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Immunology

Sequence dependent hypermutation of the immunoglobulin heavy chain in cultured B cells

(antibody/somatic mutation/hot spot/ELISA spot assay/nonsense mutation)

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ABSTRACT **The variable (V) regions of immunoglobulin heavy and light chains undergo high rates of somatic mutation during the immune response. Although point mutations accumulate throughout the V regions and their immediate flanking sequences, analysis of large numbers of mutations that have arisen *in vivo* reveal that the triplet AGC appears to be most susceptible to mutation. We have stably transfected B cell lines with γ2a heavy chain constructs containing TAG nonsense codons in their V regions that are part of either a putative (T)AGC hot spot or a (T)AGA non-hot spot motif. Using an ELISA spot assay to detect revertants and fluctuation analysis to determine rates of mutation, the rate of reversion of the TAG nonsense codon has been determined for different motifs in different parts of the V region. In the NSO plasma cell line, the (T)AGC hot spot motif mutates at rates of $\approx 6 \times 10^{-4}$/bp per generation and $\approx 3 \times 10^{-5}$/bp per generation at residues 38 and 94 in the V region. At each of these locations, the (T)AGC hot spot motif is 20–30 times more likely to undergo mutation than the (T)AGA non-hot spot motif. Moreover, the AGA non-hot spot motif mutates at as high a rate as the hot spot motif when it is located adjacent to hot spot motifs, suggesting that more extended sequences influence susceptibility to mutation.**

B cells generate antibodies with a highly diverse repertoire of antigen binding sites by rearranging germ line V(D)J and VJ genetic elements to create the heavy and light chain variable (V) regions (1–4). In spite of the enormous diversity that is created by this combinatorial rearrangement, junctional diversity and the introduction of untemplated bases during rearrangement, the "germ line" encoded antibodies are of relatively low affinity (1). To generate high-affinity antibodies, the V regions of immunoglobulin genes are further modified by somatic mutation (1, 3, 5). It has been estimated that the rate of V region somatic mutation *in vivo* is as high as 10^{-4} to 10^{-3}/bp per generation (1, 3).

The analysis of databases of mutations both in the V region coding exon and its 3′ flanking sequences reveals that there are motifs that are preferred targets of the mutational process *in vivo* (6–9). These hot spots can be deduced from the sequence analysis of passenger transgenes and from silent mutations in endogenous genes, so they are not the result of antigenic selection (6, 10). The most prominent of these intrinsic hot spot motifs is RGYW [(A/G)G(T/C)(A/T)] and the AGC triplet is a frequent representative of this motif (9). AGC is also a preferred target for mutation *in vivo* in non-Ig coding sequences that have been substituted for the coding sequences of the V region in transgenes (11, 12). The importance of this motif is confirmed by the fact that the AGY triplet encodes serine in complementarity-determining regions (CDRs) more frequently than the other serine codons. This suggests that there has been evolutionary selection in the antigen binding sites for codons that will mutate more frequently (13). In addition to AGC and TAC and their inverse repeats, GTA and GCT (9), TAA is also a preferred substrate for somatic mutation (8). While AGC was found to be a preferred target of mutation in all of immunoglobulin V region gene databases analyzed, there are AGCs and RGYW motifs in the V region that do not undergo high rates of mutation, suggesting that either the location within the V region or surrounding sequences are also important (8). The role of more distant surrounding sequences is difficult to explore through database analysis (9) and requires an easily manipulatable system in which mutation rates can be accurately quantified and compared and specific hypotheses can be tested.

We and others have recently developed model systems in which transfected heavy chain genes undergo reversion in cultured B cells (14–17). In the *in vitro* system used in the studies described here, stably transfected heavy chain constructs containing (T)AGC nonsense mutations undergo V region mutation at rates of 10^{-4}–10^{-3}/bp per generation that are as high as the rates of V region mutation *in vivo* (1–3). Mutation is assayed by reversion of the nonsense codon using the ELISA spot assay (15–17). Reversion is through single base changes and the associated base changes in other parts of the V region are all point mutations (15–17). Because rates of mutation can be determined by fluctuation analysis (18, 19) and many different constructs can be compared, this system provides a powerful tool to compare the susceptibility to mutation of different targets *in vitro* in the absence of T cells, germinal centers, and selection by antigen, and to determine if the rate of mutation of these targets is position-dependent. We therefore designed several pairs of constructs with nonsense mutations containing (T)AGC or (T)AGA in different locations in V and measured their reversion rates in the NSO plasma and 18.81 pre-B cell lines.

MATERIALS AND METHODS

Cell Lines. NSO is a non-Ig producing fusion partner for making hybridomas and is derived from the P3 myeloma line (20). NSO–LC1 is a κ light chain transfectant of NSO (16). 18.81 is an Abelson transformed pre-B cell line (21). 18.81–LC is a κ light chain transfectant of 18.81 (15). The cell lines were grown and cloned as described (15, 16).

Mutagenesis and DNA Constructs. An *Eco*RI fragment containing a V(D)J region of R45 heavy chain (17) was cloned into pBKS which lacks a *Bam*HI site to make Vwt/pBKS. A *Bam*HI fragment containing 1.3 kb of the V(D)J was cut out

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Abbreviation: CDR, complementarity-determining region.

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and cloned into pAlter (Promega). Mutagenesis was done according to the instructions in the pAlter mutagenesis kit. The following primers were used for mutagenesis: Vn38A, 5′-GTTCAGGCCTCTTCTAGACCCAGTGCATA-3′; Vn50A, 5′-CAGGATCAATCTAGACAATCCACTCCA-3′; Vn65A, 5′-TAGTGGCCTTGCTCTAGAACTTCGGGTC-3′; Vn94A, 5′-CTCCAGTACAGTTCTAGACGGCAGTG-3′; Vn94C, 5′-CTCCAGTACAGTGCTAGACGGCAGTG-3′. The sequences of mutated nucleotides are shown in Fig. 1. After mutagenesis, the *Bam*HI fragment was cloned back into original construct Vwt/pBKS to make Vn/pBKS. *Eco*RI fragments from Vn/pBKS containing different mutated V regions were assembled into pSV2neoIgG2a to create Vwt, Vn38C-Vn38A/γ2a, Vn94C-Vn94A/γ2a, and Vn50A-Vn65A/γ2a. All γ2a constructs were linearized with *Ksp*I and transfected into the NSO–LC1 or 18.81–LC light chain producing cell lines. The transfections and selections were performed by electroporation as described (15–17).

Detection and Quantitation of γ2a Revertants. Revertants of γ2a were detected by ELISA spot assay as described (17). Briefly, ELISA plates were coated with 2 μg/ml of goat anti-mouse IgG2a (Southern Biotechnology Associates) and blocked with 2% of BSA. NSO–LC1 cells transfected with the various γ2a constructs (see Fig. 1) were incubated on the plates at 37°C for 18–22 h. 18.81-LC cells were pretreated with lipopolysaccharide (15) and then were incubated at 37°C for 24 h. The ELISA spots were developed by sequential incubation with 1 μg/ml of biotin-conjugated goat anti-mouse IgG2a overnight, avidin phosphatase (Vectastain; Vector Laboratories) for 45 min and 1.0 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (5-BCIP; Amresco, Solon, OH) for 6 h.

Rates of reversion were determined by fluctuation analysis (18) and calculated by the method described previously (19). Because the unit used by those methods is cell/generation and the stop codon TAG can be reverted to eight different amino acid codons by single mutations (except for TAA that is another stop codon), the unit of rates presented in this paper was changed to base pair per generation by dividing the original number by 8/3. Enrichment of revertants was performed as described (16).

PCR and Sequencing. Genomic DNA for PCR was isolated from 2–5 × 10^6 cells by QIAamp Blood Kit (Qiagen, Chatsworth, CA). The primers used were 5′-GTCGAATTCCTCAGAGGTTCAGCTGCAG-3′ (5′ end of the V region) and 5′-GTCGAATTCGATCTACTACCGAAGTATC-3′ (the D region). PCR was carried out in 100 μl reactions containing 5 units of *pfu* DNA polymerase (Stratagene), 100 ng of each primer, 0.2 mM dNTP, and 5 μl of DNA. The reaction was carried out in the DNA Thermal Cycler (Perkin–Elmer/Cetus): 5 cycles at 94°C for 1 min, 72°C for 2 min, 46°C for 1 min, followed by 30 cycles at 94°C for 1 min, 72°C for 2 min, and 54°C for 1 min. To eliminate the PCR products from the unreverted copies of the V region, PCR products were digested with *Mae*I for Vn38C and *Xba*I for Vn38A since the loss of these sites was associated with a base change in the stop codon. After gel purification, products were cloned into pCR-script vector using a cloning kit (Stratagene). Colonies were hybridized with an oligonucleotide containing the nonsense mutation to distinguish revertants from the nonsense sequence for Vn38C as described (17). Automatic sequencing was carried out with an Applied Biosystems model 377 DNA sequencer.

RESULTS

(T)AGC Mutates at a Higher Rate than the (T)AGA. We have previously reported that a TAG nonsense codon at residue 38 of the V region of the γ2a gene construct mutates at a high rate in the NSO cell line and somewhat lower rates in the 18.81 and S107 cell lines (17). In those studies, the TAG was followed by a C, forming an AGC triplet in the context of a TAGCA and creating an RGYW hot spot motif. We renamed this construct with the TAG**C** hot spot motif Vn38C and created the Vn38A construct by replacing the C with an A to form a TAG**A** non-hot spot motif at the same location (Fig. 1).

These two heavy chain constructs (Fig. 1), differing by only a single base, were transfected into NSO–LC1 cells that produce and secrete large amounts of a κ light chain (16). Fresh transfectants were analyzed for revertants expressing IgG2a by the ELISA spot assay (17, 22) and reversion rates were calculated based on a multiple mutation accumulation model for fluctuation analysis (19). The 22 independent fresh transfectants with the Vn38C/γ2a construct reverted the TAG(**C**) nonsense codon at a rate of 6.64 × 10^{-4}/bp per generation (Fig. 2, Vn38C, first panel marked T for fresh transfectants). To see whether these transfectants were phenotypically stable, we subcloned three of them (filled circles in the Vn38C-T panel) and determined their rates of reversion.

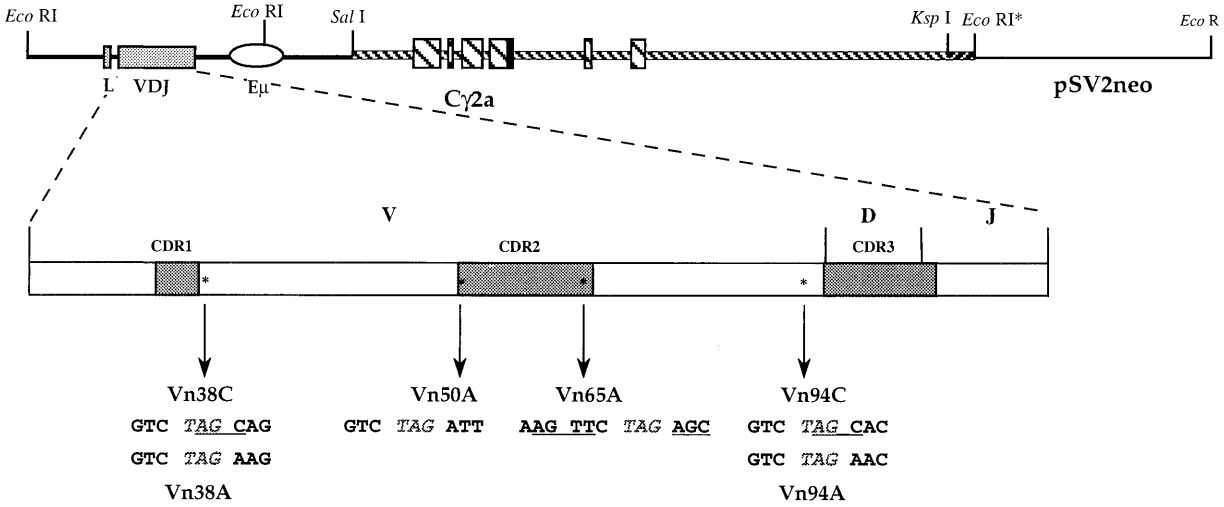

FIG. 1. The six γ2a constructs used for transfections. The leader sequence and V(D)J are denoted as grey bars; γ2a constant region as striped boxes; and intronic enhancer as an oval Eμ. The restriction endonuclease sites are indicated; the *Eco*RI* site was filled in during the cloning process. Vn38C and Vn38A are one pair of constructs containing a TAG codon at codon 38. They differ only by 1 bp in the next codon. Vn94C and Vn94A are another pair of constructs containing a TAG codon at codon 94 and also differ by 1 bp in the neighboring sequence. Hot spot motifs AGC or RGYW [(A/G)G(T/C)(A/T)] are underlined.

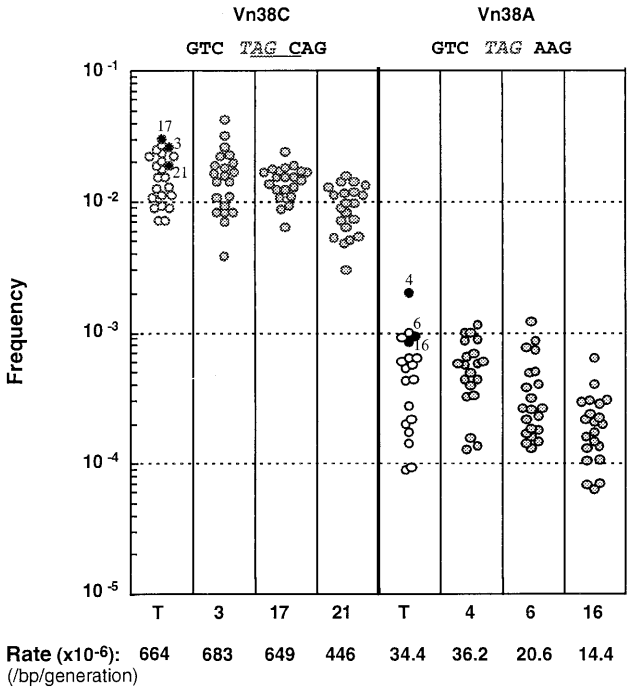

FIG. 2. Comparison of the mutation rates of Vn38C and Vn38A in the NSO cell line. NSO cells were transfected with either Vn38C or Vn38A constructs. Fresh transfectants (T, open circles) and selected subclones (shaded circles) were tested for revertants by the ELISA spot assay. Each circle represents the reversion frequency of a single transfectant or a subclone. Filled circles are the clones selected for further subcloning. Sequences around the stop codons are shown and the hot spot motifs are underlined. The mutation frequencies of the individual transfectants and subclones were converted to rates using a formula derived from a multiple mutation accumulation model (19).

All three subclones reverted at rates that were similar to the fresh transfectants, and the average reversion rate for the three Vn38C subclones was 5.93×10^{-4}/bp per generation (Fig. 2 and Table 1). The distribution of frequencies of revertants in the 22 fresh transfectants, each with a different integration site and copy numbers that ranged between 3 and 16 (data not shown), was similar to the distribution of frequencies of revertants in each of the Vn38C subclones (numbers 3, 17, and 21 in Fig. 2). This indicates that the rate of mutation of these transfected genes was not significantly affected by the integration site or the ≈5-fold difference in the copy number.

The fresh Vn38A/γ2a transfectants with the (T)AGA non-hot spot motif mutated at a lower rate of 3.44×10^{-5}/bp per generation (Fig. 2). The difference in reversion rates between the fresh transfectants with Vn38C and Vn38A was about 19-fold. The average mutation rate for the Vn38A subclones was 2.37×10^{-5}/bp per generation (Table 1), and the difference in average rates between Vn38C and Vn38A subclones was about 25-fold. The difference in the reversion frequencies between Vn38C and Vn38A was highly significant by the Mann–Whitney test ($P < 0.001$). Revertants of both Vn38C and Vn38A had the same size ELISA spots so the detection of revertants was comparable. The steady-state levels of mRNA, as examined by Northern blot analysis, differed for different subclones but did not correlate with the rates of mutation (data not shown).

To see whether (T)AGC and (T)AGA mutated at different rates in another B cell line, we transfected Vn38C/γ2a and Vn38A/γ2a into the 18.81 pre-B cell line that was previously shown to be able to mutate Vn38C/γ2a construct (17). In 18.81, Vn38C mutated at 1.52×10^{-5}/bp per generation and Vn38A mutated at 9.1×10^{-7}/bp per generation (Table 1). Although the rate of mutation was ≈40-fold lower in 18.81 than in NSO, the difference in mutation rates between AGC and AGA was 17-fold (Table 1), very similar in magnitude to their difference in NSO.

The Rate of Mutation Varies in Different Locations Within the V Region. To compare the rates of mutation of the AGC and AGA motifs at a different location within the V region, a second set of constructs was made with TAG**C** (Vn94C/γ2a) and TAG**A** (Vn94A/γ2a) in which the TAG nonsense mutation replaced residue 94 in the third framework region just 5′ to CDR3 (Fig. 1). As in the previous experiments, the NSO–LC1 cells were transfected with Vn94C/γ2a and Vn94A/γ2a and their reversion rates were determined. The reversion rate for (T)AG**C** in fresh Vn94C transfectants was 3.75×10^{-5}/bp per generation (Fig. 3), which is about 18-fold lower than the (T)AG**C** of Vn38C/γ2a at codon 38 (Fig. 3 and Table 1). Subclones of transfectants containing each of the motifs were also examined. One subclone, Vn94C.13, had a lower rate of mutation than that of the fresh transfectants and of the two other subclones, but the average rate of mutation of the three subclones was similar to the rate calculated from the fresh transfectants (Table 1).

The (T)AG**A** Vn94A/γ2a mutated at a rate that was less than 1.11×10^{-6}/bp per generation, which was more than 30-fold lower than the AGC rate at the same location (Fig. 3 and Table 1). Selected transfectant subclones had rates of mutation that were close to the rate obtained from the fresh transfectants (Table 1). The difference between AGC and AGA mutation rates was ≈30-fold. Here too, this difference did not correlate with different copy numbers or with different steady-state levels of mRNA (data not shown).

Mutation in CDR2. The CDRs that form the antigen binding site accumulate large numbers of replacement substitutions during the immune response (23). This has been attributed to selection for higher affinity antibodies (1, 23, 24), but it is also possible that there is a greater intrinsic susceptibility to

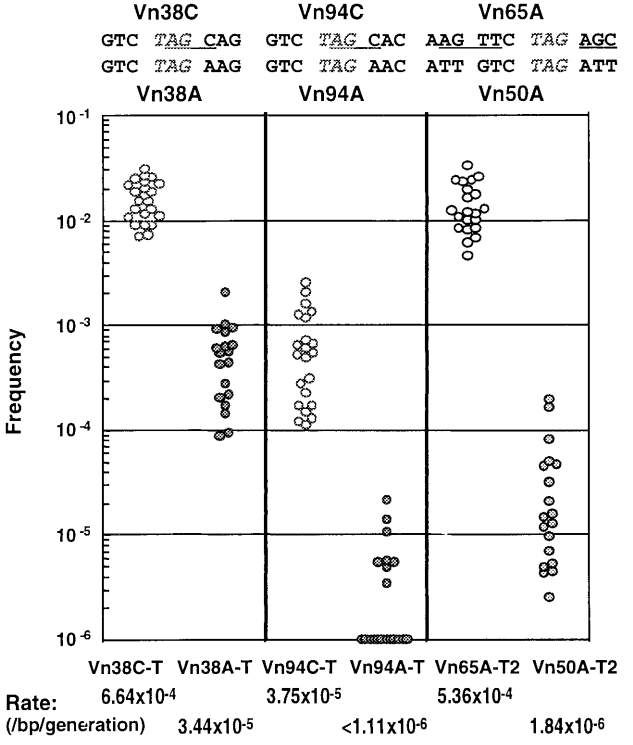

FIG. 3. Comparison of mutation rates of the six different γ2a constructs in the NSO cell line. NSO cells were transfected with each of six different γ2a constructs and fresh transfectants (T) were tested for revertants by the ELISA spot assay. Each circle represents the reversion frequency of a single transfectant. Sequences around the stop codons are shown, and the hot spot motifs are underlined.

Table 1. Comparison of reversion rates of different Vn constructs in NSO and 18.81 cells

Transfectant	Sequence	Rate, bp/generation
NSO/Vn38C-T	TGG GTC *TAG* **C**AG AGG	6.64×10^{-4}
Subclones		
3		6.83×10^{-4}
17		6.49×10^{-4} } 5.93×10^{-4}
21		4.46×10^{-4}
NSO/Vn38A-T	TGG GTC *TAG* **A**AG AGG	3.44×10^{-5}
Subclones		
4		3.62×10^{-5}
6		2.06×10^{-5} } 2.37×10^{-5}
16		1.44×10^{-5}
18.81/Vn38C-T	TGG GTC *TAG* **C**AG AGG	1.52×10^{-5}
18.81/Vn38A-T	TGG GTC *TAG* **A**AG AGG	9.10×10^{-7}
NSO/Vn94C-T	GCC GTC *TAG* **C**AC TGT	3.75×10^{-5}
Subclones		
6		3.79×10^{-5}
13		6.15×10^{-6} } 2.26×10^{-5}
16		2.38×10^{-5}
NSO/Vn94A-T	GCC GTC *TAG* **A**AC TGT	$<1.11 \times 10^{-6}$
Subclones		
1		7.88×10^{-7}
4		4.24×10^{-7} } 7.80×10^{-7}
11		1.13×10^{-6}
NSO/Vn50A-T1	ATT GTC *TAG* **A**TT GAT	3.73×10^{-6}
NSO/Vn50A-T2		1.84×10^{-6}
Subclones		
8		2.41×10^{-6}
13		3.22×10^{-6} } 2.58×10^{-6}
18		2.11×10^{-6}
NSO/Vn65A-T1	AAG TTC *TAG* **A**GC AAG	6.49×10^{-4}
Subclones		
1		6.41×10^{-4}
2		8.21×10^{-4} } 5.96×10^{-4}
3		3.25×10^{-4}
NSO/Vn65A-T2		5.36×10^{-4}
Subclones		
7		5.78×10^{-4}
21		6.49×10^{-4} } 6.15×10^{-4}

$P < 0.001$ for both Vn38C/Vn38A and Vn94C/Vn94A. RGYW motifs are underlined.

mutation in the CDRs (7, 13). To increase the chance of detecting higher mutation rates, the Vn50A/γ2a and Vn65A/γ2a constructs were designed with the non-hot spot TAG**A** stop codons at the 5′ and 3′ ends of CDR2, respectively (Fig. 1). NSO–LC1 cells were transfected and reversion rates were determined as before. Two separate transfections were carried out with Vn50A and gave low rates of 1.84×10^{-6}/bp per generation (Fig. 3) and 3.73×10^{-6}/bp per generation (Table 1) that were not statistically different from each other ($P = 0.37$). The average rate of mutation of the subclones from one

Amino Acid	---	15	---	26	---	38	39	40	---	46	---	62	---
Vn38C sequence	---	GGG	---	GGC	---	TAG	CAG	AGG	---	GAG	---	CCG	---
						Stop							
Vn38C-21. 1R1	---	---	---	---	---	--C	---	---	---	---	---	---	---
-3. 19R2	---	---	---	---	---	-G-	---	---	---	---	---	---	---
-17. 2N1	---	---	---	-A-	---	---	---	---	---	---	---	---	---
Vn38A sequence	---	GGG	---	GGC	---	TAG	AAG	AGG	---	GAG	---	CCG	---
						Stop							
Vn38A-5. 15R1	---	---	---	---	---	C--	---	---	---	---	---	---	---
-4. 9R1	---	---	---	---	---	--T	---	---	---	---	---	---	---
-4. 9R2	---	---	---	---	---	--T	---	---	---	-G-	---	---	---
-4. 15R1	---	---	---	---	---	--T	---	---	---	---	---	---	---
-4. 15R2	---	-T-	---	---	---	--T	---	---	---	---	---	---	---
-4. 15R3	---	---	---	---	---	--T	---	--T	---	---	---	---	---
-4. 12. 1R1	---	---	---	---	---	--T	---	---	---	---	---	---	---
-4. 12. 3R2	---	---	---	---	---	--T	---	---	---	---	---	T--	---
-4. 12. 3R3	---	---	---	---	---	--T	---	---	---	-G-	---	---	---

FIG. 4. Revertant sequences and associated base changes of Vn38C and Vn38A. Partial Vn38C and Vn38A sequences are shown. The revertant sequences (codon 38) and associated base pair changes are shown below the Vn38C and Vn38A sequences. The prefixes of R are the subclones from which revertant sequences were derived.

of these transfections was 2.58×10^{-6}/bp per generation (Table 1), nearly identical to the average (2.79×10^{-6}/bp per generation) of the two fresh transfections.

In contrast to the low rate of mutation of Vn50A, the reversion rate of fresh transfectants of Vn65A/γ2a was 5.36×10^{-4}/bp per generation (Fig. 3). Two separate transfections were also performed with Vn65A (Table 1) and their rates of mutation were indistinguishable from each other and from their subclones (Table 1). The rate of mutation of this (T)AG**A** non-hot spot motif at residue 65 at the 3′ end of CDR2 was higher than those of AGA at residues 38, 50, and 94 and had the same high rate as the Vn38C (T)AG**C** hot spot motif at residue 38 (Fig. 3 and Table 1). Because the TAG at residue 65 is surrounded on both sides by RGYW motifs (Table 1), this observation suggests that a hot spot motif can itself affect mutations in adjacent bases.

Revertants and Associated Mutations Are Point Mutations. In previous studies of the Vn38C/γ2a construct in the NSO and 18.81 cell lines, we demonstrated that reversion was due to point mutations in the nonsense codon and was associated with additional single base changes in other parts of the V region (17). Here, some of the revertant cellular subclones identified in these studies were isolated, and the transfected V regions were amplified by PCR and sequenced. All PCRs were carried out using high fidelity *Pfu* polymerase. The PCR error with these primers and conditions was 1 in 16.8 kb per 35 cycles (data not shown), which is close to the $1–5 \times 10^{-5}$ mutations/nt per 30 cycles reported by others (25, 26). The revertant sequences were confirmed by sequencing both strands of cloned PCR products from at least two bacterial colonies. In addition, cloned PCR products from two separate cell clones were sequenced for some of the revertants (Vn38C-21.1R1, Vn38A-5.15R1, Vn38A-4.9R1, Vn38A-4.12R1) to verify that the base changes were not the result of PCR error.

The two revertants of Vn38C/γ2a characterized in these studies, like the previous revertants of Vn38C/γ2a (17), were due to single base changes (Fig. 4). One unreverted gene (Vn38C-17.2N1) was also sequenced and found to have a single base change outside of the stop codon (Fig. 4). To see whether revertants from Vn38A/γ2a, which arose at a lower rate than Vn38C/γ2a (Table 1), were also due to point mutations, four independent revertants (Vn38A-5.15R, -4.9R, -4.12R and -4.15R) were recovered from four independent transfectants. Reversion of the (T)AG**A** in these four revertants occurred by two distinct point mutations in the stop codon (Fig. 4).

Multiple subclones of the revertants were isolated to examine their V regions for associated base changes and confirm revertant sequences. Because of the relatively low rate of reversion in Vn38A/γ2a, revertants had to be recovered by sib selection (27) and required multiple steps of enrichment over a period of several months. As shown in Fig. 4, five associated base pair changes were found in the V region of Vn38A construct, one from subclone Vn38A-4.9 and two each from subclones Vn38A-4.15 and -4.12. It is possible that the revertants isolated at the end of such an enrichment arose during the enrichment process or that the different associated changes arose in different cellular subclones of the revertant during the enrichment process. In either situation, these associated base changes represent at least four and possibly five different events. For Vn38A revertants, five associated base changes were found out of nine revertants. Four out of five associated base changes were replacement mutations and only one was a silent mutation. Of the six associated base pair changes, one occurred in a RGYW motif (8) and one occurred in an AGT motif (13), with an equal number of transitions and transversions. These associated base changes confirm that there is ongoing V region mutation in the NSO cell line. Although it is impossible to completely rule out gene conversion, none of the J558 V regions in the database contain any of the base changes that are responsible for the reversions or the associated base changes that we have observed.

DISCUSSION

Motifs that are preferred targets of V region hypermutation have been identified by examining large numbers of V region mutations that have arisen *in vivo* (6–9). However, it is difficult to compare the exact mutation rate of putative hot spot and non-hot spot motifs and to examine the impact of neighboring sequences *in vivo*. We have used transfected stably integrated Ig heavy chain genes to study the relative mutation rates of AGC, one of the most common hot spot motifs, and the AGA non-hot spot motif in cultured B cells. The reliability of this model *in vitro* system is attested to by the similarity of the rates that are obtained from the fresh transfectants and the subclones of those transfectants and by the reproducibility between multiple independent transfection experiments (Table 1). The fact that the rates are the same whether they are calculated from the fresh transfectants, which include different integration sites and copy numbers, or the individual subclones, indicates that these variables do not significantly influence the rates of mutation perhaps because their effects are smaller than the clonal fluctuations.

It would be useful to compare the rate of V region mutation in this *in vitro* system to the rate of V region mutation *in vivo*. Because the rates of mutation of the hot spot and non-hot spot motifs are very different, and the rate of mutation of the hot spot motif differs in different parts of the V region, it is difficult to establish an average rate of mutation for the V region as a whole in these transfected heavy chain genes. This is further complicated by the likelihood that this is a dynamic process and the loss or acquisition of a hot spot may affect the rate of mutation of neighboring, or perhaps even more distant sequences (28). Nevertheless, the median rate for the six constructs that we have studied here is the same as Vn38A, which was $\approx 2.5 \times 10^{-5}$/bp per generation (Table 1). We have used a multiple mutation accumulation model (19) in which independent mutants accumulate base changes at the rate of $\approx 2.5 \times 10^{-5}$/bp per generation, and the fact that it took ≈4 months to enrich for and clone the revertants whose sequences are shown in Fig. 4, to calculate that the chance of finding 1–2 associated base changes after 150 generations is ≈60%. Thus, the rate of $\approx 2.5 \times 10^{-5}$/bp per generation is consistent with the number of base changes observed by sequencing. Because the same variations in the rate of mutation of different bases in different locations also exist *in vivo* (28), and true mutation rates cannot be measured in the whole animal, it is also difficult to estimate the rate of V region mutation in normal B cells. Nevertheless, because most V regions from germinal center cells from primary Ig responses have 0–7 base changes (29), we have estimated that if all copies of transgene contributed equally to the mutation frequencies, the transfected V regions that we have studied *in vitro* were mutating at a 10- to 40-fold lower rate than the rates that have been observed for positively selected germinal center B cells *in vivo* (30). The lower rate of mutation *in vitro* could be due to the fact that the cultured pre-B and plasmacytoma cells that we have used do not represent stages of B cell differentiation during which V region hypermutation is thought to occur at a maximum rate (31). It could also reflect the fact that mutation in this *in vitro* system is occurring in the absence of T cells and Ig cross-linking (32).

In this model system, the AGC triplet in the context of the AG**C**A version of the RGYW motif underwent a 17- to 34-fold higher rate of mutation than the AG**A**A motif in the same location surrounded by the same neighboring sequences. Thus, the presence of a C instead of an A greatly increased the mutation rate of this codon *in vitro*. This was true in both the NSO plasma and the 18.81 pre-B cell lines, two lines that we have previously identified as sustaining high rates of V region

mutation in a γ2a heavy chain construct (15, 17). The finding that the AGC has a different rate of mutation at residue 38–39 and residue 94–95, and that it is a preferred substrate at both locations, suggests that the difference in susceptibility to mutation is due to additional surrounding sequences or the distance from a cis-acting sequence such as the promoter. This is consistent with the data from endogenous Ig genes and transgenes where some AGCs and RGYWs do not have an increased rate of mutation (6–8) and with recent data from a transgenic mouse study in which the serine codons AGT and TCA mutated at different frequencies in the same V region location (28).

The very high rate of mutation of the AGA at residue 65 in CDR2 is best explained by the fact that it is adjacent to and surrounded by RGYW motifs (Fig. 3), confirming that neighboring sequences are very important (6–8). These data suggest that some higher order structure of the DNA may be involved (28, 33, 34). If transcription is linked to the mutation process (26, 35, 36), and basal transcription factors such as TFIIH that participate in nucleotide excision repair are involved (37, 38), motifs such as AGC could be promoting transcriptional pausing by altering the structure of the DNA or recruiting proteins that would facilitate the mutational process (39). For example, transcriptional pausing has been observed in the rabbit globulin gene where there is an AGCW followed by an inverted repeat (40), which is very similar to the core sequence around the TAG at Vn38C. We have used a DNA folding program (41) to generate the lowest energy folding structure for the V region. No correlation was found between potential palindromic structures in the different Vn constructs and the mutation rates that we have determined. Goyenechea and Milstein (28) have recently shown that removing a palindromic structure encompassing an AGC hot spot motif did not change its mutational potential in a κ transgene and have suggested that more complex structures could be involved. The *in vitro* system that we have described here, and others that have recently been described (14, 32), should make it possible to examine a variety of constructs in cultured cells to search for evidence for such structures and then to test them in transgenic mice.

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1. Berek, C. & Milstein, C. (1987) *Immunol. Rev.* **96,** 23–41.
2. French, D. L., Laskov, R. & Scharff, M. D. (1989) *Science* **244,** 1152–1157.
3. Rajewsky, K., Forster, I. & Cumano, A. (1987) *Science* **238,** 1088–1094.
4. Tonegawa, S. (1983) *Nature (London)* **302,** 575–581.
5. Weigert, M. G., Cesari, I. M., Yonkovich, S. J. & Cohn, M. (1970) *Nature (London)* **228,** 1045–1047.
6. Betz, A. G., Neuberger, M. S. & Milstein, C. (1993) *Immunol. Today* **14,** 405–411.
7. Neuberger, M. S. & Milstein, C. (1995) *Curr. Opin. Immunol.* **7,** 248–254.
8. Rogozin, I. B. & Kolchanov, N. A. (1992) *Biochim. Biophys. Acta* **1171,** 11–18.
9. Smith, D. S., Creadon, G., Jena, P. K., Portanova, J. P., Kotzin, B. L. & Wysocki, L. J. (1996) *J. Immunol.* **156,** 2642–2652.
10. Betz, A. G., Rada, C., Pannell, R., Milstein, C. & Neuberger, M. S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2385–2388.
11. Tumas-Brundage, T. & Manser, T. (1997) *J. Exp. Med.* **185,** 239–250.
12. Yelamos, J., Klix, N., Goyenechea, B., Lozano, F., Chui, Y. L., Gonzalez Fernandez, A., Pannell, R., Neuberger, M. S. & Milstein, C. (1995) *Nature (London)* **376,** 225–229.
13. Wagner, S. D., Milstein, C. & Neuberger, M. S. (1995) *Nature (London)* **376,** 732.
14. Bachl, J. & Wabl, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 851–855.
15. Green, N. S., Rabinowitz, J. L., Zhu, M., Kobrin, B. J. & Scharff, M. D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6304–6308.
16. Zhu, M., Rabinowitz, J. L., Green, N. S., Kobrin, B. J. & Scharff, M. D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2810–2814.
17. Zhu, M., Green, N. S., Rabinowitz, J. L. & Scharff, M. D. (1996) *EMBO J.* **15,** 2738–2747.
18. Luria, S. E. & Delbruck, M. (1943) *Genetics* **28,** 491–511.
19. Lin, M., Chang, C. J. & Green, N. S. (1996) *Mutat. Res.* **351,** 105–116.
20. Galfre, G. & Milstein, C. (1981) *Methods Enzymol.* **73,** 3–46.
21. Wabl, M., Burrows, P. D., von Gabain, A. & Steinberg, C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 479–482.
22. Greene, G., Hodous, J., Dintzis, R. Z. & Dintzis, H. M. (1990) *J. Immunol. Methods* **129,** 187–197.
23. Clarke, S. H., Huppi, K., Ruezinsky, D., Staudt, L., Gerhard, W. & Weigert, M. (1985) *J. Exp. Med.* **161,** 687–704.
24. Kocks, C. & Rajewsky, K. (1989) *Annu. Rev. Immunol.* **7,** 537–559.
25. Lundberg, K. S., Shoemaker, D. D., Adams, M. W., Short, J. M., Sorge, J. A. & Mathur, E. J. (1991) *Gene* **108,** 1–6.
26. Peters, A. & Storb, U. (1996) *Immunity* **4,** 57–65.
27. Spira, G. & Scharff, M. D. (1992) *J. Immunol. Methods* **148,** 121–129.
28. Goyenechea, B. & Milstein, C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13979–13984.
29. Berek, C., Berger, A. & Apel, M. (1991) *Cell* **67,** 1121–1129.
30. Milstein, C. (1991) *Immunol. Today* **12,** 93–94.
31. MacLennan, I. C. (1994) *Annu. Rev. Immunol.* **12,** 117–139.
32. Denepoux, S., Razanajaona, D., Blanchard, D., Meffre, G., Capra, J. D., Banchereau, J. & Lebecque, S. (1997) *Immunity* **6,** 35–66.
33. Golding, G. B., Gearhart, P. J. & Glickman, B. W. (1987) *Genetics* **115,** 169–176.
34. Rogozin, I. B., Solovyov, V. V. & Kolchanov, N. A. (1991) *Biochim. Biophys. Acta* **1089,** 175–182.
35. Weber, J. S., Berry, J., Manser, T. & Claflin, J. L. (1994) *J. Immunol.* **153,** 3594–3602.
36. Maizels, N. (1995) *Cell* **83,** 9–12.
37. Friedberg, E. C. (1996) *Annu. Rev. Biochem.* **65,** 15–42.
38. Wagner, S. D. & Neuberger, M. S. (1996) *Annu. Rev. Immunol.* **14,** 441–457.
39. Storb, U. (1996) *Curr. Opin. Immunol.* **8,** 206–214.
40. Vandenbergh, D. J., James-Pederson, M. & Hardison, R. C. (1991) *J. Mol. Biol.* **220,** 255–270.
41. SantaLucia, J., Jr., Allawi, H. T. & Seneviratne, P. A. (1996) *Biochemistry* **35,** 3555–3562.