

## Frequent somatic hypermutation of the 5' noncoding region of the *BCL6* gene in B-cell lymphoma

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Communicated by Paul A. Marks, Memorial Sloan-Kettering Cancer Center, New York, NY, September 8, 1995 (received for review July 26, 1995)

**ABSTRACT** The *BCL6* gene encodes a zinc-finger transcription factor and is altered by chromosomal rearrangements in its 5' noncoding region in ≈30% of diffuse large-cell lymphoma (DLCL). We report here that, in 22/30 (73%) DLCL and 7/15 (47%) follicular lymphoma (FL), but not in other tumor types, the *BCL6* gene is also altered by multiple ( $1.4 \times 10^{-3}$ – $1.6 \times 10^{-2}$  per bp), often biallelic, mutations clustering in its 5' noncoding region. These mutations are of somatic origin and are found in cases displaying either normal or rearranged *BCL6* alleles indicating their independence from chromosomal rearrangements and linkage to immunoglobulin genes. These alterations identify a mechanism of genetic instability in malignant B cells and may have been selected during lymphomagenesis for their role in altering *BCL6* expression.

Diffuse large-cell lymphoma (DLCL) accounts for 30–40% of all non-Hodgkin lymphoma (NHL) cases and often represents the final transformation stage of follicular lymphoma (FL) (1). Chromosomal translocations involving chromosome band 3q27 are found in 8–12% of NHL cases (2, 3) and have been shown to involve the *BCL6* gene (4, 5), also called *LAZ3* (6) or *BCL5* (7) (for a review, see ref. 8). Several studies have shown that *BCL6* rearrangements occur with an overall frequency of 30–40% in DLCL and 6–14% in FL and can be found in cases without cytogenetically detectable 3q27 abnormalities (9–11). *BCL6* rearrangements are associated with a subset of DLCL characterized by extranodal presentation and favorable clinical outcome (12).

The *BCL6* gene encodes for a protein containing six C-terminal zinc-finger motifs homologous to members of the *Krüppel* subfamily of zinc-finger proteins (13). The N-terminal portion of *BCL6* contains a ZiN (for Zinc-finger N-terminal)/POZ (POx/Zinc-finger) domain that is found in other *Drosophila* and mammalian zinc-finger proteins as well as in some poxvirus proteins (14). The *BCL6* gene is expressed in mature B cells within germinal centers, but not in immature B-cell precursors or in differentiated plasma cells (5, 7, 8, 15). The structural features and the pattern of expression of the *BCL6* protein suggest that it may function as a DNA-binding transcription factor, presumably involved in the control of B-cell differentiation and lymphoid organ development.

In DLCL and FL, chromosomal rearrangements affecting the *BCL6* gene cluster within a 4-kb region spanning the promoter and the first noncoding exon. In most cases, the chromosomal breakpoints are located immediately 3' to the first exon and cause the juxtaposition of *BCL6* exons 2–10 (spanning the entire coding domain) downstream to heterologous promoters derived from other chromosomes (5, 8, 11). These alterations lead to the production of chimeric transcripts

which, however, encode for normal *BCL6* proteins, suggesting that the functional consequence of these translocations is the deregulation of *BCL6* expression by promoter substitution (16).

To determine whether the *BCL6* gene may be altered by mechanisms other than chromosomal rearrangements, evidence for mutations in either the coding or regulatory sequences of *BCL6* was sought in DLCL, FL, and other tumor types by PCR/single-strand conformation polymorphism (SSCP) analysis. The results indicate that the *BCL6* 5' noncoding region, but not the coding domains, is frequently mutated in lymphoma.

### MATERIALS AND METHODS

**Tissue Samples and Genomic DNA Isolation.** Tumor biopsies were collected at the time of diagnosis which was based on histopathologic and immunophenotypic analysis (17). The fraction of malignant cells in the pathologic specimen was at least 70% as determined by cytofluorimetric analysis of cell-surface markers and/or rearrangement analysis of antigen receptor genes (17). Genomic DNA was isolated from the tumors and the *BCL6* genomic configuration was assessed as reported (9). Normal control DNAs were isolated from Epstein-Barr virus-immortalized lymphoblastoid cell lines (LCLs) established from the tumor biopsies of 25 of the cases studied (18). Twenty-two LCLs (a gift from Richard F. Ambinder, Oncology Center, Baltimore) were obtained from peripheral blood lymphocytes of healthy individuals. BJAB is a B-cell lymphoma cell line (19) and Ly1 is a DLCL cell line (20).

**PCR and SSCP Analysis.** Fifty-eight PCR primers (Fig. 1) were used corresponding to *BCL6* sequences within the cDNA (GenBank accession no. U00115) and/or unpublished intron sequences. Primer sequences and experimental conditions used for SSCP analysis are available upon request.

**Sequencing Procedures.** PCR products E1.10, E1.11, and E1.12 were subjected to direct sequence analysis as described (21). In addition, a unique PCR product encompassing the same genomic region (nucleotides +413 to +1141), amplified by primers E1.21C (upstream; 5'-ATGCTTTGGCTCCAA-GTT-3') and E1.26 (downstream; 5'-CACGATACTTCA-TCTCATC-3'), annealing temperature = 54°C, was subcloned into the pGEM-T vector (Promega), and DNA minipreps (Wizard DNA purification system, Promega) were sequenced using forward and reverse primers with the ABI373A DNA sequencer (Perkin-Elmer, Applied Biosystems Division).

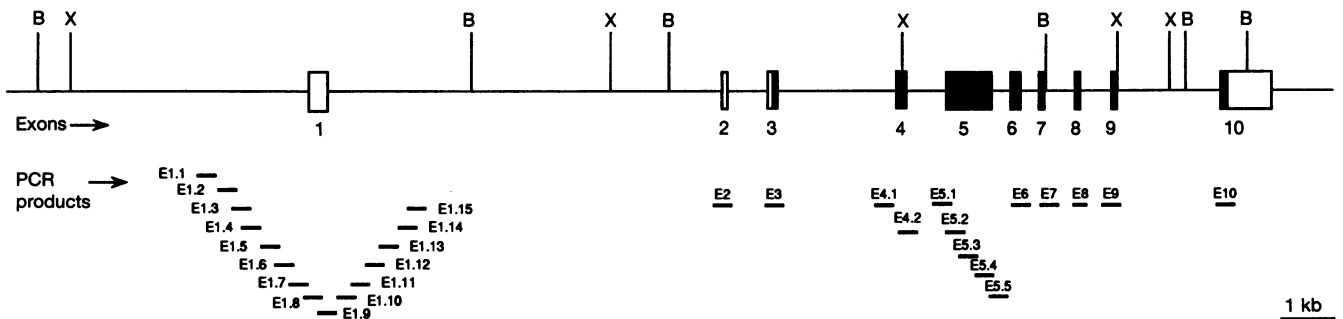


FIG. 1. Schematic representation of the *BCL6* sequences analyzed by PCR/SSCP. *BCL6* coding and noncoding sequences are represented by filled and empty boxes, respectively. PCR fragments used for SSCP analysis are approximately positioned below the map. B, *Bam*HI; X, *Xba* I.

## RESULTS

**Frequent Somatic Mutations in 5' Noncoding Sequences of *BCL6* in DLCL and FL.** A panel of 45 NHLs representative of DLCL (30 cases) and FL (15 cases) was selected for this study (Table 1). This panel included cases displaying germ-line as well as rearranged *BCL6* alleles as previously characterized by Southern blot analysis of genomic DNA (9). Karyotype information was available for 13 cases which carried normal or abnormal chromosomal band 3q27, the site of the *BCL6* gene (4).

*BCL6* sequences representing exons 2–10 and spanning the entire coding region of the gene were examined by PCR/SSCP analysis using the set of primers illustrated in Fig. 1. No SSCP variants were observed in these sequences in 22 DLCL cases tested (data not shown). Thus, in agreement with a recent report (11), we concluded that mutations in *BCL6* coding sequences do not occur at any appreciable frequency in NHL.

In contrast, a large number of SSCP variant patterns was detected in a 3.5-kb region spanning the first noncoding exon (Fig. 1) in many NHL cases (Table 1 for a summary of the data; Fig. 2 for representative PCR/SSCP results). A subset of these variants was classified as germ-line polymorphisms since they were recurrent (homozygous or heterozygous) in 22 control DNAs from Epstein–Barr virus-immortalized LCLs established from peripheral blood lymphocytes of healthy individuals (PCR fragments E1.2, E1.5, E1.6, E1.11, E1.12, E1.13; Fig. 2 for examples). An additional 71 variant SSCP patterns were noted which were unique to individual tumor DNAs. These variants were distributed in 29 tumor cases (64%), including 73% of DLCL and 47% of FL. These cases included tumors carrying unrearranged *BCL6* genes (63%) as well as 7/10 tumors carrying a rearranged *BCL6* allele (Table 1). In the latter cases, the allele affected by the mutation(s) was not identified. However, the presence of a biallelic SSCP variant in a case with a single rearranged *BCL6* allele (not shown) indicated that mutations could occur on the rearranged as well as the unrearranged allele in some cases. Although most SSCP variants (67/71) were heterozygous with retention of the normal allele, homozygous shifts with little representation of the normal allele (most likely from normal DNA contaminating the tumor biopsy) were also observed in 4/71 cases, suggesting either the loss or the mutation of the second allele (see below).

Table 1. Frequency of NHL cases with *BCL6* mutations

Sample	Total*	3q27			<i>BCL6</i> status	
		Normal	Altered	ND	Normal	Rearranged
<b>NHL</b>						
DLCL	22/30	7/8	1/5	14/17	15/20	7/10
FL	7/15	0/0	0/0	7/15	7/15	0/0
LCL	0/25	ND	ND	ND	ND	ND

ND, not determined.

\*The frequency of mutations is reported as number of cases mutated/no. tested.

To determine whether the observed mutations were of somatic origin, the DNA of Epstein–Barr virus-immortalized LCLs established from nonneoplastic B cells of 25 of the cases studied (see *Materials and Methods*) was similarly analyzed. Except for those SSCP variants previously identified as population polymorphisms, all other SSCP variants were absent in the control DNA (Fig. 2). Thus, most of the mutations affecting *BCL6* were of somatic origin and associated with DLCL and FL.

Fig. 3 summarizes the distribution of SSCP variants in the panel of cases analyzed with the exception of those attributed to polymorphisms. Mutations were found throughout the 3.5-kb sequences examined, with the majority of cases displaying multiple variant fragments. However, a stretch of 730 bp (represented by PCR products E1.10, E1.11, E1.12) located  $\approx$ 100 bp downstream the first noncoding exon could be

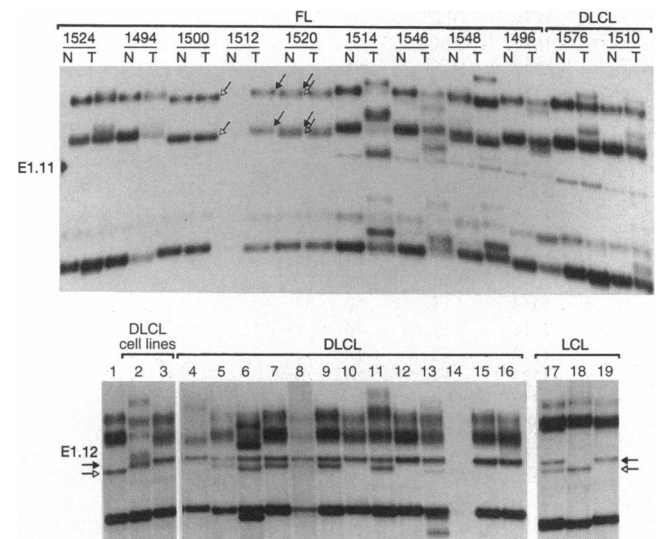
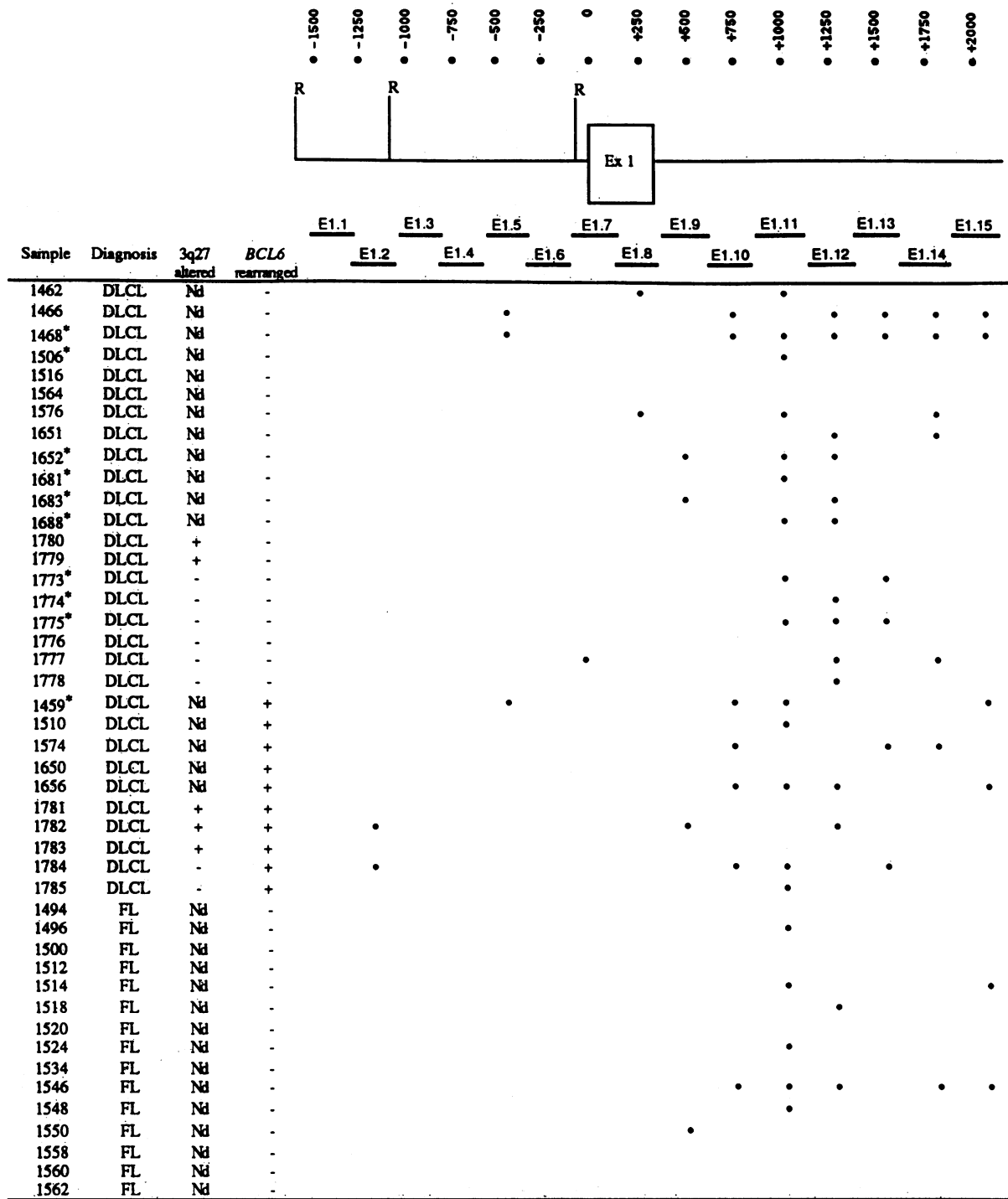


FIG. 2. Mutations in the 5' noncoding region of *BCL6* detected by PCR/SSCP analysis. Representative results obtained for PCR products E1.11 and E1.12 in cell lines and DLCL and FL biopsies are shown. (Upper) The number of the tumor specimen or the name of the cell line is indicated at the top of each lane; for selected cases tumor (T) and LCL (N) DNAs were analyzed. (Lower) Lanes: 1, LCL; 2, BJAB; 3, LY1; 4, 1468; 5, 1462; 6, 1774; 7, 1780; 8, 1683; 9, 1576; 10, 1779; 11, 1652; 12, 1773; 13, 1688; 14, 1785; 15, 1783; 16, 1784; 17–19, LCL lines. For each PCR product analyzed, several bands are usually detected representing different denatured conformers and partially denatured products. Arrows (open or closed) point to the two alleles of germ-line polymorphisms. For fragment E1.11, patients 1500, 1512, and 1520 represent examples of the two homozygous and the heterozygous patterns, respectively (sequence analysis demonstrated that this polymorphism was G–C at position +753; not shown). For fragment E1.12, homozygous and heterozygous patterns are detectable in tumor as well as in normal control DNAs (T insertion at +876; not shown).



\*Tumor case that has been sequenced in the region from E1.10 to E1.12.

FIG. 3. Distribution of SSCP variants within the 5' noncoding region of *BCL6* in 45 NHL tumors studied. The *BCL6* 5' noncoding region spanning the first exon (box) is represented at the top (R, *EcoRI* site). Below and aligned with the map are listed the PCR products analyzed by SSCP. Each dot represents a variant SSCP pattern.

identified as a major cluster since 28/29 (96%) of the samples with mutations were altered in this region.

**Sequence Analysis of Mutated *BCL6* Alleles.** To confirm and characterize the mutations affecting the 5' region of the *BCL6* gene in NHL, we performed a sequence analysis of the most frequently mutated *BCL6* region (PCR products E1.10, E1.11, E1.12) in 10 DLCL cases (indicated by the asterisks in Fig. 3). A two-step approach was followed in order to obtain information on the nature of the mutations as well as their allelic distribution. For each case, the PCR products E1.10, E1.11, and E1.12 were subjected to direct sequencing; a single PCR

product spanning the same three regions was then subcloned into plasmid and at least four independent subclones were sequenced.

In the 10 cases studied, a total of 59 alterations was detected by direct PCR sequencing including single base-pair substitutions ( $n = 55$ ), small deletions ( $n = 3$ ), and one insertion (Table 2 for a summary of data; Fig. 4 for representative results). For each tumor case studied, all PCR fragments which appeared abnormal by SSCP analysis were found to contain mutations by DNA sequencing; in addition, in 4 cases, PCR products which appeared normal by SSCP analysis were found

Table 2. Mutations in the 5' noncoding region of *BCL6* in DLCL

Sample	Allele	Mutation (position*)
1468	A	G → A (440), C → G (479), A → C (558), G → A (428), Δ <sup>†</sup> TCGCTCTT (776–783), G → A (793), G → C (797), G → A (799), A → G (811), C → T (916), C → T (1052), T → C (1095)
	B	A → T (472), C → T (479), A → C (527), T → C (545), C → A (546), T → C (559), G → C (600), G → A (733), G → T (793), C → G (798), T → A (800), Δ (1104)
1652	A	T → C (554), T → A (574), T → G (622), T → G (631), A → G (638)
	B	T → C (431), A → G (452), G → C (549), C → G (779), T → C (908), A → G (964)
1681	A	G → A (655), G → A (665), C → T (785), G → A (804), G → A (1022)
	B	A → T (577), G → C (753)
1683	A	T → C (1032)
	B	—
1506	A	T → G (789), G → C (790), T → G (836)
	B	+T (1104)
1688	A	C → T (934)
	B	—
1773	A	C → A (745)
	B	—
1774	A	T → C (1092)
	B	—
1775	A	Δ <sup>†</sup> TCGGG (719–723), C → A (743), A → G (1076)
	B	—
1459	A	C → G (434), T → A (499), G → A (567), T → C (717), C → G (785)
	B	—

\*The first nucleotide of the *BCL6* cDNA is arbitrarily chosen as position +1.

†Δ, Deletion.

to contain mutations upon sequencing, confirming the limited sensitivity of the SSCP assay. All mutations found by direct sequencing of the PCR products were found in the subcloned products where they were distributed in one or two subcloned alleles (Table 2), suggesting that they derived from a single clonal population and that no major subclonal variation was present in the tumor biopsies. Four additional mutations (distributed in 4 cases), which were not detectable by direct PCR sequencing, were found in subcloned products; however, none of these 4 mutations was confirmed upon analysis of additional (4) clones derived from an independent PCR reaction, suggesting that they might have been caused by *Taq* polymerase errors. In 4 of 10 cases both alleles carried mutations, although all mutations were heterozygous (Table 2; Fig. 4 for an example). No bias in the type of single base-pair substitution was detected (not shown), although the number of mutations studied may not be sufficient for statistically informative analysis. The number of lesions per allele was variable, ranging between 1 and 12 over 730 bp analyzed, corresponding to an incidence of  $1.4 \times 10^{-3}$ – $1.6 \times 10^{-2}$  per bp.

***BCL6* Is Not Mutated in Other Tumor Types.** To determine whether the observed *BCL6* mutations were specific for NHL, a panel of 123 nonhematologic tumor cases was screened for mutations in the sequences most frequently mutated in NHL (E1.10, E1.11, E1.12; Fig. 1) by PCR/SSCP analysis. Breast ( $n = 20$  cases), prostate ( $n = 20$ ), stomach ( $n = 13$ ), colon ( $n = 20$ ), liver ( $n = 10$ ), and kidney ( $n = 20$ ) carcinomas and glioblastomas ( $n = 20$ ) displayed no SSCP variant, except for the previously detected population polymorphisms.

## DISCUSSION

This study reports that the 5' noncoding region of the *BCL6* gene is altered by somatic mutations in a significant fraction of

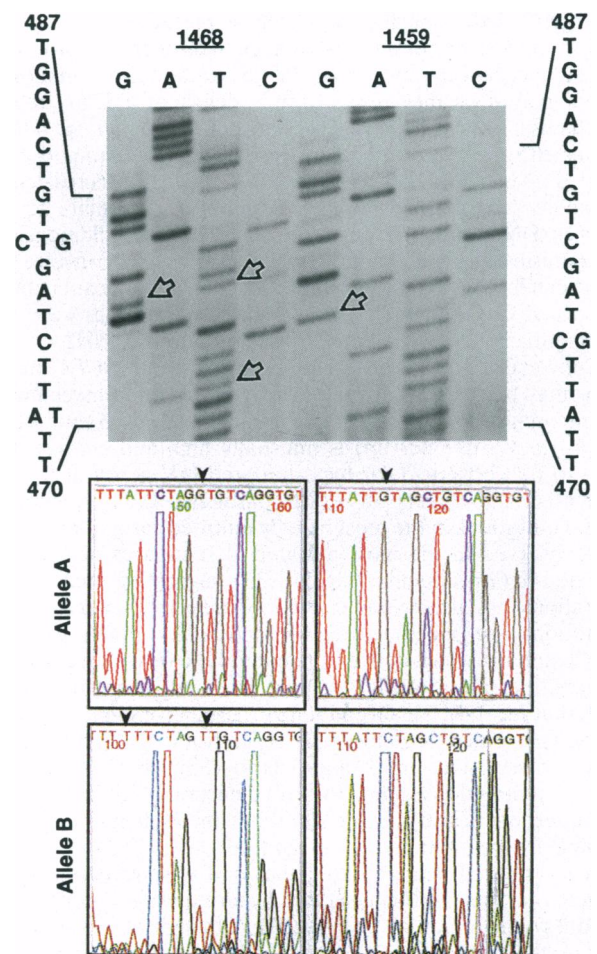


FIG. 4. *BCL6* mutations detected by nucleotide sequencing in DLCL. Representative results of direct sequencing of PCR product E1.10 in two DLCL cases are shown. Arrows point to mutations. Shown below the autoradiograms is the chromatogram generated by automatic sequencing of the same fragments after cloning into plasmid. Note that all mutations detected by direct sequencing are found after cloning and are distributed in two alleles (tumor 1468) with each mutation being heterozygous.

NHL including 73% DLCL and 47% FL. Since this region was previously shown to be involved in chromosomal rearrangements in a smaller fraction of the same tumors (33% DLCL, 0% FL; Fig. 3), our findings indicate that the same domain of the *BCL6* gene can be altered by different types of alterations. Mutations and rearrangements can occur independently in different tumor cases or concomitantly in the same tumor. Considering their combined frequencies, the underestimated frequency of mutated cases due to the limited sensitivity of the SSCP assay, and the incomplete definition of the 3' boundary of the mutated region (see Fig. 3), our results suggest that virtually all DLCL and the majority of FL may contain structural alterations of the *BCL6* gene.

Mutation frequencies similar to those detected for *BCL6* in NHL were previously observed for the *MYC* (22, 23) and *BCL2* (24) genes when translocated and juxtaposed to immunoglobulin (Ig) loci in lymphoma. In both these cases, these mutations were attributed to the Ig variable region (IgV) gene somatic hypermutation mechanism acting on sequences that had become linked to the Ig locus following chromosomal translocation (22–26). In contrast with these previous observations, the *BCL6* mutations reported here frequently occur independently of translocation to Ig loci and in the absence of any recognizable chromosomal abnormality affecting band 3q27 or rearrangement of the *BCL6* locus. Thus, *BCL6* mutations

represent a locus-specific somatic hypermutation event occurring in the absence of physical linkage to antigen receptor loci.

The mechanism underlying *BCL6* mutations is unknown. These mutations may result from a defect in a DNA repair mechanism which, however, must be distinct from the DNA mismatch repair pathway defective in various solid tumors (27) since the same NHL cases used for this study lack microsatellite instability (unpublished results), the molecular hallmark of defects in DNA mismatch repair enzymes (28). In addition, the mechanism causing *BCL6* mutations does not seem to involve the short-patch DNA repair enzymes that correct the deamination-associated G-T mispairs (29), since these mismatches were not preferentially observed among *BCL6* mutations in NHL.

Conversely, several observations suggest that *BCL6* mutations may be the result of the IgV hypermutation mechanism acting on non-Ig loci. First, the frequency of *BCL6* mutations ( $0.14\text{--}1.6 \times 10^{-2}$  per bp) is unusually high and comparable only to that observed for hypermutated IgV genes in B cells ( $0.4\text{--}10.6 \times 10^{-2}$ ; refs. 30–32). In particular, the frequency of *BCL6* mutations in the most heavily mutated cases approaches that observed in the most mutated V sequences in antigen-selected IgG-producing B cells (33) suggesting that *BCL6* mutations may also be under selective pressure. Second, *BCL6* mutations are specifically associated with tumors such as DLCL and FL, which derive from mature B cells within germinal centers, the site where IgV sequences undergo somatic hypermutation (33, 34). Finally, although the relatively small sample studied prevented the detection of a significant bias in transitions versus transversions and strand polarity (35), which often characterize mutated IgV genes, the rare detection of small deletions and insertions is also consistent with the involvement of the IgV somatic hypermutation mechanism (36).

A recent study has reported that non-Ig sequences can be targeted by somatic B-cell-specific hypermutation in an experimental system in transgenic mice (37). Although, at variance with *BCL6*, the non-Ig sequences were embedded in an Ig gene, the two sets of results may be related. In fact, *BCL6* mutations may represent the first naturally occurring example of an ectopic activity of the hypermutation mechanism involving sequences which display no homology with IgV genes. If this is the case, it remains to be determined whether *BCL6* mutations derive from a tumor-associated *misfunction* or a normal, presently unrecognized, activity of the IgV hypermutation mechanism on sequences outside the Ig loci. The absence of *BCL6* mutations in nontumorigenic B cells (LCL; Fig. 2) does not allow us to exclude the latter hypothesis since these cells also lack IgV hypermutation due to their immature pregerminal center phenotype (18). The two hypotheses (abnormal versus normal activity of the IgV hypermutation mechanism) can be discriminated by comparative analysis of the frequency of mutations within IgV versus *BCL6* sequences in purified normal germinal center B cells (34). Regardless of the nature of the mechanism involved, the existence of hypermutation events in non-Ig sequences identifies a mechanism of genetic instability with general implications for lymphomagenesis. In fact, if hypermutation can occur outside Ig loci, *BCL6* may be only one of many possible targets of physiologic or pathologic significance.

The frequency, tumor association, and clustering of *BCL6* mutations strongly suggest that they may have been selected based on their functional role during tumorigenesis. The genomic sequences most frequently involved in the mutations are adjacent to the *BCL6* promoter region and may therefore contain regulatory signals for transcription. Notably, the region involved in the mutations also represents the major cluster of chromosomal breakpoints and is either truncated or removed in most rearranged *BCL6* alleles in NHL (5, 9, 11). Thus, mutations and rearrangements may be selected for their ability to alter this region which may be important for the normal regulation of *BCL6*, possibly its down-regulation during differentiation of B cells into plasma cells (8, 15). Studies

involving *in vitro* cell transformation systems and *in vivo* expression in transgenic mice are needed to determine whether mutated *BCL6* alleles play a role in lymphomagenesis.

This work was supported by National Institutes of Health Grants CA-44029 (to R.D.-F.), CA-34775 (to R.S.K.C.), and CA-48236 and EY-06337 (to D.M.K.). A.M. and B.H.Y. are supported by fellowships from Associazione Italiana Ricerca sul Cancro and the Leukemia Society of America, respectively. K.O. is a recipient of a Clinical Oncology Center Development Award from the American Cancer Society.

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