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Novel Secondary Ig V<sub>H</sub> Gene Rearrangement and In-Frame Ig Heavy Chain Complementarity-Determining Region III Insertion/Deletion Variants in De Novo Follicular Lymphoma<sup>1</sup>

Carol B. Kobrin,<sup>2,3,4</sup> Maurizio Bendandi,<sup>2,†</sup> and Larry W. Kwak<sup>†</sup>

Human germinal center B cell tumors retain the ability of their nontransformed counterparts to somatically hypermutate Ig V genes by nucleotide substitution. Among a survey of 60 primary previously untreated, clonal, follicular lymphomas we have identified a rare V<sub>H</sub> rearrangement variant and two other in-frame nucleotide insertion/deletion variants within complementarity-determining region III of the Ig heavy chain. The neoplastic origin of the V<sub>H</sub> rearrangement variant was directly demonstrated in cells isolated by microdissection from malignant follicles. In all three cases a common clonal origin for the variants was demonstrated by complementarity-determining region III nucleotide sequence homology and shared somatic mutations in germ-line encoded positions in framework region IV. The monoclonal nature of the tumors was independently confirmed by demonstrating a single t(14;18) translocation breakpoint in the two cases with a detectable translocation. All the variants occurred in functional V<sub>H</sub> rearrangements, which in two cases were directly shown to encode functional Ab molecules. Both recombination-activating genes 1 and 2 were expressed in lymph node tumor cells containing the V<sub>H</sub> rearrangement variant, although recombination-activating gene expression among a panel of lymphomas was not limited to this variant. The Journal of Immunology, 2001, 166: 2235–2243.

**Materials and Methods**

**Clinical specimens**

Primary lymph node biopsies from 60 patients with previously untreated FL confirmed by the Laboratory of Pathology, National Cancer Institute (Dr. Elaine Jaffe), were cryopreserved as single-cell suspensions. Nonneoplastic human B lymphocytes were obtained from tonsillectomy patients (gift from Dr. Nanping Weng, National Institutes of Health, Bethesda, MD).

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<sup>4</sup>Abbreviations used in this paper: FL, follicular lymphoma; Bx, biopsy; CDR IIIH, complementarity-determining region III of the Ig heavy chain; FR, framework region; RAG, recombination-activating gene; UPN, universal patient number; U, upper band; L, lower band; VH, variable region of the Ig heavy chain; oligo, oligonucleotide.

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Oligonucleotides (oligos)

All oligos used in this paper were designed for recognition of human genes and were synthesized by Midland Certified Co. (Midland, TX). The sequences of the individual oligos will be made available upon request. A short description of the oligos is as follows: 15, IgM constant region (C; antisense) (25); Fani 1, JH consensus (antisense, a gift from M. Campbell, Stanford University, Stanford, CA); 48, pan C, Fr III (26); 102, pan V, Fr II (V, Fr IIA) (27); 73, V, Fr III family specific (starting at codon 23) (28); 62, V, Fr I family specific (29); 85, same as oligo 73, but with an Xho I restriction site instead of EcoRI; 123, universal patient number (UPN) 13-specific antisense JH oligo; 131, RAG-1 untranslated region; 121, RAG-1 (antisense); 118, RAG-1 sense for probe amplification; 137, RAG-2 (antisense); 175, IgM C region spanning domains CH1/CH2; 176, IgM C region, CH3 domain (antisense); 197, RAG-2 (antisense, inner); and 199, RAG-2, exon 1A.

Probes

Standard PCR and molecular methodologies were used to generate DNA probes for detecting RAG-1, RT-PCR products, and rearrangements to the IgG constant region (23). RT-PCR products were radioactively tagged by kinasing at the 5′ phosphate with [32P]ATP or [33P]ATP (10 mCi/ml, 3000 Ci/mmol; Redivue, [γ-32P]ATP or [γ-33P]ATP, Amersham Pharmacia Biotech), using T4 polynucleotide kinase (Promega, Madison, WI) according to standard techniques (30). PCR-generated fragments were fractionated on 1% agarose gels. Probe elution was confirmed by autoradiography before applying other probes to the blot.

Affinity purification of hybridoma Ab

Tumor-derived Ig secreted from heterohybridomas was purified by passing culture supernatant over a Sepharose column conjugated with the anti-human IgM mAb 1D12 (gift from R. Levy, Stanford, CA). Bound Ab was eluted with glycine-HCl buffer, pH 2.4, immediately neutralized with 1.0 M Tris, pH 8, and analyzed by electrophoresing 3 μg of purified Ab together with protein m.w. standards (High Range, Life Technologies, Gaithersburg, MD) through a 14% Tris-glycine polyacrylamide gel (NOVEX, San Diego, CA) under reducing conditions (30).

Nucleic acid isolation

Total cellular RNA was isolated from 2.5 × 10⁶ cells using the RNAIAD kit (BIO 101, Vista, CA). Genomic DNA was isolated from 1 × 10⁶ cells using the Qiagen Genomic DNA Blood and Cell Culture Purification Kit (Qiagen, Valencia, CA).

cDNA synthesis

First-strand cDNA was synthesized essentially as described by Timblin et al. (32).

Microdissection of cells from neoplastic follicles

This technique was conducted as previously described (33). Neoplastic follicles in frozen sections of lymph node biopsies (provided by Dr. Elaine Jaffe, Laboratory of Pathology, National Cancer Institute) were visualized following staining with hematoxylin and eosin.

PCR amplifications

Unless indicated otherwise all amplifications were conducted in 15- to 20-μl reaction volumes using a GeneAmp PCR System 9600 Thermocycler (Perkin-Elmer, Foster City, CA). Reaction mixtures contained 1–5 μl of cDNA reaction mixture, 1.0–0.5 μg of genomic DNA template; 0.1 μl of 10× PCR II buffer (Perkin-Elmer), 2.0 mM MgCl₂, 0.2 mM dNTP mixture, 2 μM each of a sense and an antisense oligo, 0.06 U/μl of AmpliTaq DNA polymerase (Perkin-Elmer), and sufficient sterile water to achieve the final reaction volume. All amplifications were started with a denaturing step at 95°C for 5 min; were continued with 30 cycles of the general cycling program 95°C for 30 s, annealing for 30 s (temperature determined by oligos in use as specified below), and 72°C for 30 s; and were terminated with a 72°C incubation for 7 min. All amplifications designed to detect transcribed sequences from the IgM constant region exons and the RAG-1 and RAG-2 genes from cDNA template used amplifiers that spanned genomic introns. Unless indicated otherwise all amplification products were analyzed by agarose gel electrophoresis (30).

The t(14;18) translocations in neoplastic B cells were amplified using published procedures (34). CDR IIIH amplification from cDNA or purified genomic DNA template was conducted as previously described (35) using oligos 15, Fani 1, and 48 and an annealing temperature of 53°C. CDR IIH amplification from genomic DNA template isolated from microdissected cells was achieved using 60 rounds of nested PCR. The first 30 cycles of amplification were conducted using sense oligo 102 and antisense oligo 123. The second 30 cycles of PCR were performed using oligos 48 and Fani 1. An annealing temperature of 53°C was used throughout. IgH germline analysis was conducted using the CDR IIH amplification from cDNA or purified genomic DNA template procedure with one modification: 1.34 ng/μl of antisense oligo was mixed with 0.66 ng/μl of the same oligo that was radioactively tagged by kinasing at the 5′ phosphate with [32P]ATP or [33P]ATP (10 mCi/ml, 3000 Ci/mmol; Redivue, [γ-32P]ATP or [γ-33P]ATP, Amersham Pharmacia Biotech), using T4 polynucleotide kinase (Promega, Madison, WI) according to standard techniques (30). CDR IIH amplification products were electrophoresed through 5–8% denaturing polyacrylamide gels (30). Gels were dried and autoradiographed on XAR film (Eastman Kodak, Rochester, NY); Amplification of the tumor-associated Vᵣ gene was obtained using antisense oligo 15 and sense oligos 62, 85, and 73 for UPN 17, UPN 49, and UPN 13, respectively, with a 53°C annealing temperature. Amplification to show IgM mRNA was conducted using oligos 175 and 176. An annealing temperature of 53°C was used throughout.

Amplification of transcribed RAG-1 sequence was performed with oligos 131 and 121 at an annealing temperature of 53°C. Transcribed RAG-2 sequences were detected using PCR-generated fragments or the Prime-It II Random primer Labeling Kit (Stratagene, La Jolla, CA).

Southern blotting

Southern blotting and subsequent hybridization of the resulting blots were conducted according to standard methodologies (30). PCR-generated fragments were fractionated on 2% agarose gels. Probe elution was confirmed by autoradiography before applying other probes to the blot.

Molecular cloning of Vᵣ sequences

Standard molecular methodologies (30) were used to clone PCR-amplified Vᵣ sequences from 100- to 200-μl amplification reactions into pUC 19 via restriction enzyme sites engineered into the sense and antisense PCR amplifiers.

Nucleotide sequencing

All nucleotide sequencing experiments were performed using the fmol DNA Sequencing System (Promega) with [33P] end-labeled oligos (10 mCi/ml, 3000 Ci/mmol; Redivue, [γ-33P]ATP, Amersham Pharmacia Biotech). All sequencing templates were purified using the Wizard PCR Prep (Promega) for PCR-generated fragments or the Wizard Miniprep DNA Purification System (Promega) for plasmids.

Bcl 2 nrh PCR fragments were sequenced in both directions with the same pair of oligos used in the second round of amplification of these fragments. Cloned Vᵣ genes were sequenced using oligo 15 and the M13/pUC forward 23-base sequencing primer (Life Technologies) and plasmid purified from 3- to 10-ml overnight cultures of bacterial transformants grown under antibiotic selection. PCR-amplified CDR IIIH-coding fragments were sequenced following purification from denaturing polyacrylamide gels as previously described (35). Assignment of germline gene expression was made using DNAPlot software with the Vbase database (J. M. Tomlinson et al., Medical Research Center Center for Protein Engineering, Cambridge, U.K.; http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html) and the Entrez database of the National Center for Biotechnology Information (Bethesda, MD).
Statistical analysis of mutations

A binomial distribution model was used to calculate the probability of statistically significant increases in replacement-type somatic mutations occurring in CDR I and II of designated V<sub>H</sub> rearrangements (36, 37). In carrying out these calculations, the numbers of R mutations in the framework regions were doubled (36), and multiple nucleotide substitutions within a single 3-bp codon were assumed to have occurred sequentially to compute numbers of replacement-type vs silent-type somatic mutations.

Results

Two codominant CDR IIIH-encoding sequences by IgH fingerprinting analysis

Application of the IgH fingerprinting technique to primary lymph node specimens isolated from 57 previously untreated FL patients consistently resulted in the amplification of a single, dominant size class of CDR IIIH-encoding species. Subsequent nucleotide sequencing analysis of the DNA contained within these bands showed the uniform expression of single CDR IIIH-coding regions, consistent with the identification of unique V<sub>H</sub> rearrangements expressed by the clonal progeny of a B cell tumor (35).

However, FL biopsies from three additional patients unexpectedly exhibited two codominant size classes of CDR IIIH-coding sequences. In UPN 13 and UPN 17, two CDR IIIH species, separated by 6 and 12 nucleotides, respectively, appeared in single biopsy specimens obtained at diagnosis (Fig. 1). The same dominant CDR IIIH-encoding fragments were amplified from cDNA as well as genomic DNA template isolated from these biopsy specimens. Only the lower m.w. fragment was isolated from the UPN 13 hybridoma used to isolate tumor-derived Ig for the vaccine and from the progression biopsy (Bx2) from this patient.

UPN 49 showed a single species in each of four serial biopsies. However, the size of the dominant CDR IIIH-encoding fragment at diagnosis was three nucleotides larger than the species obtained from subsequent lymph node biopsies, 3, 4.5, and 6.25 yr later, after relapses from chemotherapy and immunotoxin therapy (Fig. 2). In UPN 17 and 49, the same germline V<sub>H</sub> segments were expressed by both CDR IIIH variants; namely, V<sub>H</sub>14, a member of the V<sub>H</sub>3 family (Fig. 2C, clone 20). In UPN 13, this variation was manifested by demonstrating a single IgL rearrangement in tumor cells corresponding to the codons encoding aa 92–113 (Fig. 2). Alignment of the CDR IIIH codons for maximal sequence homology revealed that the two dominant CDR IIIH-encoding fragments represented clonal variants created by the in-frame insertion/deletion of multiples of three nucleotides in each case. Specifically, sequence alignment was observed after an insertion/deletion of six nucleotides within codon 94 for UPN 13 (Fig. 2A), 12 nucleotides for UPN 17 (Fig. 2B), and three nucleotides for UPN 49 (Fig. 2C). Uniquely in UPN 13, this insertion/deletion occurred precisely at the V-D recombination joint. These nucleotide insertions/deletions most likely do not represent D minigenes, since they do not show any obvious homologies to previously described sequences. Furthermore, the U and L CDR IIIH coding species shared single base substitutions that represent mutations from published germline encoded J<sub>H</sub> sequences and their allelic forms (38) (Fig. 2A, codons 108, 109, and 113; Fig. 2B, codons 109, 110, and 113; and Fig. 2C, codon 110). However, we cannot formally rule out the expression of rare allelic JH segments as the basis for these sequence differences.

The nucleotide sequences of the germline V<sub>H</sub> segments (codons 16–94 for UPN 13 and 49; codons 10–94 for UPN 17) expressed by each variant pair were determined by using PCR amplification to generate a library of V<sub>H</sub>-encoding DNA fragments from the primary tumor specimen. Cloned V<sub>H</sub> sequence expressing each CDR IIIH variant present at diagnosis and progression was obtained. Unexpectedly, the UPN 13 variants expressed two different germline V<sub>H</sub> gene segments. Both expressed segments derived from the V<sub>H</sub>13 family, with the longer CDR IIIH variant rearranged to a V<sub>H</sub>DP-49-derived gene, while the shorter variant present at diagnosis and progression was rearranged to a V<sub>H</sub>DP-35 gene (Fig. 2A). In UPN 17 and 49, the same germline V<sub>H</sub> segments were expressed by both CDR IIIH variants; namely, V<sub>H</sub>14, a member of the V<sub>H</sub>1 family (Fig. 2B), and V<sub>H</sub>DP-54, a member of the V<sub>H</sub>3 family (Fig. 2C), respectively.

The possibility that the dual V<sub>H</sub> rearrangements in UPN 13 resulted from a secondary IgH recombination event was further supported by demonstrating a single IgL rearrangement in tumor cells at diagnosis. Consistent with the immunophenotype of the tumor, a single rearrangement to the JA2 segment was observed by Southern blotting (Fig. 3). Similar experiments failed to reveal any other recombination events to the JA1 and JA7 segments (data not shown).

Finally, intraclonal diversity, characteristic of FL (17–22), was also apparent among the variant pairs (Fig. 2A, codon 100B; Fig. 2B, codons 10, 23, 26, 44, 61, 67, 69, 70, 71, 78, 83, 84, 96, 97, and 99; and Fig. 2C, codons 25, 28, 31, 35, 53, 58, 65, 82B, 98, 99,
FIGURE 2. Nucleotide and translated protein sequences of the V<sub>H</sub> rearrangement associated with each CDR III<sub>H</sub>-encoding species revealed by IgH fingerprinting analysis in UPN 13 (A), UPN 17 (B), and UPN 49 (C). For each patient, the U and L designations at the left of each line of sequence, respectively, refer to V<sub>H</sub> region sequence associated with the U and L band CDR III<sub>H</sub> encoding DNA fragments shown in Fig. 1. The sequence derived from the U band in all three cases is written as the consensus sequence against which the sequence derived from the L band is aligned. The V<sub>H</sub> region sequence was obtained from cloned PCR-amplified fragments using antisense oligo 15 with oligos 62, 73, and 85 for UPN 17, UPN 13, and UPN 49, respectively. V<sub>H</sub>3 and D<sub>H</sub>3 J<sub>H</sub> recombination joints are, respectively, indicated as gaps separating residues 94/95 and 100B/100C (A), 100A(G)/100A(GT) (B), and 100C/100D (C). Codon 94 encodes the amino acid Arg in sequences L, Lmicro, and LBX2 in A. Dashes indicate identity with the consensus sequence, and base substitutions and amino acid replacements are indicated. Residues representing apparent somatic mutations in germline-encoded J<sub>H</sub> sequences are highlighted on a black background. No homologies to any D minigenes were found using the criteria of Corbett et al. (58).
and 100A) as well as between the shorter variant in Bx1 and Bx2 from UPN 13 (Fig. 2A, codons 56, 58, 61, 68, 78, 82C, 85, and 91).

Both UPN 13 CDR IIIH variants are expressed by cells isolated directly from neoplastic follicles

The association of two different germline V H segments with the two UPN 13 CDR IIIH variants prompted us to examine CDR IIIH expression in B cells isolated directly from neoplastic follicles. This experiment was undertaken to exclude the possibility that either variant was derived from an expanded, non-neoplastic B cell population present in the lymph node biopsy. Aliquots of 20–50 cells were isolated from neoplastic follicles by microdissection of frozen lymph node biopsy sections (33). IgH fingerprinting analysis performed on genomic DNA isolated from these cells yielded the same two dominant CDR IIIH species as observed with the unfractionated biopsy (data not shown). Furthermore, sequence analysis of the Umicro and Lmicro bands obtained from IgH fingerprinting of the microdissected population revealed CDR IIIH sequences that were identical to the two sequences previously obtained (Fig. 2).

Demonstration of tumor monoclonality by t(14;18) translocation

An independent determination of whether the UPN 13 variant pairs derived from a single malignant clone was obtained by characterizing the t(14;18) translocation breakpoints on derivative chromosome 14 in the lymphomas present at Bx1 and Bx2. Translocation breakpoints amplified by PCR revealed single comigrating DNA fragments in the anticipated size range (data not shown). Nucleotide sequence analysis of these DNA fragments yielded a single, unique breakpoint sequence (Table I). In addition, Southern blotting analysis of genomic DNA isolated from the Bx1 tumors showed single, uniquely sized restriction fragments that hybridized both to a genomic probe containing the JH complex as well as to PCR-amplified genomic sequences mapping upstream of the mbr on chromosome 18 (data not shown). Taken together, these results are consistent with a single clonal origin for this pair of variants. Similar results were observed for UPN 49 (Table I), but the UPN 17 tumor failed to exhibit a t(14;18) translocation by either method of detection and thus could not be subjected to this analysis.

Expression of RAG-1 and RAG-2 mRNA by UPN 13 and a panel of FLs

As recent reports have described coincident RAG expression and secondary Ig V gene recombination in normal germinal center B cells (1–4, 39–42), we examined RAG-1 and RAG-2 expression by RT-PCR to determine whether recombination expression by human lymphomas was associated with the ability to generate the UPN 13 variants that exhibited dual V → DJ rearrangements. Human tonsil served as a positive control for nonneoplastic germinal center B cells, which are generally present as minor subpopulations in FL biopsies. Relative levels of RAG-1 mRNA expression for each specimen were evaluated by comparing the levels of amplification achieved using three concentrations of cDNA template, representing the input of approximately 1.43, 2.86, and 7.14 × 10^3 cell equivalents, respectively. First, consistent with a reactivation of RAG-1 expression in human germinal center B cells, amplification of RAG-1 mRNA sequences from tonsil was achieved at a minimum level of 2.86 × 10^3 cell equivalents of cDNA template (Fig. 4). Then, for each FL sample, the maximum number of contaminating nonneoplastic cells that could contribute cDNA template to this assay was calculated from the total cell equivalents using Ig light chain restriction. Detectable RAG-1 expression in biopsy specimens was attributed to tumor cells if the calculated representation of cDNA deriving from nonneoplastic cells was below the threshold of 1.43–2.86 × 10^3 cell equivalents.

Thus, amplification of RAG-1 mRNA sequences from the UPN 13 biopsy was detected at a minimum input of 2.86 × 10^3 total cell equivalents of cDNA (Fig. 4). Because the calculated representation of cDNA deriving from nonneoplastic B cells (0.342 × 10^3) was well below the threshold levels of detection established for tonsil, these data suggest that the RAG-1 sequences were derived from the FL, rather than nonneoplastic cells. By these criteria, RAG-1 expression attributed to tumor cells was also detected in UPN 49 Bx2 cells, but not in Bx1 cells. Specifically, the end point of detection of 2.86 × 10^3 total cell equivalents in the Bx2 sample was also calculated to contain levels of nonneoplastic cell equivalents (0.28 × 10^3) that were below the limit of RAG-1 detection in tonsil. The lack of RAG-1 expression by the UPN 49 Bx1 cells was confirmed by repeating this analysis on Bx1 tumor cells that were enriched by positive selection with an anti-idiotypic mAb (Fig. 4). RAG-1 sequences were also amplified from the UPN 17 biopsy, although a contribution from nonneoplastic cells could not be excluded, because their representation in this sample (1.78 × 10^3 cell equivalents) was not clearly below threshold levels of detection in tonsil.

Because current information on RAG expression by FL is limited, this analysis was extended to FL from eight other previously numbered sequencing products according to the convention of Kabat and Wu (59). Additional sequences in A, Umicro and Lmicro, were the IgH fingerprinting amplification products obtained from cells microdissected from neoplastic follicles. Two separate amplification reactions yielded two CDR IIIH-encoding bands, which comigrated with the two CDR IIIH fragments corresponding to the U and L variants shown. Their identities were confirmed by nucleotide sequencing analysis. L Bx2 describes sequence derived from the sole IgH fingerprinting amplification product isolated at progression in UPN 13. In C, the clone 20 designation refers to the sequence obtained from a single cloned VH sequence isolated from a molecular library of VH rearrangements expressed by the UPN 49 tumor at diagnosis, which was generated by PCR amplification with oligos 85 and 15. The VH sequence shown for clone 20 is limited to codons 82B–113, the only region that unequivocally establishes identity with the L tumor variant and where the expected VH sequences were not obtained.

FIGURE 3. Southern blotting analysis of Ig light chain rearrangements to the J-C2 segment in UPN 13 tumor cells. A single blot containing genomic DNA isolated from UPN 13 tumor cells at diagnosis (UPN 13) or human placenta (Pla) that was digested with a combination of the EcoRI and HindIII restriction enzymes was sequentially hybridized to the J-C2/ J-C3 and J-C2 probes, respectively. The J-C2/J-C3 probe will detect rearrangements to either J-C3 from the Pla sample establish the migration of unrearranged segments. All band sizes are calculated from their migration relative to the λ HindIII m.w. marker (MW marker). The 5.4-kb J-C2 hybridizing band has been observed in other patients and most likely corresponds to a genetic amplification polymorphism in the CA2-CA3 region (31).
untreated patients which exhibited single V\textsubscript{H} and CDR III\textsubscript{H} species with somatic point mutations (conventional variants). RAG-1 expression that could be clearly attributed to tumor cells was observed in five of these eight cases (Fig. 4, UPN 35, UPN 6, and Kor, and data not shown).

Relative levels of RAG-2 mRNA expression were also evaluated by the same criterion established for RAG-1, using a threshold level of detection from nonneoplastic tonsil cells of $7.14 \times 10^{3}$ cell equivalents of cDNA template (Fig. 4). RAG-2 mRNA sequences attributed to tumor cells were amplified from UPN 13 as well as UPN 17 and 49, including positively selected UPN 49 Bx1 cells and six of the eight biopsies in the conventional panel (Fig. 4, UPN 6 and Kor, and data not shown).

The presence of tumor-derived cDNA template in all samples assayed for RAG expression was confirmed by amplifying IgM C region exon sequences mapping to the CH1, CH2, and CH3 domains and the mbr breakpoint resulting from the t(14;18) translocation where applicable (Fig. 4).

Each CDR III\textsubscript{H} variant can be independently amplified and encodes a functional V\textsubscript{H} gene

Independent amplification of each CDR III\textsubscript{H} variant was conducted to exclude the possibility that variants were generated artificially during the PCR process by a mechanism such as internal base pairing of nucleotides within CDR III\textsubscript{H}. Furthermore, the functional status of each variant V\textsubscript{H} gene was addressed by demonstrating the synthesis of structurally intact Ab molecules by tumor cells expressing these rearrangements.

Specifically, UPN 17 tumor cells expressing both CDR III variants were independently immortalized as individual B cell hybridomas. IgH fingerprinting analysis of the CDR III structures expressed by these hybridomas revealed that each cell line yielded a single CDR III\textsubscript{H} amplification product that, respectively, comigrated with the U and L bands amplified from the biopsy specimen (Fig. 5A). Nucleotide sequencing analysis of the fusion-derived bands confirmed that they encoded a single CDR III\textsubscript{H} species whose sequences were identical to the U and L variants shown in Fig. 2B (data not shown). Furthermore, the functional status of each variant rearrangement was demonstrated by the synthesis of intact Ab protein, affinity purified from the culture supernatants of each hybridoma. Reduced SDS-PAGE analysis revealed two bands with electrophoretic migrations consistent with that of Ig heavy and light chains, respectively (Fig. 5B).

Independent PCR amplification of the two CDR III\textsubscript{H} variants was also achieved for the UPN 13 and 49 tumors from specimens obtained at serial time points (Fig. 1). In addition, in UPN 49 the formal demonstration of membrane Ig on tumor cells obtained from Bx1 and Bx2 confirmed the functional nature of each variant. By FACS analysis, Abs specific for the \kappa light chain, the known immunophenotype of the patient’s tumor, stained 80 and 89% of cells, respectively (data not shown).

**FIGURE 4.** Expression levels of RAG-1 and -2 in biopsy specimens and nonneoplastic tonsil cells. RT PCR was used to identify transcribed sequences deriving from the RAG-1, RAG-2 (exon 1A-associated promoter), t(14;18) mbr, and IgM C region exons from the three CDR III\textsubscript{H} length variants, three tumors exhibiting conventional variants, and nonneoplastic tonsil cells. The immunophenotype of all the tumors was IgM. Amplification reactions where amplification of RAG-1 and/or -2 can be attributed to nonneoplastic B cells are indicated by enclosure in a box. The RAG-1 amplification fragments are autoradiographic images obtained by hybridizing a radiolabeled RAG-1 probe to a Southern blot of the amplification products. All other PCR fragments are visualized by ethidium bromide staining using analytical agarose gels. No transcription products were detected in any of the specimens from the exon 1B-associated promoter of the RAG-2 gene. N.A., Not applicable.

### Table 1. Nucleotide sequences of the t(14;18) mbr breakpoints amplified in the UPN 13 and 49 tumors

<table>
<thead>
<tr>
<th>Patient</th>
<th>BCL2 Intron</th>
<th>N Region</th>
<th>J\textsubscript{H} Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN 13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bx1</td>
<td>AAGAAACGGAAACC</td>
<td>GGTCTC</td>
<td>CTACTACGTTATG (JH6)</td>
</tr>
<tr>
<td>Bx2</td>
<td>AAGAAACGGAAACC</td>
<td>GGTCTC</td>
<td>CTACTACGTTATG (JH6)</td>
</tr>
<tr>
<td>UPN 49</td>
<td>GAAATGCAGTGGCT</td>
<td>CTGTTGCAAG</td>
<td>AACTGGTTGC (JH5)</td>
</tr>
</tbody>
</table>

*The sequence consists of the N region nucleotides, flanked on the 5' and 3' sides by bcl-2 intronic and J\textsubscript{H} complex-derived nucleotides. The breakpoints in the bcl-2 intronic region occurred at positions 3140 and 3111, respectively.*
Discussion

Among a survey of 60 primary FLs, we have identified novel, rare clonal variants containing a secondary rearrangement of different VH segments and/or in-frame nucleotide insertion/deletions. In UPN 13 the neoplastic origin of the two rearranged VH variants was directly demonstrated by isolation from malignant follicles by microdissection. In addition, a monoclonal origin for these variants was independently supported by the demonstration of a single t(14;18) translocation breakpoint on derivative chromosome 14. The detection of these variants in biopsies obtained before any chemotherapy suggests that they may reflect a physiological process occurring during lymphomagenesis rather than artifactual changes resulting from prior treatment.

To our knowledge, secondary VH gene rearrangement has not been previously described in human FL. The UPN 13 VH expression variants displayed a striking structural similarity to V gene rearrangement variants described in B precursor cell acute lymphoblastic leukemia (43, 44), a malignancy of pre-germinal center origin. Both acute lymphoblastic leukemia as well as the UPN 13 variants appear to be generated by joining different germline VH segments to a common DJH rearrangement, with limited nucleotide heterogeneity at the VH-D junction between individual variants from a single patient. However, FL is classically thought of as arising in the germinal center stage of normal B cell differentiation (7). In support of a germinal center origin for the UPN 13 variants, demonstration of a single Vα-Jα rearrangement and possible shared somatic mutations from germline in Jh collectively suggest that this dual VH gene rearrangement occurred in a mature progenitor cell after IgL recombination and after it had already been subjected to the somatic hypermutation process. However, it now appears that RAG genes, which mediate V gene recombination during earlier stages of B cell differentiation, are expressed in germinal centers in the mouse and in humans (39–42). Although evidence for germinal center RAG expression in human lymphomas has been equivocal (45, 46), our RT-PCR analysis, which was designed to specifically identify transcribed sequences, clearly demonstrated RAG-1 and RAG-2 expression in UPN13, as well as other FLs in a randomly selected panel. Both UPN 13 variant VH genes express an embedded heptamer-like RAG-associated recombination signal sequence (TA[C/T]TGTG) within codons 91–93 (Fig. 2A), which could support a VH gene replacement event (47). However, the precise mechanism of secondary VH rearrangements occurring in some, but not all, FLs remains to be determined (48–50).

Regarding the CDR III H insertion/deletion events, amplification of both CDR IIIH variants using genomic DNA as well as cDNA template was consistent with their derivation from a single malignant clone. Furthermore, in UPN 49 and UPN 17, the respective CDR IIIH variant pairs shared identical nucleotide sequences at the highly heterogeneous V-D junction (Fig. 2, B and C). In addition, in all three tumors shared somatic mutation by each variant pair was suggested by the common expression of

Table II. Calculation of parameters evaluating Ag-driven somatic mutation

<table>
<thead>
<tr>
<th>R Mutations in the CDRs</th>
<th>Probability Excess R Mutations Due to Chance</th>
<th>R:S Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>Expected</td>
<td>CDR Observed</td>
</tr>
<tr>
<td>UPN 13L variant</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>UPN 17 U variant</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>UPN 17 L variant</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>UPN 49 U variant</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>UPN 49 L variant</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>

*a* Insufficient numbers of mutations precluded this analysis in the UPN 13 U variant.

*b* Expected ratio was calculated based on a median FR R, of 0.7394 (37).
nucleotide sequence substitutions in reported germline JH segments and their alleles. These molecular findings were supported by the direct demonstration of production of a structurally intact Ab protein by each variant. This was achieved by isolation of Abs secreted from individual heterohybridomas produced from UPN 17 tumor cells (Fig. 5) and the demonstration of membrane Ig on serial biopsies of UPN 49 tumor cells, respectively. Several occurrences of V gene nucleotide insertion/deletion events have been previously observed in FL (51–56). However, technical limitations precluded directly demonstrating production of a functional Ab molecule by these variants. Moreover, in the majority of these cases, the insertion/deletions did not map to CDR IIIH, but were encoded by germline VH gene segments. Because the VHg genes exhibiting these putative nucleotide addition/deletion events were not shown to be absent from the germline of the respective patients, the possibility remains that they represented rare allelic forms of germline VHg genes. Finally, the role of prior chemotherapy treatment was unclear in these studies. Rare nucleotide insertion/deletion mutations also have been observed in nonneoplastic germinal center B cells (5, 6). Taken together, these previous reports support the phenomenon of nucleotide insertion/deletion mutations in productively rearranged VHg genes in FL.

Receptor revision (secondary IgL V gene recombination) is a proposed mechanism by which B cells can increase the affinity of their Ag receptors and prevail during affinity maturation of an immune response (4, 41). Several features of our cases are reminiscent of receptor revision and suggest that the generation of secondary VHg gene rearrangement and/or nucleotide insertion/deletion variants in FL is not random and appear to be driven to maintain the expression of a functional Ig molecule. These include maintenance of the open reading frame with the insertion/deletion events and association of a functional Ig protein with each variant. Localization of these events to the CDR IIIH and the apparent stable evolution in predominant size variants exhibited by UPN 13 and 49 over time without the appearance of additional VHg gene replacements or CDRIIIH insertions/deletions at progression suggest an Ag-driven process that confers a selective growth advantage to the tumor (18, 22). However, analysis of two parameters suggestive of Ag-directed mutation, an increase in the absolute number of R mutations as well as in the ratios of R/S mutations in CDRs I and II over the levels anticipated from the inherent mutability of the codons encoding those segments, revealed that only the UPN 49 L variant displayed a statistically significant excess of R mutations in CDR I and II (p < 0.05) over what would be anticipated in the absence of selective pressure (36, 37) (Table II). Thus, in contrast with nucleotide substitutions, which continue to occur throughout the course of the disease, secondary VHg gene rearrangements as well as nucleotide insertion/deletion events may be more restricted, even in the face of selective pressure elicited by active immunization against the Ig receptor protein (57).

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