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Chromosomal breakpoints and structural alterations of the c-*myc* locus differ in endemic and sporadic forms of Burkitt lymphoma

(c-*myc* oncogene/chromosomal translocation)

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ABSTRACT **We have examined the position of the chromosomal breakpoint relative to the human c-*myc* gene (*MYC*) and the presence of other structural alterations of the same locus in 19 fresh samples of Burkitt lymphoma (BL) and 13 BL-derived cell lines. This panel includes the two pathogenetic forms of BL: the endemic (African-type) BL (eBL) and sporadic (American-type) BL (sBL). In all cases tested, including fresh samples and cell lines, structural alterations of the 5′ portion of the gene were detected, suggesting that they may be necessary for c-*myc* activation. However, the site of chromosomal breakpoint and the type of structural alterations differ in eBL and sBL. In 16 of 18 sBL cases, chromosomal translocation truncates the gene within a 5′ region that includes the first intron, the first exon, and 5′ flanking sequences. Conversely, in 14 of 14 eBL samples, the chromosomal breakpoint is located outside the c-*myc* locus, yet the same 5′ sequences are affected by several mutations identifiable by restriction enzyme polymorphisms. Different genetic mechanisms may therefore be involved in chromosomal translocation/c-*myc* activation, and these differences may be a function of differences in the stage of differentiation of eBL versus sBL.**

Burkitt lymphomas (BLs) are characterized by specific reciprocal chromosomal translocations that involve the c-*myc* oncogene (*MYC*) locus on chromosome 8 and immunoglobulin loci on chromosome 2, 14, or 22 (refs. 1–3; for reviews, see refs. 4–6). The consistent occurrence of these specific recombination events in BL and the presence of analogous recombinations involving c-*myc* in similar tumors in other species (7) suggest that the c-*myc* gene plays a role in the pathogenesis of BL. The mechanism(s) by which chromosomal translocation(s) affects the function of the *myc* gene leading to its abnormal activation is still largely unknown.

Detailed molecular analysis of the genomic segments involved in the reciprocal recombinations has indicated that the position of the chromosomal breakpoints differs within both the immunoglobulin and c-*myc* loci in different tumors. In the most frequent translocation [t(8;14)], which involves the immunoglobulin heavy chain locus, the breakpoints may be variably located both within the constant region genes for the μ, α, and γ heavy chains and also within the variable regions (4, 5). The variant t(8;2) and t(8;22) translocations involve either the κ or the λ light-chain loci (4, 5). At the reciprocal translocation site on chromosome 8, breakpoints have been mapped at different sites relative to the c-*myc* locus: (*i*) at an undefined distance 5′ of c-*myc* (3, 4); (*ii*) in a region including the 5′ flanking sequences of the gene and the first exon (4); (*iii*) within the first intron (4, 8); and (*iv*) at various locations in the 3′ flanking regions of the gene (4, 5). Some studies have also indicated the occurrence of small rearrangements, such as duplications, insertions, deletions, or point mutations, both in noncoding and in coding portions of the translocated c-*myc* gene (4, 5).

Based on these findings, several models have been proposed for the mechanism of c-*myc* activation in BL, including (*i*) inactivation of putative 5′ regulatory sequences by truncation or mutation (9–11); (*ii*) transcriptional activation of the c-*myc* oncogene by nearby immunoglobulin enhancer elements (12); and (*iii*) transcriptional activation by putative "long-distance" enhancer elements that normally control immunoglobulin genes (5). Although detailed molecular analysis of individual cases of BL provides circumstantial evidence supporting these models, direct evidence for the involvement of any of these mechanisms is still lacking, mainly because of the heterogeneity of recombination events and the multiplicity of alterations within individual cases. We therefore reasoned that a comprehensive study of many cases would lead to the identification of common patterns and would indicate the functional importance of certain regions of the c-*myc* locus, providing insights into the mechanism(s) of c-*myc* activation.

We examined the position of the chromosomal breakpoint and the presence of other structural alterations relative to the c-*myc* locus in a large panel of fresh tumors and BL-derived cell lines carrying the t(8;14) chromosomal translocation. Moreover, we wished to investigate whether the different patterns of c-*myc* translocation would correlate with the epidemiologically, phenotypically, and pathogenetically distinct forms of BL—i.e., the endemic (eBL) and the sporadic (sBL) forms (for review, see refs. 6 and 13). The former is characterized by its restricted distribution in high-incidence areas, namely equatorial Africa and Papua New Guinea, and by its virtually complete association (96% of cases) with the Epstein–Barr virus (EBV). sBL is characterized by a much lower incidence, its worldwide distribution, its different organ distribution, and its only occasional association with EBV (6, 13).

Our results indicate that the position of the chromosomal breakpoint on chromosome 8 and the structural alterations of the c-*myc* locus differ in sBL and eBL. These data have implications for the mechanism of c-*myc* activation and for a pathogenetic classification of different BL forms.

MATERIALS AND METHODS

Pathologic Samples, Cell Lines, and Diagnostic Criteria. Representative tumor biopsies and/or involved bone marrow were collected from untreated patients during the course of diagnostic procedures. Diagnosis of BL was established in each case by standard clinical and cyto- and histopathologic criteria and, in some cases, by cell marker and cytogenetic

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Abbreviations: BL, Burkitt lymphoma; eBL, endemic BL; sBL, sporadic BL; EBV, Epstein–Barr virus; kb, kilobase(s).

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Table 1. Frequency and type of structural alterations of the c-*myc* locus in eBL and sBL

	c-*myc* locus rearrangements*	5′ restriction enzyme polymorphisms†
eBL		
Fresh samples	0/12	8/8
Cell lines	2/6	2/2
sBL		
Fresh samples	7/7	0/3
Cell lines	7/7	1/7

*Values express the relative frequency of rearrangements detectable by both *Eco*RI and *Hin*dIII restriction enzyme digestions.
†Values express the relative frequency of 5′ restriction site polymorphisms detected by one or more of the following enzymes: *Pvu* II, *Hae* III, *Mbo* I, *Sma* I, *Msp* I, *Dde* I, *Hin*fI (see text and Fig. 3*B* for sites and relative frequencies for each enzyme).

analysis. eBL cases are defined as BL occurring in endemic regions of equatorial Africa. All the fresh eBL samples analyzed in this study originated from Ghana and were obtained through the National Cancer Institute's Burkitt Tumor Project. sBL cases are defined as occurring outside endemic areas, and sBL samples originated from Europe and North America. The phenotypic, cytogenetic, and immunophenotypic features, as well as the geographical origin of most of the BL cell lines used in this study, have been described (14, 15).

DNA Extraction and Southern Blot Analysis. DNA was prepared by cell lysis, digestion with proteinase K, extraction with phenol, and precipitation with ethanol (16). DNA (15 μg) was digested with the appropriate restriction endonuclease, electrophoresed in 0.8 to 1.6% agarose, denatured, neutralized, transferred to a nitrocellulose filter, and hybridized according to established procedures (16) in 50% formamide/3× SSC, at 37°C. Filters were washed in 0.2× SSC/0.5% $NaDodSO_4$ at 60°C for 2 hr. SSC (standard saline citrate) is 0.15 M NaCl/15 mM sodium citrate, pH 7.

DNA Probes. The c-*myc* probes used are represented in Fig. 2*B*. Probe MC413RC is a 1.4-kilobase (kb) *Cla* I–*Eco*RI DNA fragment representative of the third exon of the human c-*myc* locus (17, 18). Probes Pv–Pv, Pv–X, and X–Pv were obtained by restriction enzyme digestions of recombinant plasmid pMC415PP (3) and purification of the corresponding fragments (see Fig. 2*B*). DNA fragments for use as probes were labeled with ^{32}P by nick-translation (16).

RESULTS

c-*myc* Rearrangements in eBL and sBL. We first determined the approximate location of the breakpoint on chromosome 8 by distinguishing cases in which the c-*myc* locus was truncated from the ones in which it appeared intact. For this purpose, we analyzed BL DNAs with restriction enzymes that generate large c-*myc*-containing DNA fragments. Both *Eco*RI and *Hin*dIII identify DNA regions containing the entire c-*myc* locus, including the three coding exons and the evolutionarily conserved 5′ and 3′ flanking sequences of possible functional significance. *Hin*dIII- and *Eco*RI-digested DNAs were analyzed by Southern blot hybridization with probes representative of the first c-*myc* exon (Pv–Pv probe, see Fig. 2*B*). Results of this analysis are summarized in Table 1, and representative cases are illustrated in Fig. 1.

All of the 12 freshly isolated eBL and 4 of 6 eBL cell lines displayed apparently unrearranged c-*myc* loci. These results were confirmed by hybridization (not shown) with a second probe spanning the third c-*myc* exon (MC413RC fragment, see Fig. 2*B*). By analogy with previously reported studies, the chromosomal breakpoint in these 16 cases is assumed to be located at an undefined distance 5′ of c-*myc* in all cells carrying a t(8;14) translocation. These cases include all the eBL cell lines (Table 1) and most fresh samples (4, 5). In the cases for which cytogenetic data were not available, the chromosomal breakpoint can be assumed to be located either 5′ [t(8;14) translocation] or 3′ of c-*myc*, as shown in the variant translocations t(2;8) and t(8;22) (4, 5).

Conversely, all 7 fresh tumor sBL samples and all 7 sBL cell lines displayed rearrangements of one c-*myc* allele. The presence of rearranged alleles of different sizes indicates that in sBL the position of the breakpoint varies within c-*myc*. We conclude that, although in most eBLs the chromosomal breakpoint is located outside the c-*myc* locus, sBLs are invariably characterized by rearrangements directly involving c-*myc* or its immediately flanking sequences.

Mapping of Chromosomal Breakpoints in sBL. The c-*myc* rearrangements detectable in sBL were further examined by Southern blot analysis using a combination of different restriction enzymes and probes that enabled us to explore different segments of the c-*myc* locus. By this approach, it is possible to map approximately the recombination site corresponding either to the breakpoints of a reciprocal chromosomal translocation, as previously demonstrated in several cases (4, 5), or to a major internal rearrangement, as shown in a single case (9). Preliminary experiments, involving *Sst* I-digested sBL DNAs and hybridization with 5′ and 3′ c-*myc* probes (MC415PP and MC413RC, respectively; see Fig. 2*B*), showed that in all cases, the rearrangements were detectable only by the first probe, indicating that they occurred 5′ of the *Sst* I fragments containing the second and third exons. Within this region of the c-*myc* locus, the breakpoints were further mapped by various combinations of the restriction enzymes and probes shown in Fig. 2*B*. Representative data relative to the tumor DK179 and to the cell line EB3 are illustrated in Fig. 2*A*. In DK179 DNA, the 2.8-kb *Pst* I fragment was left intact, whereas rearrangements were detected in one allele within the 13.0-kb *Sst* I fragment. These data [together with the data from *Eco*RI and *Hin*dIII digestions (Fig. 1*C* and Table 1)] indicate that the recombination site in DK179 is located between the 5′ *Hin*dIII site and the most 5′ *Pst* I site (Fig. 2*B*). Similarly, EB3 DNAs displayed rearranged alleles of the 2.8-kb fragments and of the 0.8-kb *Pvu* II fragments with Pv–X probe. That two rearranged fragments are detect-

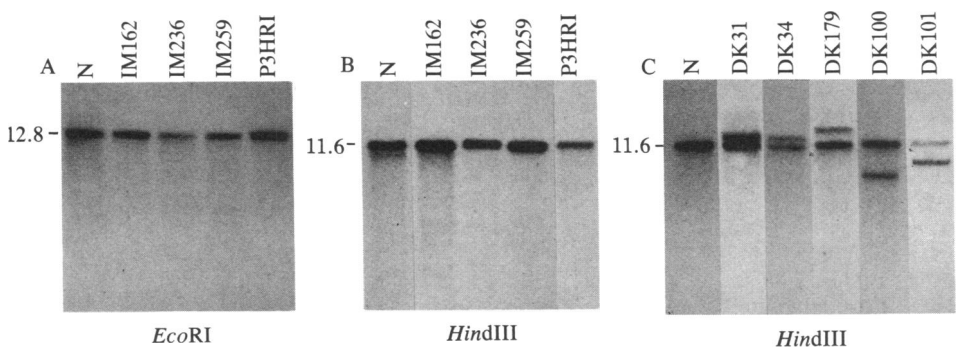

FIG. 1. c-*myc* rearrangements in eBL and sBL. DNA samples from the indicated BL cases and from normal human fibroblasts (lanes N) were digested with *Eco*RI (*A*) or *Hin*dIII (*B* and *C*) and hybridized with a Pv–Pv c-*myc* probe (see Fig. 2*B*). Fragment sizes are given in kb.

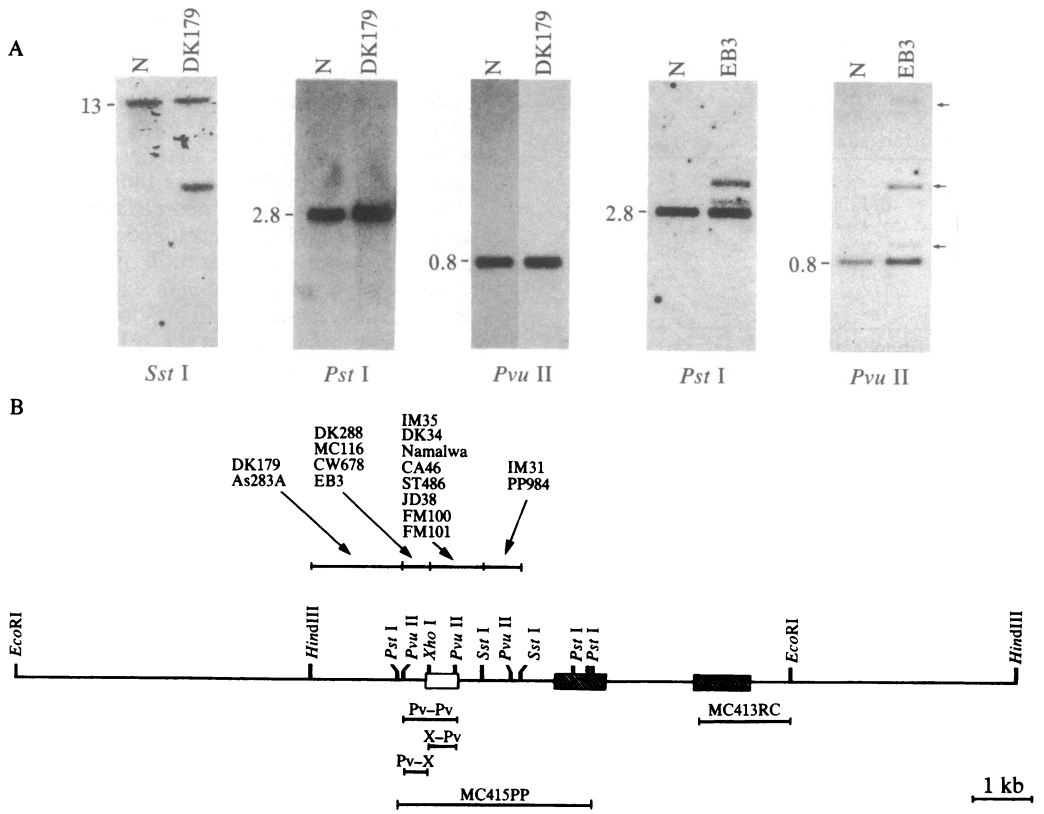

FIG. 2. Mapping of chromosomal breakpoints in sBL. (*A*) DNAs from the indicated BL cases and from normal human fibroblasts (lanes N) were digested with the indicated restriction enzymes and hybridized with the MC415PP (case DK179) or Pv–X (case EB3) c-*myc* probes. Sizes of major fragments are given in kb. Weakly hybridizing bands are indicated by arrows. (*B*) Restriction enzyme map of the human c-*myc* locus; the noncoding exon is represented by an open box, and the coding exons, by hatched boxes. Above the map, the approximate location of the breakpoints is indicated for each case. Probes are represented below the map.

ed by this probe (Fig. 2*A*) but only one is detected by the X–Pv probe (data not shown) suggests that the 0.4-kb *Pvu* II–*Xho* I fragment contains the crossover point of the reciprocal translocation. By this approach the approximate site of recombination was mapped in all the sBLs (Fig. 2*B*). These results suggest that the chromosomal breakpoints and/or rearrangements are clustered in a region of the c-*myc* locus spanning the first exon, first intron, and nearby 5′ flanking sequences.

The 5′ Region of Unrearranged c-*myc* Loci in eBL Contains Mutations. Several reports have demonstrated the occurrence of point mutations or other small internal rearrangements in the c-*myc* locus (10, 11, 19). We sought to analyze the frequency and the approximate position of these structural alterations in our collection of BLs, particularly in those with c-*myc* loci that appeared intact by *Eco*RI and *Hin*dIII restriction analysis. As an alternative to determining the entire nucleotide sequences of the c-*myc* loci, an impractical approach for the analysis of so many cases, we analyzed parts of the c-*myc* locus by Southern blot hybridization, using a combination of restriction enzymes cutting within these genomic areas and thereby allowing an overall, albeit approximate, estimation of the frequency and position of mutations. No deviation from the normal pattern was observed when *Pvu* II, *Hae* III, *Mbo* I, *Msp* I, *Dde* I, *Sma* I, and *Hin*fI restriction endonucleases were used in combination with a third-exon probe (MC413RC) (data not shown). However, several alterations were detectable with several of the restriction enzymes when probes specific for the 5′ region of the gene were used. The restriction sites involved and the relative frequency of involvement are schematically illustrated in Fig. 3*B*. Data for all BL cases tested are summarized in Table 1.

Of particular relevance is the high frequency of polymorphisms detected by *Pvu* II, for which representative data are shown in Fig. 3*A*. Of 13 eBL cases tested, 10 displayed an allelic variant of the 0.8-kb fragment detected by the Pv–Pv probe. The sizes of the rearranged segments vary in different cases (Fig. 3*A*). Digestion of the *Pvu* II fragment with *Xho* I restriction enzyme and hybridization with the Pv–X c-*myc* probe spanning the 5′ *Pvu* II–*Xho* I fragment showed this fragment to be intact in both alleles (data not shown). Hybridization with the X–Pv c-*myc* probe spanning the 3′ *Pvu* II site within the first exon is lost, so that either a new site has appeared in close proximity to it in one allele or small rearrangements, such as insertions, substitutions, and inversions, have altered the position of the *Pvu* II site. The relatively small size of the rearrangements is indicated by the intact size of the *Pst* I fragment (Fig. 3*A*).

A number of observations indicate that these restriction-site alterations, and in particular the one involving the *Pvu* II site within exon I, are not due to inherited polymorphisms but rather to somatic mutations specifically occurring in translocated c-*myc* alleles. First, no alterations of the 0.8-kb *Pvu* II fragment have been detected in a survey of 60 DNA samples from either normal individuals (peripheral blood lymphocytes, 15 cases tested) or tumors other than B-cell neoplasias (45 cases tested). Second, as opposed to common inherited restriction-site polymorphisms, these alterations vary from case to case, involving the loss of a site and the generation of a new one, and appear to be invariably monoallelic, since no homozygosity for an altered *Pvu* II fragment has been detected. Third, alterations of the *Pvu* II fragment are not detected in DNA from BL in which the chromosomal translocation truncates the c-*myc* locus, leaving the first exon on chromosome 8 (e.g., JD38, CA46, ST486; see Fig. 3*A*). We therefore conclude that muta-

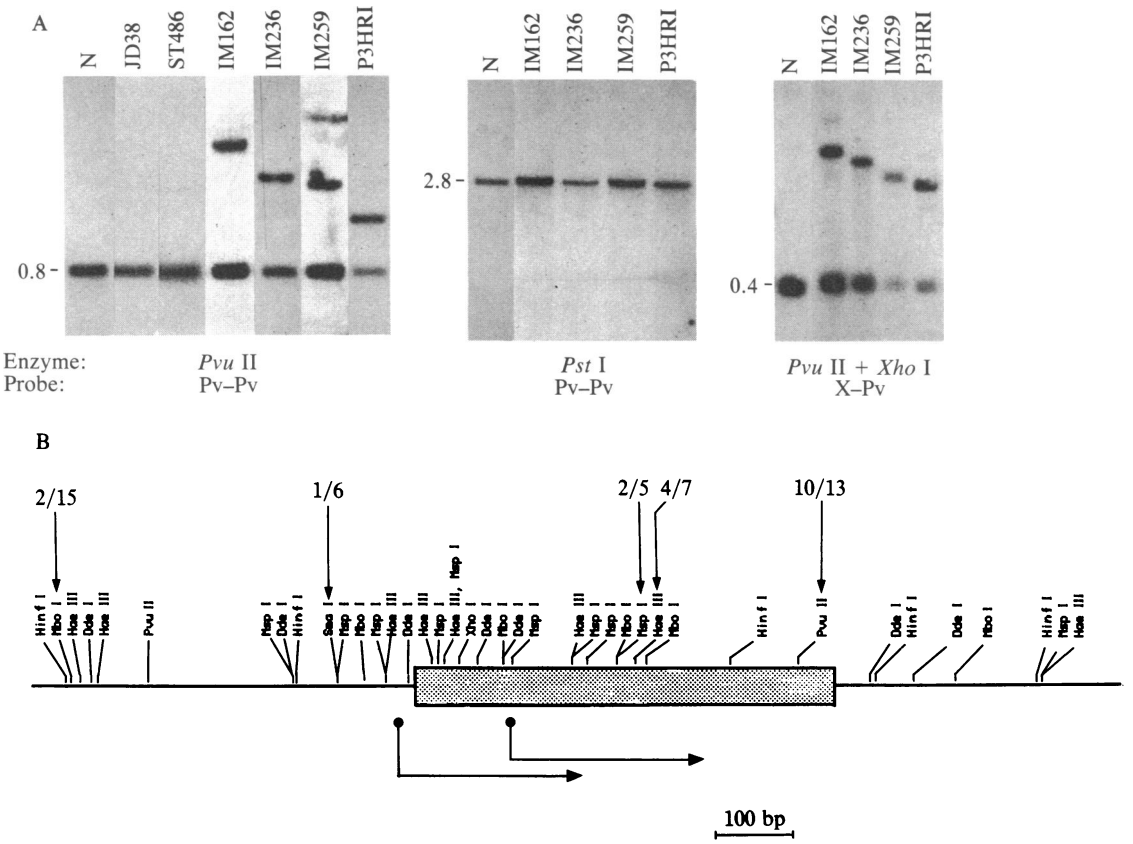

FIG. 3. Restriction enzyme site polymorphisms within the 5′ c-*myc* region in eBL. (*A*) DNAs from BLs and from normal human fibroblasts (lanes N) were digested with restriction enzyme and hybridized with c-*myc*-specific probe as indicated. (*B*) Restriction enzyme map of the 5′ flanking sequence, first exon (stippled box) and part of the first intron of c-*myc* (20). For polymorphic restriction enzyme sites, numbers indicate the frequency of polymorphism among the cases tested. The positions of the two c-*myc* promoters and the direction of transcription (arrows) are indicated below the map. bp, Base pairs.

tions and/or small internal rearrangements are a specific and frequent feature of translocated c-*myc* alleles in eBL.

DISCUSSION

The frequency and the clustering of structural alterations detectable in the translocated c-*myc* loci have implications for the mechanism of activation of this oncogene. Since chromosomal translocation brings together c-*myc* and immunoglobulin loci, it has been proposed that regulatory elements of immunoglobulin genes may contribute to the transcriptional activation of c-*myc*. These elements are represented by the known immunoglobulin transcriptional enhancer, which has been found in close proximity to c-*myc* in only a small minority of cases, or by still-undefined, long-distance-acting enhancers (5), for whose existence there is, as yet, no proof. Alternatively, the finding of alterations, either truncations or mutations of c-*myc* in a few BLs analyzed, raised the possibility that these structural changes in the gene are the primary cause of the deregulation of the translocated gene. This latter theory appears to receive strong support from our comprehensive analysis of many BLs, since we detected a structural alteration of the c-*myc* locus in all cases tested. It remains possible that an immunoglobulin enhancer element may contribute to the regulation pattern of the translocated gene in some cases. However, based on the data presented here, one can argue that structural alterations of defined portions of the c-*myc* locus may represent a necessary, if not sufficient, event in altering c-*myc* function following translocation.

Additional information about the mechanism of activation can be derived by examining the clustering of either truncations or mutations in a region spanning the entire first exon, the first intron, and a few hundred base pairs of 5′ flanking sequences. This region either is totally removed from the rest of the gene or is truncated or altered by several point mutations and/or small rearrangements, suggesting that one or more regulatory sequences may be present in this portion of the gene and that these may be inactivated by any of the observed structural alterations. The pattern of c-*myc* expression during different phases of normal cell growth (21) and the frequently increased levels of c-*myc* expression observed in BL (5, 19, 22) point toward the presence of a negative regulatory element. The existence of such an element has been suggested before, and a binding site for a putative repressor protein has been mapped in a region ≈1.5 kb from the 5′ border of the first exon (23). In this respect, in gene-transfer experiments using 5′ c-*myc* constructs containing 5′ or internal deletions in the 5′ flanking region, we have mapped a 200-base-pair region immediately adjacent to exon I; the removal of this region results in an increase in the transcription of the transfected genes (L. Lanfrancone, E. Cesarman, and R.D.-F., unpublished work). Further, c-*myc* expression may also be regulated posttranscriptionally, as recent studies suggested that the removal of the first noncoding exon from the c-*myc* gene may affect c-*myc* expression by increasing mRNA stability (24) or the efficiency of translation (12, 25). In this context, the significant frequency of mutations affecting defined sites of the gene—e.g., the *Pvu* II site (see Fig. 3*B*), which is altered in 10 of 10 cases tested, remains to be explained. While it is possible that the sequences defined by this site may be part of a regulatory element, it is also conceivable that, given the high rate of mutations present in the translocated gene, some sequences devoid of

strict functional significance may be more permissive for the accumulation of a relatively high number of mutations. Comparative functional analysis of transfected c-*myc* clones derived from normal or mutated alleles represents an obvious experimental approach to address these questions directly.

The second issue that emerged from our study is the strict correlation between the different sites of chromosomal breakpoint and the different pathogenetic forms of BL. Of 18 eBLs, 16 displayed an unrearranged c-*myc* gene, indicating that the chromosomal breakpoint lies outside the c-*myc* locus; whereas in 14 of 14 sBLs, the chromosomal breakpoint is variably located in the 5′ portion of the gene or in its immediately flanking sequences. These correlations did not emerge in previous studies (4, 5, 20), which most likely involved the selection of cases carrying a rearranged allele and were less strictly defined in terms of geographical origin. In this respect, it is important to note that no exception was observed within the group of fresh BL samples from the highly endemic region of equatorial Africa, while two exceptions were observed among cell lines derived from BL cases from different areas of North Africa. These exceptions, as well as the ones present in the literature, may represent sporadic cases occurring in endemic areas.

In an attempt to understand the significance of the correlation between BL type and site of chromosomal breakpoint, it is important to consider several studies suggesting that eBL and sBL may derive from lymphocyte precursors at different stages along the B-cell differentiation pathway. This distinction has been based on different cytomorphologic, cell surface marker, and immunologic characteristics (26–28). Most notably, most eBLs, but not sBLs, express Fc, C3 (complement component 3), and EBV receptors and sBL are characterized by significant IgM secretion, which is virtually undetectable in eBL (27, 29). Recently, we have expanded these observations on IgM secretion to include all cell lines used in this study and found that the type of molecular lesion of the c-*myc* locus correlates with the capability to secrete IgM. In particular, no IgM secretion was detectable in eBL, with the exception of the two cases (Namalwa and EB3) that displayed the truncated version of c-*myc* typical of sBL (unpublished results). These observations suggest that a correlation may exist between the site of the chromosomal breakpoint and the stage of B-cell differentiation; in this respect, IgM-secreting sBL, carrying truncated c-*myc* loci, may represent more mature cells. Consistent with this possibility is the notion that mouse plasmacytomas, which represent relatively more mature, immunoglobulin-secreting cells, most often carry truncated c-*myc* genes (30).

In addition to its implications for the pathogenesis of different forms of BL, the data presented here have practical significance for the diagnosis, classification, and clinical monitoring of BL. The observation that an alteration of the c-*myc* locus is detectable in virtually all cases by a combined restriction enzyme Southern blot analysis suggests that this technique may be useful in identifying BL cells and/or following their presence during therapy and the clinical course of the disease. This method appears more practical, more sensitive, and more objective than the histopathologic, immunophenotypic, or cytogenetic methods currently in use.

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