



The *Bcl6* locus is not mutated in mouse B-cell lineage lymphomas

Mitsuo Hori^{a,*}, Chen-Feng Qi^a, Ted A. Torrey^a, Konrad Huppi^b, Herbert C. Morse III^a

^a Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, 7 Centre Drive, Room 304, MSC 0760, Bethesda, MD 20892, USA

^b Laboratory of Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract

In normal human germinal centre (GC) B-cells and post-GC B-cell lymphomas, a region in the first intron of the *BCL6* gene, termed the major mutations cluster (MMC) exhibits somatic point mutations and deletions with patterns very similar to those seen in the variable regions of immunoglobulin (*Ig*) genes. In studies of mouse post-GC diffuse large cell lymphoma, Burkitt lymphomas, and plasmacytomas, direct sequencing or cold SSCP analyses revealed no mutations within a 686-bp region in *Bcl6* intron 1 with 72% identity to the human MMC. The mouse *Bcl6* locus must be inaccessible to the mutational machinery responsible for somatic mutations of *Ig* and *BCL6* in humans. Published by Elsevier Science Ltd.

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1. Introduction

BCL6 is a sequence-specific transcription factor expressed primarily in B- and T-cells of the germinal centre (GC) [1–3]. It was originally discovered as the product of a gene involved in translocations in certain types of human B-cell non-Hodgkin's lymphomas (NHL), most prominently those of diffuse large B-cell lymphoma (DLBCL) [4–6]. The translocations effect the replacement of *BCL6* 5' regulatory sequences with those of other genes on the translocation partner while leaving the coding region intact. Of interest, a high proportion of DLBCL as well as other B-cell lymphoma types exhibit mutations within the 5' non-coding region in the presence or absence of translocations [7–12]. Detailed studies of the mutations have shown that they have most if not all features of those within immunoglobulin gene variable region (*IgV*) sequences [7]. These include: (a) an overwhelming predominance of single base-pair substitutions, although single base insertions and deletions are observed; (b) a preference for transitions over transversions; (c) the RGYW motif as a target for increased mutational activity; (d) localisation within 2 kb of the tran-

scriptional start site but not in more distal regions; and (e) biallelic changes [7–12]. The frequency of *BCL6* mutations in lymphomas was one to two orders of magnitude lower than that for *Ig* mutations in the same tumours [7], although *BCL6* mutations can occur in the absence of *IgV* region mutations [12]. In a human B-cell lymphoma model system for inducible *IgV* region mutations, however, the rate of *BCL6* mutations was essentially the same in pattern and frequency as that for *Ig* [13].

Remarkably, studies of the same *BCL6* sequences from normal memory or tonsillar GC B-cells isolated from human peripheral blood or tonsils showed that they exhibited the same pattern and frequency of mutations as those in *Ig* sequences [14,15]. *MYC*, *SI4*, α -fetoprotein, or β -globin genes from the same cells were not mutated [14], indicating specificity for *BCL6* as a target. This suggested that the same mutation machinery that operates on *IgV* region sequences also targets *BCL6* for mutation. Unexpectedly, similar studies of normal GC cells from mice showed that they were not mutated, while *IgV* region sequences from heavy chain genes in the same cells were heavily mutated [16]; however, the genomic regions scanned for mutations in the mouse cells were less extensive than those studied for the human tumours.

Understandings of the pathogenesis of mouse B-lineage lymphomas have recently undergone major changes with the realisation that they comprise several histologically distinct entities that have parallels with human lymphoma or leukaemia [17–22]. Although issues of nomenclature are yet to be fully resolved, lymphomas derived from GC or

Abbreviations: CB, centroblastic; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; GC, germinal centre; IB, immunoblastic; *IgV* region, immunoglobulin gene variable region; MMC, major mutations cluster; MuLV, murine leukaemia virus; NHL, non-Hodgkin's lymphoma; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism

* Corresponding author. Tel.: +1-301-496-9529; fax: +1-301-402-0077.

E-mail address: mhor@niaid.nih.gov (M. Hori).

post-GC cells include those similar to human DLBCL and plasmacytoma [19–22]. The present studies were undertaken to determine whether mutations of *BCL6* might be found in lymphomas if one examined the regions of the mouse locus homologous to the major mutation complex in intron 1 of the human gene. The results demonstrate that no mutations were found in a large panel of cell lines derived from pre- and post-GC B-cell lymphomas as well as primary DLBCL.

2. Materials and methods

2.1. Lymphomas and cell lines

Primary lymphomas were from NFS.V⁺ mice, NFS/N mice congenic for ecotropic murine leukaemia virus (MuLV) induction loci of AKR and C58 [17,23]. Tissues obtained at autopsy were fixed in formalin, sectioned, and stained with hematoxylin and eosin. Histologic diagnoses of DLBCL of centroblastic (CB) and immunoblastic (IB) subtypes were made using established criteria [18,19]. Samples of involved spleen and lymph were frozen at -80°C for later preparation of DNA.

A series of cultured cell lines has been generated from primary lymphomas appearing in NFS.V⁺ mice; NFS/N, FVB/N, or NIH Swiss mice infected with different MuLV; and C57BL/6 mice carrying a human *MYC* transgene driven by human Ig λ light chain 3' regulatory sequences [21] or Ig κ light chain regulatory sequences (Morse III, unpublished data). The cell lines, associated diagnoses, and prior references when applicable are listed in Table 1. Pellets of cultured cells were frozen at -80°C for later preparation of DNA.

2.2. DNA polymerase chain reaction (PCR) and cold single-strand conformational polymorphism (SSCP) analysis

DNA was prepared from frozen tissue samples of primary tumours or pellets from cell lines using standard techniques. Primers for different regions of the mouse *Bcl6* locus were

prepared based on the sequence of 10 kb of the NFS/N *Bcl6* locus covering 2 kb upstream of the start site and the entire first exon and intron [22]. Sequence comparisons with the human *BCL6* locus identified a region with highest homology to the sequences in the human major mutations cluster (MMC) in intron 1 as well as 5' of the first exon (Fig. 1). The two sets of primers amplified overlapping fragments from the first intron covering a total of 686 bp with 75.6% identity to the human sequence from this region. The amplified fragment from the 5' flank of exon 1 covered 854 bp with a comparable level of identity to the human sequences. For comparison, the identity score for exon 1 is 81.2%.

For DNA PCR, 50 μl PCR reaction mixture comprised MgSO₄ (2.0 mM), Tris (pH 8.3, 20 mM), KCl (50 mM), dNTP (0.2 mM), platinum *Taq* polymerase high fidelity (2.5 U) (Gibco-BRL), appropriate primer pairs (0.2 μM each), and 50 ng genomic DNA. PCR mixtures without polymerase were first heated to 96°C for 5 min before adding 0.3 μl of *Taq* per 50 μl reaction at 80°C . Cycling conditions were: 96°C for 30 s; 56°C for 30 s; 72°C for 30 s; for 30 cycles followed by 4 min at 72°C .

SSCP analysis was conducted utilising a nonradioactive technique [24]. To detect and re-amplify the mutant band directly from the gel, we used the pre-cast 10 cm \times 10 cm 20% TBE gel and the ThermoFlowTM electrophoresis temperature control system (Novex Experimental Technology) to rapidly identify mutant bands under constant, optimised temperature conditions. PCR samples are prepared for SSCP as follows: 6 μl PCR product, 6 μl 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol. The mixture was incubated at 98°C for 5 min, then placed on ice prior to loading the entire 12- μl sample. Each sample was analysed at 17, 22, 27, and 32°C . The gels were run at 150 V constant voltage with TBE running buffer until the xylene cyanol reached the bottom of the gel. The gels were stained with SYBR Green II.

2.3. Molecular cloning and nucleotide sequencing

The PCR products were sequenced after subcloning into the TA plasmid vector (Invitrogen). All clones were

Table 1
Mouse lymphoma cell lines used to study *Bcl6* mutations

Histologic diagnosis of primary tumour	Cell line(s)
Precursor B-cell lymphoblastic lymphoma	NFS-5, -70, -105, -112, -1252, -1437
Follicular lymphoma	NFS-202, -203
Burkitt lymphoma	B6.003-1, -1MLN, -2, -3, -4, -5, -6Spl, -6ILN, -6MLN, -EB2ILN, -EB2Spl, B6.KH3485-1, -2, B6.T1, B6.T2
Burkitt-like lymphoma	NFS-8, -806, FVB-1, NIH SW-1, NIH SW, WEHI 231
Diffuse large cell lymphoma	
Centroblastic	NFS-201
Immunoblastic	NFS-890
Plasmacytoma	TEPC-2241, -2242, -2243, -2245, -2246, -2247, -2248, -2249, -2250, -2251, ABPC-18, -22, -23, -48, -52, -65, -71, -100, -102, -105, MOPC-104E, -167, -1734, -348, -460, SIPC-5581, -5634, -5976, -348, -460, SIPC-5581, -5634, -5976

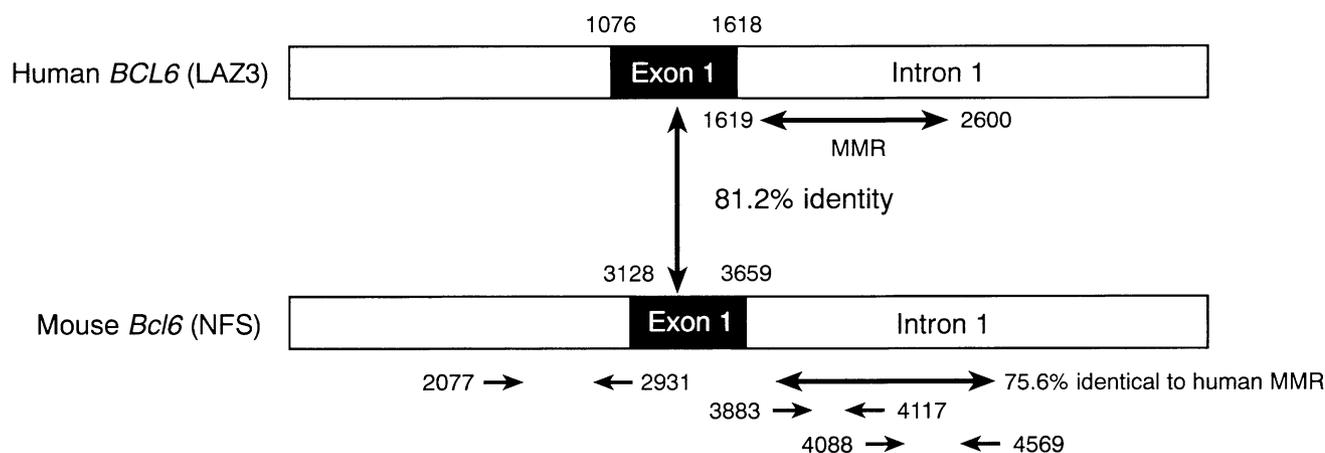


Fig. 1. Comparative structure of the 5' non-coding regions of the human LAZ3 *BCL6* and mouse *Bcl6* sequences indicating the location of the first exon and intron. The numbers for the human sequence are from Bernardin et al. for LAZ3 [30]. The mouse sequence numbers are for NFS/N, which differs from the published *Bcl6* sequence for NIH Swiss mice.

sequenced from both directions using the M13 forward and reverse primers. The sequencing reactions were performed using IRDye™ 800 termination mixes kit (LI-COR) according to the manufacturer's instructions. Fragments were analysed with the NEN® Global IR² DNA sequencer (LI-COR).

3. Results and discussion

3.1. DLBCL in mice

Studies of human NHL showed the highest frequencies of cases showing mutations of *BCL6* were follicular (FL), DLBCL, and Burkitt [7]. Although neoplasms with some features of human FL have been recognised to occur in mice [17–22], there are insufficient histologic and molecular similarities to allow them to be defined as true homologs of the human disease. This is not the case for Burkitt lymphoma, as a recently developed, molecularly engineered mouse model exhibits compelling parallels to the human disease [21].

Mouse lymphomas diagnosed as CB and IB (Fig. 2) have many similarities to human lymphomas now included under the umbrella of DLBCL in the proposed World Health Organisation classification of human lymphomas [25]. Mouse CB comprises cells with a moderate amount of basophilic cytoplasm and a round vesicular nucleus with conspicuous nucleoli attached to the nuclear membrane. It has been suggested that an origin from follicular or marginal zone cells can be discerned in some cases, but not in those where the normal splenic and lymph node architecture is completely obscured by exuberant growth of the tumour. The cases of primary CB chosen for this study include only those with the diffuse growth pattern (Fig. 2A and B).

Mouse IB is a high-grade tumour as shown by a high mitotic index (Fig. 2C). The cells are large and feature a

nucleus with a dispersed chromatin pattern and often a single bar-shaped nucleolus (Fig. 2D). As noted previously, most cases with greater than 50% cells of this phenotype are from CFW mice [19]. The cases studied here were primarily from NFS.V⁺ mice in which 30–50% of cells were immunoblasts admixed with centroblasts and some centrocytes.

We have previously made a case for inclusion of mouse lymphomas with lymphoblastic morphology but expressing surface *Ig* within the category of DLBCL [20,22]. This lymphoma type has been found to exhibit structural alterations in *Bcl6* sequences in two large series of lymphomas [20,22]. Nonetheless, the histologic picture can be indistinguishable from that of mouse Burkitt lymphoma but would never be confused with subsets of DLBCL. Consequently, an international nomenclature committee decided to apply the diagnosis of Burkitt-like lymphoma to these neoplasms.

3.2. Analyses of mouse *BCL6* for mutations

DNA amplified from genomic sequences just upstream from exon 1 and in the regions homologous to the human MMC was cloned and sequenced for five cell lines including one precursor B-cell lymphoblastic lymphoma and four DLBCL. All sequences were germline, including that for WEHI 231, which carries a T(5;16) *Bcl6* translocation [22]. No mutations were found among sequences from the 5' region of *Bcl6* in an additional four cell lines and six primary lymphomas. Together, approximately 19,000 bp of germline sequence were found among the lymphomas including 6175 bp from the MMC region.

To further evaluate *Bcl6* sequences in post-GC B-cell neoplasms, we performed cold SSCP analyses of the overlapping sequences within the MMC region for 28 different plasmacytomas and 15 Burkitt lymphoma samples from 12 mice (Table 1). This technique has been successfully employed to detect point mutations and deletions within

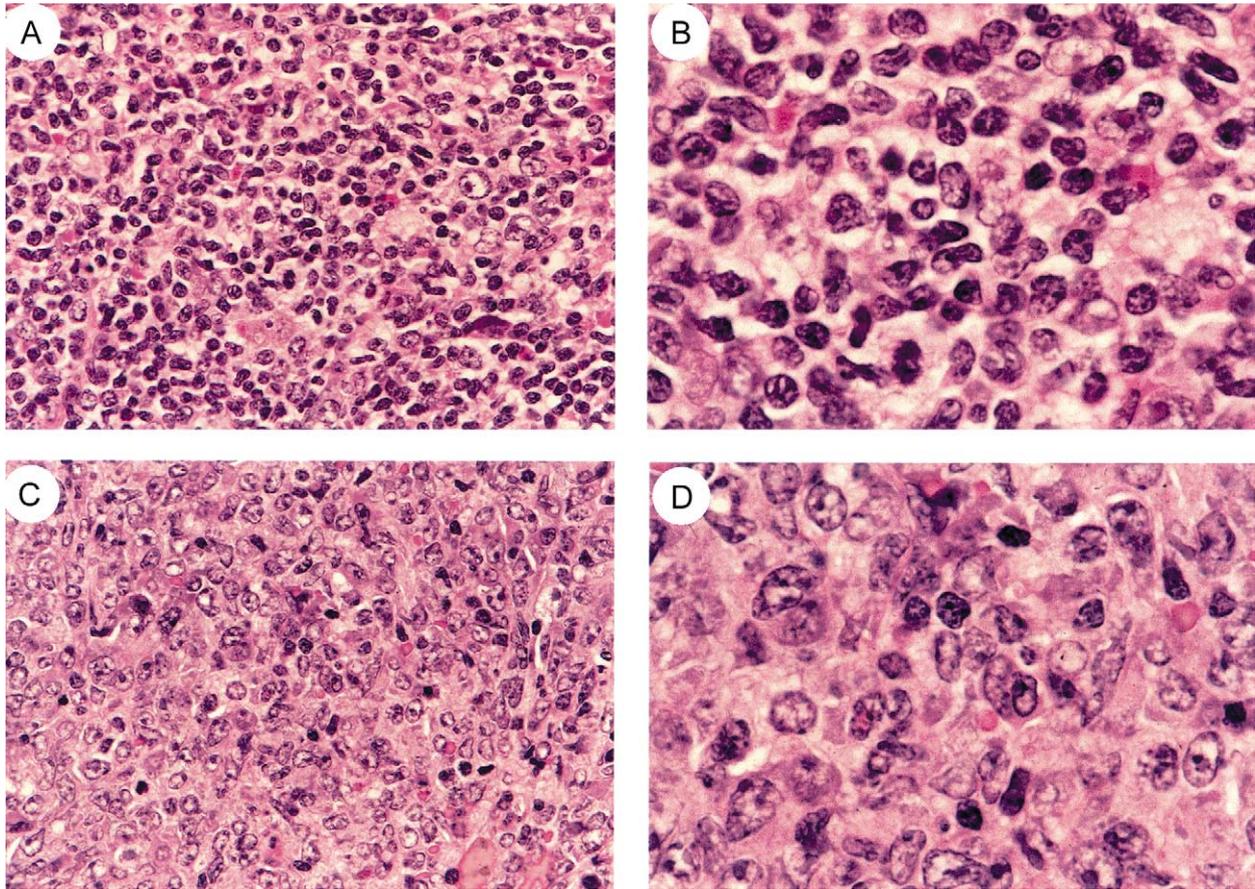


Fig. 2. Histologic features of mouse DLBCL. Low power (20 \times) and high power (80 \times) views of (A and B) mouse CB lymphoma and of (C and D) IB lymphoma.

p53 in the mouse model of Burkitt lymphoma (Hori et al., manuscript in preparation). No *Bcl6* polymorphisms were detected.

It has been shown that the frequency of *BCL6* mutations in human NHL is approximately 10-fold lower than for *Ig* variable region sequences in the same tumour [7–11]; however, mutations of *BCL6* have been found to occur in the absence of *Ig* hypermutation in CLL [11]. In addition, recent studies have shown that *BCL6* is not the only gene targeted for *Ig*-like mutations in human NHL. Mutations in the proto-oncogenes *PIMI1*, *MYC*, *RhoH/TTF*, and *PAX5* were found in more than 50% of DLBCL [26]. It was therefore important to know whether the lymphomas and PCT studied here had mutations in *IgV* region sequences or in the genes listed above. SIPC3336 and SIPC3370 have been shown to have low levels of somatic mutations with high replacement to silent ratios in V_{κ} and V_{λ} [27]. The light chain genes of MOPC167 and MOPC460 are also mutated, while those of MOPC104E and ABPC22 are germline [28]. Information regarding the *IgV* region sequences of the other tumours is not available. Of interest, although *Ig* sequences are not available, TEPC2251 and TEPC2245 both have mutations in the *Myc* transactivation domain [29].

These studies demonstrate that mouse *Bcl6* sequences with high homology to the human *BCL6* MMC are not mutated in mouse NHL of multiple histologic types including those with strong parallels to human post-GC NHL. The reasons for this species difference are not clear. The many similarities between the types of mutations seen in human *IgV* regions and the *BCL6* MMC strongly suggest that the same mutational machinery is operating on both sequences. Because mouse *IgV* region sequences also undergo somatic hypermutation, our results suggest that mouse *Bcl6* sequences are not recognised by the machinery responsible for *Ig* mutations, due to sequence differences, or that they are inaccessible to the machinery due to chromatin configuration, methylation status, or other epigenetically determined features. Studies designed to directly test this model by introducing mouse *Bcl6* genomic sequences to a human B-cell lymphoma cell line that can be induced to mutate both human *IgV* and *BCL6* sequences [13] are in progress.

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