Histologic Transformation of Follicular Lymphoma to Diffuse Lymphoma Represents Tumor Progression by a Single Malignant B Cell

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Summary

To investigate the clonal relationship between follicular lymphoma (FL) and transformed diffuse lymphoma (tDL), we examined the expression of tumor idiotype, immunoglobulin (Ig) gene rearrangements and sequence of Ig variable genes in paired tissue specimens. All 16 cases analyzed expressed surface immunoglobulin (sIg) on both the FL and the tDL, though the immunophenotype of one case of FL could not be definitively determined. In 14 of 15 cases, the surface immunophenotype was preserved; the exception was likely secondary to a class switch from IgM to IgG. In 12 of 13 cases, antiidiotypic monoclonal antibodies prepared against the FL reacted with the paired tDL. Analysis of Ig gene rearrangements in four cases by Southern blot hybridization showed evidence of clonal relationships in all cases though concordance was not seen with all probes tested ($C\kappa$, $C\lambda$, J_H, PFL1, and PFL2). In the one case that had a discordant L chain rearrangement, sequence analysis of the L chain demonstrated a common mature B cell origin for both the FL and tDL. To determine whether tDL arose from one or more FL cells, the sequences of the H chain variable genes were analyzed. Individual clones of the V region gene of the FL showed a random distribution of changes throughout the sequence. In contrast, individual clones of the V region gene from tDL shared numerous nonrandom sequence alterations, implying a common single cell origin. In conclusion, tDL is a mature B cell and arises by transformation of a single FL cell.

Follicular low-grade non-Hodgkin's lymphoma (FL)[1] has a relatively indolent course with the median survival of 8-10 yr (1, 2). However, histologic conversion to a more aggressive diffuse lymphoma (DL) is frequently observed at relapse or progression and portends a very poor survival (3, 4). The actuarial risk of histologic conversion is 44% at 5 yr and 67% at 10 yr (2). Histologic conversion is not monomorphic, a variety of intermediate and high grade tumor histologies have been observed. (In this study, lymphomas will be classified according to the Working Formulation; reference 1.) Antemortem studies suggest that the most common conversion is to diffuse large cell lymphoma (DLCL) (3, 4) though small noncleaved lymphoma (Burkitt's [SNC-B] and non-Burkitt's [SNC-nB]) (4-6) and acute lymphoblastic leukemia (ALL) have been observed (7-9). A postmortem study (10) of 56 patients initially diagnosed with FL found that the final

diagnosis was diffuse mixed lymphoma (DML) or DLCL in 41% of the cases and SNC-nB in 20%. No cases were classified as Burkitt's or lymphoblastic lymphoma/leukemia. Given these diverse outcomes it is of interest to investigate whether they represent tumor progression of the original FL or a de novo malignant lymphoma as others have done with divergent conclusions (5, 7-9, 11, 12).

We have now reexamined the relationship between FL and histologically transformed lymphoma by a combination of approaches. Using antiidiotypic mAbs we find that the surface immunoglobulin (sIg) expressed by the transformed diffuse lymphoma (tDL) to be the same as the antecedent FL in 12 of 13 cases. Southern hybridization analysis in four cases (three cases in which antiidiotypic antibodies were not available, and one case in which the tDL failed to bind an antiidiotypic antibody reactive with the FL) demonstrated evidence for a clonal relationship between the FL and tDL. To further prove the clonal relationship we performed nucleotide sequencing of the L and H chain V region genes expressed by the two forms of tumor in one patient. The results prove a common cell of origin for both tumors within a given patient and suggest that tDL arises as a subclone of a single mature B cell member of the FL tumor.

[1] *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; DL, diffuse lymphoma; DLCL, diffuse large cell lymphoma; DML, diffuse mixed lymphoma; FL, follicular lymphoma; FLCL, follicular large cell lymphoma; FML, follicular mixed lymphoma; FSCL, follicular small-cleaved cell lymphoma; LL, lymphoblastic lymphoma; MBR, major breakpoint cluster region; SNC-B, small noncleaved Burkitt's lymphoma; SNC-nB, small noncleaved, non-Burkitt's lymphoma; sIg, surface immunoglobulin; tDL, transformed diffuse lymphoma; TdT, terminal deoxynucleotidyltransferase.

Materials and Methods

Tumor Material and Cell Lines. Patients with FL were biopsied for preparation of antiidiotypic antibodies. Tumor material was stored as either single cell suspension or tissue blocks as previously described (13). Subsequent biopsies were obtained at various intervals when there was a clinical suspicion of histologic conversion. We established cell lines from the tDL for case JP by the method of Tweeddale et al. (14) Numerous colonies of cells grew initially in methylcellulose. Colonies from independent plates were transferred to IMDM supplemented with 20% FCS, 5 × 10^{-5} M 2-ME and adapted to liquid culture. Three independent cell lines — JP-DLCL-1, JP-DLCL-3, and JP-DLCL-4 — were thus derived. Further characterization by flow cytometry demonstrated they all expressed a μ/λ sIg as did the initial FL.

Antiidiotypic Antibodies. Antiidiotypic mAbs were prepared by the method of rescue fusion from FL tumors as previously described (15).

Immunohistochemistry. Frozen sections of biopsy material were stained as previously described (16) using biotinylated goat anti-mouse Ig followed by horseradish peroxidase–coupled avidin.

Flow Cytometry. Single cell suspensions of tissue specimens were stained with monoclonal or polyclonal antibodies either directly or indirectly and analyzed on the fluorescence-activated cell sorter (FACS 440; Becton Dickinson & Company, Mountain View, CA) as previously described (17). The antibodies used for direct staining included Leu 4-FITC (Becton Dickinson & Co.); goat F(ab')$_2$ anti–human κ-FITC, goat F(ab')$_2$ anti–human λ-FITC, goat F(ab')$_2$ anti–human IgG-FITC, and goat F(ab')$_2$ anti–human IgM-FITC (Tago Inc., Burlingame, CA). Cells were stained with specific or class-matched irrelevant antiidiotypic mouse mAbs in a two-step assay with a goat F(ab')$_2$ anti–mouse Ig-FITC second step (Tago Inc.).

Cellular DNA and RNA Isolation. Total cellular RNA was prepared by the guanidinium isothiocyanate/CsCl method (18) and poly(A)$^+$ RNA was selected on oligo-dT-cellulose spin columns (Pharmacia Fine Chemicals, Piscataway, NJ) as recommended by the manufacturer. Genomic DNA was isolated from the CsCl gradient as follows: the upper layer of the step gradient was transferred to a 15-ml conical bottom tube; 2 vol of room temperature ethanol were layered onto the DNA solution; and the DNA was spooled onto a glass rod. DNA was further purified by incubation with proteinase K (50 μg/ml) followed by three extractions with phenol/chloroform (1:1) and one extraction with chloroform. DNA was recovered by ethanol precipitation after the addition of sodium acetate to 0.1 M.

Southern Hybridization Analyses. DNA transfer to a positively charged nylon membrane (OptiBlot, IBI, New Haven, CT) was performed by vacuum blotting as recommended by the manufacturer (LKB Instruments Inc., Bromma, Sweden) with the exception that the transfer solution was 1× SSC. Probes for Ig gene rearrangements were prepared from J$_H$ (19), Cκ (20, 21), Cλ 2.5, and Cλ 3.5 (22). Additional probes were prepared for the major and minor breakpoint cluster regions characteristic of the t(14; 18) in FL, PFL1 (23), and PFL2 (24). DNA was labeled with [α-^{32}P] dCTP by random hexamer priming (25). Hybridization and washing conditions were as recommended by the membrane manufacturer.

Oligonucleotides. Defined oligonucleotides were prepared in a DNA synthesizer (model 380 B; Applied Biosystems, Foster City, CA) and purified by HPLC chromatography. Oligonucleotides T3 and T7 were obtained from Strategene (La Jolla, CA). The oligonucleotides used in this study are listed in Table 1.

Preparation of SfiI.A and SfiI.B Adaptors. The SfiI.A (or SfiI.B) adaptor was formed by annealing equimolar quantities of phosphorylated oligionucleotides Sfi/A.I (or Sfi/B.I) and Sfi/C as previously described (26).

cDNA Cloning of λ Variable Genes. Directional cDNA libraries were prepared from the FL and DL of cases JP and MT as previously described (26). Libraries were screened at high density (27) using a Cλ probe. Candidate clones were picked and rescreened at lower density until a single isolate was obtained.

PCR Amplification and Cloning of H Chain Variable Genes. PCR was performed in a DNA thermal cycler as recommended by the supplier (Perkin-Elmer Corp., Norwalk, CT) with minor modification. Primer concentration was reduced to 0.5 μM and cycling was as follows: first cycle of 2 min at 94°C (denaturation), 30 s at 55°C (annealing), 30 s at 72°C (extension); 35 cycles with the denaturation time reduced to 15 s; and a 5-min final extension at

Table 1. *Oligonucleotides Used in this Study*

Designation	Sequence
V$_H$1-L	5'-CCATGGACTGGACCTGGAGG
V$_H$2-L	5'-ATGGACATACTTTGTTCCAC
V$_H$3-L	5'-CCATGGAGTTTGGGCTGAGC
V$_H$4-L	5'-ATGAAACACCTGTGGTTCTT
V$_H$5-L	5'-ATGGGGTCAACCGCCATCCT
V$_H$6-L	5'-ATGTCTGTCTCCTTCCTCAT
J$_H$-SfiB	5'-GTGGCCACAGTGGCCACCTGAGGAGACGGTGACCAGGGT
Cμ17	5'-CAGGAGACGAGGGGGAA
Sfi/C	5'-TGGCCATGG
Sfi/A.I	5'-CCATGGCCAGCA
Sfi/B.I	5'-CCATGGCCACTG
Hλ8	5'-CGGGTAGAAGTCACT
T3	5'-ATTAACCCTCACTAAAG
T7	5'-AATACGACTCACTATAG

72°C. Initial templates were either 1 μg of genomic DNA or first-strand cDNA prepared from 0.5–3 μg of poly(A)$^+$ RNA (28). For sequencing of PCR products, asymmetric amplifications were performed with primer concentrations of 0.5 and 0.01 μM using gel-purified primary amplification products as template. PCR products to be cloned were extracted with 3 vol of chloroform followed by phenol/chloroform (1:1) and chloroform extractions. The DNA was then precipitated after the addition of 0.1 vol of 3 M Na acetate, pH 5.5, 20 μg glycogen (Boeringer Mannheim Biochemicals, Indianapolis, ID) and 2.5 vol of ethanol. DNA was resuspended in double-distilled H_2O and ligated to 100 pmol of SfiI.A adaptor (or 100 pmol of both SfiI.A and SfiI.B adaptors) in 0.5× KGB (1× = 100 mM potassium glutamate, 25 mM Tris acetate, pH 7.6, 20 mM magnesium acetate, 100 mg/ml BSA, 1 mM 2-ME) (29) buffer supplemented with 1 mM ATP and 2,000 U T4 DNA ligase (New England Biolabs, Beverly, MA) in a final reaction of 10 μl and incubated at 15°C for 12–16 h followed by heat inactivation of the DNA ligase at 70°C for 10 min. (When the J_H SfiB primer adaptor was used as the 3' amplimer, the reaction was adjusted to 100 μl of 0.5× KGB buffer and digested with 100 U SfiI and incubated for 90 min at 50°C.) The band of interest was resolved on a 3% NuSieve GTG–agarose gel (FMC Bioproducts, Rockland, ME) and cut out with a sterile scalpel blade. The gel slice was melted at 65°C and 1–2 μl of the gel combined with 10 ng of pLIB:AZ or pLIB:ZA (26) and ligated for 2–16 h at 15°C. The ligation reaction was diluted to 100 μl, extracted, and precipitated as above. The ligated DNA was washed three times with 70% ethanol to remove residual salt. The DNA was resuspended in 10 μl double-distilled H_2O and 1 μl was used to electrotransform (30) E. coli XL1-Blue (Stratagene).

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

Sequencing. Double-stranded plasmid DNA was sequenced (31) after denaturation of the template with 0.4 N NaOH for 10 min at room temperature followed by neutralization with 1 vol of 0.9 M Na acetate, pH 5.5, and ethanol precipitation. Template was annealed with 0.5 pmol of sequencing primer at 37°C for 30 min. Primers were removed from asymmetric PCR templates by chromatography on an exchange resin (Qiagen) using the protocol for M13 DNA recommended by the manufacturer. Asymmetric PCR templates were annealed with 1 pmol of the primer that was limiting in the PCR reaction and sequenced. Sequences were analyzed with the assistance of the University of Wisconsin Genetic Computer Group programs (32) installed at the VAX computer facility in the Department of Cell Biology at Stanford University (Stanford, CA).

Results

FL Idiotype Is Expressed on Transformed Lymphoma. To investigate the relationship between FL and tDL we examined the immunophenotype of serial specimens from 16 cases of histologic conversion by immunohistochemistry and/or flow cytometry. The results, shown in Table 2, demonstrate that there was concordance of immunophenotype in 14 of 15 cases in which it could be determined. The exception, case FB, expressed an IgM κ on the FL and an IgG κ on the tDL and is likely explained by an H chain class switch. The FL of case BH demonstrated high back-ground staining with L chain–specific antibody reagent; hence, an L chain determination could not be made in that case.

The immunophenotype is only a rough measure of relatedness between the tumor types. However, antibodies directed against idiotypic determinants of the sIg provide a highly specific marker for B cell lymphoma (15). In 13 of 16 cases, one or more antiidiotypic mAbs were available that had been prepared against the idiotype rescued from the FL (13). In 12 of 13 cases, the tDL was also reactive with the antiidiotypic mAb reagents. Even in case BH, in which the FL L chain type could not be determined, the antiidiotype was found to react with both the FL and tDL. In cases BE and RT, multiple tissue specimens showed a spectrum of histologies from FSCL progressing to FLC and finally to DLCL; all the samples expressed the tumor idiotype. Case EC was the sole instance of a transformed lymphoma failing to react with the antiiodiotypic reagent. In three cases—JP, MT, and FB—antiidiotypic mAbs were unavailable.

Gene Rearrangements Suggest Common Clonal Origins of FL and tDL. The analysis of Ig gene rearrangements can be used as a marker for monoclonal B cell proliferation (33). If multiple tumor isolates demonstrate identical rearrangements, then they may be suspected to have derived from a common progenitor. The results of Southern blot analyses are shown in Fig. 1 for cases of JP, MT, FB, and EC. DNA was prepared from the FL and tDL tissue samples listed in Table 2 with the exception of case JP tDL where the tDL cell lines were used (see Materials and Methods). In case MT there was concordance of the rearranged L chain band detected with the λ constant region probe between EcoRI-digested DNA extracted from FL and tDL (Fig. 1 A, lanes 5 and 6). When the same samples were digested with HindIII and hybridized with a J_H probe, two rearrangements were seen in both the FL and tDL; however, only the 5.5-kb band was concordant (Fig. 1 B, lanes 5 and 6). In the FL, a second rearrangement was seen at 6 kb in addition to a germline fragment of 12 kb. The germline band arises from normal cells that frequently infiltrate these tumor specimens (33). In the tDL, the second rearrangement was 8 kb and only a trace amount of the germline DNA is seen. Since MT produces a sIg, one of the J_H rearrangements must be productive; furthermore in FL, the second J_H rearrangement is often involved in the t(14;18) chromosomal translocation in which the J_H segment is juxtaposed to the bcl-2 protooncogene (23). To determine if one of the J_H rearrangements was involved in the t(14;18), the HindIII blot was rehybridized with a chromosome 18 probe, PFL1, for the major breakpoint cluster region (MBR) of the t(14;18) (23). This demonstrated a concordant 5.5-kb band (Fig. 1 C, lanes 5 and 6) at exactly the same position as was observed with the J_H probe (Fig. 1 B, lanes 5 and 6). Hence, the 5.5-kb band observed with the J_H and MBR probes must be derived from the t(14; 18); whereas, the nonidentical rearrangement detected with the J_H probe in the MT FL and tDL specimens must be the productive allele.

In case JP, a single rearrangement of the λ constant (Cλ) could be detected in the FL at 6.2 kb (Fig. 1 A, lane 1). The prominent germline bands in this sample indicates substantial infiltration of the tumor by cells without clonal rearrangements; nonetheless, a prolonged exposure failed to reveal a second rearrangement (data not shown). The three cell lines

Table 2. *Surface Immunoglobulin and Antiidiotypic mAb Reactivity of Paired FL and tDL*

Case	Date	Site*	Diagnosis‡	sIg	ID	(%)§	Case	Date	Site	Diagnosis	sIg	ID	(%)
BH	5/24/84	l-cerv	FML	μ	+		JP	8/31/88	l-cerv	FML	μλ	N/A	
	11/4/88	r-axil	DML	μλ	+	(92)		1/27/89	PBL	DLCL	μλ	N/A	
BL	1/27/84	spleen	FSCL	μκ	+	(100)	LV	10/15/86	l-ing	FML	μκ	+	(53)
	10/6/87	breast	DLCL	μκ	+			10/27/88	l-ing	F/DLCL	μκ	+	(97)
CL	8/23/88	l-axil	FSCL	μκ	+		MW	9/19/81	l-cerv	FML	μλ	+	
	6/13/89	l-scln	SNC,nB	μκ	+	(100)		10/31/83	r-ing	DML	μλ	+	
FB	6/2/83	r-axil	FSCL	μκ	N/A‖		RF	11/6/86	r-iliac	FML	μκ	+	(100)
	12/13/84	r-ing	IBL	γκ	N/A			10/11/88	pao	DL	μκ	+	
JC	10/6/83	spleen	FSCL	γλ	+	(92)	RS	1/19/88	l-ing	FSCL	αλ	+	(100)
	10/9/86	skin	DLCL	γλ	+			6/24/88	r-ing	DLCL	αλ	+	(88)
BE	9/21/84	r-axil	FSCL	μκ	+	(92)	RT	11/15/85	l-fem	FSCL	μλ	+	(100)
	6/4/87	LN sns	FLCL	μκ	+			11/22/88	r-ing	FLC	μλ	+	(100)
	4/14/88	r-cerv	F/DLCL	μκ	+	(94)		1/5/90	chest	DLCL	μλ	+	(92)
EC	3/26/87	l-cerv	FSCL	μκ	+	(100)	RW	3/27/84	r-ing	FML	γλ	+	(100)
	4/6/89	r-axil	F/DLCL	μκ	−			1/8/87	l-cerv	DML	γλ	+	(95)
	8/23/89	l-ing	F/DLCL	μκ	−			6/21/88	r-cerv	DML	γλ	+	
MT	5/7/87	r-axil	FSCL	μλ	N/A		SG	11/18/86	spleen	FSCL	γκ	+	(82)
	12/29/88	gluteal	DLCL	μλ	N/A			4/28/88	l-ing	DML	γκ	+	(100)
	1/18/89	PBL	DLCL	μλ	N/A			10/7/88	l-scln	FML	γκ	+	

* Site of tissue biopsy. *l*, left; *r*, right. LN sites: *axil*, axillary; *cerv*, cervial; *fem*, femoral; *ing*, inguinal; *pao*, paraaortic; *scln*, superclavicular; *LN sns*, lymph node, site not specified. Chest, breast, and gluteal are soft tissue extranodal sites.

‡ Surgical pathologic diagnosis according to the Working Formulation (reference 1).

§ Reactivity of antiidiotypic mAb on tissue or by flow cytometry. When analysis by flow cytometry the percent of tumor cells staining is shown in parentheses. % Tumor cells = % staining with correct L chain − % staining with incorrect L chain.

‖ *N/A*, Antiidiotypic mAb not available.

derived from the tDL had two rearrangements detectable at 3.8 and 8.6 kb; they were concordant among the three cell lines though distinct from the FL. Analysis of the H chain locus with the J_H probe revealed the FL and tDL cell lines to be concordant for two rearranged bands at 2.7 and 9.0 kb (Fig. 1 *B*, lanes *1–4*). Rehybridization of this blot with probes for the t(14;18) MBR (PFL1, Fig. 1 *C*, lanes *1–4*) or the minor cluster region (PFL2; data not shown) failed to show evidence of a chromosomal translocation in this tumor.

In case FB, analysis of the BamHI-digested DNA with a probe for the κ constant (Cκ) region revealed a concordant band >15 kb between the FL and tDL (Fig. 1 *D*, lanes 7 and *8*). Both the H chain alleles were rearranged in the FL and tDL though only the 7.2-kb band was concordant (Fig. 1 *E*, lanes 7 and *8*). Again, a germline band was detected because of infiltration of the specimen with nontumor cells. In the FL sample J_H crosshybridizing bands could be seen at 3.8 and 4.5 kb as previously described (33). Analysis with the PFL1 probe (Fig. 1 *F*, lanes 7 and *8*) and the PFL2 probe

(data not shown) did not demonstrate rearrangements; therefore, assignment of the productive J_H allele could not be done.

Finally, the analysis of case EC of BamHI-digested DNA with the Cκ probe revealed a concordant rearrangement of 7.9 kb between the FL and tDL (Fig. 1 *D*, lanes *9* and *10*). FL DNA digested with HindIII and probed with J_H demonstrated two bands of 3.8 and 4.6 kb (Fig. 1 *E*, lanes *9* and *10*). Similar analysis of the tDL demonstrated a 6.1-kb band in addition to the bands seen in the FL. In the tDL, the 3.8-kb band is decreased in intensity relative to the new 6.1-kb band. Since the tDL tumor sample was an admixture of follicular and diffuse large cell lymphoma, it is possible that the faint 3.8-kb band is arising from the residual follicular DNA. Reprobing of this blot with the PFL1 probe (Fig. 1 *F*, lanes *9* and *10*) and the PFL2 probe (data not shown) revealed only the germline configuration.

Sequence of the L Chain Proves a Common B Cell Origin of FL and tDL. The Southern hybridization analysis of the L chain rearrangement(s) of the FL and tDL in case JP demon-
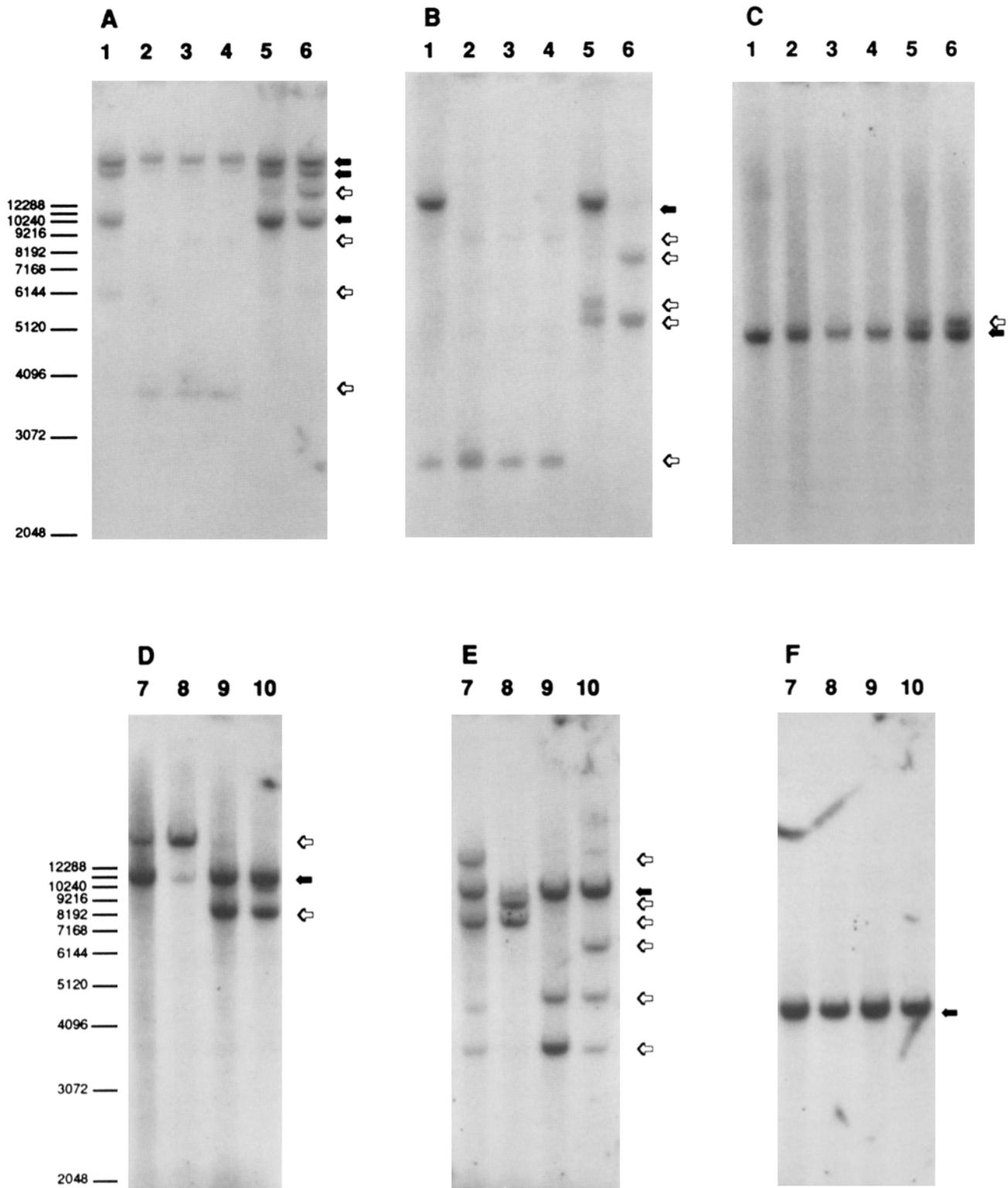
Figure 1. Analysis of Ig gene rearrangements and t(14;18) translocation by Southern blot analysis. DNA was isolated from paired FL and tDL specimens and designated as follows: (lanes *1*) JP FL [8/31/88]; (lanes *2*) JP-DLCL-1; (lanes *3*) JP-CLCL-3; (lanes *4*) JP-DLCL-4; (lanes *5*) MT FL [5/7/87]; (lanes *6*) MT DL [1/18/89]; (lanes *7*) FB FL [6/2/83]; (lanes *8*) FB DL [12/13/84]; (lanes *9*) EC FL [3/26/87]; and (lanes *10*) EC DL [4/6/89]. DNA was digested with EcoRI and probed with C_λ (*A*), HindIII and probed with J_H (*B* and *E*) or BamHI and probed with C_κ (*D*). Filters from *B* and *E* were stripped of probe and rehybridized with either a PFL1 (*C* and *F*) or PFL2 probe (not shown). The migration of germline (*solid arrow*) and rearranged (*open arrow*) DNA as well as molecular weight markers are indicated.

strated bands of different sizes. Since the H chain rearrangements were identical in the FL and tDL, this result could be explained if the FL and tDL lymphomas had arisen from a common pre-B cell clone subsequently giving rise to distinct mature B cell subclones. Alternatively, this difference could have been generated secondary to somatic mutation with alteration of restriction sites. Since L chain rearrangement is the final event defining a mature B cell (34), sequencing of the L chain rearrangements of FL and tDL was done to determine the relationship of the tumor B cells. cDNA libraries were prepared in the vectors pLIB:AZ and pLIB:ZA from oligo(dT)-primed poly(A)⁺ RNA derived from the FL and tDL of case JP (26). The sources of RNA were as follows: JP FL, single-cell suspension of lymph node and JP tDL, cell line JP-DLCL-3. The libraries were screened with a Cλ probe as described in the Materials and Methods section. Four candidate clones were isolated from each library. The cDNA clones were sequenced and 5 of 8 clones were found to span the V-J join; the sequences are shown in Fig. 2 A. The three sequences obtained from the JP-DLCL-3 cell line library were identical and are shown as a single sequence. The similarity in the L chain sequences demonstrate that in case JP the FL and tDL are derived from the same mature B cell. The V gene segments of both the FL and tDL are from the Vλ I family (35). The isolates from the FL library demonstrated

somatic mutations as has been previously described (36); the tDL sequence showed a similar distribution of mutations though they were more numerous. The Jλ segments used in both the FL and tDL are most similar to the human germline Jλ minigenes HUMJL2 or 3, whose sequences are identical (35). As illustrated in Fig. 2 B, both the FL and tDL Jλ minigenes have diverged from the germline sequence by four identical somatic mutations; both the FL and tDL have additional sequence changes likely the result of somatic mutation. These shared mutations demonstrate a common clonal origin. Both tumors use the same constant region though the tDL has a single base change at position 405. When compared with the consensus sequence the FL Vλ gene segments are 99 and 97% identical whereas the DL Vλ gene segment is 91% identical. This extent of alteration in the sequence between the FL and tDL can be explained by somatic mutations occurring between the time of initial diagnosis of FL and the subsequent development of tDL (36). A search of Vλ sequences available in GenBank revealed no sequence to be more closely related to the FL and tDL sequences than those sequences are to each other.

Patterns of Somatic Hypermutation in the H Chain Gene Demonstrate a Single-Cell Origin of FL and tDL. If V genes expressed by FL undergo active somatic mutation during clonal expansion, each cell within a tumor population will have a

Figure 2. (A) Nucleotide sequences of the λ light chain from the FL and tDL of case JP. The leader, CDR1 and 2, joining segment (J), and constant region (C) are indicated according to Kabat et al. (35) and are demarcated with solid horizontal lines. Individual base positions are marked by dots (.) and every 10 base pairs are marked by pluses (+). The sequences designated JP-FL-λ were derived from the FL of case JP; the sequence designated JP-DL-λ was derived from the DL of case JP. (B) Nucleotide sequence of the germline Jλ segment HUMJL2,3 aligned to the Jλ segments of the consensus sequences of JP-FL-λ and JP-DL-λ sequences. The germline sequence is from Kabat et al. (35).

slightly different Ig V gene sequence (36). Thus, the patterns of mutations can be exploited as markers of clonal evolution. If tDL arose from a single FL cell, then there should be common sequence alterations in the V_H or V_L among independent tDL isolates. On the other hand, if transformation arose from multiple FL cells the pattern of somatic mutation would be random in independent tDL isolates.

Ig V genes were cloned and sequenced after amplification in a PCR assay (Campbell, M., A. Zelenetz, S. Levy, and R. Levy, manuscript submitted for publication). Briefly, 5' amplimers were prepared from consensus leader sequences of the known V_H gene families (38) (VH-L1-6) (Table 1) and used in conjunction with either a J_H (37) or $C\mu$ 3' amplimer to identify the rearranged H chain Ig allele. For a given template only one of the six reactions yielded appropriately sized PCR products (~550 bp from DNA and ~450 bp from cDNA) reflecting the dominant tumor clone present (data not shown).

Using this technique the H chain V genes were amplified, cloned, and sequenced from JP FL tumor RNA, JP-DLCL-1,3,4 cell line DNA and RNA, and DNA derived from the original DLCL specimen (see Table 2). Only the V_H3-L primer amplified a product of the correct size. The sequences are shown in Fig. 3 A; to facilitate comparison the intron in the variable gene segment of clones derived from genomic DNA templates is not shown. All sequences represent V_H3 family genes on the basis of sequence comparison with data available from GenBank, release 62. The J_H segment used in
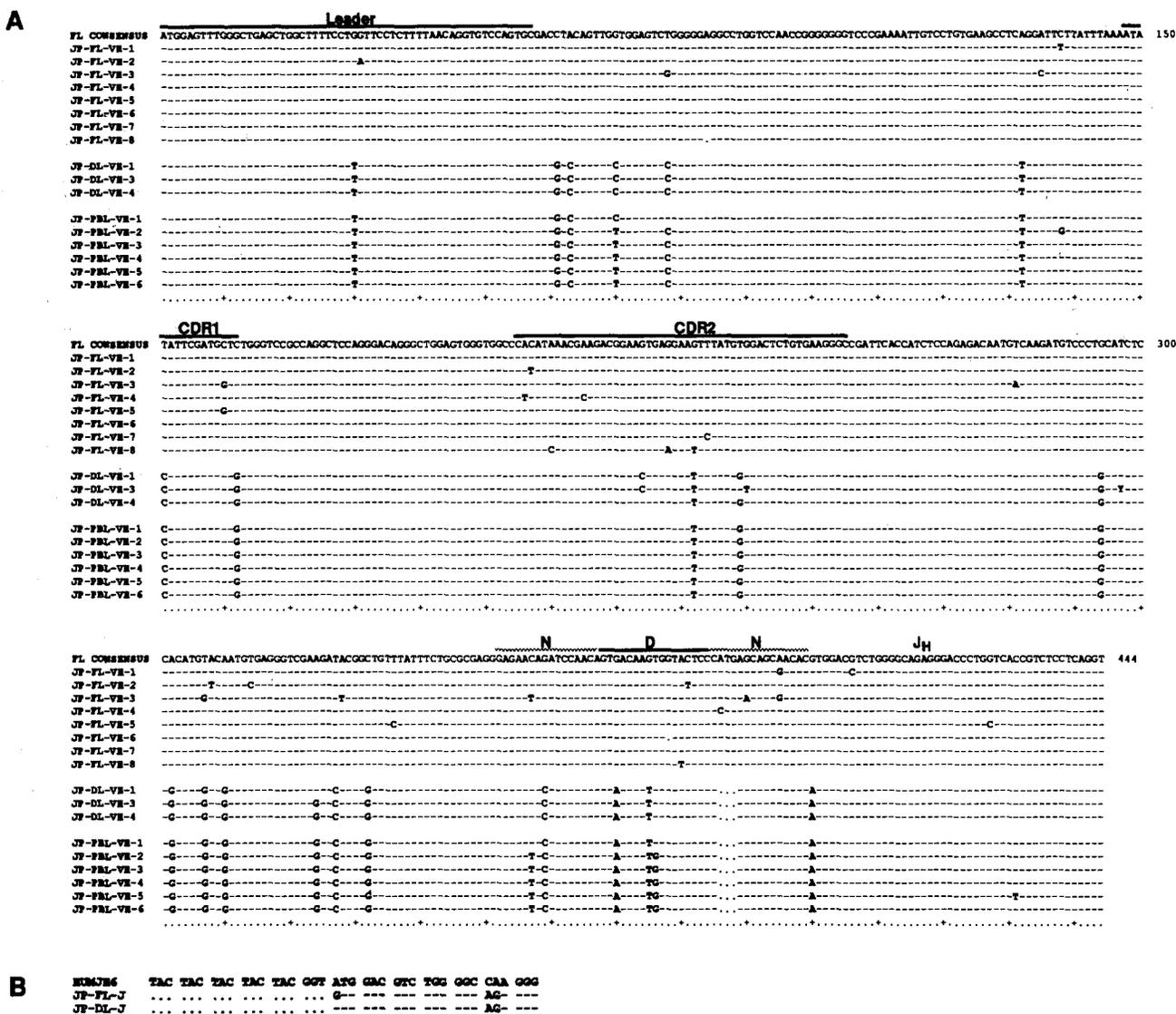
Figure 3. (A) Nucleotide sequences of the H chain V genes from the FL and tDL of case JP. Various regions are indicated as in Fig. 2. Differences from an FL consensus sequence are indicated by appropriate base changes. Y indicates a C or T (see text). Individual base positions are marked by dots (.) and every 10 base pairs are marked by pluses (+). The sequences designated JP-FLVH were derived from the FL in case JP; the sequences designated JP-DLVH were derived from the cell lines JP-DLCL1,3, and 4; and those designated JP-PBLVH were derived from the leukemic phase of the DL in case JP (see Table 1 and Materials and Methods). (B) Nucleotide sequence of the germline J_H segment HUMHJ6 aligned to the consensus sequences of JP-FLVH and JP-DLVH. The terminal 21 bp of the J_H segment have been omitted since this is the region that is derived from the PCR 3' oligonucleotide (see Table 1). The germline J_H sequences is from Schroeder and Wang (52).

both the FL and tDL is most similar to the germline segment HUMJH6 (35) as shown in Fig. 3 B. Both the FL and tDL have used an identically foreshortened form of the J_H segment. The FL and tDL J_H sequences have diverged from the germline by two shared sequence alterations; the FL has an additional mutation not seen in the tDL. The common evolution of these sequences demonstrates they arose from the same clonal origin. The D segment was most similar to DM1 (39) though somatic mutation and lack of sequence information about all D segments makes this assignment tentative. Eight sequences were derived from JP FL. They are characterized by 28 random sporadic mutations throughout the gene. JP-FL-VH-6 and JP-FL-VH-8 each have a 1-bp deletion at positions 378 and 86, respectively, which result in nonfunctional genes secondary to termination codons in the new reading frame. tDL sequences were derived from the three independent JP-DLCL cell lines and six clones derived from the original tumor specimen. The sequence from each cell line was obtained from at least two clones. All independent clones derived from a given cell line yielded identical sequences except position 297 of sequence JP-DL-VH-3 which was found to be C in a clone derived from genomic DNA template and T in three other clones (one from a genomic DNA template and two from cDNA templates). This may have arisen either as an artifact of cloning or PCR amplification or from ongoing somatic mutation in the JP-DLCL-3 cell line. The independent tDL sequences are highly conserved, sharing 17 differences from the FL consensus sequence. At an additional three positions — 78, 239, and 324 — eight of the nine tDL sequences share common differences; the exceptions may be a result of back mutation. Furthermore, they all share a 3-bp deletion in the region of the D-J join. The markedly nonrandom distribution of the differences between the FL and DL sequences strongly suggests that the tDL cell lines were representative of the original tumor and furthermore that the tDL arose from a common origin. There are several differences among the nine tDL sequences at positions 138, 224, 240, and 297 where there are changes affecting only one or two sequences most likely representing ongoing somatic mutation after the transformation event.

Discussion

Histologic conversion of follicular to diffuse lymphoma occurs frequently in the natural history of this disease and is associated with poor prognosis. There are three possible models to explain the relationship between these different tumors within the patient. First, the tumors could be biclonal, i.e., arising from two independent transforming events. In a study of B cell lymphomas showing multiple histologic forms, Siegleman et al. (40), using Southern blot analysis of Ig gene rearrangements, found that in some instances no concordant bands were observed between distinct histologic forms of lymphoma obtained from a single individual. Thus, they concluded that in some cases biclonal lymphomas could develop. However, FL represents a stage of B cell development at which somatic mutation of the Ig genes is occurring at

a high rate (36, 41). Mutations can occur in restriction enzyme sites and alter the size of bands in a Southern analysis (11); therefore, the absence of concordant bands does not necessarily indicate multiclonal origins. In the present study, the L chain rearrangement of case JP and the productive H chain rearrangement of case MT were nonconcordant between the FL and tDL; however, sequence analysis proved the respective variable gene rearrangement were in common (Fig. 3; case MT, data not shown).

A second model for transformation proposes that the two tumors are related through a common pre-B cell which had chosen an H chain gene but had not yet chosen an L chain gene. According to this model, the FL would arise from one subclone and the tDL would arise at a later point in time but from a different pre-B subclone. Gauwerky et al. (7, 8) suggested this model to explain the development of a pre-B cell acute lymphoblastic leukemia in a patient with a prior history of FL. They found that the leukemic cells contained two chromosomal rearrangements, t(14;18) characteristic of FL and t(8;14) characteristic of sporadic Burkitt's lymphoma. The contention that this tumor arose from a pre-B cell pool harboring a t(14;18) was further supported by the finding of terminal deoxynucleotidyltransferase (TdT) in the leukemic blasts and the absence of sIg. A similar conclusion was reached by de Jong et al. (9) in a report of a case of composite lymphoma comprised of FL and lymphoblastic lymphoma (LL). Both components shared an identical t(14;18) and only the LL showed the additional t(8;14) rearrangement. The FL and LL were TdT⁻ and TdT⁺, resepectively. Neither component expressed a sIg despite the finding that both the TdT⁻ and TdT⁺ populations had concordant L chain rearrangements. However, in the current study, 16 of 16 cases of tDL expressed sIg demonstrating a mature B cell origin. Reactivation of TdT expression has been observed in a murine B cell lymphoma in which ongoing L chain rearrangements occur (42). Therefore, an alternative explanation for the finding of sIg⁻, TdT⁺ tDL is that in the process of isotype switching in a subclone of the FL B cell, activation of the c-myc oncogene occurred giving rise to the LL. This conclusion is supported by the finding of Gauwerky et al. (8) that the translocation of chromosome 14 to chromosome 8 occurred in a sequence near the C_γ2 constant gene segment typical of switch regions. This alternative explanation was reached by Lee et al. (5) who reported a case of SNC-B developing in a patient with a one-year history of untreated FL. In that case the original FL had L chain rearrangements but was sIg⁻ because the only J_H rearrangement was the t(14;18) translocation, the second allele was in a germline configuration. The inability to produce a sIg because of the t(14;18) translocation has been documented in a number of cases (43). In the high grade tumor, subsequent translocation of t(8;14) resulted in activation of the c-myc oncogene.

The third model for the development of tDL is the sequential transformation of a mature FL B cell to a higher grade of malignancy (Fig. 4). This model is supported by the evidence presented here demonstrating the retention of idiotype expression in 12 of 13 cases of histologic conversion. However, in four cases either antiidiotypic reagents were
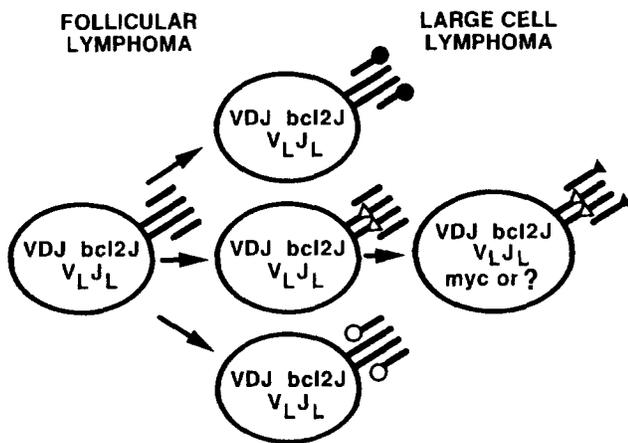
FOLLICULAR   LARGE CELL
LYMPHOMA   LYMPHOMA

Figure 4. Schematic model for histolgic transformation. Malignant cells in follicular lymphoma are characterized as mature B cells expressing sIg and having the t(14;18) translocation. As a result of somatic mutation the tumor population becomes heterogeneous with individual subclones marked by mutation of the Ig V gene segments. The data presented show that the tDL has a nonrandom pattern of mutation compared with the FL, supporting the conclusion that the tDL arises from a single FL subclone.

unavailable (JP, MT, FB) or the reagent failed to bind to the sIg of the tDL (EC). Analysis of these cases by Southern hybridization revealed clonal J_H rearrangements supporting a clonal origin for the FL and tDL. Since L chain rearrangement is the final step in B cell maturation, a common mature B cell origin was demonstrated in three cases (EC, FB, MT) by identity of L chain rearrangements. In case JP, the L chain rearrangements were nonidentical, suggesting either that FL and tDL had rearranged to different V_λ gene segments or that somatic mutation had altered restriction banding pattern (11). Sequence analysis of the rearranged L chains in the FL and tDL in case JP demonstrated the genes to be very similar. The J_λ segment from both the FL and tDL diverged from the germline J_λ minigene by four common mutations proving the common clonal origin of these tumors from a mature B cell. This sequential model can explain both the cases described here as well as those previously reported cases discussed above.

The V_H gene sequence data allow us to take this model one step further and to prove that transformation occurs as the result of changes in a single FL cell (Fig. 4). In V gene segments, the distribution of mutations is relatively random (41, 44). Therefore, the finding of repetitive mutations among independent cells in the tumor suggests that they have a common cell of origin (45). In this study, the FL V gene clones for case JP were obtained from a single tumor mass. Since there was a unique clonal immunoglobulin gene rearrangement based on the Southern blot analysis (Fig. 1, A–C, lanes 1–4) this mass represented clonal expansion of a single progenitor cell. However, as a result of ongoing somatic mutation individual cells within this tumor mass had generated subclones marked by different patterns of mutations. Among the 8 FL V_H clones examined only two positions along the

sequence showed a mutation more than once (Fig. 3). In contrast, the tDL V_H gene sequences from the JP tDL tumor and cell lines were highly homologous among themselves and had strikingly nonrandom and repetitive differences from the FL V gene consensus sequence. The changes seen in the tDL included a 3-bp deletion in the D-J join raising the alternate possibility that these tumors represent independent tumors using a restricted V_H gene pool. Several lines of evidence suggest this is not the explanation. First, the J_H segments of the FL and tDL were both derived from an identical fore-shortened form of the germline J_H6 and both had diverged from it by two common mutations, strongly suggesting a clonal origin of these two tumor populations. Similar analysis of the L chain sequences also supported a common clonal origin. Second, the V-D join which is composed of randomly added nucleotides and which follows in D-J join in B cell ontogeny, is identical in length and differs in sequence by only 2 bp. Furthermore, one of the changes in this sequence is also seen in the FL clone JP-FL-VH-3 suggesting this area has been subject to somatic mutation. Third, the D-J join that contains the 3-bp deletion in the tDL is otherwise identical to the FL consensus sequence for this region. Fourth, somatic mutation in Ig genes can include deletion of an entire codon (46, 47). The finding that 4 of the 28 mutations observed among the eight FL clones occurred in this 15-bp stretch where only one would have been expected if the mutations were distributed evenly suggests that the D-J join is a target for active mutation. Finally, there is no evidence for restricted V_H gene usage in FL; in more than 40 cases of FL V_H genes sequenced in this laboratory, no rearranged V_H gene segment is as similar to the JP-FL V_H as is the JP-DL V_H (Bahler, D., M. Campbell, S. Levy, and R. Levy, manuscript in preparation). Thus, the 3-bp sequence difference in the D-J join is most likely the result of somatic mutation and not an independently derived join. The nonrandom sequence changes among the tDL could have arisen only if tumor progression occurred in a single FL subclone that had previously accumulated a large number of sequence alterations by mutational events. The same conclusion can be reached from the sequence analysis of the H chain in case MT FL and tDL (data not shown). Thus, tDL results from the transformation of a single FL cell.

The sequence data also suggests that somatic mutation can occur in DLCL. In addition to the common, nonrandom mutations, the tDL V_H clones from case JP show several randomly distributed mutations which likely have occurred subsequent to the transformation event. Somatic mutation appears to occur at specific stages of B cell development. In FL, active somatic mutation is well described (36). In contrast, somatic mutation has not been observed in ALL, SNC-B, and small lymphocytic lymphoma, lymphoid malignancies representing other stages of B cell development (48–51). In DLCL it is not known if somatic mutation is ongoing. The three tDL cell lines developed from case JP provide a possible in vitro model system to study somatic mutation in DLCL.

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