

Clonal Expansion in Follicular Lymphoma Occurs Subsequent to Antigenic Selection

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Summary

The genesis of human follicular lymphoma (FL) is a multistep process. The initial event is thought to be the chromosomal translocation t(14;18)(q32;q21) juxtaposing the *bcl-2* proto-oncogene with the immunoglobulin (Ig) H chain locus joining segment (J_H) as an error of D-J or V-D joining in the pre-B cell. However, FL is recognized clinically as a tumor of surface Ig (sIg)-positive B cells with morphologic and phenotypic similarities to the centrocyte of the secondary immune response. Thus, additional steps must be involved in the clonal expansion of the FL tumor cell beyond the activation of *bcl-2* as a consequence of the t(14;18) translocation. Like the normal centrocyte, somatic mutations accumulate in the variable (V) genes of FL tumor B cells. To determine if clonal expansion of FL occurs before or after the development of the malignant follicle, we sought to examine the evolution of the FL V gene from its unmutated germline (GL) counterpart. To obtain the GL gene we first cloned the productively rearranged V gene of patient MT FL and obtained the clone rMTF. A hybridization probe derived from the 2.1-kb region upstream of the V gene in clone rMTF identified a single band in Southern blot hybridization of GL DNA. This probe was used to screen a size-selected library, and candidate GL V genes were isolated. Two identical clones, MTGL1 and 2, proved to have upstream regions (USRs) that were colinear with the USR of the rMTF. Thus, the MTGL clones represent the unmutated GL V genes, which were productively rearranged in the MT FL. Comparison of the GL V gene sequence to a consensus of MT FL V gene sequences revealed 42 mutations, demonstrating that malignant clonal expansion occurred subsequent to the activation of somatic mutation, presumably in the malignant follicle. Furthermore, the individual FL V gene sequences segregated into two distinct patterns of mutation. The major population represented 71% of the clones, and the minor population 29%. To investigate possible mechanisms for the parallel selection of distinct tumor cell populations, we analyzed the pattern of silent and replacement mutations within the V gene sequences. We found that in the framework regions (FRs) of both populations there were significantly fewer replacement changes than expected, suggesting that negative selective pressure was maintaining the structural integrity of the sIg. In contrast, the complementarity determining regions (CDRs), which make up the antigen binding domain of Ig, had an excess of replacement changes, suggesting positive selection for altered ligand binding. The findings of positive selection of CDRs and of negative selection of FRs support the hypothesis that the sIg was under selective pressure, presumably by antigen in both subpopulations of this tumor. Thus, we propose a model of stepwise development of FL beginning with the t(14;18) translocation followed by antigen-driven clonal expansion.

Human follicular lymphoma (FL)¹ is a low-grade indolent B cell lymphoma (1) with a propensity to progress to diffuse large cell lymphoma in up to 65% of patients within 10 yr of diagnosis (2–5). Cytogenetic and molecular studies

have demonstrated that 90% of these tumors possess a specific chromosomal translocation, t(14;18)(q32;q21), which juxtaposes the joining segment (J_H) of the Ig H chain locus on chromosome 14 and the *bcl-2* proto-oncogene on chromosome 18 (6–12). Data suggest that FL originates in the bone marrow (13) where the chromosomal translocation occurs in a pre-B cell as an error in D-J or V-D joining (14–18). The frequency of the t(14;18) in FL suggests that activation

¹ Abbreviations used in this paper: FL, follicular lymphoma; GC, germinal center; GL, germline; mbr, major breakpoint region; R/S, replacement to silent mutations; sIg, surface immunoglobulin.

of the *bcl-2* proto-oncogene is a critical event in follicular lymphomagenesis. However, several lines of evidence suggest the t(14;18) translocation alone is not sufficient to produce the malignant phenotype. Transgenic mice expressing a dysregulated *bcl-2* demonstrate polyclonal follicular hyperplasia and extended B cell survival (19). A clonal, malignant phenotype requires either a stochastic second event (20) or coexpression of a second proto-oncogene (21, 22). Furthermore, FL is invariably a tumor of mature B cells with clonal H and L chain rearrangements. Provocatively, a recent report suggested that the t(14;18) translocation can be detected in some normal tonsil tissue by sensitive PCR assays (23), suggesting its presence does not inevitably lead to lymphomagenesis.

The malignant cell in FL has a number of parallels to the centrocyte of the secondary immune response. Morphologically and phenotypically the two cell populations are very similar. The architecture of the malignant follicle is strikingly similar to the germinal center (GC) of the secondary immune response; both are characterized by the presence of follicular dendritic cells (FDC) and CD4⁺ T cells (24). Like the centrocyte, the FL cell expresses the integrin very late antigen 4 (VLA-4), which mediates binding to follicular dendritic cells via interaction with vascular cell adhesion molecule 1 (VCAM-1) (25). They share the expression of CD10 and LFA-1 as well as surface immunoglobulin (sIg) (26–29). In both the normal and malignant cells, the Ig V genes are altered by somatic hypermutation (30, 31). Given the striking similarity of the FL cell and the normal centrocyte, we wanted to determine if expansion of the FL tumor clone occurred in the malignant follicle. Though B cells have the capacity to undergo clonal expansion at several stages of development, our focus was the expansion of the FL tumor clone, which results in clinically detectable disease (malignant clonal expansion). To examine the stage of B cell ontogeny during which malignant clonal expansion occurs, we sought to exploit the observation that as a result of ongoing somatic mutation of Ig genes, each FL cell in the tumor population has a slightly different Ig V_H and V_L sequence. Thus, the sequences of these V genes from individual cells within a tumor can provide data regarding clonal relatedness. Previously, we used this tool to demonstrate that transformed diffuse lymphoma arose from a single FL cell (5). In the current study, we compared the pattern of mutation in the FL V gene to the germline (GL) V gene. A probe, upstream region sequence 2 (USR2), isolated from a region between 1.2 and 2.1 kb upstream of the productively rearranged H chain Ig allele from the FL, identified a unique GL V gene. Using this USR probe the GL V gene was cloned. The comparison of sequences obtained from the GL with those isolated from the FL demonstrates that malignant clonal expansion of FL occurred when somatic mutation was ongoing; thus, it was presumably in the GC. This finding supports the conclusion that events secondary to the t(14;18) contribute to the development of FL occurring in the B cell, not the pre-B cell. Strikingly, the case of FL analyzed in this paper is composed of two populations with distinct patterns of nonrandom mutation relative to the GL. Application of the mutational analysis proposed by Shlomchik et al. (32, 33) to the current data

supports the conclusions that the evolution of the GL to FL involved antigenic selection.

Materials and Methods

Tumor Material. Patient MT underwent biopsy at various points in time and the tumor material was stored as a single cell suspension as previously described (34). The initial FL was biopsied in May 1987.

Cellular DNA and RNA Isolation. Total cellular RNA and genomic DNA was prepared by the guanidinium isothiocyanate/CsCl method (35) as previously described (5). GL DNA was obtained from semen as previously described (36).

Southern Hybridization Analyses. DNA transfer to a positively charged nylon membrane (ZetaBind; CUNO, Meriden, CT) was performed by vacuum blotting as recommended by the manufacturer (LKB, Bromma, Sweden), with the exception that the transfer solution was 1× SSC. Probes were prepared for the Ig H chain joining region (J_H; 37), and the major breakpoint cluster region characteristic of the t(14;18) in FL, pFL1 (7). Additional probes were obtained from regions 5' of the rearranged V gene as described in Results. DNA was labeled with α-[³²P]dCTP by random hexamer priming (38). Hybridization and washing conditions were as recommended by the membrane manufacturer.

Oligonucleotides. Defined oligonucleotides were synthesized by Operon Technologies (Berkeley, CA) and were used without purification. The oligonucleotides used in this study have been described (5). Additional oligonucleotides used are indicated in Results.

Oligonucleotide Hybridizations. Oligonucleotides were labeled with γ-[³²P]ATP with polynucleotide kinase (39) for use as hybridization probes. Colonies were grown in situ on hybridization membranes as described below. Filters were prehybridized at 42°C for 3 h in a solution of 6× SSPE (1× = 180 mM sodium chloride; 10 mM sodium phosphate, pH 7.7; 1 mM EDTA), 10× Denhardt's solution (1× = 0.02% Ficoll 400 [Sigma Chemical Co., St. Louis, MO], 0.02% BSA [fraction V; Sigma Chemical Co.], 0.02% polyvinylpyrrolidone [Sigma Chemical Co.]), 1% SDS, 20 μg/ml *Escherichia coli* tRNA, 50 μg/ml sonicated, single-stranded, salmon sperm DNA (Sigma Chemical Co.). Hybridization with radiolabeled oligonucleotides was performed at 42°C in 6× SSPE and 1% SDS. After 12–16 h, filters were washed in 6× SSPE, 1% SDS three times for 10 min at 42°C. This was followed by a final 3-min wash in either 0.1× or 1× SSPE, 1% SDS at 42°C. Filters were exposed to x-ray film (X-AR; Kodak, Rochester, NY) at -70°C with intensifying screens (Lightening Plus; DuPont Co., Wilmington, DE) for 3–16 h.

Plasmid Isolation. Plasmid DNA was prepared on exchange resin (Qiagen, Chatsworth, CA) as recommended by the manufacturer.

PCR Amplification and Cloning of Ig H Chain V Genes. Productively rearranged Ig H chain V genes were amplified via the PCR using family-specific 5' amplimers and a consensus J_H primer (40) as previously described (5, 36). Initial templates were either 1 μg of genomic DNA or first-strand cDNA prepared from 0.5–3 μg of A⁺ RNA (41). The amplified products were either cloned into pLIB:AZ or pLIB:ZA or directly sequenced as previously described (5, 42, 43). Additional clones of the rearranged MT FL V_H gene were obtained by PCR amplification using a 5' amplimer from the upstream region sequence, USR1, and J_H-S as the 3' amplimer. The material was cloned into the SmaI site of pBluescript KS(-) (Stratagene, La Jolla, CA), which had been dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals [B-MB], Indianapolis, IN).

Molecular Cloning from Size-selected Libraries. 20 μg of genomic

DNA was digested to completion with the appropriate restriction endonuclease in buffer supplied by the manufacturer. The DNA was resolved in a 0.8% agarose gel (International Biotechnologies, Inc., New Haven, CT) run in 1× TAE buffer (40 mM Tris acetate, pH 8.0, 1 mM EDTA; 39) flanked by 1-kb ladder DNA markers (Gibco-BRL, Gaithersburg, MD). A gel slice containing the fragment size of interest \pm 1.5 kb was isolated. The size-selected DNA was purified from the gel using GlassMilk™ (Bio101, La Jolla, CA) and concentration estimated by ethidium bromide fluorescence (39). To determine if the proper size fraction had been selected, one half of the DNA was resolved by electrophoresis, transferred to Zetabind, and hybridized with the appropriate probe as described above. The pBluescript II KS(-) phagemid DNA (Stratagene) was digested with the appropriate enzyme and dephosphorylated with calf intestine alkaline phosphatase as recommended by the manufacturer (B-MB). Approximately 20 ng of size-selected DNA was combined with 10 ng of dephosphorylated vector DNA and ligated 2–16 h at 15°C. The reaction was diluted to 100 μ l with TE (10 mM Tris, pH 8.0, 1 mM EDTA) and extracted sequentially with phenol-chloroform and chloroform alone. To insure quantitative precipitation, 20 μ g of glycogen (B-MB) was added as a carrier molecule. Sodium acetate, pH 5.5, was added to a final concentration of 0.3 M. DNA was precipitated by the addition of 2.5 vol of ethanol and the precipitate was collected by centrifugation. The pellet was washed three times with 500 μ l 70% ethanol and the precipitate was recollected by centrifugation after each wash. The DNA was resuspended in 10 μ l of Milli-Q H₂O (Millipore, Bedford, MA) and 1 μ l was used to electrotransform (44) *E. coli* SURE (Stratagene). Libraries were screened at high density with the appropriate probe as previously described (45). Candidate clones were picked and rescreened at low density until a single isolate was obtained.

Sequencing. Double-stranded plasmid and asymmetric PCR DNA were sequenced as previously described (5, 46). Sequences were analyzed with the assistance of the University of Wisconsin Genetic Computer Group programs (47) installed at the VAX computer facility in the Department of Cell Biology at Stanford University.

Results

The IgH V Gene of Case MT Is Intraclonally Diverse as the Result of Somatic Mutation. In FL, the productively rearranged Ig V genes demonstrate intraclonal diversity (reviewed in reference 30). To determine if this was also true in the FL of case MT, the rearranged V region gene was amplified by the PCR using consensus V_H leader primers and the J_H-Sfi primer (5, 36). Both cDNA and genomic DNA were used as templates. Products of the expected size were obtained only with the V_HIII leader primer, suggesting that the productively rearranged V_H gene was from the V_HIII family (48). The PCR product was cloned into the pLIB:AZ vector as previously described (5). Sequences were determined for clones derived from independent amplifications. In Fig. 1, the sequences have been aligned relative to a consensus FL sequence. The sequences of five independently derived FL clones (MTFC3,8,11; MTFJ1,2) were extremely homologous to each other but there were randomly distributed somatic mutations, as has been previously described in FL. The sequence-labeled rMTF is derived from a genomic clone of the rearranged allele, as discussed below.

Assembly of a productive H chain Ig gene rearrangement involves two joining steps: the juxtaposition of a D segment with one of the J_H segments followed by the juxtaposition of a V_H gene with the D-J join (49, 50). The joins are imprecise because of the removal of a variable number of base pairs as well as the nontemplated addition of a variable number of base pairs (51). Therefore, the V-D-J join is an important marker of clonality in Ig H chains. In all the clones the N-D-N sequence was identical in length; and in three clones (MTFC3, MTFC11, MTFJ1) the sequence of this region was identical. In clone MTFC8 one difference with the consensus was seen, and in clone MTFJ2 three differences were observed. This

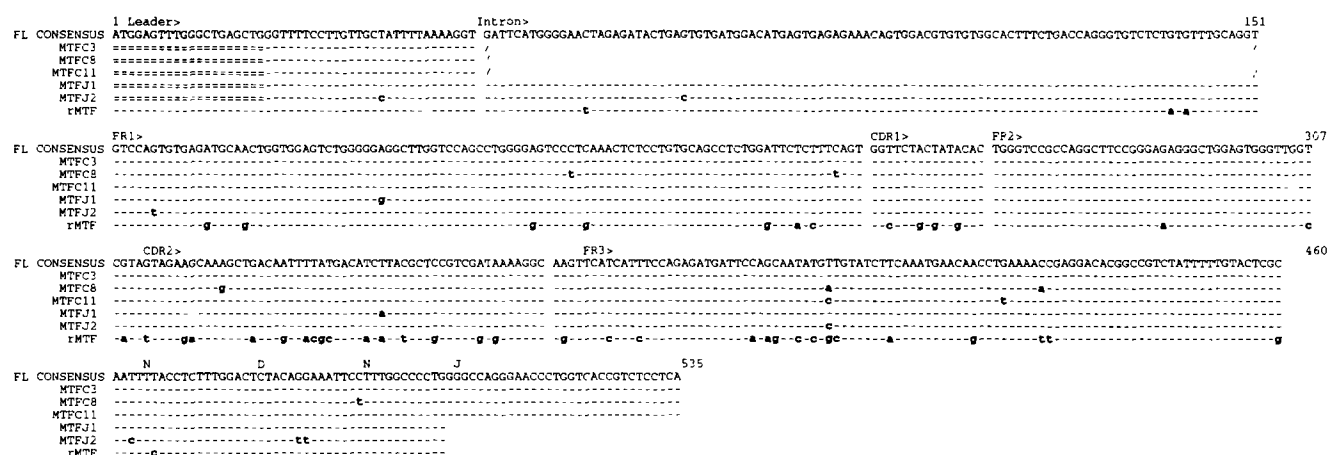


Figure 1. Nucleotide sequences of the H chain V gene from the FL case MT. Framework segments (FR1,2,3), complementarity determining regions (CDR1,2), diversity segment (D), and joining segments (J) are indicated according to Kabat et al. (67). Sequences are aligned relative to a consensus sequence. Clones MTFC3, 8, and 11 are derived from PCR of cDNA and therefore have no intron sequences. Clones MTFJ1 and 2 are clones of the PCR product obtained using genomic DNA as template. Clone rMTF is a molecular clone of the productively rearranged V gene isolated from a size-selected HindIII library. Differences from the consensus are indicated by the appropriate base change.

degree of sequence homology is unlikely to arise as the result of independent recombinational events and is similar to the variability of this region in other cases of FL (5). Thus, these clones are clonally related and therefore must be derived from the rearranged IgH allele in the FL tumor cells. The sequence differences among the individual FL clones probably are the result of somatic mutation.

Sequential Probing of MT FL DNA with J_H and $bcl-2$ Probes Identifies the Productively Rearranged IgH Allele. Rearrangements of the Ig H chain gene and the $bcl-2$ proto-oncogene in case MT were analyzed by Southern blot hybridization (Fig. 2). A probe for J_H detected non-GL bands of 4.7 and 5.5 kb in DNA digested with HindIII. Only a single rearrangement was detected at 17 kb in EcoRI-digested DNA. The filters were stripped and rehybridized with a probe for the major breakpoint region (mbr) of the $bcl-2$ proto-oncogene. In HindIII-digested DNA, a non-GL fragment of 4.7 kb was detected that comigrated with one of the bands detected with the J_H probe, indicating this band was derived from the J_H allele juxtaposed to $bcl-2$ as a consequence of the t(14;18) translocation. Thus, the 5.5-kb HindIII fragment detected with the J_H probe had to be derived from the productively rearranged Ig allele. Only GL fragments were detected with the mbr probe in EcoRI-digested FL DNA, thus with this analysis, it not possible to unequivocally assign the 17-kb EcoRI fragment to the productive IgH allele.

An Upstream Region Probe from the Productively Rearranged Ig Allele of Case MT Identifies a Unique V Gene. Since the 5.5-kb HindIII fragment identified with the J_H probe was derived from the productively rearranged IgH allele, a size-selected (5–6 kb) library of HindIII-digested MT FL genomic DNA was prepared in the phagemid pBluescript SK⁻ and screened with the J_H probe. A clone with a 5.5-kb HindIII insert was isolated and designated rMTF. The Ig V gene was mapped within the fragment by a PCR assay using 5' amplimers from both the T3 and T7 promoters of the pBluescript SK⁻ and a 3' amplimer from a consensus J_H sequence (40).

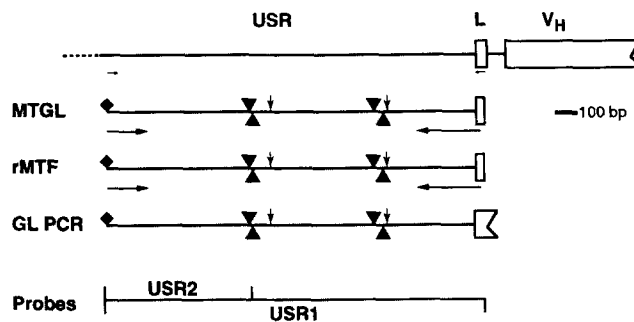


Figure 3. Restriction endonuclease map of the 2.1-kb USR of the H chain V genes from GL and FL of case MT. The USRs of the MTGL and rMTF clones were amplified by the PCR using the MTVH5' and MTIdr-com primers; the amplified fragments were both 2.1 kb. The GL PCR fragment was generated by PCR using the MTVH5' and V_H III-sig primers yielding a fragment of 2.6 kb, only the restriction map of the USR is shown. The restriction maps were generated using standard techniques. The small left and right arrows beneath the schematic of the locus indicate the position of the PCR primers. (◆) HindIII; (▼) AvaII; (▲) FokI; (↓) RsaI.

A 2.6-kb fragment was amplified with the T7 and J_H primers demonstrating that the 5' end of the V gene was downstream of the T7 promoter and the clone had a region of ~2.1 kb upstream of the V gene. Clone rMTF proved to be representative of the rearranged IgH allele in the MT FL since the sequence of the N-D-N region was identical, albeit with a single T→C point mutation, to the previously sequenced MT FL V_H isolates (Fig. 1). Like the other sequences, there were scattered differences relative to the consensus sequence. This intraclonal diversity was investigated further, see below.

A restriction map of the USR is shown in Fig. 3. A probe, USR2, was prepared from the 1-kb HindIII-AvaII fragment. This probe detects a single band in GL DNA digested with HindIII and EcoRI of 6 and 10 kb, respectively (Fig. 2). The

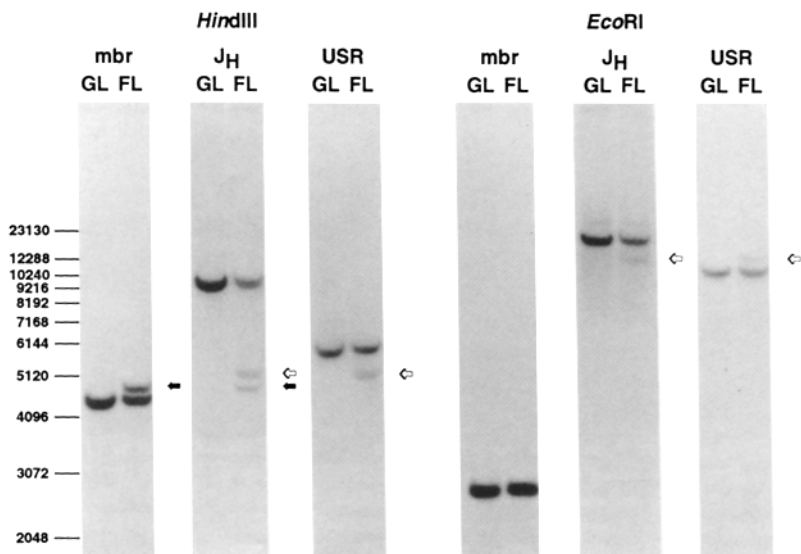


Figure 2. Analysis of Ig gene rearrangements and t(14;18) translocation in case MT FL by Southern blot analysis. DNA was isolated from normal sperm (GL) and the MT FL tumor specimen. DNA was digested with either HindIII (H) or EcoRI resolved by electrophoresis in agarose, transferred to Zetabind hybridization membrane, and sequentially hybridized to: a t(14;18) mbr probe, pFL1; a J_H probe; and the USR2 probe (see text and Fig. 3). The migrations of translocated (→) and productively rearranged (⇨) Ig alleles are indicated.

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                                     -523                               -501
MTGL                               AAATGGTCAAGTAGACAATCAG
                                     MTUSR3
rMTF                               -----

-500                               -401
MTGL  GTAATTAGGCTGTCGAAGGCAACACTGCCAAATGACAGCATTTCGGTGGAAACTGGGTGTACATCCAGCACTCCACCTGTGAACGATGACATCATACCCT
rMTF  >-----

-400                               -301
MTGL  TCACAGTGTGAGGAAAAGAGACATCACTCAAACAGACAAGCCAAAGGACTTCAGAAAATATAAGGGAAATACAGTGTGCAAATATGTAAAAATGCATAAG
rMTF  -----

-300                               -201
MTGL  ATGATTACTCCTAAATGAATATCAAGACACAATCACATAATATGAAATAAATTTTCCTGAATGATAGGAATACTACCAATCACCCCCAGXACACCCCTC
rMTF  -----

-200                               -101
MTGL  ATCTACTCTGTGCACAGCCTTCTCGTCAGGCGTCCCAGCCAGACCTTGCTATGTAGCAGAAGACATGCAAATAAGACCCCCCTTTTGCTGATGAAAAG
rMTF  -----
                                     *****
                                     MTUSR1>
-100                               -1
MTGL  CAGCCAGCCCTGACCCCTGCAGCTCTGGGAGAGGAGCTCCAGCCTGGGATTCCCAGCTGTCTCCACTCGGTGATCGGCACTGAATACAGGAGACTCACC
rMTF  -----
                                     C-----

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Figure 4. Nucleotide sequences of the USRs of the H chain V genes from clones MTGL and rMTF. Base -1 is the first base 5' of the ATG in the leader sequence. The position of the Oct2 binding site is indicated by the asterisks. The single underline indicates the position of the MTUSR1 PCR primer; the double underline indicates the position of the MTUSR2 PCR primer.

MT FL DNA demonstrated an additional, non-GL band in both HindIII- and EcoRI-digested DNA that comigrated with the rearranged alleles detected with the J_H probe of 5.5 and 17 kb, respectively (Fig. 2). This result demonstrates that the 17-kb EcoRI band detected with the J_H probe was the productively rearranged allele.

Candidate GL V Gene Clones Were Selected by Homology to the Region Upstream of the rMTF Clone. The USR1 probe was prepared from the entire 2.1-kb region upstream of the rMTF clone (Fig. 3). USR1 was used to probe a size-selected HindIII library (5.5–6.5 kb), and two independent clones were obtained designated V_{HIII} -GLA and B; however, other than orientation in the cloning vector, they proved to be identical and are therefore referred to as V_{HIII} -GL. Restriction mapping and sequencing (data not shown) demonstrated that the USR of V_{HIII} -GL was not colinear with the rMTF clone. Thus, these clones do not represent the GL V gene and were likely identified on the basis of the partial homology to conserved regulatory and promoter elements immediately 5' of the V gene.

Since the utility of the USR1 probe proved to be limited by homology to other V regions, the USR2 probe (Fig. 3) was used to screen the size-selected library of MT FL genomic DNA. Two independent clones were isolated and designated MTGL1 and 2, and proved to be identical by restriction mapping and partial sequencing; they are referred to as MTGL. The cloned fragment was 6 kb in length, consistent with that expected from the Southern blotting data (Fig. 2). The restriction map of the USR of the GL clone was identical to that of the FL (Fig. 3). Partial sequencing of the region immediately upstream of the V gene for MTGL is shown in Fig. 4. There is near sequence identity in the region -1 to -523 bp between the MTGL and rMTF clones. A single difference from the USR of rMTF was observed at base -7 consisting of an A→C; this presumably arose from somatic mutation of the GL sequence. Based on the virtual identity of the USRs of the MTGL clones and the rMTF clone, we

concluded that MTGL1 and 2 were strong candidates to encode the GL version of the V gene rearranged in MT FL.

The Molecular Clones Homologous to the USR Probes Encode V_{HIII} Family GL V Genes. To understand the nature of the evolution of the GL V gene to the FL V gene, the sequence of the V gene of MTGL was compared with those obtained from the productively rearranged allele in the FL. The sequences are shown in Fig. 5, which includes the previously presented sequence data (Fig. 1); however, all the sequences have been aligned relative to the MTGL sequence. Included in the data area the sequences of the V genes from four additional, independently derived MT FL sequences, MTFU2, 4, 5, and 6 (see below). The homology between the MTGL V gene sequence and the FL V gene consensus sequence was 90.7%. The MTGL V gene was functional, as there were proper open reading frames and a heptamer-nonamer signal sequence commencing at base 463. Clone MTGL represented a new V_H gene with >80% homology to genes of the V_{HIII} family (48).

The USR of MTGL Is Associated with a Single V Gene. Since the putative GL V gene was quite divergent from the FL consensus, it raised the possibility that despite the virtually identical USRs between MTGL and the rMTF clones, they represented different V genes. The hypothetical alternative V gene would have to share nearly identical USRs. To determine if there were multiple V genes that shared the common USR of MTGL and rMTF, genomic DNA was amplified by PCR using a 5' amplimer, MTVH5', which was common to both the rMTF and MTGL clones, and a 3' amplimer based on a consensus V_{HIII} heptamer-nonamer signal sequence. The PCR product was 2.6 kb, as expected from the molecular clones, and had the identical restriction maps (Fig. 3). The PCR product was cloned and the library was sequentially hybridized with oligonucleotides specific for the CDR2 of the V genes from MTGL or the FL consensus (boxed sequences in Fig. 5). In addition, the colonies were hybridized to an USR oligonucleotide that would identify clones

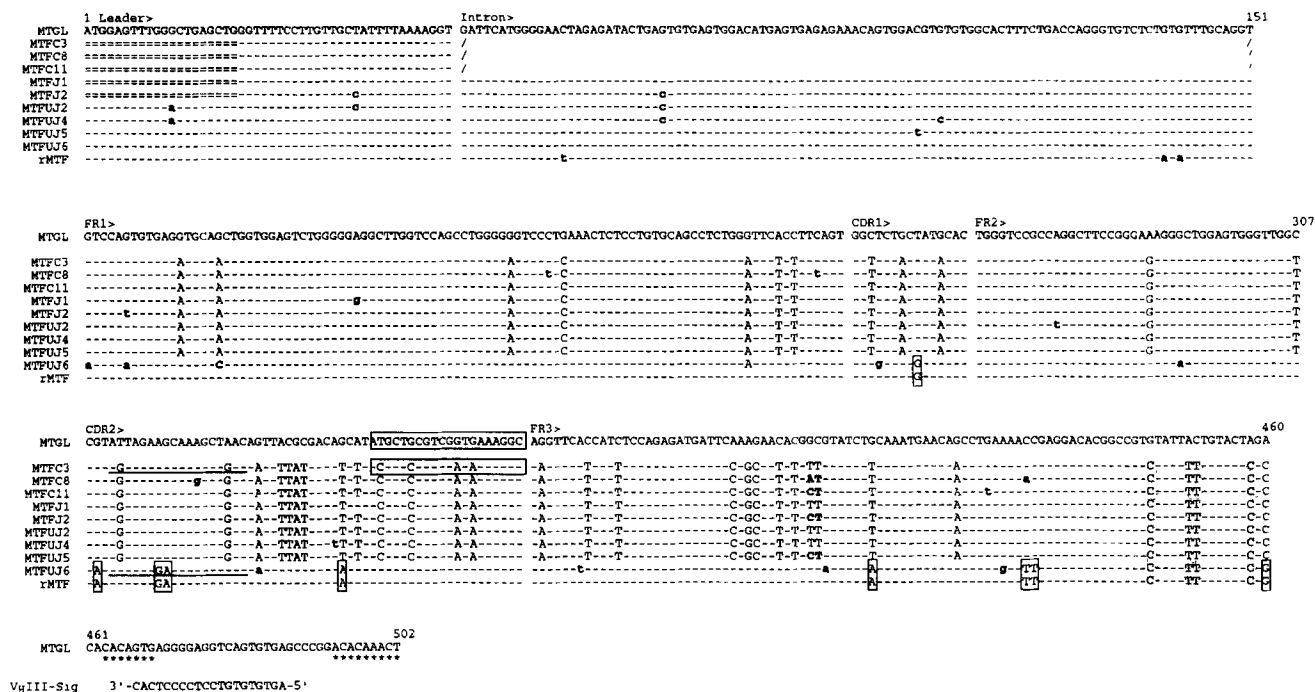


Figure 5. Nucleotide sequences of the H chain V genes from clones MTGL and rMTF. Framework segments (FR1,2,3) and complementarity determining regions (CDR1,2), are indicated according to Kabat et al. (67). Sequences are aligned relative to the GL V gene clones MTGL1 and 2, which were identical and are shown as a single sequence. Differences from the MTGL sequence are indicated by appropriate base changes. The boxed bases in clones rMTF and MTFUJ6 denote shared changes in the minor FL clone (see text). Clones MTF3, 8, and 11 were derived from cDNA, and therefore there is no intron sequence. The asterisks denote the position of the heptamer-nonamer sequence. An equal sign denotes sequencing through the PCR amplicon. Dots denote missing base pairs. The sequences of the CDR2-specific oligonucleotides used to demonstrate the unique relationship between the USR and the MTGL V gene are boxed. The sequences of the CDR2-specific oligonucleotides used in Table 1 are underlined. The position of the V_HIII-sig PCR primer is indicated.

containing the PCR insert. All the colonies hybridizing with the USR oligonucleotide also hybridized to the MTGL CDR2-specific probe (107/107). No colonies hybridized to the FL CDR2-specific probe (0/107). Thus, the USR of MTGL is present upstream of a single V gene; the differ-

ences in the V gene sequences of the MTGL clones and the FL clones must be a consequence of somatic mutation.

The FL Appears to Be Comprised of Two Dominant Sub-clones. The sequence of the clone of the rearranged allele, rMTF, appeared to be much closer to the GL sequence than

Cell Stage	pre-B Cell t(14;18)+	Virgin B Cell t(14;18)+	Centroblast t(14;18)+	Centrocyte t(14;18)+	FL Cell
V Gene	Not Rearranged	Rearranged but Unmutated	Random Mutation	Random Mutation	Selected, Shared Mutations

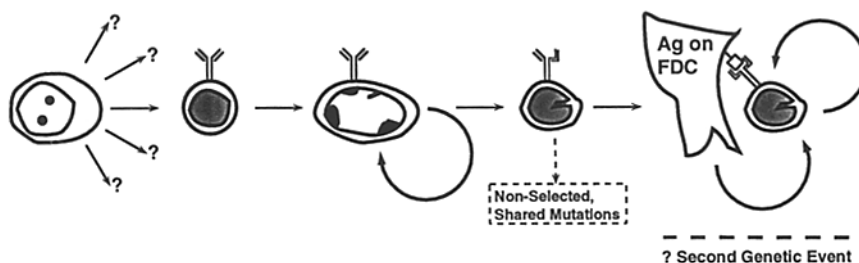


Figure 6. Schematic model for the follicular lymphomagenesis. FL has its origin in the pro-B cell (13) where the t(14;18) arises as an error of D-J_H (15, 16) or V_H-D (17) recombination. The pro-B cell then undergoes H and L chain Ig gene rearrangements. Potentially, an oligoclonal population could arise with multiple H and L chain rearrangements (indicated by ?) though they have yet to be unequivocally demonstrated. A t(14;18)⁺ B cell with unmutated V genes enters the circulation and homes to the LN in a process akin to the secondary immune response. Within the node,

a hypothetical t(14;18)⁺ centroblast proliferates introducing somatic mutations into the V genes. If transformation occurred at this point (as indicated by the dotted line), the V genes of the tumor cells would have shared mutations relative to the GL, but there would be no evidence positive selection on the CDRs as in the current data and the data presented by Bahler and Levy (57a). The FL cell is selected, presumably by binding to Ag presented on the follicular dendritic cell (FDC) or possibly infiltrating T cells; as in the current data, multiple clonotypes may be selected. The data presented here suggest a role for antigen in the process of monoclonal expansion; however, whether continual presence of antigen is required remains an unresolved question. The malignant clonal expansion of the tumor cell may result from autocrine or paracrine stimulation, as indicated by the arrows.

Table 1. Hybridization of CDR2 Oligonucleotides Specific to MTF V Gene Segment Library

Oligo	Template	Positive colonies	Percent*
FR1con	FL	62	–
MTFCDR2 (major)	FL	44	71
rMTFCDR2 (minor)	FL	18	29

MT FL genomic DNA was amplified by PCR using V_H-3-ldr as the 5' amplimer and J_H-Sfi as the 3' amplimer. The PCR product was cloned into pBluescript II SK⁻ and screened as described in Materials and Methods using 5'-labeled oligonucleotide probes as indicated in the table. * Percent of colonies hybridizing with the CDR2-specific probe and the consensus framework 1 primer, FR1con.

the other sequences obtained from the FL. Homology of rMTF with the GL was 96.5% (16 differences) compared with the 91.1% (41 differences) homology of the FL consensus sequence. Examination of the sequence revealed that rMTF shared only 4 of the 41 changes seen between the consensus FL and the GL sequence. These differences all clustered in the 3' end of the V gene between bases 445 and 460 immediately before the heptamer-nonamer signal sequence. These alterations could have arisen during V-D recombination rather than as a result of somatic mutation. These differences, shared by all the V gene sequences, support a common clonal origin for the various tumor cell populations; however, the rMTF sequence seemed to have evolved independently from the dominant population. To determine if rMTF was a unique sequence, additional clones were generated from a PCR of MT FL DNA between an MTUSR3 amplimer (sequence indicated in Fig. 4) and the J_H primer. Four clones were isolated and sequenced, MTFUJ2, 4, 5, and 6, and are shown in Fig.

5. Clone 6 was very similar to rMTF. The MTFUJ6 and rMTF clones shared nine mutations with each other in addition to the four mutations shared with the major clone as noted above. The other clones, 2, 4, and 5, were all very similar to the dominant population sharing all the changes from GL as the FL consensus sequence.

To quantitatively determine the proportion of the population represented by this variant clone, oligonucleotides were prepared corresponding to sequences in CDR2 unique for either the minor or dominant clones (underlined sequences in Fig. 5). The rearranged V gene from MT FL was amplified by PCR with V_H-3 L and J_H amplimers; the resulting PCR product was cloned, screened with the CDR2-specific oligonucleotides. Additionally, the FR1 oligonucleotide was used as a positive control that should hybridize to the V genes from both the major and minor FL sequences. The results are shown in Table 1. Of the 62 that hybridized to one of the CDR2 primers, 44 (71%) hybridized to the major clone oligonucleotide and the remaining 18 (29%) hybridized to the minor clone oligonucleotide. All 62 clones hybridized to the universal primer. Thus, the FL clones were composed of two major subclones each representing a significant portion of the tumor cell population.

The V Genes of the Two FL Populations Have Undergone Antigenic Selection. In a randomly mutated sequence the expected ratio of replacement to silent mutations (R/S) is 2.9 (52). Shlomchik et al. (32) have argued that if a mutated segment of DNA has a R/S ratio significantly lower than this value it is likely that there was selection to maintain the protein sequence (negative selection), as would be expected for Ig framework regions. However, R/S ratios >2.9 imply positive selection, as expected for mutations in CDR sequences resulting from antigenic selection. The FL has undergone clonal selection as indicated by the nonrandom patterns of mutations through the V gene when compared with the GL sequence. To determine if this selection could be on the basis of antigen, the pattern of R and S mutations were quantitated; the results are presented in Table 2. As would be ex-

Table 2. Distribution of Replacement and Silent Mutations in the Ig Heavy Chain Variable Gene Segments of the MTF Relative to the GL Gene

Tumor		FR1	FR2	FR3	Total FR	Expected FR*	CDR1	CDR2	Total CDR	Expected CDR
FL major	Replacement	3	1	11	15	24	2	12	14	7
	Silent	3	1	6	11	8	1	1	2	3
	R/S	1.33	1.00	1.43	1.36		2.00	∞	7.00	
FL minor	Replacement	0	0	3	3	8	0	4	4	2
	Silent	0	0	5	5	3	1	0	1	1
	R/S	–	–	1.00	0.60		0.00	∞	4.00	

* The expected number of mutations is given by: R_{FR-EXP} = N × proportion of total sequence in FR × proportion R; S_{FR-EXP} = N × proportion of total sequence in FR × proportion S; R_{CDR-EXP} = N × proportion of total sequence in CDR × proportion R; S_{CDR-EXP} = N × proportion of total sequence in CDR × proportion S; where N is the total number of mutations observed, the proportion of R or S as calculated from the genetic code (52) is 0.74 and 0.26, respectively, and proportion of sequence as FR is 0.77 (237/309) and proportion as CDR is 0.23 (72/309).

pected for a functional Ig, the FR regions have R/S ratios <2.9 , which would be expected for random mutation. However, the CDRs of the two FL cell subpopulations had R/S ratios >2.9 . Since the CDRs are involved in direct contact with antigen (53), the finding of high R/S ratios suggests the clonal selection observed in these sequences resulted from positive selection on the basis of binding ligand by the sIg. Furthermore, there are more R mutations in the CDRs than would be expected by chance. Given a total of n mutations, the expected number of replacement mutations in the CDR is found by: $n \times R_f \times CDR_f$; where R_f is the expected proportion of replacement mutation (0.74) and CDR_f is the fraction of the V gene encoding the CDR. In both FL cell populations, the observed R mutations in the CDRs exceed the expected number of R mutations: FL major, 14:7 (observed/expected); and FL minor, 4:2. To determine if the excess of R mutations in the CDRs could have arisen by chance, the probabilities were determined by calculating the binomial distribution (33). The likelihood that these mutations arose by chance are $p = 0.003$ and $p = 0.10$ for the FL major and minor sequences, respectively. Furthermore, the probabilities that the dearth of R mutations in the FRs arose by chance were $p = 0.002$ and $p = 0.01$ for the FL major and minor sequences, respectively. Thus, there is evidence of strong selection for maintenance of a functional sIg (negative selection on the FRs) and support for selection of an altered antigen binding domain (positive selection of the CDRs).

Discussion

Development of FL almost invariably involves the t(14;18) chromosomal translocation in which the *bcl-2* proto-oncogene becomes deregulated as a consequence of its translocation to the IgH locus. This translocation is thought to arise in the pre-B cell as an error of D-J or V-D joining (13, 15–17). However, the disease is recognized clinically as a tumor of a mature B cell having undergone H and L chain Ig rearrangements (54). Furthermore, the recent identification of the translocation in benign tonsil (23) also suggests that the activation of *bcl-2* is not sufficient to develop the malignant phenotype. The data presented in this paper shed light on the process of follicular lymphomagenesis. The finding of nonrandomly distributed mutations in the FL V gene in comparison with the GL V gene demonstrates that malignant clonal expansion occurred after the initiation of somatic mutation. This would suggest that the additional signals necessary for clonal expansion are occurring in the malignant follicle at a stage of B cell ontogeny akin to the secondary immune response. Furthermore, the pattern of mutation in the FL suggests that the V gene has undergone mutation and selection, presumably by an antigen.

These conclusions rest upon the proper identification of the GL V gene. Several lines of evidence suggest that the MTGL clones encode the bona fide GL V gene clone, which gave rise to the FL. First, the USR2 probe derived from the rMTF clone identified only a single GL band with EcoRI, HindIII (Fig. 1), and BamHI (data not shown). Furthermore,

this probe failed to detect any polymorphism in four individuals when genomic DNA was digested with BamHI, HindIII, or EcoRI (data not shown), suggesting that this is not a highly polymorphic locus. The USR2 probe was derived from the region -1.2 to -2.1 kb upstream of the V gene, which proved to be highly specific compared with a probe consisting of the entire 2.1-kb upstream region because it lacked conserved regulatory regions immediately 5' of the V gene. When the entire 2.1-kb region was used as a probe (USR1), V genes were cloned (V_H III-GLA and B) on the basis of homology in the region of -1 to -300 bp, but were otherwise not colinear with the USRs of the FL. Using the USR2 probe, two independently derived clones were obtained that were identical to each other. In these clones, the 2.1-kb region between the HindIII site and the V gene were colinear with that of the FL rMTF clone, differing in only a single base pair mutation (Fig. 4). The MTGL clones were functional GL genes and, like the productively rearranged IgH allele in the FL, they were members of the V_H III family by sequence homology (48). Furthermore, a one-to-one correlation was established between molecular clones expressing this USR sequence and also expressing the MTGL V gene. Thus, the data support the conclusion that the MTGL clones represent the GL sequence for the V gene rearranged in the MT FL, and that the differences present in the tumor-derived V gene sequence represent somatically derived variations.

An understanding of follicular lymphomagenesis requires an explanation that accounts for the development of a mature B cell tumor from a pre-B cell in which the t(14;18) has occurred (Fig. 6). The *bcl-2* gene product is a membrane protein that is thought to interfere with programmed cell death (55). However, expression of the *bcl-2* protein alone does not result in a fully transformed phenotype (56). Thus, cells in which the protein is dysregulated by the t(14;18) chromosomal translocation are at a survival advantage, but additional stimuli are necessary for malignant clonal expansion. The finding of nonrandom changes in the FL as compared with the GL support the conclusion that a cell destined to become the tumor clone was selected at a time after the initiation of somatic mutation. In the course of normal B cell development, somatic mutation is felt to occur principally in the GC during the secondary immune response. Thus, by analogy to normal B cell development, malignant clonal expansion occurs within the GC.

The finding of two distinct subclones within a single FL tumor mass suggests that selection occurred in parallel in at least two cell populations. By analysis of the nature of the mutations within a V gene, it is possible to infer that selection on the basis of antigenic binding has occurred (32, 33, 57). The R/S ratios for the CDRs of the two FL subclones demonstrate there has been positive selection, whereas the FRs have been negatively selected, as would be expected if the sIg were selected by antigen binding. Similar results have been obtained in another case of FL that expressed the V_H -4-21 V gene. In that case, comparison of the GL sequence with the V gene sequence from a series of biopsies over a number of years demonstrates emergence of several clonotypes. Fur-

thermore, the pattern of mutations in the CDRs of the dominant clone supported a role for antigenic selection in the clonal expansion of the tumor (Bahler and Levy, 57a). Recently, Friedman et al. (58) described the evolution of V gene clonotypes in the pathogenesis of an autoreactive human B cell lymphocytic lymphoma. Over time, subpopulations of the malignant clone emerged and became the dominant clone. Furthermore, comparison of the V gene sequences in the lymphoma to the autologous GL V gene suggested that negative selective pressure resulted in maintenance of autoantigen binding. These similar findings in a distinct histologic subtype of human lymphoma suggest that antigenic selection may be a critical event in the pathogenesis of a variety of subtypes of human lymphomas.

In the present case the nature of the antigen is unknown. The data suggest positive selective pressure on the cell surface Ig receptor. This could be in the form of exogenous antigen, autoantigen, or a regulatory autologous antiidiotypic antibody. In the case reported by Friedman et al. (58), the tumor-derived IgM was specific for a red cell carbohydrate antigen, Pr₂ (59). In that case negative selection was necessary to main antigenic binding encoded by GL-encoded V genes with the potential for autoreactivity. However, in other cases of autoreactivity, such as RFs or anti-DNA antibodies, positive selection for autoreactive clones has been observed (32, 57). Interestingly, tumor-derived idiotypes from a series of FL were shown to frequently react with a panel of autoantibodies (60) and thus may be involved in pathogenesis of FL.

Furthermore, it is impossible from the current data to determine if the continual presence of antigen is necessary in the genesis of the tumor. It is possible that antigen is continually required for the maintenance of the FL clone. That the FL is composed of two positively selected, clonally related subpopulations (Table 1) argues that antigenic selection occurred as the FL mass expanded. However, the finding by others of FL tumors that were sIg negative suggests that continual presence of antigen is not always necessary (61–63). Additional genetic or epigenetic events could result in malignant clonal expansion independent of antigen. For instance, expansion of the malignant clone beyond a critical mass could allow the tumor to maintain its expansion via an autocrine mechanism. Furthermore, it is known that additional cytogenetic abnormalities can coexist with the t(14;18) translocation without resulting in histologic transformation. These additional changes can portend more aggressive clinical behavior (64), which is reflected on the cellular level as a selective growth advantage. Furthermore, as the FL cell mass expands additional genetic alterations can occur. At some point a single cell within the FL population undergoes further transformation, giving rise to a histologically distinct transformed diffuse lymphoma (5).

In conclusion, we propose that FL undergoes malignant clonal expansion under selection by antigen in a process akin to B cell maturation during the secondary immune response. Our current model for follicular lymphomagenesis is presented in Fig. 6. As an error of D-J or V-D joining, the *bcl-2* proto-oncogene is activated as a consequence of the t(14;18) translocation in a pre-B cell. Since there is proliferation in the pre-B cell compartment during V gene rearrangement (65), potentially multiple productive H and L chain rearrangements could occur. However, FL invariably originates from a cell bearing a single V_H and V_L rearrangement (54; our unpublished observations). Cells with other Ig V gene rearrangements derived from the same pre-B cell in which the t(14;18) translocation occurred probably do not undergo malignant clonal expansion because the subsequent steps necessary in lymphomagenesis have not occurred. A virgin B cell with unmutated V genes and the t(14;18) translocation enters the circulation and migrates to the LN. Drawing a parallel to normal B cell ontogeny, the cell develops into a t(14;18)-bearing centroblast capable of somatic mutation and proliferation. At this point the rearranged V genes are randomly mutated. Some cells in this population are mutated in such a way that they can bind to antigen and are therefore selected. The finding of two selected populations in the current data suggest that more than one combination of mutations can result in selection. After selection, the cell then undergoes clonal expansion, either the result of exogenous (paracrine) or endogenous (autocrine) signals, and possibly as the result of additional genetic abnormalities. Previous work has demonstrated that in vitro proliferation of FL is dependent on T cell factors and contact (66), possibly reflecting an in vivo dependence on a similar environment. The progeny of this clone retain the ability to home to the LN and bone marrow, possibly as the result of expression of VLA-4 or other adhesion molecules (25), and thus are disseminated to other nodes. Within the LN, further clonal expansion results in the clinical presentation typical of FL. The alternative model, which would also account for shared mutations, hypothesizes that acquisition of additional genetic changes after initiation of somatic mutation, but before antigenic selection, gives that clone a growth advantage. In this alternative model, the shared mutations would be randomly distributed with respect to, and would not provide evidence for, positive selection of the CDRs and negative selection of the FRs. The model, as supported by the data, does not preclude another critical genetic event occurring subsequent to the t(14;18) translocation; however, it does support a role for antigenic selection of the malignant clone before expansion into a clinically evident mass.

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