

Genomic instability in mouse Burkitt lymphoma is dominated by illegitimate genetic recombinations, not point mutations

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λ -*MYC*-induced mouse Burkitt lymphoma (BL) harboring the shuttle vector pUR288, which includes a *lacZ* reporter gene to study mutagenesis, was employed to assess genomic instability associated with *MYC* deregulation. The frequency of *lacZ* mutations in lymphomas was elevated only 1.75-fold above that in normal tissue, indicating that mouse BL does not exhibit a phenotype of hypermutability. However, the nature of *lacZ* mutations was strikingly different in normal tissues and lymphomas. While point mutations comprised approximately 75% of the mutations found in normal tissues, apparent translocations, deletions and inversions constituted the majority of mutations (~65%) in lymphomas. Genomic instability in mouse BL thus seems characterized by a preponderance of illegitimate genetic rearrangements in the context of near-background mutant frequencies. SKY analyses of cell lines from primary BL tumors revealed substantial changes in chromosomal structure, confirming the *lacZ* studies. Bi-allelic deletions of the tumor suppressor p16^{Ink4a} were detected in six out of 16 cell lines, illustrating cellular selection of advantageous mutations. Together, these approaches indicate that *MYC* may contribute to lymphomagenesis through the dominant mutator effect of inducing chromosomal instability. The results further suggest that a phenotype of hypermutability (elevated mutant frequency) may not always be required for oncogenesis to occur.

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Deregulation of *MYC*, a proto-oncogene central to the coordination of cellular growth, proliferation, differentiation, and apoptosis, is a common feature of many

forms of cancer. Although the precise mechanisms by which *MYC* drives the oncogenic process are not understood (Cole and McMahon, 1999), it is becoming increasingly clear that one consequence of deregulated *MYC* expression with a major impact on cell transformation is genomic instability (reviewed in Mushinski and Mai, 2002). *MYC* induced genomic instability appears to favor gene amplifications, gene rearrangements, and chromosomal aberrations (Felsner *et al.*, 2000). This spectrum of mutations suggests that the contribution of *MYC* to tumor development may be primarily mediated by structural modifications of the genome, rather than point mutations. To test this hypothesis in a mouse model of *MYC* induced neoplasia, we decided to generate λ -*MYC*/pUR288 doubly transgenic mice. The λ -*MYC* transgene utilizes the 3' enhancer and other regulatory elements of the human immunoglobulin light-chain λ locus to enforce the expression of human *MYC* in B cells. All λ -*MYC* mice develop lymphomas with striking similarities to human Burkitt lymphoma (BL) (Kovalchuk *et al.*, 2000). The pUR288 transgene harbors a shuttle vector with a *lacZ* reporter gene to examine mutagenesis *in vivo* with greater ease than studying endogenous genes (Boerrigter *et al.*, 1995). The plasmid-based pUR288 vector also permits, in contrast to previously developed shuttle vectors based on phage λ (Gossen *et al.*, 1989; Kohler *et al.*, 1991), the detection of a broad range of recombination mutations, including rearrangements of the reporter gene with mouse genomic sequences and large deletions within the reporter gene. The combined features of the λ -*MYC* and pUR288 transgenes rendered λ -*MYC*/pUR288 mice uniquely valuable for assessing *MYC*-induced genomic instability in a mature B-cell lymphoma (mouse BL).

The mutant frequency of *lacZ*, a mutagenesis reporter gene that confers neither positive nor negative selective pressure on the cell in which it resides, was determined in liver, spleen, and mesenteric lymph node (MLN) of three control mice (Table 1, column 5, lines 1–9), and liver, spleen, and lymphoma tissue from enlarged lymph nodes (MLN and peripheral lymph nodes [PLN]) of three lymphoma-bearing mice (lines 10–21). Histologic analysis (results not shown) revealed that the enlarged lymph nodes were completely infiltrated with highly aggressive lymphomas that

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Table 1 *LacZ* mutant frequencies in control and BL bearing λ -MYC mice

Mouse ^a	Tissue ^b	Mutants ^c	Total ^d	MF ^e	Exp. ^f	Mean MF ^g	SD ^h
1-C	Liver	72	13.2	5.44	3		
2-C	Liver	76	10.5	7.26	2		
3-C	Liver	69	10.9	6.36	4	6.35	0.91
1-C	Spleen	61	10.7	5.69	2		
2-C	Spleen	44	8.49	5.18	3		
3-C	Spleen	53	12.5	4.24	3	5.04	0.73
1-C	MLN	40	6.99	5.72	3		
2-C	MLN	57	8.56	6.66	3		
3-C	MLN	32	7.54	4.24	3	5.54	1.21
1-MYC	Liver	180	14.2	12.6	3		
2-MYC	Liver	227	23.4	9.41	3		
3-MYC	Liver	94	14.3	6.07	3	9.36	3.27
1-MYC	Spleen	115	15.1	7.35	4		
2-MYC	Spleen	109	11.2	9.20	4		
3-MYC	Spleen	110	18.6	5.82	4	7.46	1.69
1-MYC	BL-MLN	65	5.82	11.2	4		
2-MYC	BL-MLN	59	7.05	8.37	4		
3-MYC	BL-MLN	48	7.24	6.63	4	8.73	2.31
1-MYC	BL-PLN	94	5.52	13.0	4		
2-MYC	BL-PLN	76	5.44	11.8	4		
3-MYC	BL-PLN	39	4.50	7.11	4	10.6	2.95

^a“C” designates normal controls and “MYC” refers to λ -MYC transgenics harboring mouse BL. ^bMLN, mesenteric lymph node; BL-MLN, BL tumor in MLN; BL-PLN, BL tumor in peripheral lymph node. ^cNumber of mutant colonies not expressing β -galactosidase (β -Gal). ^dNumber of wild type colonies expressing β -Gal, as multiples of 10^5 . ^eMF, mutant frequency. Ratio of mutant to wild type colonies, as multiples of 10^{-5} . ^fNumber of pooled experiments to determine MF. ^gMean MF, as multiples of 10^{-5} . ^hSD, standard deviation of the mean MF, as multiples of 10^{-5} .

were studied with metallophilic macrophages containing apoptotic bodies, yielding the typical ‘starry sky’ appearance of BL. The presence of numerous mitotic figures reflected the high rate of cell proliferation in the tumor. Unlike BL lymph nodes, liver and spleen showed early stages of tumor cell infiltration (<10%), but otherwise retained their typical architecture. Surprisingly, regardless of the degree of tumor invasion, tissues infiltrated with lymphoma cells demonstrated a similar, moderate elevation of mean *lacZ* mutant frequencies over the background levels determined in control tissues (Table, 1 column 7): 1.47-fold in liver, 1.48-fold in spleen, 1.58-fold in BL-MLN, and 1.91-fold in BL-PLN. The BL-PLN sample was compared to normal MLN because normal PLN were too small to be used as controls in the pUR288 assay. When the mean mutant frequency of the pooled LN tumor samples from mice with BL (9.69×10^{-5}) was compared to that of MLN from normal mice (5.54×10^{-5}), the increase was 1.75-fold. None of these increases were significant by Student’s *t*-test. This indicated that BL in mice is not characterized by the dramatic increase in mutant rates observed in many human and mouse tumors, such as mismatch repair-deficient thymic lymphomas (Baross-Francis *et al.*, 1998).

Restriction enzyme analysis of *lacZ* mutants was performed to differentiate between point mutations (identified by the wild type pattern of pUR288) and

illegitimate recombinations (IR; identified by a deviant pattern; Figure 1). In *lacZ* mutants obtained from control mice, point mutations were the dominant category of mutation. Thus in these mice, recombination mutations comprised only 15 out of 145 (10.3%) mutations in liver, 29 out of 97 (29.9%) in spleen, and 16 out of 63 (25.4%) in MLN. Recombinations also constituted the minority of mutations in liver (26 out of 149, 17.4%) and spleen (31 out of 154, 20.1%) from the λ -MYC mice. Strikingly, the mutational pattern was reversed in tumor tissues, where recombinations were dominant, constituting 54 out of 94 (57.5%) mutations in the BL-MLN sample and 94 out of 134 (70.1%) mutations in the BL-PLN sample. The prevalence of recombination mutations was significantly higher in the pooled MLN plus PLN tumor samples from BL-bearing mice (148 out of 228, 64.9%) when compared to the MLN sample from normal mice (16 out of 63, 25.4%). These observations indicated that although point mutations in *lacZ* were the

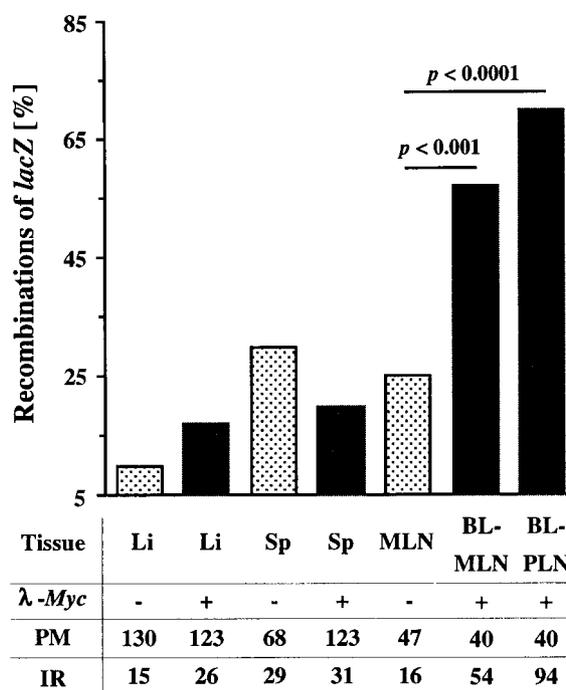


Figure 1 Marked increase in structural alterations of *lacZ* in mouse BL. The prevalence (%) of illegitimate recombinations (IR) in liver (Li), spleen (Sp), mesenteric lymph node (MLN), and BL tumor nodes (BL-MLN and BL-PLN (peripheral lymph node)) is plotted both for three λ -MYC transgenic mice (+, dark bars) and three non-transgenic littermates (-, light bars). The total number of point mutations (PM) and IR analysed for each tissue is indicated at the bottom. IR in tumor tissues of λ -MYC transgenics were significantly elevated compared to IR in MLN of normal mice (*P* values of χ^2 analyses are indicated). Plasmids were extracted from overnight cultures of mutant colonies from the pUR288 rescue assay using QIAprep 96 Turbo miniprep kits. Plasmids were doubly digested with *Hind*III and *Pst*I, fractionated by agarose gel electrophoresis, and analysed by comparison with the wild type banding pattern. Point mutations of *lacZ* have a wild type pattern; differing patterns result from IR mutations of *lacZ* (i.e., translocations, deletions, and inversions, as shown in Figure 2)

dominant class of mutation in normal tissue, the majority of mutations in the lymphomas resulted from structural alterations of *lacZ*.

The molecular structures of 40 recombination mutations of *lacZ* found in lymphoma tissue were elucidated by DNA sequencing. These mutations took two forms, rearrangements of *lacZ* with mouse genomic sequences (29 out of 40) (Figure 2a) with one breakpoint in *lacZ* and one in the mouse genome, and deletions within *lacZ* (11 out of 40) (Figure 2b). The chromosomal origin, precise breakpoint, and orientation of the mouse sequences recovered in mutant pUR288 plasmids were identified by BlastN searches of the complete mouse genome using the Celera Discovery System. The mouse sequences involved in the rearrangements were clearly defined in 25 out of 29 cases (searches of the databases failed to identify the incoming mouse sequences in the four remaining cases (yellow symbols)). Fourteen out of 25 rearrangements (56%) were the result of apparent translocations of the reporter gene loci at 59.9 Mb on Chr 3 and 87.2 Mb on Chr 4 with other chromosomal partners (red symbols). There were three translocations with Chr 1, two translocations each with Chrs 14, 17 and 19, and single translocation events with Chrs 2, 7, 8, 9 and 16. Eleven out of 25 rearrangements (44%) were the result of apparent deletions (light blue) and inversions (dark blue) recombining *lacZ* with chromosomal sequences from Chrs 3 or 4. In 14 out of 29 (48%) *lacZ* recombination mutations, the mouse genomic sequences included common DNA repeats (indicated by squares in Figure 2a; lack of repeats is indicated by circles). Repeat elements included L1 elements in eight cases, LTRs in four cases, SINEs in two cases, and L2 in one case. It is not known whether the frequent involvement of repeats reflects the abundance of these elements in the mouse genome, or, as suggested by findings in *Saccharomyces cerevisiae*, the preferred involvement of endogenous repeats in double-strand break repair (Moore and Haber, 1996). Six rearrangements interrupted genes, five of which are computationally predicted without confirmed functions or names (not shown). The significance of the only identified gene, *Scp2* (sterol carrier protein 2), for the pathogenesis of BL is unknown.

The molecular structures of 11 internal deletions in the *lacZ* transgenic concatamer are illustrated in Figure 2b. Internal deletion mutations displayed a considerable amount of microhomology at the breaksite, in contrast to the sample of rearrangements shown in Figure 2a. Thus, while 10 out of 11 (91%) internal deletions in *lacZ* were characterized by the occurrence of two to eight basepair long homologies at the breaksite (mean 4 ± 0.719 bp), only four out of 14 (29%) translocations and six out of 11 (55%) deletions/inversions exhibited one to four basepair long homologies. This translated into mean values of 0.571 ± 0.309 bp for translocations and 1.00 ± 0.394 bp for deletions and inversions. The difference in the mean length of microhomology between the internal dele-

tions (Figure 2b) and the rearrangement mutations (Figure 2a) was highly significant in the two-tailed Student's *t*-test ($P=0.0032$). Since microhomologies at genomic junction sites are widely considered earmarks of DNA double-strand breaks repaired by non-homologous end joining, NHEJ (Roth and Wilson, 1988), our data suggest that microhomology-mediated NHEJ may be more important for the repair of internal deletions than illegitimate recombinations. Remarkably, none of the 40 sequenced mutations from tumor tissues shown in Figure 2 shared the same breakpoint in *lacZ*. This indicated that all changes were unique, that *lacZ* mutations tend to occur relatively late during BL development, and that the mutational analysis of our sample was not distorted by clonal amplification of cells with particular mutations, in spite of the extremely high proliferative activity of BL tumors.

The existence of translocations, deletions, and inversions rearranging *lacZ* suggested that primary BL tumors are characterized by the persistent generation of new chromosomal variations that also involve endogenous loci. To determine if chromosomal variations of this sort can be detected in individual cell lines derived from primary BL tumors, we developed a series of BL cell lines and assayed 14 of them by SKY. The cell lines were hyperdiploid with a modal chromosome number of 47 (range 39–68). The most common aberrations were translocations, predominantly non-reciprocal, seen in nine tumors (see Figure 3a for some examples). Translocations involved almost all autosomes (Chrs 1, 2, 3, 4, 5, 6, 8, 10, 12, 13, 16, 17 and 19) and X, and included, interestingly a Chr 5F translocation, T(12;5)(D;F), previously found in 50% of primary peritoneal BALB/c plasmacytomas (Coleman *et al.*, 2000). Trisomies, mostly involving Chr 14 (four cases), Chr 1 (three cases) and Chr 11 (two cases), were identified in seven cell lines. Unique aberrations included two Robertsonian translocations, two large multi-band deletions, one dicentric chromosome (Dic(3A,3A)), and extrachromosomal material derived from Chrs 18 or 11 (two cases). Two cell lines derived from different tissues of the same mouse (one from a cervical LN and one from the mesenteric LN) illustrated chromosomal instability during tumor progression. Thus, while two translocations and one insertion common to both lines clearly indicated the shared clonal ancestry, a unique third translocation, T(1;8)(D-E;A-B2) in the line from cervical LN and T(1;3)(D;E1-2) in the line from the mesenteric LN, demonstrated the clonal diversification. These and other data, to be published in greater detail elsewhere, indicated that mouse BL exhibits true chromosomal instability. Furthermore, the multitude of sporadic cytogenetic changes identified by SKY are a likely correlate of the mutator phenotype of chromosomal instability identified by molecular analysis of *lacZ*.

To provide evidence in support of widespread deletional mutagenesis at the molecular level, we performed Southern analysis of BL cell lines for V(D)J rearrangements of the immunoglobulin heavy-

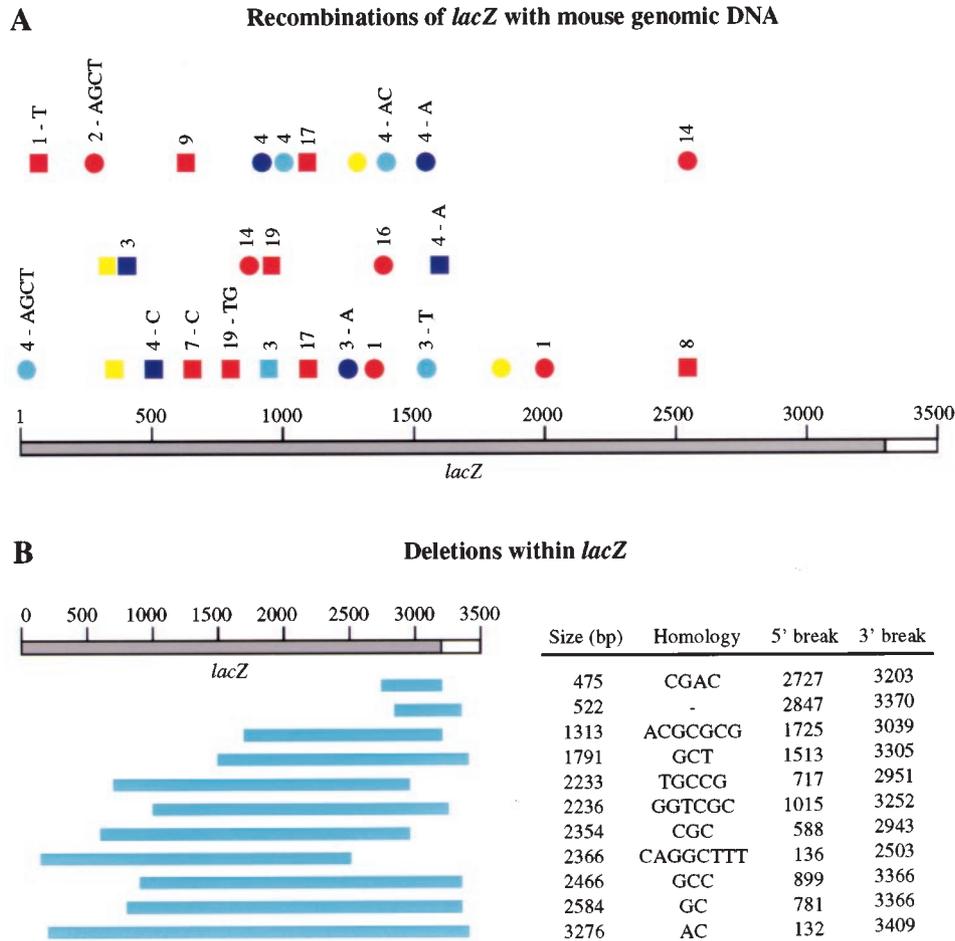


Figure 2 Fine structure of illegitimate recombinations of *lacZ* in mouse BL. (a) Presents a map of pUR288 containing the 3,282-bp *lacZ* gene (grey rectangle). The nucleotide sequence is numbered according to SYNPUR288V (GenBank, L09147). Breakpoints in *lacZ* of 29 recombinations are indicated by the position of the symbols above *lacZ*. Blue indicates intra-chromosomal rearrangements of *lacZ* on Chr 3 (four cases) or Chr 4 (seven cases). Deletions (five cases) and inversions (six cases), are distinguished by light and dark blue, respectively. Red indicates trans-chromosomal rearrangements of *lacZ* (chromosomal translocations; 14 cases). Yellow indicates unidentified recombination partners (four cases). The number above the symbol identifies the recombination partner chromosome followed by homologous basepairs at the breaksite. A circle denotes the presence of such repeats. A square indicates that a repeat element was identified at the recombinational breaksite (14 out of 29 cases, 48%). (b) Illustrates 11 deletions within *lacZ* depicted to scale in blue below the map of *lacZ*. The table to the right indicates the minimum number of deleted basepairs (assuming that deletions took place within the same copy of pUR288; see additional comments below), microhomologies at the deletion breakpoints, and the positions of the 5' and 3' breakpoints in *lacZ*. Mutant plasmids were sequenced using a set of 24 primers spanning overlapping regions of both strands of *lacZ*. Sequencing reactions were performed with the BigDye Terminator Cycle Sequencing Kit and read on an ABI Prism 377 Cycle Sequencer. Analysis of the sequences was performed using the Celera Discovery System and associated Celera databases. Although it does not change the overall interpretation of the data presented here, it should be acknowledged that the designation of rearrangements as intra-chromosomal events in a is based on the likely but unproven assumption that rearrangements on Chrs 3 or 4 occurred in *cis* rather than *trans*. Intra-chromosomal rearrangements are generally favored over inter-chromosomal rearrangements; however, we cannot exclude the possibility that some of the 'deletions/inversions' on Chrs 3 or 4 are in fact translocations between *lacZ* on one chromosome with sequences on the other. Furthermore, the pUR288 assay cannot differentiate deletions that affect one copy of the transgenic concatamer (intragenic deletions) from deletions that span several copies of the concatamer (intergenic deletions). It is thus possible that some or all of the deletions shown in b are larger than indicated

chain (*IgH*) locus on Chr 12. V(D)J rearrangements offer a unique opportunity to screen BL tumors for genomic deletions in a specific endogenous locus, because the tumors are derived from mature B cells expressing surface Ig, which requires productive V(D)J rearrangement of either one *IgH* allele (retaining the second allele in germ line configuration) or V(D)J rearrangement of both *IgH* alleles (if the first

rearrangement is non-productive). In either case, the two alleles can be distinguished by Southern blotting of genomic DNA, since V(D)J rearrangements are almost invariably unique (clonotypic). Figure 3b illustrates that three of 12 BL cell lines contained apparent deletions of one *IgH* allele, presumably the germ line or non-productively rearranged allele (indicated by red asterisks). The high incidence of deletions (25%) was

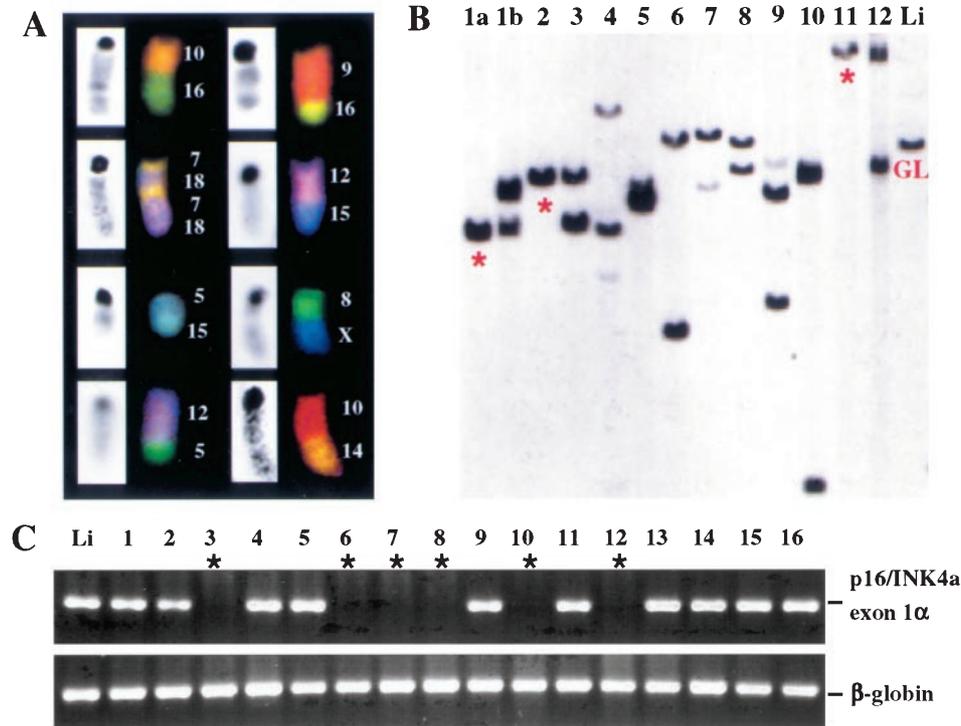


Figure 3 Analysis of BL cell lines for chromosomal instability. (a) Cytogenetic aberrations revealed by spectral karyotyping (Liyanage *et al.*, 1996). Eight examples of sporadic chromosomal translocations are shown in SKY display colors, with inverted DAPI images to the left. Rearrangements are labeled. (b) Apparent monoallelic deletions of *IgH* in three out of 12 cell lines (red asterisks), as revealed by Southern hybridization of genomic DNA with a J_{H4} probe. Lanes 1a and 1b contain related cell lines derived from MLN and spleen of the same mouse; lanes 2–12 contain independent BL cell lines, and lane 14 contains a control tissue (liver) indicating the unrearranged *IgH* germ line (GL) allele. (c) Detection of bi-allelic deletions of exon 1 α of p16^{Ink4a} by PCR (asterisks). The following primers flanking exon 1 α were used; forward, 5'-AGG AAA GCG AAC TCG AGG A-3'; reverse, 5'-GGG AGA AGG TAG TGG GGT C-3'. PCR detection of mouse β -globin was included as an internal control, using these primers: forward, 5'-CCA ATC TGC CAC AGG ATA GAG AGG GCA GG-3'; reverse, 5'-CCT TGA GGC TGT CCA AGG ATT CAG GCC ATC G-3'. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Lane 1, normal liver; lanes 2–17, BL cell lines; lanes 12 and 13 are derived from two different BL tumors of the same mouse

striking, particularly when one considers that monoallelic loss of germ line or non-productively rearranged *IgH* does not confer a selective advantage to the BL cell. This result is consistent with the deletional mutator phenotype of BL revealed by analysis of *lacZ*.

To obtain evidence of somatic mutagenesis at the level of an endogenous gene that may be important for the pathogenesis of mouse BL, we analysed the state of the *Cdkn2a* locus in BL cell lines. The choice of *Cdkn2a*, which encodes the important tumor suppressors p16^{Ink4a} and p19^{Arf}, was guided by reports that $E\mu$ -*Myc* induced B-cell tumors, whose pathogenesis is likely similar to the pathogenesis of λ -*MYC* induced BL tumors, almost invariably exhibit a disruption of the ARF-Mdm2-p53 tumor suppressor pathway (Eischen *et al.*, 2001). Postulating that complete loss of *Cdkn2a* confers a strong selective advantage to BL cells, we hypothesized that bi-allelic deletions of *Cdkn2a* may be frequent in BL cell lines. This hypothesis was confirmed by DNA PCR analysis of *Cdkn2a*'s exon 1 α , which encodes the aminoterminal portion of p16^{Ink4a}. Figure 3c shows that six out of 16 (37.5%) cell lines exhibited bi-allelic deletions of exon

1 α . Studies of RNA prepared from the lines indicated that in some cases the deletion involved only exon 1 α without affecting the 5' flanking exon, 1 β (encoding the aminoterminal portion of p19^{Arf}), or the 3' flanking exons, 2 and 3 (encoding the carboxyterminal portions of p16^{Ink4a} and p19^{Arf}), but included the flanking exons in other cases (data not shown). These results indicated that genomic deletions, both small and large, effected mutations of the *Ink4a/ARF* tumor suppressors, an example of selection of advantageous mutations likely critical to the development of BL in λ -*MYC* mice. Further studies of these cell lines, to be reported in detail elsewhere, indicate that they also exhibit mutations of p53 and disruptions of the ARF-MDM2-p53 axis that are functionally like those seen in pre-B and immature B cell lymphomas that develop in $E\mu$ -*Myc* transgenic mice (Eischen *et al.*, 1999).

The results presented here show that somatic mutagenesis in the highly proliferative and aggressive mouse BL tumor is characterized by a preponderance of structural genomic alterations in the context of a surprisingly low intrinsic mutation rate. A mutator phenotype of this sort, which has not been described

thus far in other mouse models of cancer, adds support to the growing evidence that deregulated *MYC* may act as a structural modifier of the genome. According to this model, *MYC* promotes tumorigenesis by facilitating translocations, deletions, and inversions throughout the genome (Mushinski and Mai, 2002), but does not cause elevated mutation rates (no hypermutability). Similar to our results with mouse BL, normal or near normal mutant rates *in vivo* were found in p53^{-/-} thymic lymphomas (Buettner *et al.*, 1996), polyoma middle T induced adenocarcinomas (Jakubczak *et al.*, 1996), and *Myc* induced liver cancers (Davis *et al.*, 1996). However, since in these studies the mutant rate was determined with the assistance of phage λ assays that cannot detect chromosomal rearrangements, it is possible that the true mutant rate in these tumors was underreported. Re-evaluation with the pUR288 assay may thus demonstrate higher mutant rates, and may furthermore reveal whether the proclivity to gross chromosomal alterations is unique for mouse BL or common to various forms of cancer. In conclusion, our

findings in mouse BL clearly indicate that a mutator phenotype of hypermutability (Loeb, 1991) may not always be required for oncogenesis to take place. The powerful force of cellular selection (Tomlinson and Bodmer, 1999) coupled with the exceptionally disruptive mutator phenotype of chromosomal instability appears sufficient to drive the development of this type of lymphoma.

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