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Immunology

A well-differentiated B-cell line is permissive for somatic mutation of a transfected immunoglobulin heavy-chain gene

(reversion analysis/spot ELISA/fluctuation analysis)

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ABSTRACT **pSV2neo plasmids containing an IgM heavy-chain gene with nonsense mutations in either the variable (V) or the constant (C) region were transfected into four differentiated mouse plasma cell lines: S107 and the NSO fusion partner (myeloma cell lines) and 2C3 and 36.65 (hybridoma cell lines). The frequencies of reversion of the nonsense mutations in multiple independent transfectants were determined with the spot ELISA and rates of reversion were calculated by fluctuation analysis. Mutations in both V and C regions were confirmed by sequence analyses. In the S107 cell line, spontaneous point mutations occurred in the V region at a rate of $\approx 5 \times 10^{-5}$/bp per cell generation, >400-fold higher than the rate of V-region mutation in the NSO cell line and considerably higher than the rates in 2C3 and 36.65 hybridoma cell lines. These studies suggest that S107 is a relatively permissive cell line in which V-region mutations can occur constitutively, even though it represents a late stage of B-cell differentiation. Further, the results show that the construct used contains sufficient information in its flanking and coding sequences to allow a relatively high rate of V-region mutation, at least in the S107 cell line.**

Every individual expresses an enormous repertoire of antibodies capable of binding to a vast array of antigens. This diversity is initially created in the immunoglobulin (Ig) heavy (H) chain by assembling variable (V), diversity (D), and joining (J) gene segments from the many different genetic elements available in the germ line to form a functional V region (1). As individual B cells differentiate in response to antigen and accessory cells, the rearranged V-region genes undergo further diversification through extensive introduction of single base changes (2, 3). *In vivo*, V-region somatic hypermutation is estimated to occur at rates of 10^{-3} to 10^{-5}/bp per cell generation (reviewed in refs. 4 and 5) and appears to be confined to the coding and immediate flanking sequences of the V region (6, 7). Although point mutations are found throughout the V region and are believed to occur relatively randomly in the absence of antigen selection, motifs have been identified that appear to be "hotspots" for unselected mutations in the light (L) chain (8, 9). The process of V-region hypermutation is thought to commence in germinal centers in B cells expressing surface Ig and is believed to turn off as the B cell differentiates into a plasma cell (10–14).

Although a great deal has been learned about the impact of somatic mutation on the affinity and specificity of the antibody response, little is known about the biochemical and molecular events responsible for the high rate of mutation, its restriction to the V region and its immediate flanking sequences, and its regulation during B-cell differentiation (4, 5). Studies using transgenic mice have shown that sequences in or near both the intronic and 3′ L-chain enhancers are required for mutation of the κ L-chain V region *in vivo* (15–17) and that sequences within a 2.5-kb region 5′ of the H-chain V (V_H) region are required for V_H mutation (18). If cell lines undergoing somatic mutation in culture could be identified and transfected with manipulated Ig genes, it would greatly facilitate the examination of the role of cis-acting DNA sequences and the identification of trans-acting factors involved in V-region hypermutation. Although many highly differentiated myeloma and hybridoma cell lines have been examined for V-region mutation (reviewed in ref. 19), V-region hypermutation confirmed by sequence has been reported for the endogenous Ig genes only in 18.81, an Abelson virus-transformed murine pre-B-cell line (20, 21), and S107, a mouse myeloma cell line (22, 23). Paradoxically, constant (C)-region mutations have been observed in many antibody-forming cell lines in culture and are usually due to small deletions, although point mutations in C regions have also been reported (19).

The accurate determination of the actual rate of somatic mutation in the V and C regions in cultured cells requires a detection system that will be equally likely to reveal mutations in different parts of the Ig gene. Since Ig transgenes undergo somatic mutation *in vivo* (15–18, 24, 25), it should be possible to transfect Ig genes into antibody-forming cell lines to study these processes. We have therefore transfected IgM heavy-chain constructs containing the same amber nonsense mutations in either the V or the C region into several cultured cell lines. Mutations that "revert" this amber stop codon to sense allow the production and secretion of IgM antibody by the cells. Using this approach, which is similar to that used by Wabl *et al.* (21) to study mutation of the endogenous heavy-chain gene of 18.81, we have compared the frequencies and rates of mutation in the V and C regions in four mature B-cell lines. The results show that spontaneous mutations occur in the V region of the S107 mouse myeloma cell line at a rate >400 times that in the NSO myeloma and considerably higher than those in the 2C3 and 36.65 hybridoma cell lines. This suggests that the S107 mouse myeloma cell line is permissive for a relatively high rate of V-region somatic mutation and that the construct contains at least some of the sequences required for mutation in permissive cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. S107, an IgA(κ)-producing mouse myeloma cell line (26) was kindly provided by Melvin Cohen (Salk Institute). 2C3 was kindly provided by S. K. Ghosh and R. B. Bankert (Roswell Park) and is an IgG1(κ)-producing hybridoma generated by the fusion of the nonsecreting myeloma X63-Ag8.653 with spleen cells from a

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Abbreviations: V, variable; D, diversity; J, joining; C, constant; H, heavy; L, light.

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BALB/c mouse immunized with phthalate conjugated to keyhole limpet hemocyanin (27). 36.65 is a hybridoma producing IgG1(κ), generated from a *p*-azophenylarsonate-immunized A/J mouse (28). NSO is a non-Ig-producing fusion partner derived from the P3 myeloma line (29). These cell lines were grown in Dulbecco's modified Eagle's medium (GIBCO) with 10–20% (vol/vol) fetal bovine serum (HyClone), 5% NCTC109 (BioWhittaker), 1% nonessential amino acids (GIBCO), and 1% penicillin/streptomycin solution (GIBCO). The transfectants from these cell lines were grown in the feeding medium plus G418 (GIBCO) at 2 mg/ml (for S107), 1 mg/ml (for 2C3), 1.2 mg/ml (for 36.65), or 1.5 mg/ml (for NSO).

DNA Construction and Transfection. pSV2neoIgM (a gift of S. Morrison, University of California, Los Angeles) contains all of the J–C intervening sequence, including the intronic enhancer and 1.4 kb of the 4-kb μ switch region, the murine IgM H-chain (μ) C region (C_μ) and its intervening sequences and secretory and membrane exons, and 1.6 kb of 3′ flanking sequences cloned into the *Eco*RI site of pSV2neo (30). A productive V region that contains 1.7–1.8 kb of 5′ V_H sequence, a J558 V segment, a presumed D segment, and a J_{H1} segment encoding a ricin-neutralizing antibody (R45) was introduced into pSV2neoIgM to form the wild-type construct (Wt, Fig. 1). Nonsense mutations were introduced by site-directed mutagenesis (31) into the wild-type V region to form V^n and into the C region to form C^n (Fig. 1). In V^n, the TAG amber stop codon was introduced into the codon for aa 38 of the V region, while in C^n, this stop codon was introduced into codon 128 of the C_{H1} domain. In both V^n and C^n, an additional silent base substitution was introduced in the third base of the proceding codon to mark the mutant gene (Fig. 1). Other than these single base changes, the V^n and C^n constructs differ only by an additional 100 bp in the C^n construct, 1.7 kb 5′ of VDJ (where this sequence joins pSV2neo). DNA transfection was performed with the Gene Pulser transfection apparatus (Bio-Rad) to electroporate plasmid DNAs (5–20 μg) that had been linearized at a unique *Ksp* I site 3′ of the membrane exon (Fig. 1) into 1–5 × 10^6 cells. Cells were seeded in 96-well plates and G418 was added 24–48 hr after transfection. Transfection frequencies were ≈10^{-5}. The L-chain construct was made by cloning the L-chain V region of the same R45 monoclonal antibody into pSV2gptKappa (a gift of S. Morrison). This construct was transfected into NSO cells and selected with medium containing xanthine (125 μg/ml; Sigma), hypoxanthine (7.5 μg/ml; Calbiochem), and mycophenolic acid (1 μg/ml; GIBCO). Preliminary studies were done on fresh transfectants from single wells. All quantitative studies were done on cells that had been cloned at least twice in soft agar (32).

Detection and Isolation of μ^+ Revertants. The spot ELISA (33, 34) was used to detect IgM-secreting revertants. ELISA plates (96 well; Corning) were coated with goat anti-mouse IgM antibody (1–2 μg/ml; Southern Biotechnology Associates) in coating buffer (34) and then blocked with coating buffer containing 2% bovine serum albumin. Approximately 10^5 cells were added to each well and incubated at 37°C overnight. The cells were then removed by extensive washing with washing solution. Spots were developed either by sequential incubation (for the S107 and 2C3 cell lines) with biotin-conjugated goat anti-IgM antibody (1 μg/ml; Southern Biotechnology Associates), avidin-conjugated alkaline phosphatase (0.4 μg/ml; Southern Biotechnology Associates), and 5-bromo-4-chloro-3-indolyl phosphate (1 mg/ml; Amresco, Solon, OH) or by sequential incubation (for 36.65 and the NSO cell line) with rabbit anti-mouse IgM (0.5 μg/ml; Zymed), biotin-conjugated goat anti-rabbit IgG (1.5 μg/ml; Vector Laboratories), Vectastain avidin-phosphatase amplification system (Vector Laboratories), and 5-bromo-4-chloro-3-indolyl phosphate.

To clone revertants, cells from wells containing the largest number of spots were sib selected to enrich for revertants and cloned in soft agar (32). For cell lines that reverted at low frequencies, about 10 rounds of sib selection were required.

Fluctuation Analysis. Eighteen to 25 soft-agar clones from each transfectant were randomly picked and seeded in a 96-well plate. When 70–80% confluence was achieved, the cells were transferred to 24- or 6-well plates. Spot ELISA was performed to test the reversion frequency of each clone, using 5–40 × 10^6 cells per clone. The rate of mutation was calculated according to Lea and Coulson (35).

RNA Extraction, cDNA Preparation, PCR Amplification, and Sequencing. Total RNA from V^n or C^n transfectants and revertants was isolated with guanidinium isothiocyanate/phenol (36). For reverse transcription (RT) (37), 5–10 μg of total RNA was incubated for 50 min at 50°C with 20 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL), 3 mM dNTPs, and primer (0.5 ng/ml; 5′-ATAAGCTTGTTCCAGGTGAAGGAAAT-3′, a sequence from the μ C_{H1} domain) in 60 mM Tris·HCl, pH 8.3/12 mM dithiothreitol/12 mM $MgCl_2$/12.5 mM KCl with actino-

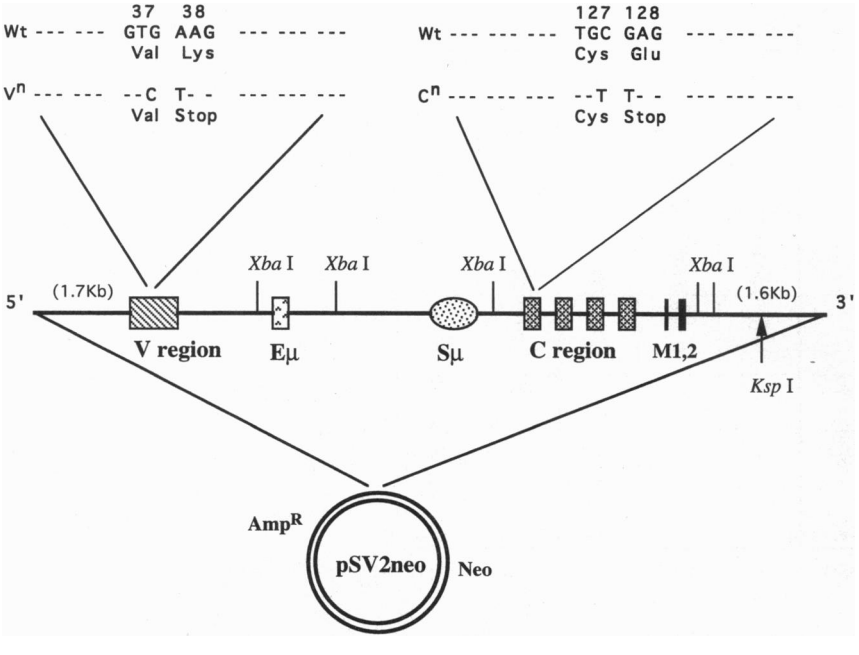

FIG. 1. Constructs used for transfection. Amp^R, ampicillin-resistance gene; Neo, neomycin-resistance gene. The R45 V region and the μ C region gene [including enhancer (E_μ), switch (S_μ), and membrane exons (M1,2)] were inserted into pSV2neo plasmid. Wild-type (Wt), V nonsense (V^n), and C nonsense (C^n) constructs were used. Differences in nucleic acid and amino acid sequences of V^n and C^n compared with Wt are shown. Constructs were not drawn to scale.

mycin D (60 μg/ml). Samples were heated at 82°C for 5 min both before and after RT. After RT, 3 μl of product (cDNA) was used in 100-μl PCR mixtures containing 200 μM dNTPs, 2 units of *Taq* DNA polymerase (Boehringer Mannheim), and 0.1 ng of each primer (coding strand, 5′-ATCTCGAGTCA-GAGGTTCAGCTGCAG-3′, aa −3 in the leader to aa 5 of the V region; noncoding strand, the same oligonucleotide used for RT, aa 161–165). These primers could be used to sequence mutational events at both the V- and C-region nonsense codons. cDNA PCR was performed in a DNA thermal cycler (Perkin–Elmer/Cetus) for 5 cycles of 94°C for 1 min, 46°C for 1 min, and 72°C for 1 min, and 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. There was a final extension at 72°C for 10 min. DNA-based PCR was performed by using the same 5′ primer as for cDNA PCR and 5′-TAGGATCTAC-TACCGAAGTACC-3′, corresponding to the D element, as 3′ primer. The PCR products were cloned into the pCR vector with the TA cloning system (Invitrogen). Sequencing using either T7 or SP6 promoter as primer was carried out by the chain-termination method with a Sequenase version 2.0 kit (United States Biochemical) or on a 373A DNA sequencer (Applied Biosystems). RNAs and DNAs from multiple cellular subclones of each revertant were used for RT–PCR and DNA-based PCR. After each PCR and TA cloning, multiple bacterial colonies were used for sequencing in order to rule out RT- or PCR-induced error.

RESULTS

Comparison of Mutation Rates in the V and C Regions. Somatic mutation of transfected Ig genes in cultured antibody-forming cells was examined by using constructs that contain all of the coding sequences and almost all of the intervening sequences of the rearranged R45 μ antibody gene (Fig. 1). These studies were initiated with hybridoma and myeloma cells that secrete large enough amounts of antibody to be reliably detected at a single-cell level with the spot ELISA. In preliminary studies, the wild-type construct (Wt) was electroporated into the S107 (6), 2C3 (27), and 36.65 (28) hybridoma cell lines, which produce their own antibodies. Seventy to 80% of the G418-selected transfectants secreted enough IgM encoded by the transfected μ gene to be easily detected in medium by routine ELISA and at the single-cell level by the spot ELISA (Fig. 2), indicating that the transfected R45 μ chain could assemble and be secreted with the endogenous L chain in these cell lines. No spots were detected with untransfected cell lines. Further, almost all of the cloned transfected cells continued to express IgM when the cells were maintained in G418, and many stably expressed the IgM even when grown in the absence of selection.

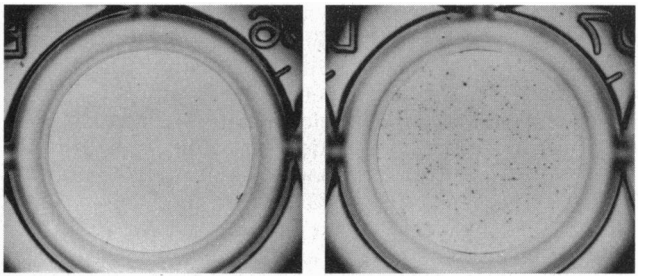

FIG. 2. Spot ELISA for identification and enrichment of cellular revertants. Spots formed on the bottom of the well of a 96-well ELISA plate by the revertants from 2C3 cell line before (*Left*) and after (*Right*) enrichment. About 10^5 cells were tested per well.

The spot ELISA was shown to be quantitative in reconstruction assays. A single IgM-producing cell could be detected among 1×10^5 non-IgM-producing cells in a well, and 10^7 cells could be screened in a single 96-well plate. In cloned revertants, virtually all of the cells gave spots. Since this appeared to be a sensitive and reliable assay for IgM-producing cells, the V nonsense (V^n) and C nonsense (C^n) constructs shown in Fig. 1 were separately transfected into each of the cell lines. For S107, Southern analysis of *Eco*RI-digested DNA with a J probe revealed that most transfectants had one to three copies of the transfected gene (data not shown). Clones from different transfectants that had integrated only a few copies of the transfected gene and had different integration sites as judged by Southern analysis of *Xba* I-digested DNA (data not shown) were chosen for further study.

The relative rates of reversion of the V^n and C^n constructs in the S107, NSO, and 2C3 cell lines were determined by fluctuation analysis (38). Independent transfectants from these cell lines were tested. Fig. 3 shows the distribution of reversion frequencies of the V^n in three different transfectants of the S107 cell line and in representative transfectants of 2C3 and NSO. S107 cells transfected with the V^n construct generated IgM-producing cells at an average rate of 4.6×10^{-5}/bp per generation (frequency range, 2×10^{-3}–2×10^{-5}) (Fig. 3 and Table 1). Although there are some differences in the

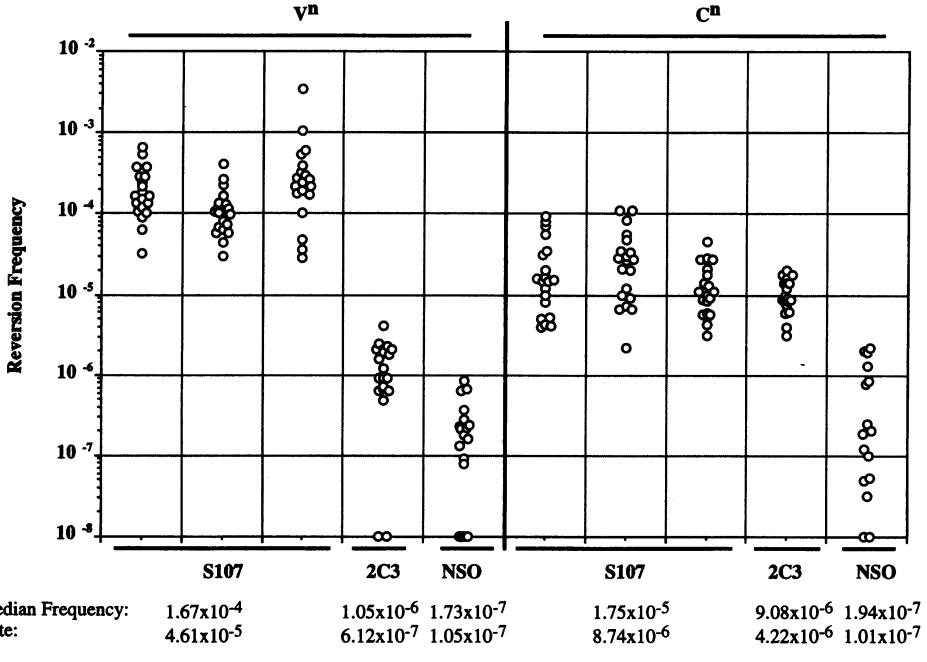

FIG. 3. Mutation frequencies (no. of revertants/no. of cells tested) and rates (per base pair per generation) of V^n (*Left*) and C^n (*Right*) in three different cell lines. The circles on the line of 10^{-8} mean that no revertants were seen when 10^7 cells were tested.

Table 1. Reversion rates of V^n and C^n constructs in mature B-cell lines

Cell line	Construct	Rate, no./bp per generation: Individual transfectants	Average
S107	V^n	4.16×10^{-5}	4.61×10^{-5}
		2.84×10^{-5}	
		6.83×10^{-5}	
	C^n	8.10×10^{-6}	8.74×10^{-6}
		1.22×10^{-5}	
		5.91×10^{-6}	
2C3	V^n	6.12×10^{-7}	5.61×10^{-7}
		5.18×10^{-7}	
		5.52×10^{-7}	
	C^n	4.22×10^{-6}	6.09×10^{-6}
		6.51×10^{-6}	
		7.53×10^{-6}	
NSO	V^n	1.05×10^{-7}	1.05×10^{-7}
	C^n	1.01×10^{-7}	1.01×10^{-7}

distribution of frequencies (Fig. 3), as might be expected from independent fluctuation analyses, the mutation rates calculated from these distributions are similar (Table 1). The V^n construct reverted at a 9-fold higher rate than C^n in the S107 cell line (Fig. 3 and Table 1). Both V^n and C^n reverted at lower rates in 2C3, and V^n reverted even less frequently than C^n in this cell line (Fig. 3 and Table 1). This was confirmed in a larger sample of independent 2C3 transfectants, where revertants were always found among 10^7 cells from subclones of C^n transfectants, while some subclones of the V^n transfectants did not contain revertants among 10^7 cells (data not shown). One set of the NSO V^n and C^n transfectants was also analyzed, and they showed very low mutation rates (Fig. 3 and Table 1). Other independent NSO transfectants gave similar results (data not shown). Rates of reversion for 36.65 were not determined, but the average frequency of reversion in three independent V^n transfectants was 1.08×10^{-6}, and in three independent C^n transfectants it was 1.7×10^{-6}.

Comparison of Mutated Sequences in the V and C Regions in S107 and 2C3 Cell Lines. To confirm that the cells producing IgM spots were due to mutation of the amber (TAG) nonsense codon, revertant cells were recovered after sib selection and soft-agar cloning. Sequencing of revertant V^n genes from four independent S107 revertants (Fig. 4) revealed that three of the four reversion events were due to point mutations of the guanine that is the hotspot in one of the motifs that others have shown to mutate frequently *in vivo* (8, 9).

Since mutation of both V^n and C^n occurred at a low rate in 2C3 and NSO cell lines, revertant cells were difficult to isolate, and only one V- and one C-region revertant of 2C3 were recovered and sequenced (Fig. 4). The additional silent base changes that were introduced in the codon preceding the termination codon (Figs. 1 and 4) allowed us to distinguish these true revertants from possible wild-type cellular or PCR contaminants. No other base changes were observed in the V region of S107 or in the C_{H1} domain of the C region in S107 and 2C3 cell lines, consistent with the reversion rates measured by spot ELISA.

DISCUSSION

We have directly compared the rates of mutation in both V and C regions of transfected Ig genes in mature B-cell lines. The S107 cell line was studied because, as noted above, it originally generated both V- and C-region point mutations in its endogenous H-chain gene at a relatively high frequency (22, 23). The studies reported here reveal that this is also true for transfected genes. The average of median reversion frequencies of the V^n gene is 1.67×10^{-4}. This frequency is in the range that has been reported for the endogenous genes in B cells *in vivo*

a

	32	33	34	35	36	37	38	39	40
WT	ACC	TAT	ATG	CAC	TGG	GTG	AAG	CAG	AGG
	Thr	Tyr	Met	His	Trp	Val	Lys	Gln	Arg
V^n	---	---	---	---	---	**GTC**	**TAG**	---	---
						Val	*stop*		
R^v1 (S107)	---	---	---	---	---	**GTC**	**TAC**	---	---
						Val	*Tyr*		
R^v2 (S107)	---	---	---	---	---	**GTC**	**TAT**	---	---
						Val	*Tyr*		
R^v3 (S107)	---	---	---	---	---	**GTC**	**TAT**	---	---
						Val	*Tyr*		
R^v4 (S107)	---	---	---	---	---	**GTC**	**TGG**	---	---
						Val	*Trp*		
R^v5 (2C3)	---	---	---	---	---	**GTC**	**TTG**	---	---
						Val	*Leu*		

b

	121	122	123	124	125	126	127	128	129	130
WT	GTC	TTC	CCC	CTC	GTC	TCC	TGC	GAG	AGC	CCC
	Val	Phe	Pro	Leu	Val	Ser	Cys	Glu	Ser	Pro
C^n	---	---	---	---	---	---	**TGT**	**TAG**	---	---
							Cys	*stop*		
R^c (2C3)	---	---	---	---	---	---	**TGT**	**AAG**	---	---
							Cys	*Lys*		

FIG. 4. Partial nucleotide and amino acid sequences of four revertants (R^v1–R^v4) from V^n transfectants of the S107 cell line and one from the 2C3 cell line (R^v5) (*a*) and one C^n transfectant of 2C3 cell line (R^c) compared with V^n, C^n, and wild type (WT) (*b*). Mutated nucleotides and amino acids are in bold and italic print, respectively. The hotspot motif (8, 9) is AGCA starting in the codon for aa 38.

on the basis of the frequency of mutations in monoclonal antibody genes and PCR-amplified V regions (4, 5, 10, 11). Using fluctuation analysis, we have calculated rates of V-region mutation of 4.6×10^{-5} bp per generation. This is at the lower end of the range of the rates that have been deduced by sequencing V regions that have mutated *in vivo*, at least 10-fold lower than rates of 10^{-3}–10^{-4}/bp per generation in B cells that are being selected by antigen (4, 5, 10, 11) and 100-fold lower than the rate of V-region mutation calculated for germinal-center B cells from Peyer's patches (17) that are under intense antigenic stimulation. Even if silent mutations and passenger genes are examined, these *in vivo* rates may overestimate the average rates of mutation *in vivo*, since the B cells carrying these V regions may have been selected for hypermutation through the interaction of antigen with the expressed Ig genes. The rates of mutation of the transfected V^n in S107 are roughly comparable to the rates of reversion of a TAG nonsense in the endogenous V region of 18.81 *in vitro* (20, 21). Further, we may be underestimating the rates of V-region mutations in S107 because the calculations to analyze fluctuation analyses were originally developed for very rare events in bacteria (38). For this reason we have presented the raw data, frequencies, and calculated mutation rates in Fig. 3.

In S107, V-region mutations occur almost 10 times more frequently than C-region mutations. However, the interpretation of this observation is complicated because the amber mutation that was introduced into the V region is part of a motif (Fig. 4) that has been reported to be a hotspot for V-region mutation in light chains *in vivo* (8, 9), while the same TAG introduced into the C region is not in such a hotspot. In S107, three of the four V-region revertants studied mutated the guanine in the motif. This suggests that this motif might also be a hotspot in S107 and that the mechanism of V-region mutation in this cell line may be similar to that which occurs *in vivo*.

The relatively similar rate of V and C mutation is not what would be expected from *in vivo* studies where C-region mutations have not been reported. Since T cells and the microenvironment of the germinal center (14) are required for V-region mutation *in vivo*, the lack of such signals could

explain why V-region mutation is not occurring at as high a rate as has been reported *in vivo* and is not restricted to the V region in these cultured cells. It is also possible that our construct lacks some of the sequences that are required for a maximal rate of V-region mutation and restriction to the V region and its flanking sequences. Nevertheless, these studies with S107 suggest that the construct used here contains sufficient information to allow a relatively high rate of somatic mutation in a permissive cellular environment *in vitro*. In studies to be published elsewhere, we have shown that mutations occur in the V region of this construct at a high rate in a pre-B-cell line and in transgenic mice. S107 is an unusual mature B-cell line in that it does not express NF-κB (39), a transcription factor that is usually found in plasma cell lines, and does express BTK kinase (40), which is not expressed in other plasma cell lines. Both of these factors are believed to be important in B-cell differentiation. This aberrant expression raises the possibility that S107 may be different from other mature B-cell lines in its regulation of Ig expression and possibly of mutation.

When the 2C3 hybridoma cell line was transfected with the V^n and C^n constructs, the rates of V- and C-region mutation were 5.6×10^{-7} and 6.1×10^{-6}/bp per generation, respectively. Both rates are low, but the C-region mutations are about 10-fold more frequent than V-region mutations. The 36.65 hybridoma, which is derived from A/J mouse splenocytes fused to a BALB/c SP2/0 fusion partner, also generated V- and C-region revertants at a lower frequency than S107. NSO, a cell line routinely used as a fusion partner for the production of hybridomas (29), has a V-region mutation rate of 1.1×10^{-7}/bp per generation and a similar rate of C-region mutation. Other V^n and C^n transfectants of NSO have been analyzed and have similar low rates of mutation (data not shown). The rates of mutation in NSO are similar to rates of reversion from drug resistance in other plasma cell lines (41).

While the low frequencies of V-region mutation in 2C3 and 36.65 are consistent with past difficulties in identifying V-region mutants in hybridomas (19), many C-region mutants with deletions and point mutations have been isolated in the past (19). A mutation rate of 10^{-6}/bp per generation converts to roughly 4×10^{-4} mutations per Cμ region per generation if all of the four C-region domains are at equal risk. Considerable clonal variation was observed in the rate of C-region mutation (see C^n in Fig. 3), and subclones of 2C3 that reverted C^n at a still higher rate can easily be enriched for (unpublished data). This could not be done for V^n transfectants. This suggests that there may be strong negative regulation of V-region mutation in some mature B-cell lines and could, at least in part, explain the relative ease with which C-region mutants were obtained in the past (19). The inability of the NSO cell line to carry out V-region mutation is particularly interesting, since it and related cell lines also derived from the P3 myeloma (29) have been used to make virtually all of the hybridomas that have been generated. If these cells express a negative regulatory element for V-region hypermutation, it could explain why hybridomas do not mutate their V regions at a high rate. This possibility has been suggested by Wabl *et al.* (21), who reported that a single hybrid between 18.81 and a hybridoma (Ag.8.653) was unable to revert the V-region nonsense in the endogenous gene of 18.81.

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