Receptor Revision of Immunoglobulin Heavy Chain Variable Region Genes in Normal Human B Lymphocytes

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Abstract

Contrary to the general precepts of the clonal selection theory, several recent studies have provided evidence for the secondary rearrangement of immunoglobulin (Ig) genes in peripheral lymphoid tissues. These analyses typically used transgenic mouse models and have only detected secondary recombination of Ig light chain genes. Although Ig heavy chain variable region (V_H) genes encode a substantial element of antibody combining site specificity, there is scant evidence for V_H gene rearrangement in the periphery, leaving the physiological importance of peripheral recombination questionable. The extensive somatic mutations and clonality of the IgD^+Strictly-IgM^-CD38^+ human tonsillar B cell subpopulation have now allowed detection of the first clear examples of receptor revision of human V_H genes. The revised VDJ genes contain "hybrid" V_H gene segments consisting of portions from two separate germline V_H genes, a phenomenon previously only detected due to the pressures of a transgenic system.

Key words: receptor revision • immunoglobulin heavy chain • variable region • recombination • receptor editing

Introduction

V(D)J rearrangement of Ig and TCR genes has the capacity to generate an immense repertoire of immune receptors capable of recognizing virtually any foreign substance (1). The stochastic nature of this process also results in many unsuccessful rearrangements or in immune receptors displaying specificity to self-molecules. Lymphocytes with unsuccessful VDJ rearrangements can be rescued by continued rearrangement of the second allele, and for the light chain loci, the same allele. Similarly, it is now recognized that in the bone marrow, B lymphocytes displaying self-specificity can either alter their specificity by further V gene rearrangement, referred to as receptor editing, or they are eliminated (2–4). A similar mechanism has been observed for peripheral T lymphocytes (5).

Rather than generating the largest possible nonautoimmune repertoire, it is believed that the selective forces acting on mature B cells in the periphery, unlike the bone marrow, hone the repertoire by selecting only clones with the highest affinity for antigen, referred to as clonal selection (6, 7). Central to peripheral selection of B lymphocytes is the formation of germinal centers (GCs)[1] through the massive proliferation of antigen-activated clonotypes (8, 9). In GCs, a new round of diversification is initiated by somatic hypermutation of Ig V genes (10–12). It has recently become evident that the recombination machinery can also be used to adjust the specificity of clonotypes in GCs, especially if the repertoire is limited such as in transgenic models (13, 14). This peripheral secondary recombination is apparently diversity driven (15, 16) and has come to be referred to as "receptor revision" to distinguish it from the tolerance-driven secondary recombination in the bone marrow, referred to as "receptor editing" (2–4, 17).

Most work on receptor revision has used transgenic mouse models to generate examples of the process. To this point, most examples of receptor revision in the periphery have involved the light chain locus (15–25). Additionally, recombination activating gene (RAG) expression and recombination intermediates have been detected in GC cells of both normal mice and humans, suggesting a physiological role for receptor revision (15–17, 19, 21, 23, 24), and

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[1]*Abbreviations used in this paper:* CP, clonal pool; GC, germinal center; GFP, green fluorescent protein; RAG, recombination activating gene; RSS, recombination signal sequence(s); UTR, untranslated region.

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several recent studies have suggested a role of this process in autoimmune disease (18, 19, 25).

Analyses of site-specific transgenic animals carrying V_H transgenes with anti-DNA specificity inserted into the J_H portion of the endogenous heavy chain locus demonstrated replacement of the V_H portion of the VDJ transgene by endogenous 5′ V_H genes using an internal cryptic recombination signal sequence (RSS) (26). This observation was similarly made in other site-directed transgenic models (27–30), and previous studies suggested that V_H replacement might occur in other instances of severely limited repertoire such as in a B cell lymphoma (31, 32). Although detection of edited VDJ rearrangements in these experimental systems suggested that such a mechanism could be active in normal B cells, the unique nature of the experimental systems left doubts, and the many years of study have not led to the detection of VDJ editing of previously rearranged heavy chain genes from normal B cells. It has therefore been a long-standing question if V_H gene editing plays any role in the generation of the antibody repertoire.

In this report, clear examples of Ig heavy chain receptor revision resulting in hybrid V_H genes were cloned from the tonsil of a normal child. Although primarily found in specific mature B cell subpopulations, examples of recombined V_H gene clones were detected in various tonsillar B cell subpopulations from several individuals. The only revised VDJ genes detectable by DNA sequencing are formed by the recombination of two V_H segments in the central portion of the genes. Analysis of the hybrid junctions suggests that the process is RAG mediated. The formation of hybrid V_H genes by receptor revision as reported here is a low frequency event that may appear insignificant; however, it should be realized that these products of recombination are the only products immediately detectable. Antibody protein structural constraints will likely preclude the formation of a functional protein by the combination of portions of two unique V_H genes in most instances; however, complete V_H or J_H replacements would not be as subject to such constraints and likely occur at a much greater frequency. However, complete V_H or J_H replacement in a previously rearranged VDJ gene would be essentially invisible. Additionally, recombination of two V_H elements to produce nonfunctional proteins may be an important mechanism for deactivation of one allele and activation of the second V_H allele; this process would also be essentially invisible by standard analyses. It is therefore highly significant that recombination-generated hybrid V_H genes are detected, as these products of receptor revision are likely only the tip of the iceberg of the total contribution of receptor revision of V_H genes to the generation of a secondary immune response.

Materials and Methods

B Cell Separation Procedures. All B lymphocyte subpopulations were isolated from normal human tonsils removed during routine tonsillectomy. The various B lymphocyte subpopulations were isolated by separation protocols using cell depletion, density gradient centrifugation, magnetic bead depletion, and FACS® based on the cell surface phenotypes indicated in Table I as described previously (12, 33–36).

Cloning and Sequencing of Ig V_H Genes. V_H4 using VDJ gene cDNAs was isolated by reverse transcription and PCR of mRNA from the isolated B cell subpopulations using V_H4 family–specific and isotype-specific oligonucleotide PCR primers as described previously (34, 37). V_H4DJ cDNAs were blunt cloned using either pBluescript plasmid vectors (Stratagene) or the PCR-Blunt cloning kit (Invitrogen), and transformed into *Escherichia coli*. V_H4 or isotype-positive colonies were detected and picked as described previously (34, 37).

Isolation of the Recombinant V_H Hybrid Clones. 36 clonal pool 1 (CP1) isolates, including 2 of the 7 hybrid recombinants, and 41 CP2 isolates including 1 of the pair of hybrids from CP2 were cloned at random from a total of 141 IgD^+Strictly-IgM^-CD38^+ GC (hereafter IgD^+GC) cDNAs from a single tonsil preparation. Screening colony lifts of the IgD^+GC V_H4DJ library with CP1 hybrid CDR3, and CP2 CDR3 specific oligonucleotide probes labeled with ^{32}P produced 29 additional CP1 isolates and 36 additional CP2 isolates, including the additional hybrid sister clones from each clonal pool. The CDR3-specific probe sequence (5′-GGGATCGGGGTGCTTTTGA-3′) used to isolate these additional CP1 clones spans the DJ junction, and that for the CP2 CDR3 spans the VD junction (5′-AGGCCTCTCGG[A/G]TA[G/C]TGCA-3′).

DNA Sequence Analysis. DNA sequencing was performed on an ABI-377 automated DNA sequencer (Advanced Biotechnologies, Inc.). Germline V_H genes for comparison were identified using the DNAPLOT search components of the VBASE (http://www.mrc-cpe.cam.ac.uk/imt-doc/, coordinated by I.M. Tomlinson, MRC Centre for Protein Engineering, 1999) and the international ImMunoGeneTics (IMGT [38]) Ig databases. The background mutation rate for Ig V_H gene analyses attributed to experimental error was 0.79 mutations per clone (per 300 nucleotides) by analysis of ~15,000 C region nucleotides sequenced along with the V_H genes (each clone consisted of a VDJ gene and a portion of the C region). Ig V_H gene sequences from this study are available from EMBL/GenBank/DDBJ under accession nos. AF262069–AF262209.

Computations. Statistical analyses described in the text were performed using SYSTAT for Windows, Rel. 8.0.0 (SPSS, Inc.). Phylogenetic analysis was performed using the DNAPENNY component of the PHYLIP (Phylogeny Inference Package) v3.5c software (distributed by the author, J. Felsenstein, Department of Genetics, University of Washington, Seattle, 1993) and was drawn using TreeView v1.5.2 (39). Software for the detection of hybrid V_H genes was written for the Microsoft Windows 95/98 platform using Microsoft Visual Basic v6.0 and is available from the authors for analysis or use.

Results

In a random cloning of 141 IgD^+GC transcripts from a single human tonsil, 7 VDJ genes contained hybrid V_H segments that consisted of from 1/3 to 2/3 of their length from one V_H gene, and the remainder from a second V_H gene. Typically in V_H gene analyses, such clones are set aside, since it is impossible to discern their origin as either a physiological event or an experimental error that occurred during the PCR or cloning process due to homology between the various V_H genes (40, 41). However, 2 of these

hybrid clones were clonally related to each other and to 33 additional nonhybrid clones and 1 additional unique hybrid clone, but all were differentiable by their patterns of somatic hypermutation. Any set of V_H gene clones isolated that share CDR3 sequences but are differentiable by the pattern of somatic hypermutations is considered to have been derived from the clonal expansion of the same founder clone during an immune response (clonally related). In fact, extensive statistical modeling has previously determined that it is very unlikely that even 2 unrelated B cells with different CDR3s could occur at random, and 65 or 77 (see below) as in this study is virtually impossible (42). Additionally, as discussed below, these transcripts were cloned from the IgD^+GC subpopulation, which may be particularly prone to receptor revision (see Discussion). To further investigate these interesting clonotypes, oligonucleotide probes were designed that specifically bind the unique CDR3s from 1 pool of 36 related clones (hereafter referred to as CP1), and used to screen colony lifts of the original IgD^+GC V_H gene library. Additionally, a second CDR3 probe was designed to screen a second large clonal expansion (designated CP2) that contained examples of hybrid V_H genes. In this directed cloning, an additional 29 CP1 clones were sequenced of which an additional 5 highly similar hybrid clones were isolated, totaling 7 "sister" hybrids in a total of 65 CP1 isolates (Fig. 1 A). Similarly, a total of 77 unique clones was sequenced from CP2, including a pair of sister hybrid clonotypes (see Fig. 2 A).

As depicted in Fig. 1 A, the 7 sister hybrid clones from CP1 and the consensus of the remaining 57 nonhybrid CP1 clones (labeled Consensus in the figure) share CDR3 regions consisting of the 3′ end of the V_H4-34 gene element with the final A residue deleted, an addition of 4 N nucleotides (GATA), a D3-10 with five 5′ and twelve 3′ nucleotides removed by exonuclease activity, and a J_H3 gene segment with the first nucleotide removed. A single hybrid clone from CP1 using a different donor is not included in the CP1 consensus but also shares this VDJ junction. The unique nature of this CDR3 makes it highly probable that these clones were derived from the same founder V_H4-34/D3-10/J_H3 clonotype (42). The 5′ end of the hybrid sequences to nucleotide position C183 (black arrowhead) according to the numbering system of Chothia et al. (43) was derived from the V_H4-61 gene element based on sequence homology (V_H4-61 is the bottom sequence in the alignments of both Fig. 1, A and B). This region of the V_H4-34 gene segment is easy to differentiate from V_H4-61 because it contains a unique FW1 compared with all other V_H genes and a six-nucleotide deletion in the CDR1 (Fig. 1 A).

The junction between the V_H4-61 gene–encoded donor region and the V_H4-34 gene–encoded recipient region is suspected to be a well-conserved cryptic RSS at position C201 (Fig. 1 A, white arrowhead) shared by both germline genes in the FW3 after the last position of hybrid homology to V_H4-61 at C183 (black arrowhead). However, if the mechanism of recombination turns out not to be RAG mediated, such as a homologous recombination event, the region of homology between the V_H4-34 recipient and the V_H4-61 donor sequences stretches from position C183 to C261 and the junction of recombination can be anywhere in this region. The region of recombination can be further reduced to the mutated A241 shared by the hybrid recombinants and the consensus of the remaining 57 nonhybrid CP1 clones. Although many somatic mutations are shared between the seven hybrids, indicating a phylogeny (discussed below), the individual clones differ from each other by an average of 22.5 ± 9.0 mutations, far exceeding the background mutation rate determined for this study (Materials and Methods). This differential mutation indicates both unique clonality for each of the seven hybrid clones, and extensive divergence and mutation after the recombination event.

Similar to the CP1 clones described above, the 72 nonhybrid clones from CP2 (Consensus in Fig. 2 A) plus the pair of related hybrid clones isolated share CDR3 regions consisting of V_H4-34, an N nucleotide element (5′-CCTCTCG-3′), a fusion of a D6-6 D gene with an apparent cryptic D gene identical for a contiguous 10-nucleotide stretch to a motif 271 nucleotides upstream in the D locus which is flanked by cryptic RSS (locus information from reference 44), and J_H6 with the first 15 nucleotides removed by exonuclease activity (Fig. 2 A). The pair of hybrid sister clonotypes from CP2 were also produced by the invasion of a V_H4-61 germline gene (bottom sequence in alignment) into a V_H4-34 using CP2 recipient at exactly the same junction as for the CP1 hybrids (position C201, white arrowheads; Fig. 2 A). In CP2, there were three additional hybrid clones isolated, but none of these had a clonally related sister hybrid to verify their somatic origin. The pair of sister hybrids from CP2 also shared 12 mutated nucleotide positions, indicative of their clonality, but they were differentially mutated far beyond the background mutation rate (differing at a total of 27 nucleotide positions), indicating both clonal uniqueness and further mutation after the recombination event (Fig. 2 A).

Phylogenetic Analysis. The seven hybrid sequences in Fig. 1 A are arranged based on phylogenetic similarity from top to bottom. Based on the shared mutations between these individual hybrids, they can be grouped into three subphylogenies after the receptor revision event, including clones CP1-HB1-1, CP1-HB1-2, and CP1-HB1-3 in one set, clones CP1-HB1-4, CP1-HB1-5, and CP1-HB1-6 in a second branch, and clone CP1-HB1-7 as a unique branch in the phylogeny of the seven recombinants. Based on several sequence similarities between CP1-HB1-7 and the CP1-HB1-4, -5, and -6 subphylogeny, CP1-HB1-7 may have arisen early on in that branch as well.

The phylogeny of the entire set of 65 CP1 clones is depicted in Fig. 1 B and the phylogeny of CP2 in Fig. 2 B. These analyses were based solely on the regions 3′ to the proposed recombination junction (nucleotide position C201, white arrowheads; Figs. 1 A and 2 A) so that differences between the germline donor and recipient genes in the hybrid sequences would not effect the outcome. The seven related hybrid clones (boldface type) from CP1 ap-

pear to have been derived relatively early in this phylogeny; however, it should be noted that all of these clones are extensively mutated with an average mutation frequency of 29.14 ± 6.11 mutations per nonhybrid CP1 clone, and an average of 19.71 ± 3.41 mutations per hybrid clone, indicating that all clones had diverged extensively from the original V_H4-34/D3-10/J_H3 founder. Note that all seven of the CP1 clonotypes were placed in the same branch of the phylogeny by the DNAPENNY program's algorithm (see Materials and Methods), thus verifying that these are sister clonotypes. In addition, several of the nonhybrid clonotypes share the same branch in the phylogeny demonstrating the affinity maturation that occurred before the recombination event.

The phylogeny of CP2 in Fig. 2 B indicates that the pair of sister hybrid clonotypes (CP2-HB1-1 and CP2-HB1-2) was generated relatively late in the phylogeny, and is part of a greatly extended branch consisting of over half of the 77 total CP2 isolates. The program's algorithm also placed clones CP2-HB1-1 and -2 immediately adjacent to one another, verifying their relation. Based on mutational similarity to the germline V_H4-34/D-D fusion/J_H6 (and consensus junctional nucleotides), the pair of related hybrid clonotypes were preceded by at least 21 of the clonotypes

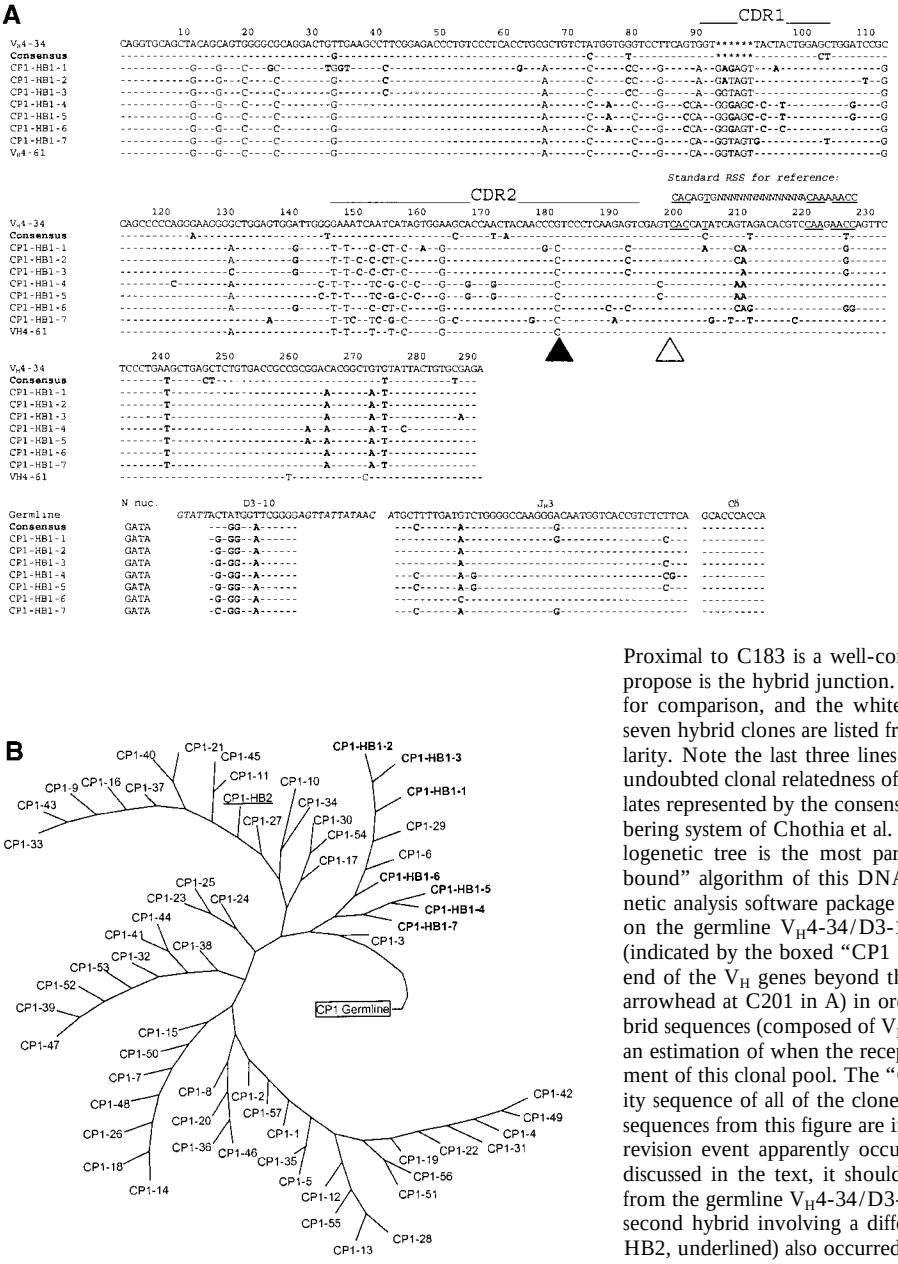

Figure 1. 7 related recombinant hybrids were isolated from the 65 clones of CP1. A total of 65 clonally related sequences referred to as CP1 were isolated by either random or directed cloning of V_H4DJ_H transcripts from IgD$^+$GC B cells. The proposed germline genes encoding the CP1 founder clonotype consist of V_H4-34, D3-10, and J_H3 (first line of comparisons). Nucleotides homologous to these germline genes are indicated as dashes. Base exchanges resulting from somatic hypermutation are indicated in boldface type. Asterisks indicate deletional differences between the V_H4-34 and V_H4-61 germline genes. (A) A comparison of the 7 sister hybrid V_H segments of the revised clonotypes with the consensus sequence of the remaining unrevised 57 CP1 clones (second line) and with the V_H4-34 (top line) and V_H4-61 hybrid that used a different donor V_H gene than the seven sister hybrid clones is not shown or included in the consensus. The last nucleotide of homology of the hybrids to V_H4-61 is C183 (black arrowhead). Proximal to C183 is a well-conserved cryptic RSS beginning at C201 that we propose is the hybrid junction. A canonical heptamer-nonamer RSS is included for comparison, and the white arrowhead marks the proposed junction. The seven hybrid clones are listed from top to bottom in order of phylogenetic similarity. Note the last three lines of the sequence comparison, demonstrating the undoubted clonal relatedness of the hybrid clonotypes to the rest of the CP1 isolates represented by the consensus. Ig sequence numbering is based on the numbering system of Chothia et al. (reference 43). (B) Phylogeny of CP1. This phylogenetic tree is the most parsimonious tree implied using the "branch and bound" algorithm of this DNAPENNY component of the PHYLIP phylogenetic analysis software package (see Materials and Methods). The tree is rooted on the germline V_H4-34/D3-10/J_H3 genes with consensus junctional regions (indicated by the boxed "CP1 Germline"), and was produced using only the 3′ end of the V_H genes beyond the point of the proposed hybrid junction (white arrowhead at C201 in A) in order to negate effects of the 5′ portion of the hybrid sequences (composed of V_H4-61) on this comparative analysis, and allowing an estimation of when the receptor revision event occurred during the development of this clonal pool. The "Consensus" sequence in A is based on the majority sequence of all of the clones in normal print on this tree. The seven hybrid sequences from this figure are indicated in boldface type. Note that the receptor revision event apparently occurred quite early in the phylogeny; however, as discussed in the text, it should be noted that all clones were highly diverged from the germline V_H4-34/D3-10/J_H3 sequence of the original CP1 founder. A second hybrid involving a different 5′ V_H donor from this clonal pool (CP1-HB2, underlined) also occurred early in the phylogeny.

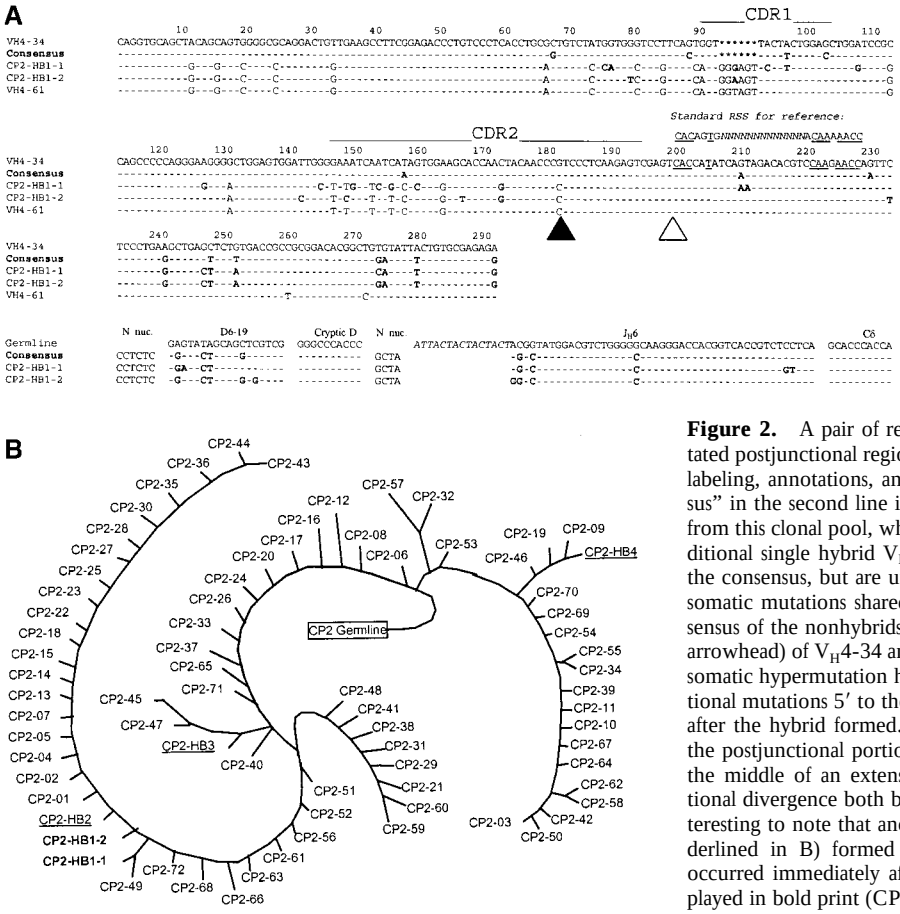

Figure 2. A pair of related hybrid recombinants with extensively mutated postjunctional regions were isolated from 77 total clones of CP2. All labeling, annotations, and methods are standardized to Fig. 1. "Consensus" in the second line in A of the comparison represents 72 nonhybrids from this clonal pool, which are displayed in normal print in B. Three additional single hybrid V_H isolates in this clonal pool are not included in the consensus, but are underlined in the phylogeny in B. Note the eight somatic mutations shared by the two hybrid recombinants and the consensus of the nonhybrids in the postjunctional regions (after C201, white arrowhead) of V_H4-34 and J_H6. These mutations are highly indicative that somatic hypermutation had begun before the hybrid formation, and additional mutations 5′ to the junction demonstrate continued hypermutation after the hybrid formed. A similar point is evident by the phylogeny of the postjunctional portions of all CP2 clones in B. The hybrids occur in the middle of an extensive branch of the phylogeny, indicating mutational divergence both before and after the recombination event. It is interesting to note that another single hybrid recombinant (CP2-HB2, underlined in B) formed with a different donor gene (V_H4-39 donor) occurred immediately after the pair of related hybrid recombinants displayed in bold print (CP2-HB1-1 and CP2-HB1-2).

in this branch alone, and share several somatic mutations with clones in divergent branches of the tree as well. As discussed below, this phylogeny is of extreme interest, as it suggests that the recombination event generating these clonotypes occurred after the process of somatic hypermutation was initiated, and that somatic mutation then continued after the event.

Specific Nucleotide Changes Provide Evidence That Receptor Revision Occurred during Affinity Maturation. The shared VDJ junctions between the 7 hybrid clones of 58 total CP1 clones and the 2 related hybrids of 77 total CP2 isolates indicate with certainty that there was recombination of donor (V_H4-61) sequence into the respective recipient V_H4-34 founder sequence for at least 2 separate clonotypes. However, the detection of the products of recombination alone does not indicate the stage of B cell differentiation when these events occurred, although the phylogenetic analyses above are quite suggestive of recombination during affinity maturation. It is highly unlikely that these hybrid recombinants were formed in the bone marrow during normal B cell development and recombination, as the IgD^+GC population analyzed has such extensive clonal expansion and somatic hypermutation. Additionally, a careful analysis of the particular nucleotide positions mutated greatly enhances this hypothesis. 2 mutated positions in the CP1 sequences, A243 to T and G276 to T, are shared among the majority of the 57 nonhybrid clones, the single unique hybrid clone, and all 7 sister hybrid CP1 clones in the postrecombinational junction region after C201 (arrowheads in Fig. 1 A). Even more telling are the postjunctional mutations shared by the pair of CP2 sister hybrids with the remaining 72 CP2 isolates (Fig. 2 A). Note that not including the D gene elements and junctional regions, there are 8 positions similarly mutated between the pair of CP2 isolates and the consensus of the remaining 72 nonhybrids (the 3 additional hybrids not related to the pair in Fig. 2 were not included).

It should also be realized that the consensus sequences represent only the majority sequence identity at each nucleotide position, and that there is a minority of the remaining mutated positions on the nonhybrid clonotypes that are also shared with the postjunctional regions of their respective CP1 and CP2 hybrid counterparts. Similarly, there is a minority of nonhybrid clonotypes from either pool that do not share many of these mutated positions, indicating that these alterations are not simply polymorphic

variation of the V_H4-34 gene used by the different clonotypes. One drawback to working with human specimens, especially rare cell populations such as these IgD^+GC cells, is that there is limited availability and therefore direct analysis of this patients genomic V_H4 repertoire could not be done to verify that these mutations were not due to polymorphism. However, the fact that we had cloned and sequenced 230 V_H4-34 cDNAs from this individual allowed us to prove unquestionably that the nucleotide positions altered resulted from somatic hypermutation. Of these 230 V_H4-34 using clones, 176 could be placed within 14 clonal expansions, and the remaining 54 had unique CDR3s, thus totaling 68 unique V_H4-34 clonalities. Although there were particular mutational similarities in the various clonal expansions, there were no consensus positions matching the postjunctional mutations of the consensus CP1 and CP2 clonotypes. In fact, the expected mutation rate for this subpopulation, which averages 21.90 ± 8.92 mutations per V gene sequence (300 nucleotides), is 21.90 ± 8.92 per 300 nucleotides, equating to a 4.3–10.3% chance of each position being mutated at random. Of the 2 CP1 and 8 CP2 postjunctional consensus mutations that might be polymorphic variations, only the G at position 276 was found mutated in >10.3% of the 68 unique clonalities from this tonsil; however, the mutation frequency for this position was only 12.5% and therefore quite insignificant (χ^2 probability approaches 1.0). The lack of related sequences in other V_H4-34 clonalities of this tonsil donor is highly suggestive that these nucleotide alterations arose somatically. As a second test of whether these mutations could have arisen via polymorphic variation as opposed to somatic hypermutation, a comprehensive search was performed of both our own extensive database of V_H4 family genes, including 456 unique clonotypes using V_H4-34, and extensive searches of the IMGT, VBASE, and Kabat Ig databases and GenBank for V_H4-34 polymorphic variants resembling the mutated consensus sequences of CP1 and CP2. The overwhelming conclusion is that these clonotypes resulted from somatic hypermutation, not polymorphic variation. The fact that the nucleotide alterations demonstrated in the postjunctional regions were both progressively accumulated, as indicated in the phylogenetic analyses, and were undoubtedly generated somatically is an important observation as it places the timing of the recombination events during ongoing somatic hypermutation.

V_H Gene Hybrids Can Be Found in Other B Lymphocyte Subpopulations. Over a period of several years, we have cloned >700 full-length V_H4(D)J transcripts from several different tonsils, B cell subpopulations, and isotype compartments (Table I). After finding evidence that V_H gene hybrids are somatically generated in the IgD^+GC subpopulation, a computer program was written to analyze V_H genes on a nucleotide by nucleotide basis for sequence regions more similar to another germline V_H gene than the sample sequence's nearest germline counterpart. The program compares any region <100% similar to the proposed nearest-fit V_H germline gene to every human V_H gene clone in the VBASE and IMGT Ig databases. In this analysis, 14 additional transcripts appeared to be hybrid V_H gene recombinants, including an additional CP1 clone with a

Table I. *Comparison of Various B Cell Populations Compiled from Tonsils of Four Individuals**

B cell subpopulation (no. of clones)	Tonsil donor	Cell surface phenotype‡	Somatic mutation§	Percent clonality‖	J_H6 usage¶	Hybrid frequency**
				%	%	%
Follicular mantle (35)	1	$IgD^+IgM^+CD38^-$	0.08 ± 1.11	None	15	None
IgM GC precursors (94)	3	$IgD^+IgM^+CD38^+$	3.29 ± 3.45	22 (1 pool)	20	2.1 (2)
IgD^+GCs (141[240])‡‡	3	$IgD^+IgM^-CD38^+$	21.90 ± 8.92	77 (14 pools)	50	5.0 (7)
IgM GCs (145)	1, 2	$IgD^-IgM^+CD38^+$	6.51 ± 5.81	28 (10 pools)	10	2.0 (3)
IgG GCs (146)	1	$IgD^-IgM^+CD38^+$	14.96 ± 8.37	14 (10 pools)	10	None
IgM memory (60)	1, 2	$IgD^-IgM^+CD38^-$	4.46 ± 5.51	None	7	1.6 (1)
IgG memory (72)	1	$IgD^-IgM^+CD38^-$	14.48 ± 8.72	None	13	None
IgM plasma cells (29)	2	$IgM^+CD38^{++}CD20^{low}$	4.69 ± 3.81	41 (6 pools)	29	3.4 (1)

*J_H6 usage and frequency of somatic hypermutation are consistent with previous publications (references 12, 33, 49).
‡Cell surface phenotypes of the various B lymphocyte subpopulations analyzed as described previously (references 12, 33–36).
§Mean number of mutations per V_H segment of each clone analyzed minus the error rate (see Materials and Methods) ±SD.
‖Clonality is defined as clones sharing identical HDJ junctions (CDR3s), but which are differentially mutated to a degree greater than the background mutation rate (see Materials and Methods) determined for the V_H gene C regions. A clonal pool (parentheses) is a set of clonally related V_H genes based on CDR3 similarity as described in the Results.
¶For this analysis, each pool of clonally related transcripts is counted as only one instance of J_H6 utilization.
**Percentage of total clones (number of hybrids): this includes only revised VH genes cloned at random, not hybrids isolated by directed cloning as described in the text.
‡‡240 total isolates from this population were sequenced and used to produce the average mutation frequency as mutation could act equally on all clones; however, the remaining analyses were based solely on the 141 isolates that were randomly cloned.

hybrid V_H gene but derived from recombination with a different germline gene than V_H4-61 (the donor is V_H4-39), and 3 additional unique CP2 hybrids (1 with a V_H4-61 donor but a different junction, a second with a V_H4-30.1 donor, and a third with a V_H4-39 donor). 12 of 14 of the additional hybrid V_H clones isolated contained mutations comparable to the B cell subpopulation from which they were isolated, suggesting a peripheral origin for most of these recombinants as well. As indicated in Table I, the greatest number of hybrid V_H sequences was isolated from the IgD^+GC subpopulation. Although the only population to which V_H hybrid detection could be statistically linked is the IgD^+GC subpopulation (>95% confidence based on Pearson correlation analysis), hybrid clones were detected in all other populations except the naive follicular mantle cells and, surprisingly, the IgG^+ subpopulations analyzed. The inability to find V_H hybrid recombinants in the 146 IgG GC and 72 IgG memory B cell transcripts analyzed is likely due to the low frequency of these events. It should be noted that these additional hybrid V_H sequences identified were single, unique isolates and therefore have not been irrefutably proven to be the products of a recombination event as for the seven recombinants of CP1 and the pair from CP2. However, it is unlikely that these transcripts are polymorphic variants that have never before been isolated, as they were cloned only one time in our analyses of many transcripts from each of the individuals and have never been isolated in the thousands of V_H genes analyzed in this and other laboratories in the past. It is also unlikely that these events arose as errors of the experimental system, as they all formed apparently functional clonotypes.

Cryptic Recombination Sequences Are Found at the Hybrid Junctions. A striking observation in our study was the detection of cryptic RSS in proximity to every junction of every hybrid isolated, even when the region of similarity in which the junction could occur only consists of six nucleotides (Fig. 3). Granted, our definition of a cryptic RSS is minimal in that it has been shown that the minimal sequence requirements of an RSS is the 5′ CAC (or inverse 3′ GTG) of the canonical heptamer (45), and several of the proposed heptamers contained only this trinucleotide. Of particular interest is that the proposed junction used by the seven sister hybrids from CP1 is also the best junction for the CP2 hybrid sisters and one other single recombinant isolated (Fig. 3, Set 1). For the junction in Set 1, there are 4/7 conventional heptamer nucleotides, including the obligatory CAC motif, and if a spacer of 13 nucleotides rather then 12 or 23 is used, there are 7/9 conventional nonamer nucleotides. The canonical 12-bp RSS is commonly considered as 12 ± 1 bp (45), and so this RSS is nearly ideal. Another cryptic heptamer junction was used by four separate recombinant hybrids isolated in three separate cloning experiments (Fig. 3, Set 2). Two hybrid clones isolated use a third junction (Fig. 3, Set 3), and the remaining six are all unique. Another recombinant of particular interest is CP1-HB3, which contains a GTG motif at the junction followed by a deletion of 24 nucleotides (8 amino acids), leaving the gene in frame. This deletion is within the CDR2 region of the V_H gene, which is a hypervariable region and structurally quite flexible. We and others have demonstrated previously that in these hypervariable loops large insertions and deletions can be tolerated by immunoglobulins (34, 46, 47). This observation suggests exonuclease activity at the hybrid junction.

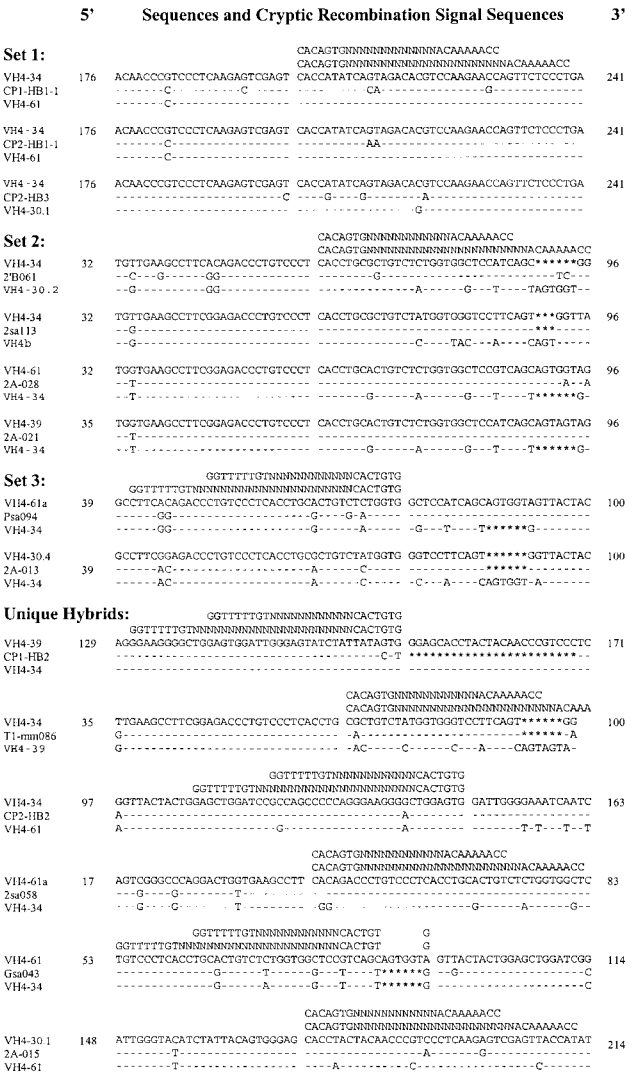

Figure 3. There are cryptic RSS at each of the V_H recombinant hybrid junctions. For each comparison, the germline counterpart of the original or recipient V_H gene is the top sequence, the bottom sequence is the germline donor V_H gene, and the middle sequence is the hybrid V_HDJ clone. Nucleotides similar to the recipient V_H gene are shown as dashes. Standard RSS containing both 12- and 23-bp spacer segments are listed at each junction for reference (the short RSS for Set 1 uses a 13-bp rather than a 12-bp spacer, as this spacer length gives a more complete nonamer; the canonical 12-bp spacer is actually 12 ± 1 bp [reference 45]). Three of the cryptic RSS were shared by greater than one hybrid V_HDJ_H clone isolated (listed as Set 1, Set 2, and Set 3). The remaining six isolates had unique junctional positions. Note that clone CP1-HB3 has a 24-nucleotide (8-codon) deletion suggestive of exonuclease activity during recombination. Complete Ig V_H gene sequences from this study are available from EMBL/GenBank/DDBJ under accession nos. AF262069–AF262209.

RAG-mediated Recombination Intermediates Could Not Be Detected. Although intermediates of recombination resembling those expected in the formation of hybrid V_H genes have not been conclusively detected to date. These intermediates may be exceedingly rare and there are many inherent difficulties in working with GC cells for techniques designed to detect the free DNA ends produced by recombination such as ligation-mediated PCR, due to the propensity of these cells to apoptosis and DNA degradation. Despite significant effort to identify the expected recombination intermediates in the form of double-strand DNA breaks at the proposed recombination junctions by ligation-mediated PCR (48), we have been unable to conclusively demonstrate these intermediates at this point (data not shown). Additionally, the IgD^+GC cells in which we detected a particular propensity to carry recombinant hybrid V_H genes have been characterized as most like centroblasts (49), which it has been demonstrated do not express the RAG proteins (16, 50); however, the IgD^+GC cells have not been tested directly. We have some evidence that a sorted IgD^+GC isolate expresses RAG transcripts; however, two factors make this information inconclusive: (a) it is not clear if these IgD GC cells have a centroblast ($CD38^+CD77^+$) and a centrocyte ($CD38^+CD77^-$) component due to the requirement of a four-marker analysis (CD38, CD77, IgD, and IgM); and (b) this population typically represents only ~1% or less of tonsillar B cells.

Finally, a set of PCR experiments was performed in an attempt to detect the predicted deletion circles of hybrid joint recombination. PCR primer binding sites for the most frequently observed hybrid recipient (V_H4-34) and donors (V_H4-61 and V_H4-39) were designed as depicted by the small arrows in Fig. 4 a. It should be noted that standard PCR amplification is particularly prone to generate false positive products for these analyses, presumably because of the linear amplification of single stranded, complimentary copies of the donor and recipient genes during the early rounds of PCR due to the abundance of "normal" (noncircle) template (see the asterisk in Fig. 4). For example, a PCR using these primers on a recombination-negative population of 500,000 cell equivalents of DNA would produce 30,000,000 single-stranded reverse compliments of the two donor alleles and the recipient alleles that could very easily combine at regions of homology to form what appears to be a deletional circle product. This indeed was the case: after multiple trials on various cell populations that are recombination competent or not, we found that apparent recombination circles could be amplified from every population; however, none of these products were somatically mutated in the recipient half, and the junctions were typically not at cryptic RSS. To improve this technology, we first used a biotinylated primer specific for the recipient gene (the right primer in Fig. 4, below the asterisk) in a one-cycle extension step using Taq polymerase to produce biotinylated template that must contain the 5′ untranslated region (UTR) of the recipient. We then captured this template using streptavidin beads and washed away all remaining remnants of nonbiotinylated DNA. This template was then amplified by PCR using an internal primer for the 5′ UTR of the recipient gene, and a primer specific for the donor 3′ UTR. Using this technique, we eliminated all false positive amplification products, while retaining amplification using a pair of recipient-specific primers as a positive control. These experiments also failed to detect the expected recombination circles (negative data not shown), leading to the conclusion that as discussed below, it may be impossible to isolate heavy chain recombination intermediates from the currently known GC cell phenotypes isolated.

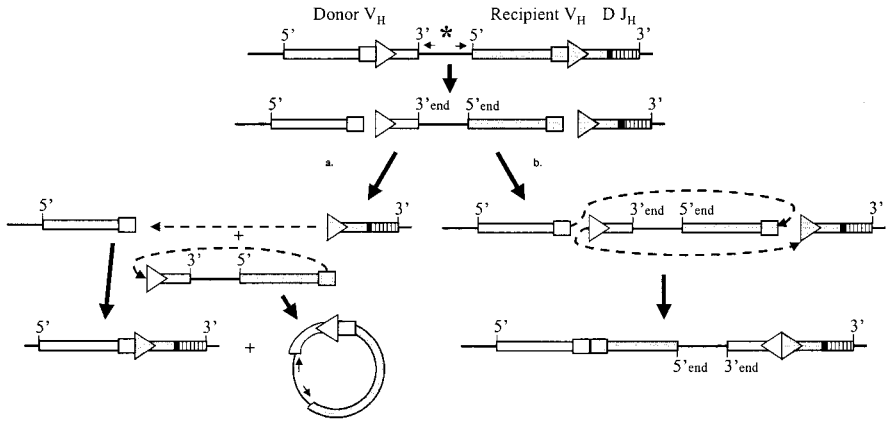

Figure 4. V_H to V_HDJ_H recombination requires hybrid joint formation. RAG-mediated cleavage occurs at the RSS producing signal ends (triangles) and coding ends (short rectangles). (a) To produce a functional hybrid recombinant, nonstandard V(D)J recombination involving hybrid joint formation with coding to signal end joining must occur (reference 26). Joining of the recipient signal end to the donor coding end produces a functional V_H gene consisting of 5′ donor sequence and 3′ recipient and DJ_H sequence. The end of the original V_HDJ_H gene 5′ to the internal cryptic RSS, and the end of the donor V_H gene 3′ to its internal cryptic RSS, plus any intervening DNA, form a circular deletional product by donor signal end to recipient coding end joining. (b) Standard V(D)J recombination involves signal to signal end, and coding to coding end joint formation. If standard recombination were to occur involving a V_H to V_HDJ_H recombination, the donor signal end and 3′ donor sequence would be joined to the recipient signal end and 3′ sequence, forming a nonfunctional hybrid consisting of the inverted 3′ end of the donor recombined to the recipient 3′ end DJ_H. Recipient to donor coding end joining would similarly disrupt the donor V_H gene sequence. Note that this inversional recombination would not damage the chromosome and may be a means to deactivate rearrangements allowing rearrangement at the other allele, as suggested by Taki et al. (reference 27). As described in the text, the small arrows in the top panel marked by an asterisk mark the position bound by primers to specifically detect the recombination circle deletion product. Note that only in the deletion circle will these primers drive an efficient reaction.

Discussion

Lack of Recombination Intermediates

There is the distinct possibility that heavy chain recombination activity may only occur in GC cells exhibiting a differential phenotype not included with typical isolates. This is particularly true for the IgD$^+$GC cells that we isolate based on IgD and CD38 surface expression and the lack of IgM. It would not be expected that heavy chains being actively rearranged would display surface Ig, and therefore our sorting strategy would fail to collect the recombinationally active cells. Furthermore, the impetus to rearrange the heavy chain genes may be loss of functional heavy chain due to mutational damage. It is also certainly feasible that GC cells lacking Ig will take on a differential phenotype. The previous examples of RAG protein expression in centrocytes only detected recombination intermediates of light chain origin in combination with surrogate light chain expression (16); heavy chain recombinants may simply not look like normal GC B cells, and therefore may not be found when standard isolation strategies are employed. Therefore, the lack of detection of the hallmarks of recombination is not particularly surprising, and the verdict must still be out on the issue of RAG mediation.

The Ideal Substrate: IgD$^+$GC B Lymphocytes

The IgD$^+$GC B cell subpopulation is of tonsillar origin and displays the interesting phenotype of having undergone a Cμ-Cδ isotype switch, and is therefore strictly IgD single positive (36, 51). In this analysis and/or as suggested previously (36), IgD$^+$GC cells display several characteristics that make them uniquely suited for both the occurrence and detection of V genes altered by receptor revision.

First, IgD$^+$GC cells display 90–99% λ light chain usage (33, 49, 51), whereas the expected light chain utilization for humans is a 2:1 ratio of κ over λ light chains (52). Detection of light chain recombination intermediates, as well as increased λ usage have been correlated to receptor editing and revision (15, 16, 21, 23, 24).

Second, IgD$^+$GC cells display an uncommonly high frequency of somatic hypermutations (33, 49). The 240 IgD$^+$GC V_H transcripts that we cloned averaged at least 1.5 times more mutations per clone then any of the other 7 mature B cell subpopulations analyzed (Table I). This high level of mutation could be related to increased receptor revision in several ways. First, the extremely large number of base substitutions could introduce excessive amino acid changes, disrupting the VDJ tertiary structure or introducing stop codons. Receptor revision could be a means to rescue these damaged clones. Second, the extensive clonality of IgD$^+$GC cells (discussed below, and see Table I) suggests that this population displays limited diversity and could therefore be driven to diversify its V_H genes to a much greater extent than other GC cells by both mutation and rearrangement. Finally, as discussed by Liu et al., IgD$^+$GC cells may be sequestered in the GC environment for prolonged lengths of time and therefore excessively subjected to the somatic mutation machinery (33). A similar prolonged exposure to factors stimulating recombination could also occur. Finally, as discussed below, we present a model in which the process of somatic hypermutation may be directly involved in the generation of the hybrid recombinants.

Third, IgD$^+$GC cells have a much higher propensity to use the J_H6 gene segment than any other B cell subpopulation analyzed (33, 49; Table I). This increased J_H6 usage could result from receptor revision of already rearranged VDJ genes by downstream J_H6 genes, as J_H6 is the most 3′ J_H gene segment. This mechanism can be envisioned as analogous to the V_H hybrid generation demonstrated here, although we have no evidence yet for this occurrence. A second possibility is a V(D)J rearrangement involving cryptic D elements within the V locus upstream to the original rearrangement that recombines with J_H6, and then to an upstream V_H gene segment, thus deleting the original VDJ rearrangement. Interestingly, we have performed extensive searches of the published sequence of the human V_H locus (44) for better fits to D elements used in the J_H6^+ IgD$^+$GC clonotypes and found several 8–16-nucleotide-long matches with sequences within the V_H portion of the locus. These "cryptic Ds" typically had at least one believable RSS proximal, for example: clone 1, 5′-GGGGAACATCACACACTGGGTCCTGTCATGGGGTGGG**GGTAGTG**GGGAGGGATACCAATTAGGATATATG-3′; clone 2, 5′-TTTAAACTCCCGATTCCTACT**CTCAGTG**GGAGGGAAT**CACAGCC**AATCACACATCACAGGACAAATCTGTAAAC-3′; and clone 3, 5′-TGTCCATGGTGGATGTGTGGTGTGGGTG**TCTGTG**TCTATTTCTGTTTACTCTGCTTGAGGTT**CTCTGTG**ATTCTAGGATCTGTAGTTCAGTGTC-3′. The underlined segment is the region of identity with a D element in a J_H6^+ clone for which no known D germline gene could be assigned. The proposed cryptic RSS are in boldface. The germline V_H gene for all three clones is V_H4-34 and is located 129 kb 5′ in the human V_H locus from clone 1, 289 kb upstream of clone 2, and 308 kb upstream of clone 3. All of these segments are located well upstream of the D genes within the V_H region. Although these observations are intriguing, we have as yet no direct evidence that these elements are truly recombined to V_H and J_H genes. Secondary rearrangement of J_H gene segments in the periphery was previously suggested by a report of J_H gene recombination signal breaks from peripheral B cells of a transgenic mouse model stimulated with LPS and IL-4 in vitro (24).

And finally, IgD$^+$GC cells display an uncommonly high number of clonally related sequences (77–83%, Table I; 33, 49). This high level of clonality allows the unambiguous detection of revised VDJ rearrangements as clones within a single clonal pool (sharing VD and DJ junctions, but with different V_H genes).

V_H Locus Considerations for Recombination by Receptor Revision

Donor V_H genes 5′ in the locus are a prediction of receptor revision involving V_H to V_HDJ recombination be-

cause all 3′ V_H elements between the recipient V_H and the D gene are deleted during the original V(D)J recombination. In Fig. 3, the recipient V_H gene is the top sequence of each comparison. Surprisingly, 8 of the 15 hybrids detected involved donors located 3′ to the recipient genes according to available V_H locus maps. However, it should be realized that there are essentially only two complete V_H loci mapped and these maps differ significantly in both V_H gene content and gene positions (44, 53, 54). Additionally, partial maps and other analyses of various alleles have detected many allelic variations between V_H loci (55). Nothing short of mapping additional V_H loci will address this discrepancy adequately and considering that the couple of loci currently available took many years to produce, such experiments are impractical and beyond the scope of this report. Another less likely possibility also exists in that there is evidence of second allele reactivation during receptor revision (24), and therefore recombination could occur using a transallelic donor V_H gene. Transallelic recombination for V_H to V_HDJ revision is physically possible, as this process requires the production of either a hybrid recombination joint (discussed below, and see Fig. 4) or a homologous recombination event leaving both chromosome 14 alleles intact, but with exchange of the 3′ ends of the chromosome pair from the point of the recombination junction.

The current study used PCR primers specific for the V_H4 gene family and therefore all of our examples were formed of V_H4 gene family donors and recipients. The seven human V_H gene families are differentiated based on 80% or greater homology between genes within each family. There is no reason to believe that V_H genes from other families don't also recombine in a similar fashion. It is doubtful however, due to protein structural constraints that hybrid V_H recombinants could form between two V_H genes that are highly divergent, such as between two different V_H families where at least 20% (and typically more) divergence exists, and still retain some semblance of their antigen-binding surface. As little as a single amino acid alteration can have drastic effects on the binding surface on an antibody, and 20% variation in the primary structure is likely disastrous (Edmundson, A., personal communication).

Frequency of V_H Gene Editing

There are several factors that make it difficult to determine a true frequency of receptor revision and its impact on the V gene repertoire in the periphery. Clones in which a successful internal rearrangement occurred forming a hybrid V_H clone are most easily identified, as rearrangements using the conserved cryptic heptamer at the 3′ end of the V_H gene, reminiscent of the V_H gene editing observed in the autoimmune transgenic mouse models, would be undetectable in most instances (26, 31, 32). Similarly, replacement of J_H gene elements as suggested by the significantly increased J_H6 gene usage in the IgD^+GC population (discussed above) relative to the other subpopulations (Table I) would be invisible to standard sequence analyses. Additionally, as suggested by Taki et al., based on observations in their transgenic model of V_H gene receptor editing, V_H to VDJ rearrangements may be more important as a means to deactivate one allele, allowing recombination to initiate on the second allele (27). As indicated in Fig. 4 A, hybrid V_H gene production requires nonstandard hybrid joint recombination as opposed to standard recombination. It has been demonstrated that RAG protein activity results in only 5% of this nonstandard recombination activity and 95% standard recombination (56). It is therefore probable that if RAG mediated, the bulk of the activity of receptor revision is to deactivate the current allele, allowing for second allele rearrangement as demonstrated in Fig. 4 B. There are reports of second V_H allele activation in the spleen of two transgenic systems (24, 30). Second allele activation would also be undetectable by previous experimental approaches. Thus, peripheral recombination of V_H genes to form hybrid V_HDJ genes is probably a minor product of receptor revision; the bulk of the activity of this process and its impact on the antibody repertoire is likely much greater. Several experimental approaches are being followed to access the true frequency of receptor revision of Ig V_H genes.

Two Possible Mechanisms of V_H Hybrid Formation: RAG versus Not-RAG

Revision of the rearranged V_HDJ_H genes by V_H gene replacement is a documented process of receptor editing in the bone marrow as a mechanism to alter autospecific transgenes (26, 27). Chen et al. (26) suggested that this process was RAG mediated, but nontraditional in that "hybrid" rather than standard recombination joints would have to be formed in order to produce functional V_H genes (Fig. 4). Nontraditional joints including hybrid joints and "open-and-shut" joints have been shown to occur at a low frequency in normal V gene rearrangement both in vitro and in vivo (56–60). Both joints involve cleavage at the heptamer signal, followed by signal to coding end joining, as opposed to signal–signal and coding–coding joint formation as in normal recombination. Open-and-shut recombination joins the coding and signal ends of a single substrate, essentially reversing the cleavage reaction, whereas hybrid joints involve the joining of the signal end of one RSS to the coding end of the second (Fig. 4). A great deal of insight into the formation of hybrid joints has been gained recently that provides important modifications and enhancements to this model.

The Ku proteins (Ku70 and Ku86) form a heterodimer that is important for the activity of DNA-PK_{CS}, which in turn plays a central role in DNA double-stranded break repair and is necessary for proper V(D)J recombination (61). Recent work has demonstrated that in Ku and DNA-PK_{CS} deficient systems, although normal recombination is essentially absent, the formation of hybrid joints and open-and-shut joints is nearly normal (59, 60). In fact, only the RAG proteins and HMG1 are required to form these nonstandard joints in vitro (57). The demonstration by Lewis and Hesse that the recombination machinery can produce open-and-shut joints on several different substrates, includ-

ing noncanonical signal sequences, single RSS substrates, and paired 23–23 substrates, have led to the hypothesis that this activity of the recombination machinery acts to reverse the cleavage reaction as a last ditch effort to circumvent improper cleavage (58). Additional support for this hypothesis derives from the observation that in the open-and-shut joints and hybrid joints produced in Ku-deficient systems, although some P nucleotide addition is present, there is a lack of N nucleotide addition and reduced exonuclease activity (60). Limiting junctional diversity would be ideal for the repair of inappropriate cleavages.

Model 1: RAG-mediated Hybrid V_H Gene Formation. The nonstandard joint-forming activity of the recombination machinery could be driven toward hybrid joint formation rather then open-and-shut joint formation when the substrates are the cryptic heptamers found within a pair of V gene coding sequences. Normal recombination might be inhibited by the cryptic nature of the RSS through disruption of the normal synaptic complex, and therefore the only reaction possible by the RAG proteins would be nonstandard joint formation. Additionally, the fidelity of these joints as described above would allow the formation of functional V_H genes upon revision. The homology between the pair of recombinants in V_H to V_H recombination may help drive hybrid joint formation. In this model, involvement of the recombinase may serve only to bring the donor V_H gene element and the recipient VDJ gene into proximity with cleavage of one of the substrates to initiate a homologous recombination event. It is feasible that the recombination machinery has evolved so that not only can it repair its own mistakes, as suggested by the formation of open-and-shut joints, but it can also serve in different capacities of recombination such as V_H gene hybrid formation, either directly or through facilitation. We are currently exploring the recombinational capacity of the cryptic RSS found within the V_H4 genes described.

Model 2: Non-RAG–mediated V_H Hybrid Formation. Two intriguing recent studies using green fluorescent protein (GFP) transgene constructs to mark RAG-2 gene expression throughout a B cell's life span have generated some intriguing insight into peripheral receptor editing (62, 63). These studies suggest that RAG protein expression is not actually reinduced in GC cells, but rather is perpetuated from the bone marrow. Adoptive transfer studies in the report from Yu et al. indicated that only cells that were RAG^+ before transfer could be induced to rearrange their V genes (62). These studies suggest that rather than reinduction of RAG protein expression in the GC environment, proper stimulation upregulates the recombination machinery only in cells where RAG expression is already present at basal levels. The authors of both reports suggested models of increased bone marrow cell migration into the GC environment after immunization.

The findings of the current report suggest extensive clonal expansion and somatic hypermutation both before and after the recombination of V_H genes. Also, RAG expression would have to perpetuate through multiple cell divisions, which is potentially disastrous if recombination breaks are present during mitosis. In addition, there is recent evidence that cell cycle regulators control RAG-2 activity before the synthesis phase of mitosis, and therefore continued RAG gene expression during a clonal expansion is not likely (48, 64–66). These observations in addition to our inability to conclusively demonstrate the predicted heavy chain recombination intermediates leave the distinct possibility that the recombinational activity observed may not be RAG mediated.

An intriguing possibility is that these events result from the activity of the somatic hypermutation machinery. As discussed in the Results section, the extensive somatic mutations found in the IgD^+GC subpopulation relative to the remaining subpopulations analyzed correlate with the frequency of hybrid V_H gene detection (Table I). Also, we and others have demonstrated previously that large insertions and deletions can result during somatic mutation (46), suggesting a propensity of the somatic mutation machinery to produce gross alterations in the DNA sequence (34, 47). There are also numerous accounts of lymphoma-associated translocations involving V sequences possibly related to the genetic alterations that occur during somatic mutation. Translocation of a portion of the VDJ element with an upstream V gene at a region of homology between the two genes would result in a hybrid VDJ element identical to the observations here. An important recent study from Sale and Neuberger has demonstrated that in a somatically mutating Burkitt's lymphoma cell line that was transfected to overexpress terminal deoxynucleotidyl-transferase (TdT), the mutating regions of the V genes become peppered with short untemplated nucleotide insertions that are not found in the C regions (67). This analysis suggests single- or double-strand breaks in the mutating V genes that could facilitate a homologous recombination event through attack of an upstream donor V gene by the free end or ends of the mutating recipient. Greater mutation activity would cause more free ends to be generated, leading to increased recombination.

Synopsis of Findings

Three key considerations in this report included: (a) B lymphocytes derived from the clonal expansion of the same founder can be identified by the highly unique junction of the V, D, and J gene elements; (b) antigen-activated B cells undergo a massive clonal expansion; and (c) the differential pattern of somatic mutations provides a simple assay to compare the many different progeny of the original founder. In this study, somatic mutation represents a cellular process that leaves a permanent and progressive mark on the various affected sequences that can be phylogenetically analyzed. The possibility that seven highly similar but mutationally different CP1 progeny using V_H4-34, and seven highly similar but differentially mutated V_H4-61 clones (V_H4-61 was the hybrid donor) in the same reaction tube could randomly come together to form hybrid V_H sequences by PCR–splicing by overlap extension (SOE) or other experimental artifacts is extremely unlikely. Similarly, the pair of CP2 sister hybrids could only have formed in a

somatic process. The differential and shared mutations of the various hybrid clones relative to each other and the remaining 58 CP1 clones or 75 CP2 clones, plus the phylogenetic analyses, indicate that these clones arose by receptor revision in the periphery. The evidence at hand leaves only one good scenario for the origin of these seven clonal hybrid recombinants: these hybrid clones were derived by secondary recombination of a portion of a germline V_H4-61 gene into the already mutated V_H4-34/D3-10/J_H3 gene of a CP1 founder or into the V_H4-34/D-D fusion/J_H6 gene of a CP2 founder, followed by additional clonal expansion and somatic hypermutation of the hybrid clonotypes and the other founder derivatives. The detection of hybrid V_H recombinants may only be a fraction of the contribution of V_H gene recombination to the peripheral repertoire, as this activity could also occur involving: (a) complete V_H gene replacements, (b) deletion or disruption of the original rearrangement with secondary rearrangement on the same allele (with cryptic D elements) or second allele activation, and (c) J_H gene replacement, as suggested by the overrepresentation of J_H6 in the IgD^+GC cells. The issue of RAG mediation of these events is still unresolved and will require additional study.

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