that, despite its small size, the Vκ1 family is expressed by a large proportion (13–26%) of naive B cells (22, 32, 33). To determine the contribution of a single member of the Vκ1 family, an oligonucleotide probe was designed to be specific for the Vκ1-A gene. The Vκ1-A-specific hybridization temperature was determined empirically by melting experiments using a panel of 96 Vκ cDNA-containing phage isolated based on hybridization with a Vκ1 family probe (22). Based on these experiments, more than one-half (54 of 96) of the Vκ1 cDNAs were Vκ1-A. Three Vκ1-A-hybridizing and one non-Vκ1-A-hybridizing cDNAs were randomly selected from the Vκ1 panel and sequenced. All three Vκ1-A hybridizing phage contained a Vκ gene segment corresponding to the published Vκ1-A germline sequence, whereas the nonhybridizing Vκ cDNA was identical with the published Vκ1-C sequence (31) (data not shown).

Using the Vκ1-A-specific oligonucleotide probe, 9.6% of approximately 19,500 Cκ hybridizing phage were found to contain Vκ1-A sequence (Table I). This figure is consistent with the finding (above) that more than half of the Vκ1-positive phage hybridized with the Vκ1-A-specific probe and that 13% of the Cκ-sequence containing cDNAs in the library have Vκ1 family sequences (22).

**Utilization of the Vκ22 gene**

Based on Southern blot analysis, the Vκ22 family consists of a single gene (34). Previous analysis of two Vκ cDNA libraries, using a digoxigenin-labeled probe, indicated that the Vκ22 gene is used at a relatively low frequency of 0.18% (22). This low frequency of Vκ22 representation was verified by screening approximately 36,000 Cκ-hybridizing phage with a 32P-labeled Vκ22 probe. Consistent with previous results, only 0.16% (59 phage) hybridized with the Vκ22 probe (Table I). To make certain that the Vκ22 probe recognized only one germline sequence, four of the Vκ22-hybridizing phage were chosen at random, and the cDNA inserts were sequenced. The rearranged Vκ genes of all four cDNAs were identical in sequence and also identical with the Vκ22 gene expressed by the plasmacytoma S107 (25).
It is possible that RSS and/or associated sequences contribute to differences in the utilization of V<sub>k</sub>1-A and V<sub>k</sub>22. To compare the RSS of these two genes, the 4.0-kbp EcoRI fragment containing the germline V<sub>k</sub>22 gene was cloned from BALB/c liver DNA. The sequence of the germline V<sub>k</sub>22 gene was determined and was found to be identical with that of the V<sub>k</sub> gene expressed by S107 and the four V<sub>k</sub>22 cDNAs from our V<sub>k</sub> phage library (Fig. 1).

The heptamer sequences of both V<sub>k</sub>1-A and V<sub>k</sub>22 match the consensus sequence, and both have 12-bp spacers. The nonamer sequence of V<sub>k</sub>22 (ACACAAACC) differs from consensus (ACAAAAACC) only at position 4, one of the least conserved nucleotides (8). Based on a recombination assay using retroviral constructs, changes in nonamer position 4 appear to be tolerable and result in a rearrangement rate of 27% relative to the consensus RSS (35). The V<sub>k</sub>1-A nonamer (ACAAAAATA) also differs from consensus at positions 8 and 9. As assayed in retroviral constructs, changes in these two positions decreased the rearrangement rate to 65% of the consensus RSS (35). Thus, the available data suggest that the V<sub>k</sub>1-A RSS might be somewhat more efficient than the V<sub>k</sub>22 RSS. However, since RSSs identical with those of V<sub>k</sub>1-A and V<sub>k</sub>22 have not been tested, this suggestion must be viewed with considerable caution.

### FIGURE 1

Sequence of the BALB/c germline V<sub>k</sub>22 gene, the rearranged and expressed V<sub>k</sub>22 gene of the BALB/c plasmacytoma S107 (25), and four V<sub>k</sub>22 cDNAs isolated from a BALB/c V<sub>k</sub> cDNA phage library (22). The heptamer-nonamer sequence of the germline gene is underlined. The S107 rearrangement and the rearrangements of cDNA clones 1, 2, and 4 are to J<sub>k</sub>5. The cDNA clone 3 is a V<sub>k</sub>22-J<sub>k</sub>2 rearrangement. Dots denote nucleotide identity with germline gene, except for the J<sub>k</sub> region, where they denote identity with the S107 sequence. Codon numbering is according to Kabat et al. (26). V<sub>k</sub>22, germline sequence was submitted to GenBank (accession number AF044198).

**Coding sequences immediately flanking the heptamer sequence** can also affect the rearrangement efficiency of gene segments, as assessed using extrachromosomal substrates. Gerstein and Lieber (9) found a hierarchy of rearrangement efficiencies correlating with the nucleotide immediately flanking the heptamer signal (C, G > A > T), where a C or G immediate to the heptamer is the most efficient, and an A or T is less efficient. The V<sub>k</sub>22-coding region ends in a T, while the V<sub>k</sub>1-A gene ends in a C. This T flanking the V<sub>k</sub>22 heptamer sequence could further contribute to a decreased rearrangement efficiency of the V<sub>k</sub>22 gene relative to that of the V<sub>k</sub>1-A gene.

**In vivo rearrangement frequency**

In addition to the RSS and flanking sequences, other features that might influence the rearrangement frequency of individual gene segments include chromosomal position (36, 37), transcriptional orientation (38), and other associated noncoding elements, such as the octamer element (39), which may affect accessibility to the recombinase. To determine rearrangement frequency in vivo, we developed a quantitative competitive PCR assay with specificity for V<sub>k</sub>1-A and V<sub>k</sub>22. Accurate determinations of the number of rearrangements in a population of cells are obtained by adding a precise amount of competitor DNA to a series of PCR reactions.
To verify the accuracy of the assay, experiments were performed with the S107 plasmacytoma cell line, which has rearranged the V<sub>k22</sub> gene to J<sub>k5</sub> on one allele. To maintain a constant number of cells per reaction, dilutions of S107 cells were made in 25A fibroblasts, which have no κ locus rearrangements. These pilot experiments demonstrated that the assay was linear between 100 and 10,000 copies. These experiments also allowed the number of V<sub>k22Δ</sub> competitor molecules to be precisely calibrated by comparison with the number of S107 cells added to a reaction (data not shown). After calibration, the signals between the same number of S107 cells and V<sub>k22Δ</sub> competitor molecules gave signals within twofold in subsequent experiments.

The concentration of the V<sub>k1-1</sub> competitor was calibrated by comparison to V<sub>k22Δ</sub>. Competitor construct dilution series were amplified using a primer pair specific for plasmid sequences common to both competitor molecules and flanking the V<sub>k</sub> sequences in V<sub>k22Δ</sub> and V<sub>k1-1</sub>. The PCR products were analyzed by Southern blots hybridized with the J<sub>k5</sub> oligonucleotide probe that contains sequence shared by both V<sub>k22Δ</sub> and V<sub>k1-1Δ</sub>

The in vivo rearrangement frequencies were determined in the absence of selection by examining κ locus rearrangements in the 103/4 cell line (23). These B-lineage cells are transformed with a temperature-sensitive mutant of Ab-MLV and have been shown to undergo a high frequency of κ locus rearrangement when maintained at the nonpermissive temperature. Since these cells do not express an Ig heavy chain protein, there is no possibility of BCR-mediated growth selection, and the resulting V<sub>k</sub> gene rearrangements should reflect the intrinsic rearrangement frequency of the joined DNA segments.

For all rearrangement frequency determinations, we compared the amplification of the competitor only to J<sub>k5</sub> rearrangements. This strategy allowed comparison of PCR products of similar size and avoided differences in the efficiencies of amplification and Southern transfer. In addition, the larger PCR products associated with the more 5' Jκ segments (Jκ1 and Jκ2) were not reproducibly detected in all experiments, most likely because of the larger sizes of those products. Figure 2 shows an experiment performed on the cell line 103/4 induced to rearrange κ by being grown at the nonpermissive temperature for 20 h. The number of V<sub>k1-1</sub> rearrangements in the 103/4 cells was 2.2-fold greater than that of the V<sub>k22</sub> rearrangement. A second, independent TS Ab-MLV-transformed pre-B cell line, DE/1, was also examined. Although the low number of copies (<100/100,000 cells) of both V<sub>k1-1</sub> and V<sub>k22</sub> rearrangements to J<sub>k5</sub> did not permit reliable quantification, both V<sub>k</sub> segments appeared to be rearranged at comparable low levels in the DE/1 cell line.

To quantify rearrangement frequency in normal B-lineage cells, Q-PCR assays were performed on three populations of cells: unfractionated bone marrow, B cell-depleted bone marrow cells as a source of Ig<sup>−</sup> pre-B cells, and IgD<sup>+</sup> splenocytes as a source of mature B cells. Figure 3 shows representative experiments for these three cell populations, and the calculated number of rearrangements in each population is summarized in Table II. Consistent with the results using TS Ab-MLV-transformed cells, the number of V<sub>k1-1</sub> rearrangements was 2.9-fold greater than the number of V<sub>k22</sub> rearrangements in Ig<sup>−</sup> pre-B cells. The numbers of both V<sub>k1-1</sub> and V<sub>k22</sub> rearrangements are greater in unfractionated bone marrow cells than in Ig<sup>−</sup> pre-B cells. However, the number of V<sub>k1-1</sub> rearrangements shows a slightly larger increase, resulting in 4.3-fold more V<sub>k1-1</sub> rearrangements than V<sub>k22</sub> rearrangements in unfractionated bone marrow. This pattern continues in mature splenic (IgD<sup>+</sup>) B cells in which V<sub>k1-1</sub> rearrangements were observed at 13-fold greater numbers than V<sub>k22</sub> rearrangements. Thus, V<sub>k1-1</sub> and V<sub>k22</sub> rearrange to J<sub>k5</sub> at similar frequencies in TS 103/4 and Ig<sup>−</sup> pre-B cells, but the V<sub>k1-1</sub>/V<sub>k22</sub> ratio increases in unfractionated bone marrow cells and even more so in splenic B cells. The finding that the V<sub>k1-1</sub>/V<sub>k22</sub> ratio increases with the frequency of Ig<sup>−</sup> cells suggests that cellular selection might play a key role in the differences in the contributions of V<sub>k1-1</sub> and V<sub>k22</sub> to the naive B cell repertoire.

**Frequency of productive rearrangements**

The extent of cellular selection for (or against) the product of particular Ig variable regions results in a distinctive ratio of productive to nonproductive rearrangements involving that V gene segment (see Ref. 14 for detailed discussion) If, as suggested above, the difference in the utilization of V<sub>k1-1</sub> and V<sub>k22</sub> is the result of differences in cellular selection for the product of those V<sub>k</sub> genes, the frequency of productive rearrangements isolated from spleen should be greater for V<sub>k1-1</sub> rearrangements than for V<sub>k22</sub> rearrangements. We chose to amplify rearrangements only to J<sub>k5</sub>, since rearrangements to the more 5' functional Jκ segments (Jκ1, Jκ2, and Jκ4) could be either deleted or relocated far upstream of the Cκ gene by deletional and inversional secondary Vκ-Jκ rearrangements. The V<sub>k1-1</sub> and V<sub>k22</sub> rearrangements might be influenced differently by multiple rearrangement events depending on the location of these Vκ genes within the locus.

V<sub>k1-1</sub> and V<sub>k22</sub> rearrangements were amplified and cloned from IgD<sup>+</sup> spleen cells. This naive B cell population was chosen to exclude isotype-switched memory cells from the analysis. Each
rearrangement was obtained from an independent PCR reaction to ensure that each clone represents a unique rearrangement event. This is important, since, in contrast to the nearly infinite D segment- and N insertion-enhanced diversity of heavy chain CDR3 sequences, V\(_k\)k junctions are somewhat limited in junctional diversity. Thus, \(\kappa\) rearrangements have no CDR3 fingerprint with which to determine clonal independence. A total of 44 V\(_k\)-A and 51 V\(_k\)-22 rearrangements were sequenced (Fig. 4). The V\(_k\)-A rearrangements were predominantly productive (84%, 37 of 44). In contrast, only 57% of the V\(_k\)-22 rearrangements were productive (29 of 51). More than 95% of the sequenced V\(_k\) genes were identical with the corresponding germline sequence, and the occasional single base changes observed most likely resulted from errors during PCR amplification.

It has been demonstrated that coding sequences of rearranging Ig gene segments can influence the joining process and possibly result in preferential junctions (40), and it has been suggested that germline V gene sequences are selected for their bias in forming particular junctions (41). We asked whether V\(_k\)-A to Jk5 rearrangements were predisposed, because of the interaction of germ-line sequences and the rearrangement process, to preferentially join in-frame. Similarly, we considered the possibility that V\(_k\)-22 to Jk5 rearrangements were biased toward nonproductive rearrangements. To survey V\(_k\)-A and V\(_k\)-22 junctions from a population of lymphocytes that should not be selected via the BCR, B220\(^+\)/IgM\(^-\) pre-B cells were purified from bone marrow and used to amplify and clone either V\(_k\)-A or V\(_k\)-22 rearrangements to Jk5. As in the experiments using mature B cells from spleen, each cloned rearrangement analyzed was isolated from an independent PCR reaction. Ten rearrangements were sequenced for each V\(_k\) gene (Fig. 5). Only three of the 10 V\(_k\)-A rearrangements were in-frame, indicating a lack of significant bias toward productive rearrangements. Four of the 10 V\(_k\)-22 rearrangements were in-frame, again showing no obvious bias in the production of either productive or nonproductive rearrangements. Taken together, seven of 20 (35%) rearrangements were productive, a value consistent with the expected one-third in-frame rearrangements for random, nondirected rearrangements. Our results for V\(_k\)-A and V\(_k\)-22 rearrangements are also consistent with those of a previous study by Ramsden et al. (42) showing that 33% of a large and diverse set of V\(_k\) rearrangements cloned from mouse fetal liver were in-frame.

### Diversity of CDR3 sequences

Our experiments provided an opportunity to examine the diversity created by the use of alternative rearrangement sites. The most striking feature of the productive rearrangements of both V\(_k\)-A and V\(_k\)-22 is the very pronounced bias for one or two particular junctions (Fig. 4). Of the 37 V\(_k\)-A productive rearrangements obtained from spleen cells, 23 have identical junctions (DP1.A). The remaining 14 junctions include a set of eight sequences (DP1.B), a set of two sequences (DP1.C), and only four unique junctions (DP1.D, DP1.E, DP1.F, and DP1.G). Interestingly, the CDR3 encoded by the predominant DP1.A junction, found in 23 independent rearrangements, is identical with the CDR3 encoded

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**Table II.** V\(_k\)-A and V\(_k\)-22 rearrangements determined by quantitative PCR

<table>
<thead>
<tr>
<th>Tissue/Cell Line</th>
<th>V(_k)-A</th>
<th>V(_k)-22</th>
<th>V(_k)-A / V(_k)-22</th>
</tr>
</thead>
<tbody>
<tr>
<td>103/4</td>
<td>288</td>
<td>129</td>
<td>2.2</td>
</tr>
<tr>
<td>Ig-negative BM</td>
<td>1177</td>
<td>411</td>
<td>2.9</td>
</tr>
<tr>
<td>Unfractionated BM</td>
<td>1658</td>
<td>389</td>
<td>4.3</td>
</tr>
<tr>
<td>Ig(^+) spleen cells</td>
<td>3185</td>
<td>240</td>
<td>13.3</td>
</tr>
</tbody>
</table>

\(^{a}\) Q-PCR experiments were performed using lysates from 10^6 cells per reaction. Calculations of copy number were performed as described by Piatak et al. (28) and described in detail in Materials and Methods. Calculated results are from the experiments shown in Figures 2 and 3. Other experiments gave similar V\(_k\)-A/V\(_k\)-22 ratios, and the calculated copy numbers were within 2 to 3-fold in all experiments analyzed using a PhosphorImager. All experiments were performed at least three times except for the analysis of Ig-negative BM for which only the experiment shown in Figure 3 was quantified by PhosphorImager analysis.
by the DP1.C junction, which is represented by only two of the 37 Vx1-A in-frame rearrangements. Since any selective forces would influence DP1.A and DP1.C rearrangements equally, the large difference in the representation of these two rearrangements must reflect a bias of the rearrangement mechanism.

Great care was taken to avoid contaminating PCR reactions with previously amplified products. For example, all amplification reactions were set up in the laboratory of a colleague not working with Ig genes. Before amplification, all PCR work used dedicated supplies and equipment never exposed in our laboratory. No contamination of Vk gene products was observed in multiple “no DNA” controls included in all experiments. To further rule out any possibility that the high frequency of repeated productive and nonproductive Vx1-A to Jx5 junctions was the result of contamination, the last 15 Vx1-A products obtained from adult spleen were amplified using a more 5' primer (Vx1-A 5') that would not be capable of priming previously amplified products. This last set of amplifications continued to yield the dominant productive

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**A. Vx1-A Rearrangements**

| GERMINE | 92 | A | C | G | T | C | T | P | V | N | P | J | G | A | C | O | G | T | G | T | G | O | G |
| PRODUCTIVE | DP1.A | --|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|
and nonproductive junctions observed for Vκ1-A to Jκ5 rearrangements.

The bias for certain junctions in the set of productive Vκ22 rearrangements is also noteworthy. Twenty-four of the 29 in-frame Vκ22 junctions obtained from spleen cells (Fig. 4) are identical with one of two repeated junctional sequences, 15 with the 22.A sequence and 9 with the 22.B sequence. The 22.A and 22.B sequences encode the identical amino acid sequence. The remaining five junctions (22.C through 22.G) are each unique. Three of the four productive Vκ22 rearrangements obtained from bone marrow (Fig. 5) also have the 22.A junction. Considering both spleen- and bone marrow-derived productive Vκ22-Jκ5 rearrangements, 27 of 33 (82%) encode the same CDR3 (22.A or 22.B).

As in this report, a number of investigators have examined nonproductively rearranged Ig loci to study rearrangement biases in the absence of BCR-mediated cellular selection (13, 14, 43). Since there is no selection for or against nonproductive rearrangements, the absence of BCR-mediated cellular selection (13, 14, 43). Since the mouse κ locus consists of about 140 Vκ exons (51), an unknown number of which are pseudogenes, 0.5% is within the expected order of magnitude for a relatively unbiased use of individual Vκ segments.

In contrast to rearrangements in pre-B cells, Vκ1-A rearrangements in mature splenic B cells were present at a 13-fold greater number than Vκ22 rearrangements based on Q-PCR. If these results are adjusted to reflect only productive rearrangements (57% of Vκ22 and 84% of Vκ1-A), there are 20-fold more Vκ1-A productive rearrangements than Vκ22 productive rearrangements in IgD+ splenic B cells. It is possible that the full 60-fold difference measured using the Vκ cDNA libraries was not observed by the Q-PCR assay, because Vκ22 may use Jκ5 more frequently than does Vκ1-A. Only 14 of 108 analyzed Vκ1-A cDNAs (13%) were rearranged to Jκ5 (our unpublished observations), whereas the frequency of Jκ5 rearrangements in the total Vκ cDNA library is 20% (30). On the other hand, three of the four sequenced Vκ22 cDNAs from our Vκ phage library (Fig. 1) and the expressed Vκ genes of both S107 (25) and TEPIC15 (52) plasmacytomas are Vκ22 to Jκ5 rearrangements, suggesting that Vκ22 may be preferentially associated with Jκ5.

We observed a striking difference in the proportions of productive and nonproductive rearrangements involving Vκ1-A vs Vκ22 gene segments in the adult spleen. Rearrangements of Vκ22 to Jκ5 were found to be in-frame 57% of the time. The ratios of productive to nonproductive rearrangements for both Vκ4 family members (53) and Vκ21 family members (40) have also been reported to approach 1.0 in adult spleen. In contrast, 84% of the Vκ1-A rearrangements were found to be in-frame. Based on this high proportion of productive rearrangements for Vκ1-A, we suggest that Vκ1-A-expressing B cells are preferentially expanded in the naïve repertoire. Although the feature of Vκ1-A chains being selected is not known, one possibility is that a BCR-ligand interaction, perhaps with self Ag, is a mandatory checkpoint in B cell development. Recent evidence suggests that the naïve repertoire contains self-reactive clones that are expanded in the primary response (54). Another possibility is that Vκ1-A can form functional associations with many heavy chains, whereas other Vκ genes can effectively pair with a more restricted set of VH regions. Although there are not yet sufficient data to test this idea, Kaushik et al. (55)
found that Vκ and Vλ families appear to associate with each other in a stochastic fashion.

The junctional diversity of κ rearrangements is more limited than heavy chain rearrangements due to the absence of D segments. In addition, mouse κ rearrangements generally have few N nucleotide additions (56, 57), presumably because terminal deoxynucleotidyl transferase expression is down-regulated as a result of μ-chain expression before most κ locus rearrangements (58, 59). Junctional variability is further restricted by a bias to form particular junctions between particular Vκ and Jκ segments. For example, both Milstein et al. (53) and Victor et al. (43) examined large sets of Vκ reorganizations involving members of the Vκ4 or Vκ21 gene families, respectively, and reported that the nonrandom deletion of nucleotides from Vκ and Jκ segments in biased junctions. Similarly, we found preferences for one or two junctional sequences for productive and nonproductive rearrangements of both Vκ1-A and Vκ22.

The finding that 23 of 37 productive splenic Vκ1-A/Jκ5 rearrangements have the DP1.A rearrangements cannot be explained solely by selection, since the identical amino acid sequence is encoded by the DP1.C, rearrangement, which accounted for only 2 of the 37 productive Vκ1-A rearrangements (Fig. 4). These data provide compelling evidence for a strong mechanistic bias in the formation of Vκ/Jκ junctions. Interestingly, the frequently formed productive junction, DP1.A, is not mediated by short regions of homology (56), suggesting that other features of the recombination are responsible for the preference for the DP1.A junction (40). The Vκ22 junctions amplified from adult spleen also show biases. The DP22.A junction occurs in a region of sequence homology between the V and Jκ5 gene segments. This sequence is also observed at a high frequency in the bone marrow-derived sequences (three of four in-frame junctions are BM22.A), indicating that this junction is favored by the rearrangement process. DP22.A and DP22.B encode identical CDR3 sequences and are expressed in both S107 and TEPC15 anti-phosphocholine light chains (25, 52). Since these junctions make up 29.83% of the splenic junctional sequences, it is likely that this CDR3 sequence is positively selected in the repertoire.

Other biases noted are the high percentage of nonproductive junctions occurring within regions of sequence homology (32 of 42). Since nonproductive junctions cannot be selected by expression of BCRs, these must represent independent rearrangement events and are the result of a biased rearrangement mechanism.

Analysis of 115 Vκ1-A and Vκ22 rearrangements revealed only a single nucleotide that is unambiguously an untemplated N addition. Rearrangements of other mouse Vκ genes have had a slightly greater frequency of N-containing junctions: 5% for Vκ4 (53) and 10% for Vκ21 (43). It must be noted that given that N insertions in κ are often a single nucleotide, there is a significant possibility for a N nucleotide to be incorrectly assigned to either V or J segments. Despite this uncertainty regarding the precise frequency of N insertions in light chains, the tight regulation of terminal deoxynucleotidyl transferase expression during B lymphocyte development appears to severely limit nontemplated additions. The paucity of N insertions in fetal heavy chain junctions has previously been postulated to restrict the early repertoire by facilitating homology-directed junction formation (60, 61). Similarly, minimizing N additions during light chain rearrangement would facilitate the coevolution of Vκ genes capable of favoring the formation of useful junctional sequences.

It has become increasingly accepted that the preimmune repertoire does not consist of a random assortment of V, D, and J segments linked by arbitrary junctions. Rather, the content of the primary repertoire can be determined by the frequency of rearrangement of individual gene segments, bias in the formation of junctions, and cellular selection based on Ig chain amino acid sequence (62). Thus, the Ig loci and V(D)J rearrangement mechanism have probably evolved together to provide the species with a preimmune Ab repertoire that represents a compromise between sufficient diversity and the expression of particularly useful heavy and light chains.

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