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Evolution of Ig DNA Sequence to Target Specific Base Positions Within Codons for Somatic Hypermutation[1]

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Ig variable (V) region genes are subjected to a somatic hypermutation process as B lymphocytes participate in immune reactions to protein Ags. Although little is known regarding the mechanism of mutagenesis, a consistent hierarchy of trinucleotide target preferences is evident. Analysis of trinucleotide regional distributions predicted and we now empirically confirm the surprising finding that the framework 2 region of κ V region genes is highly mutable despite its importance to the structural integrity and function of the Ab molecule. Interestingly, much of this mutability appears to be focused on the third codon position where synonymous substitutions are most likely to occur. We also observed a trend for high predicted mutability for codon positions 1 and 2 in complementarity-determining regions. Consequently, amino acid replacements should occur at a higher rate in complementarity-determining regions than in framework regions due to the distribution and subsequent targeting of microsequences by the mutation mechanism. Our results reveal a subtle tier of V region gene evolution in which DNA sequence has been molded to direct mutations to specific base positions within codons in a manner that minimizes damage and maximizes the benefits of the somatic hypermutation process. ***The Journal of Immunology*****, 2002, 168: 2302–2306.**

The importance of somatic hypermutation to acquired immunity is revealed by the observation that memory B cells almost invariably express mutant Abs with improved functional characteristics that include an increased affinity for cognate foreign Ag and reduced cross-reactions with self-Ags (1–7). Somatic mutations are not distributed randomly throughout Ab V region genes. While some of this unevenness is an indirect result of selection pressures acting on B lymphocytes via mutant B cell Ag receptor, much of it is a direct effect of local differences in intrinsic mutability within the V region gene (8, 9). Palindromes, repeated sequences, secondary structure of encoded transcripts, distance from the transcriptional promoter, and DNA microsequence have all been implicated in mutation targeting (10–13), but the latter two seem to be most significant. For example, sequencing studies have revealed a sharp 5′ mutation boundary delimited by the promoter, with mutation frequencies being highest in the coding exon and gradually diminishing to background levels over a 2-kb range (14–17). Inserting an Ig promoter and leader immediately upstream of the constant region targeted mutations to a previously unsusceptible location (18). Similarly, increasing the distance between the Ig promoter and the V coding region did not change the overall frequency of mutations but did alter their distribution (19). These studies, along with several other lines of evidence, have implicated the Ig promoter in broadly targeting the mutation mechanism (20, 21).

Although several studies have demonstrated the mutability of non-Ig DNA (13, 19, 22, 23), indicating no requirement for the Ig coding sequence, high resolution studies have revealed that short DNA sequences exhibit varying levels of intrinsic mutability, indicating that mutation per se or repair mechanisms that may follow are strongly influenced by local microsequence context (9). RGYW and TAA were the first highly mutable motifs identified (10), but we have found that there is a consistent hierarchy of mutability for all di- and trinucleotide sequences (24, 25). This conclusion was drawn from analyses of somatic mutations located within V gene introns or nonproductively rearranged exons to avert a mutation sample bias due to indirect, but substantial, influences of selection on the Ab sequence.

We previously demonstrated that nonproductively rearranged human V_H genes displayed regional mutability differences. Triplet sequence composition predicted and empirical data confirmed that complementarity-determining regions (CDR)[3] were more intrinsically mutable than the framework region (FR) in V_H genes (24). This result is consistent with the widely held view that CDR influence Ag binding most directly, while FR serve as a scaffold to provide overall structural integrity to the V region domain (26). This view is supported by the general observation that naturally occurring replacement mutations in FR are less frequent than in CDR (27–29) and by results of in vitro mutagenesis studies demonstrating that FR are relatively intolerant of amino acid replacements (30, 31). Our parallel analyses of light chain V region genes, however, led to the unexpected and surprising prediction that human and mouse Vκ FR2 would be as highly mutable as CDR. In this study we tested this prediction and analyzed positional mutability within V gene codons to determine whether Vκ FR2 might be inordinately prone to acquire silent mutations.

Materials and Methods

The mutability indexes for di- and trinucleotides were calculated as described previously (24, 25). Briefly, the number of times a given oligonucleotide within a segment of DNA contained a mutation was divided by the number of times the oligonucleotide was expected to be mutated for a mechanism with no bias. Mutability indexes are normalized for the di- and trinucleotide compositions of unmutated templates covering the precise regions for which mutational data were analyzed. Positional mutabilities of

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[3] Abbreviations used in this paper: CDR, complementarity-determining region; FR, framework region.

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bases in trinucleotides were calculated in an analogous fashion. The predicted mutability index for a region was calculated by determining the number of times each di- or trinucleotide occurred within each region of each gene (regardless of frame of reference) and multiplying by its mutability index. The resulting products for the 16 dinucleotides or 64 trinucleotides were summed and then divided by the total number of di- or trinucleotides in the region under consideration. The composite mutability index predicted for a type of region, for example nucleotide sequences encoding human V_HFR1, was determined by summing all di- or trinucleotide products (occurrences $\times$ mutability index) and dividing this number by the sum of all di- or trinucleotide occurrences in the region for all such sequences in the database. The predicted codon positional mutabilities were calculated in an analogous fashion. The observed mutability index for each region of the nonproductively rearranged human V_H (32, 33) and Vκ (34) genes were determined by dividing the number of mutations per nucleotide for a region by the number of mutations per nucleotide for the entire gene. In essence, this gives the observed/expected mutability ratio for a region, where the expected frequency is the average frequency of mutations for the whole gene. The length of each region is therefore considered when its composite mutability index is calculated.

Results and Discussion

Using recently published data presented in the report by Foster et al. (34), we calculated regional mutation frequencies for Kabat FR and CDR (35) in nonproductively rearranged human κ genes. As shown in Fig. 1, the regional predictions based solely on di- and trinucleotide composition and empirically derived mutability indexes for doublets and triplets accurately forecasted the overall distribution of mutations observed in human κ sequences. It is noteworthy that the doublet and triplet mutability data used in this forecast were derived from mouse intronic sequences, thus reinforcing our view that microsequence targeting of mutagenesis is similar for all parts of Ab genes in both mice and humans (24). The high mutability of Vκ FR2 is striking because it is apparently at odds with the observation that amino acid changes to FR are often damaging to V region integrity. It is also incongruous with aforementioned results of parallel analyses performed on V_H genes that revealed the CDR to be more mutable than the FR (24).

To resolve the apparent discrepancy between the high mutability of Vκ FR2 and its importance to the structural integrity of the Ab molecule, we hypothesized that bases in the third position of Vκ FR2 codons might be inordinately more mutable than those in the first two positions. This hypothesis was inspired in part by a report by Kepler (36) indicating that synonymous codon usage among CDR and FR differed in a manner favoring replacement mutations in CDR relative to FR. This preceding analysis was necessarily

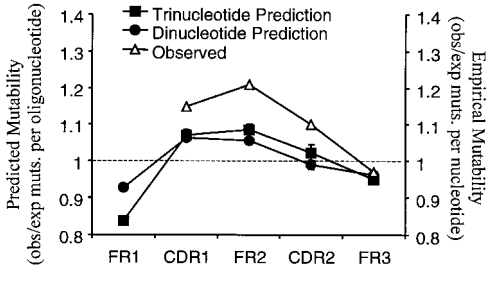

FIGURE 1. Predicted vs empirical regional Vκ mutability. Observed regional mutabilities in human Vκ genes follow the predicted pattern based on di- and trinucleotide compositions. The data are shown as observed/expected mutation ratios $\pm$ SEM (often obscured by symbols) for each region. The expected value is the average mutability for all triplets (i.e., no target bias). The average observed/expected mutability index is 1 (dashed line), while a value >1 indicates preferential targeting, and a value <1 indicates avoidance by the mutation mechanism. Somatically mutated, nonproductively rearranged, human Vκ data described by Foster et al. (34) were used to calculate observed regional mutabilities. No mutation data for FR1 are available.

Table I. *Human and murine positional trinucleotide mutability indices*

	Human V_H[a]			Murine J_H/Jκ[b]		
Trinucleotide	First position	Second position	Third position	First position	Second position	Third position
AAA	0.24	1.20	0.96	1.31	1.35	1.42
AAC	0.43	2.14[c]	2.00	1.18	2.02[c]	2.02[c]
AAG	1.11	1.90[c]	0.85	1.02	1.61[c]	1.99[c]
AAT	1.83	2.36	1.31	1.42	2.01[c]	1.03
ACA	0.82	0.52	0.89	2.02[c]	0.97	0.53
ACC	1.33	1.40	0.82	0.71	1.19	0.83
ACG	1.83	0.73	1.83	0.96	0.96	0.00
ACT	1.62	1.53	0.57	1.70[c]	2.22[c]	0.59
AGA	0.92	0.42	0.42	1.24	1.07	0.51
AGC	1.47	3.44[c]	2.58[c]	1.68	3.36[c]	3.36[c]
AGG	1.18	0.47	0.39	1.14	0.29[c]	0.33[c]
AGT	1.12	1.80[c]	0.68	0.90	1.11	0.63
ATA	0.47	2.19[c]	2.35[c]	1.08	2.07[c]	2.27[c]
ATC	2.19[c]	1.05	1.84[c]	1.74	0.22	1.19
ATG	1.26	0.28	0.98	2.37[c]	1.15	1.15
ATT	2.37[c]	0.66	1.58	1.56	0.81	0.34
CAA	0.67	0.92	1.76	0.87	0.79	2.13[c]
CAC	0.83	0.97	0.56	0.49	0.85	0.97
CAG	0.75	0.62	2.26[c]	0.36[c]	0.82	0.66
CAT	0.62	0.74	1.11	0.63	1.15	0.94
CCA	1.16	0.61	0.88	0.85	0.25	0.93
CCC	0.67	0.37	0.07[c]	1.19	0.40	0.20
CCG	1.08	0.46	0.31	0.44	0.44	0.88
CCT	0.94	0.62	0.57	1.06	0.77	0.39
CGA	0.29	0.00	1.44	0.00	0.00	0.00
CGC	0.46	0.69	0.57	0.00	0.00	0.00
CGG	0.24	0.37	1.10	0.43	0.43	0.86
CGT	0.99	1.39	0.60	0.59	0.59	0.00
CTA	2.26[c]	1.24	1.36	1.18	0.86	2.90[c]
CTC	0.52	0.33	0.26[c]	1.66	0.40	0.20[c]
CTG	1.25	0.37[c]	0.58	1.54	0.43	0.69
CTT	1.03	1.20	0.34	1.71[c]	0.68	0.55
GAA	0.49	0.33	2.62[c]	0.91	0.70	1.71[c]
GAC	0.16[c]	0.40	0.16[c]	0.09[c]	0.27	0.63
GAG	0.35[c]	0.75	1.85[c]	0.20[c]	0.45	1.01
GAT	0.94	1.61	0.85	1.63	0.96	1.48
GCA	2.09[c]	1.39	0.30	2.18[c]	1.20	1.31
GCC	0.52	1.33	0.29[c]	0.66	2.13[c]	0.49
GCG	0.51	0.26	0.51	0.00	0.00	0.00
GCT	3.83[c]	2.01[c]	0.71	2.97[c]	2.80[c]	0.79
GGA	0.58	0.62	1.07	0.40[c]	0.50	0.40[c]
GGC	0.28	1.20	0.74	0.11[c]	1.68	0.42
GGG	0.25[c]	0.59	1.09	0.13[c]	0.44	0.93
GGT	0.91	1.36	0.45	0.71	1.11	1.19
GTA	2.89[c]	1.27	3.70[c]	2.71[c]	1.08	3.43[c]
GTC	0.69	0.28[c]	0.41	0.40	0.67	0.47
GTG	1.17	0.56	0.93	1.02	0.14[c]	1.56
GTT	3.41[c]	1.00	1.00	1.98[c]	0.53	0.33
TAA	0.00	5.90[c]	0.74	0.63	2.06[c]	1.77[c]
TAC	2.51[c]	2.24[c]	2.24[c]	1.46	3.74[c]	2.93[c]
TAG	1.95	3.32[c]	2.34[c]	2.10[c]	2.18[c]	0.78
TAT	1.30	2.30[c]	1.00	0.73	2.93[c]	0.63
TCA	0.66	0.73	0.93	0.57	0.17[c]	0.85
TCC	0.41	0.65	0.89	0.52	0.31	0.31
TCG	0.65	0.32	0.00	0.00	0.00	0.51
TCT	0.43	0.85	0.43	0.29[c]	0.83	0.54
TGA	0.31	0.31	0.23[c]	0.28[c]	0.47	0.90
TGC	0.29	0.57	0.71	0.99	1.24	2.47[c]
TGG	0.48	0.44[c]	0.76	0.73	0.23[c]	1.13
TGT	0.51	1.70	0.85	0.24[c]	2.12[c]	0.24[c]
TTA	0.74	2.23[c]	2.08[c]	0.33	0.83	1.41
TTC	1.16	0.51	0.51	0.62	0.28[c]	0.35
TTG	1.00	0.50	0.00	0.77	0.17[c]	0.72
TTT	0.00	0.71	2.14	0.58	0.45[c]	0.41[c]

[a] Sequences from Dorner et al. (32) and Dunn-Walters and Spencer (33).
[b] A/J plus autoimmune plus literature sequences from Smith et al. (25).
[c] Statistically significant by χ^2 test at $p = 0.01$.

restricted to the translational reading frame and the findings could be the result of a lower overall mutability, a higher likelihood of sustaining silent mutations or a combination of both factors in FR vs CDR codons. Similarly, Chang and Casali (37) reported intrinsic sequence differences between CDR and FR codons favoring random amino acid replacements in the former over the latter, but this analysis did not take into consideration targeting biases of the mutation mechanism.

We analyzed Vκ FR2 codons to determine whether somatic mutations were predicted to be asymmetrically distributed according to the base position within the codon. Intrinsic mutabilities of each base position in all triplets (regardless of frame) were first determined using a database of somatic mutations located within nonproductively rearranged human V_H genes or murine Ig introns (24). Nonproductive genes or introns were specifically chosen to avert influences of selection on the mutation database. It is important to recognize that there is no known frame of reference for the mutation mechanism. Thus, in calculating these data any single mutation was ascribed to the last position of one triplet, the second position of the +1 triplet, and the first position of the +2 triplet. Table I lists the mutability indexes for each position of all nucleotide triplets.

Using the triplet positional mutability data from human V_H sequences, we then calculated the predicted mutabilities of each base position in Vκ FR2 codons of germline human Vκ genes. We performed this analysis on representative members of the four largest Vκ gene families. Due to the high sequence similarity among family members, these examples should adequately represent the majority of the known human κ genes. In calculating the mutability of a given base in a Vκ codon, we averaged its mutability in all three triplet reading frames, thus taking into account the three triplets that encompass a given nucleotide. This contrasts with earlier analyses (36, 38) that only considered the translational reading frame despite no evidence supporting a coincidental mutational reading frame. The example calculation in Fig. 2 demonstrates that the mutability of a base within a codon is highly dependent on the overlapping triplet composition.

The results of this analysis revealed that for each Vκ gene the average predicted mutability was highest for the third position of FR2 codons (Fig. 3). We used a test of contrasts within an ANOVA and determined that mutability of the third codon position for the combined Vκ FR2 dataset was statistically different from that of both the first and second codon positions (p values of 0.0033 and 0.0006, respectively). In more straightforward terms, the random likelihood that the third position would be the most mutable in a given Vκ FR2 is 1/3, and the chance that four independent Vκ genes share this characteristic is 1/81.

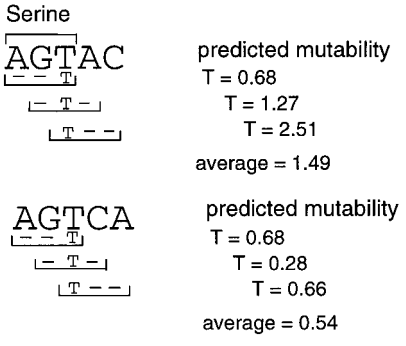

FIGURE 2. Calculating positional mutability within a codon. The mutability of a given base in a codon is taken as its average mutability in the context of the three encompassing trinucleotides. Sequence adjacent to a codon affects the intrinsic mutability of nucleotides in the codon.

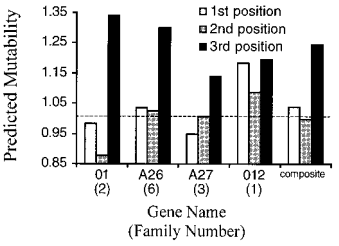

FIGURE 3. Predicted positional mutabilities for Vκ FR2 codons. Predictions are calculated as illustrated in Fig. 2, using trinucleotide mutation indexes obtained from nonproductively rearranged human V_H genes (Table I). Gene name, with family designation in parentheses, appears on the *x*-axis.

We extended this analysis to other regions of Vκ genes and then to human V_H genes. Fig. 4 shows that there is a general trend in which the first or second codon position within Vκ CDRs is the most mutable. It is interesting to note that this trend holds for all Vκ CDRs except for the two in which the mutation mechanism shuns the region (as defined by an observed/expected mutability ratio of <1 for each codon position). As predicted from the wobble effect, an analysis of the four Vκ genes demonstrates increased amino acid replacements when alterations occur within either the first or second codon position of CDRs (97%) compared with the third codon position of FR2 (40%). Similarly, in four human V_H genes representing the largest families, the most mutable bases are generally located in the first or second position of CDR codons and in the third position of FR1 and FR2 codons (Fig. 5). However, the trend does not hold for FR3 or for CDR1 of V_H 1-02, which has a high predicted mutability (observed/expected mutability >1). In some Abs the FR3 loop between the D and E β strands is located in the same solvent face as the CDRs and therefore might be preferentially targeted for somatic hypermutation. To investigate this possibility we calculated the predicted mutability of all FR3 codons for four Vκ and V_H genes. As shown in Fig. 6, no correlation between high predicted mutability and structural location is evident, although it is interesting that the predicted mutability is greatest near the 3′ ends of Vκ FR3 and V_H FR3.

Higher structural constraints, as evidenced by greater homology in Vκ, may have precluded evolution from decreasing the overall Vκ FR2 mutability in the same manner as in V_H. Yet, as indicated in Fig. 7, V_H and Vκ FR2 amino acid homology was equivalent for most codons, and no obvious correlation between the extent of chemical conservation of a residue and predicted mutability was

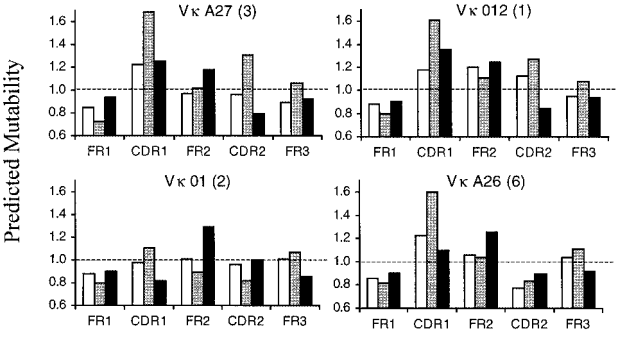

FIGURE 4. Contributions of different base positions within codons to regional mutability indexes of human Vκ genes. Predictions for base positions within codons (first bar is first position, etc.) are calculated from positional mutability indexes of trinucleotides obtained from nonproductively rearranged human V_H genes (Table I). The gene family appears in parentheses.

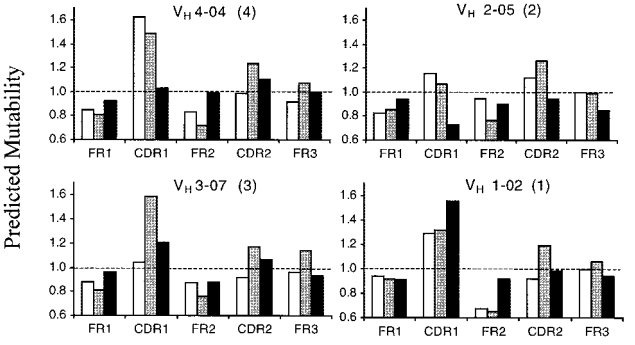

FIGURE 5. Contributions of different base positions within codons to regional mutability indexes of human V_H genes. Predictions for base positions within codons (first bar is first position, etc.) are calculated from positional mutability indexes of trinucleotides obtained from nonproductively rearranged human V_H genes (Table I). The gene family appears in parentheses.

evident. Also, Vκ FR2 codons of higher mutability were not preferentially confined to a particular form of secondary structure. Therefore, it is unclear why V_H FR2 evolved to attain a low degree of mutability while Vκ FR2 did not. It is possible that a subtle form of conservation that is not obvious from grouping chemically related amino acids is resident in Vκ FR2.

If the somatic mutator obeyed a single triplet frame of reference that was coincidental with the translational reading frame, the described mutability trend could be achieved by substituting mutationally "hot" codons with synonymous "cold" ones and vice versa. However, there is no evidence for a mutational triplet reading frame. AGC, for example, is the hottest of all triplets regardless of frame (25). Thus, for evolution to maximally enhance or diminish somatic mutation frequencies at a given codon position, particularly in the first and last positions, adjacent codons would have to coordinately evolve in a manner that would often unavoidably produce nonsynonymous changes. Conservation of protein integrity presumably enforced strict limits on evolutionary adjustments affecting positional mutability. This could explain why the somatic positional mutability trend is not always strong or absolute for every CDR and FR. FR3, for example, may have demanded more conservation than FR1 or FR2 during evolution.

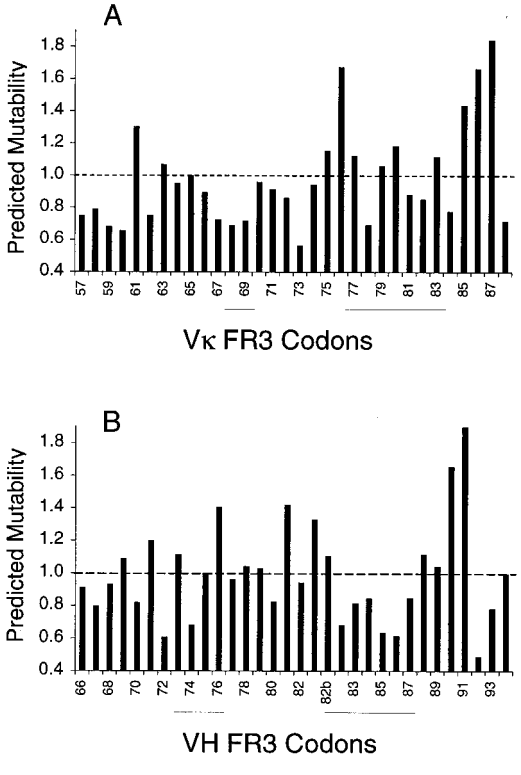

FIGURE 6. Predicted mutability for each FR3 codon in Vκ (*A*) and V_H (*B*). The analysis combines the Vκ genes 01, A26, A27, and 012 and the V_H genes 1-02, 2-05, 3-07, and 4-04. A bar under the *x*-axis indicates the codon's location within the DE or EF interstrand loop, respectively.

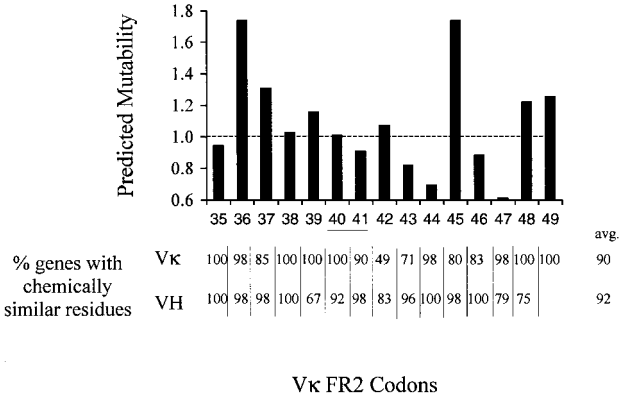

FIGURE 7. Predicted mutability for each codon of Vκ FR2. The analysis combines the Vκ genes 01, A26, A27, and 012. The percentage of all known human Vκ and V_H genes with a chemically similar amino acid in each corresponding codon (relative position correct for V_H) is indicated below the axis. The bar under the *x*-axis indicates the codon's location within the CC′ interstrand loop.

Igs have apparently evolved in two different ways to subtly increase the benefits and reduce the detriments of somatic mutagenesis. In a preceding study we demonstrated that V_H gene sequences preferentially direct somatic mutations to CDRs and away from FR regions (24). Here we provide evidence that evolution has again taken advantage of the microsequence bias of the mutation mechanism by manipulating V region gene sequences to direct mutations to specific base positions within codons. This is most obvious for Vκ FR2 sequences, where mutation frequencies are unexpectedly high and mutations are preferentially targeted to the third base position. This finding supports the idea that during an immune reaction, damage to V region structure and function caused by somatic mutagenesis may be a substantial obstacle to the rapid development of B cells expressing receptor Abs with improved affinity and specificity for Ag (39).

Acknowledgments

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