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Chromosomal translocations cause deregulated *BCL6* expression by promoter substitution in B cell lymphoma

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The *BCL6* gene codes for a zinc-finger transcription factor and is involved in chromosomal rearrangements in 30–40% of diffuse large-cell lymphoma (DLCL). These rearrangements cluster within the 5′ regulatory region of *BCL6* spanning its first non-coding exon. To determine the functional consequences of these alterations, we have analyzed the structure of the rearranged *BCL6* alleles and their corresponding RNA and protein species in two DLCL biopsies and one tumor cell line which carried the t(3;14)(q27;q32) translocation involving the *BCL6* and immunoglobulin heavy-chain (IgH) loci. In all three cases, the breakpoints were mapped within the IgH switch region and the *BCL6* first intron, leading to the juxtaposition of part of the IgH locus upstream and in the same transcriptional orientation to the *BCL6* coding exons. An analysis of cDNA clones showed that these recombinations generate chimeric IgH–*BCL6* transcripts which initiated from IgH germline transcript promoters (I_μ or $I_\gamma 3$), but retain a normal *BCL6* coding domain. In the tumor cell line, the chimeric $I_\gamma 3$–*BCL6* allele, but not the germline *BCL6* gene, was transcriptionally active and produced a normal BCL6 protein. These findings indicate that t(3;14) translocations alter *BCL6* expression by promoter substitution and imply that the consequence of these alterations is the deregulated expression of a normal BCL6 protein.

Keywords: *BCL6*/chromosomal translocation/immunoglobulin genes/lymphoma/zinc fingers

Introduction

Diffuse large-cell lymphoma (DLCL) are frequently associated with reciprocal chromosomal translocations affecting band 3q27 and various other chromosomal sites (Offit *et al.*, 1989; Bastard *et al.*, 1992). By conducting a molecular analysis of these translocations, a gene, *BCL6*, has been identified on 3q27 adjacent to the chromosomal breakpoints (Baron *et al.*, 1993; Ye *et al.*, 1993a,b; also called *LAZ3*, Deweindt *et al.*, 1993; Kerckaert *et al.*, 1993; or *BCL5*, Miki *et al.*, 1994; the *BCL6* name has been officially adopted, see McAlpine *et al.*, 1994). Rearrangements of the *BCL6* gene have also been shown in cases lacking recognizable abnormalities of band 3q27 at the cytogenetic level. Several surveys of non-Hodgkin's lymphoma (NHL) panels reported similar frequencies in various NHL subtypes, i.e. 30–40% in DLCL, 5–14% in follicular lymphoma (FL; Ye *et al.*, 1993a; Bastard *et al.*, 1994; Lo Coco *et al.*, 1994; Ohno *et al.*, 1994; Otsuki *et al.*, 1995) and 20% in acquired immune deficiency syndrome (AIDS)-associated DLCL (Gaidano *et al.*, 1994). In DLCL, cases with *BCL6* rearrangements have distinct clinical features including a favorable prognosis (Offit *et al.*, 1994).

The *BCL6* gene codes for a protein containing six C-terminal zinc-finger motifs and an N-terminal POZ/ZIN domain (Numoto *et al.*, 1993; Bardwell and Treisman, 1994) homologous to a family of zinc-finger molecules, including the *Drosophila* developmental regulators *Tramtrack* and *Broad-complex* (Harrison and Travers, 1990; DiBello *et al.*, 1991), as well as the human KUP (Chardin *et al.*, 1991), ZID (Bardwell and Treisman, 1994) and PLZF (Chen *et al.*, 1993) proteins. The *BCL6* gene is tightly regulated during B cell differentiation, being expressed in mature B cells within germinal centers but not in immature B cell precursors or differentiated plasma cells (Ye *et al.*, 1994; Cattoretti *et al.*, 1995; Flenghi *et al.*, 1995). Its features and pattern of expression suggest that BCL6 may function as a DNA-binding transcription factor involved in the control of B cell differentiation and lymphoid organ development.

Chromosomal translocations affecting band 3q27 represent reciprocal recombinations between this genomic region and >10 alternative chromosomes in different DLCL cases (Ye *et al.*, 1993a). The majority of these chromosomal breakpoints cluster within a 4 kb region spanning the first non-coding exon of the *BCL6* gene. Cytogenetic data and molecular analyses of a few cases have suggested that these translocations juxtapose sequences derived from other chromosomes to the *BCL6* coding domain (exons 2–10). However, no information is available about the functional consequences of these translocations and, in particular, about their effect on *BCL6* gene transcription and protein expression. To address these issues, we have selected three NHL cases carrying t(3;14)(q27;q32) translocations involving the *BCL6* gene and the IgH locus, with the assumption that an analysis of recombinations between *BCL6* and a well characterized locus such as IgH would facilitate the understanding of the consequences of the translocations. In these three cases, the structural and functional consequences of the IgH–*BCL6* juxtaposition have been determined by cloning the rearranged genomic loci and their corresponding abnormal *BCL6* transcripts, and by examining BCL6 protein expression using a Western blot analysis. The

results indicate that chromosomal translocations alter *BCL6* expression by promoter substitution, a mechanism which is novel for chromosomal translocations involving Ig loci.

Results

Cloning of t(3;14)(q27;q32) chromosomal junctions

Three NHL cases were selected for a molecular analysis of the t(3;14)(q27;q32) translocations. Two cases (SM1444 and KC1445) were represented by tumor biopsies from IgM-producing DLCL, whereas the third was a cell line (Ly8) derived from a IgM-producing immunoblastic lymphoma (Tweeddale *et al.*, 1987). In all three cases, the involvement of the *BCL6* locus in the translocation was confirmed by a cytogenetic analysis and by evidence of a rearranged *BCL6* allele upon Southern blot analysis of the genomic DNA (data not shown).

For cases SM1444 and KC1445, the cloning and preliminary characterization of one translocation junction, der(14), has already been reported (Ye *et al.*, 1993b). As part of this study, we have mapped the position of the *BCL6* (exon 1) and Ig sequences within the cloned sequences (Figure 1A and B). In addition, the reciprocal der(3) translocation junctions have also been cloned for both cases using *BCL6* probes located 3′ to the translocation breakpoints. In both cases, the comparative analysis of der(14) and der(3) junctions (Figure 1A and B) was consistent with the pattern expected for reciprocal translocations affecting the *BCL6* and IgH loci with breakpoints located within the first intron of the *BCL6* gene and the switch μ (S_μ) region of the IgH locus. In both cases, the der(3) chromosome carries IgH loci with rearranged D–J or V–D–J regions.

In the case of the Ly8 cell line, a phage library was constructed from genomic DNA and a single recombinant phage was isolated. Restriction enzyme digestion combined with Southern blot hybridization using *BCL6* and IgH probes indicated that the phage contained the region corresponding to *BCL6* intron 1 and exons 2–3 linked to the IgH $S_\gamma 3$ region (Figure 1C). Despite evidence from a Southern blot analysis that a reciprocal translocation product exists, no additional phages were isolated to account for such a product, suggesting the underrepresentation of the corresponding genomic region in the genomic library.

For all three cases, the position of the breakpoints was further mapped by a nucleotide sequence analysis of the der(3) chromosomal junctions. The involvement of S sequences was further confirmed by the presence of the typical S repeat motifs in the immediate proximity of the breakpoints in SM1444 and KC1445 (Figure 2B and C). The precise position of the breakpoint could not be determined within each S region because the highly repetitive nature of the S sequences involved prevented precise alignments. Within the *BCL6* locus, the position of the breakpoints clustered within 1.3 kb of *BCL6* intron 1 sequences (Figure 2A). No apparent homology was detectable between chromosome 14 and chromosome 3 sequences close to the breakpoint junctions. Although scattered S motifs were seen in the *BCL6* first intron, their frequency was not higher than that expected for random sequence matches (data not shown).

In conclusion, the structures of breakpoint junctions in all three translocation cases are consistent with Ig(S)–*BCL6* (intron 1) recombinations. Based on the orientation of the IgH locus on chromosome 14 (telomere–V–D–J–S_μ–C_μ–C_γ–centromere; Ravetch *et al.*, 1981), and assuming that long-range inversions have not occurred during these translocations, the organization of the cloned chromosomal junctions in cases SM1444 and KC1445 establishes the orientation of the *BCL6* gene on chromosome 3 as telomere–5′–3′–centromere.

Cloning of IgH–BCL6 fusion cDNAs

To determine the functional consequences of the IgH–*BCL6* juxtaposition, we examined the structure of the *BCL6* transcripts in the three NHL cases. A Northern blot analysis could not be performed for the two biopsy cases because of severe RNA degradation; a Northern blot analysis of Ly8 RNA using a *BCL6* cDNA probe showed the presence of a single *BCL6* RNA species of apparent normal size (3.8 kb; data not shown). This finding, together with the fact that the rearranged *BCL6* gene has lost its promoter region in Ly8 cells (Figure 1C), suggested that an IgH promoter was driving *BCL6* expression and that the heterologous region fused to the *BCL6* 5′ end may be too small to be detected by gel electrophoresis. Based on these observations, we constructed a cDNA library from the Ly8 cell line and, in the case of the two DLCL biopsies, we analyzed the 5′ ends of *BCL6* transcripts using the rapid amplification of cDNA ends (RACE) technique (Frohman *et al.*, 1988), which allows the isolation of short unknown 5′ RNA termini and can be applied to partially degraded RNA.

Figure 3 shows the various 5′ terminal PCR products isolated from each case using the RACE primers located within *BCL6* exons 4–5 which, based on the organization of the der(3) junctions, should be retained in both normal and abnormal *BCL6* RNA species. For both SM1444 and KC1445 RNA, two RNA species were identified based on a nucleotide sequence analysis of the cloned RACE products. The first species showed variable portions of V–D–J sequences spliced to *BCL6* exon 2 sequences, with the 5′ terminus represented by an inverted V3 region and an apparently intact D–J region in the cases of KC1445 and SM1444, respectively. The site of transcription initiation could not be established for these RNA species. A second RNA species detectable in both biopsy RNAs contained sequences from the I_μ exons correctly spliced to *BCL6* exon 2 sequences. The I_μ exons are normally found at the 5′ end of sterile transcripts initiated upstream of S_μ (Coffman *et al.*, 1993). A single species of *BCL6* cDNA was isolated from the Ly8 cDNA library which contained $I_\gamma 3$ 5′ sequences correctly spliced to *BCL6* exon 2 sequences, consistent with the structure of the der(3) junction (Figure 1C). In all three cases, the lengths of the I_μ or $I_\gamma 3$ sequences linked to *BCL6* were consistent with transcription being initiated at the described I_μ or $I_\gamma 3$ promoters (Milili *et al.*, 1991; Neale and Kitchingman, 1991). In the case of Ly8, the lengths of the I_γ sequences (177 bp) were consistent with an undetectable shift of *BCL6* RNA migration in gel electrophoresis.

Nucleotide sequencing of the IgH–*BCL6* cDNA junctions (Figure 3) provided information on the coding potential of the fusion transcripts. In all the RNA species

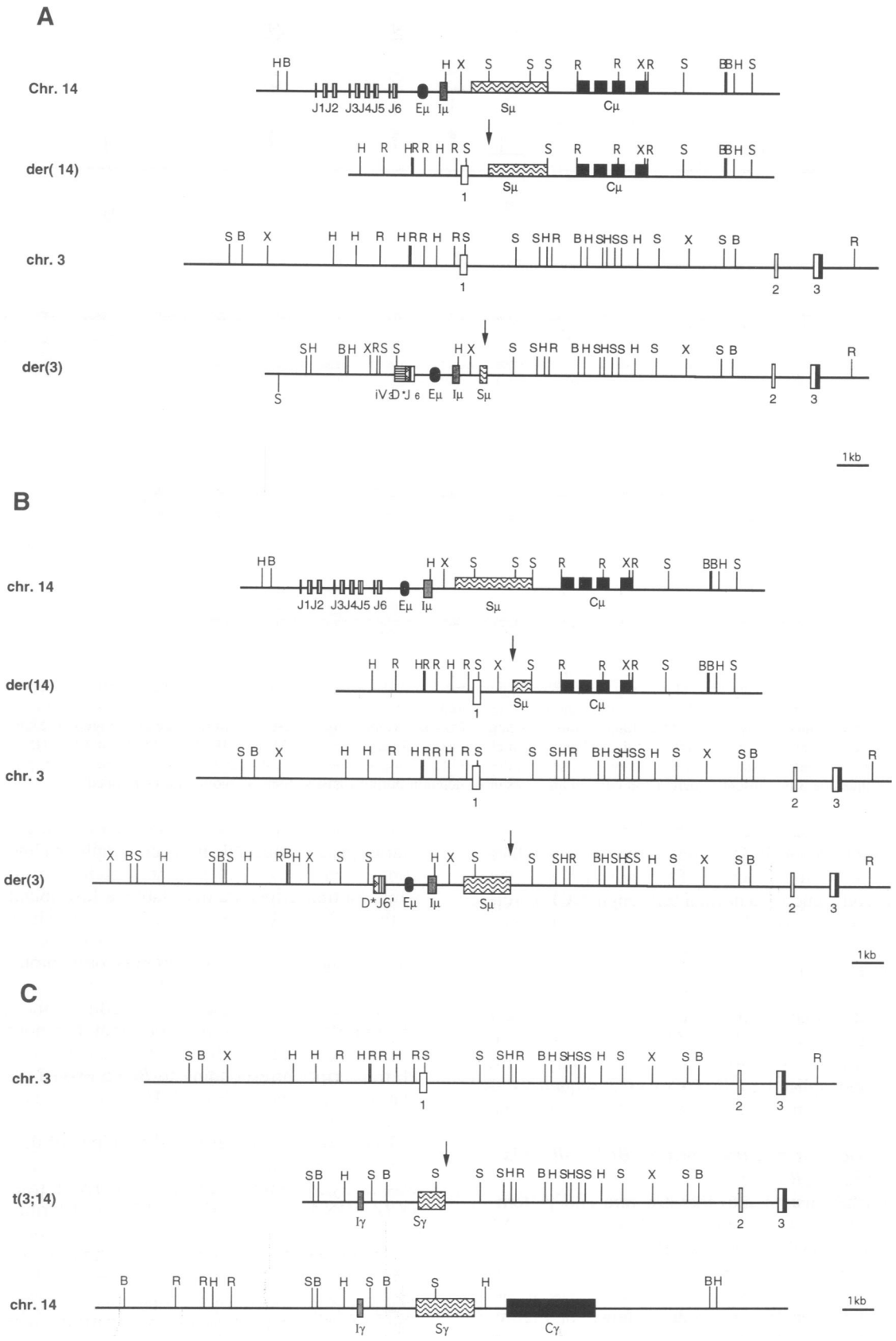

Fig. 1. Molecular cloning of chromosomal breakpoints of t(3;14) translocations in three NHL cases: SM1444 (**A**), KC1445 (**B**) and Ly8 (**C**). For each case, schematic representations of the cloned breakpoint regions [der(14) and der(3); der(3) not shown for Ly8, see text] and their respective germline counterparts (chromosomes 3 and 14) are shown. Relevant functional domains are indicated for chromosomes 14 (iV_3, inverted V_3 region; D, diversity region; J1–6, joint regions; E_μ, IgH transcriptional enhancer; I_μ, IgH germline transcript promoter region; S_μ, switch μ region; C_μ, C_μ exons) and 3 (*BCL6* exons are indicated by numbered boxes; filled boxes indicate coding sequences). Restriction enzyme abbreviations are: B, *Bam*HI; X, *Xba*I; H, *Hin*dIII; R, *Eco*RI; S, *Sac*I. Arrows indicate the positions of the chromosomal breakpoints.

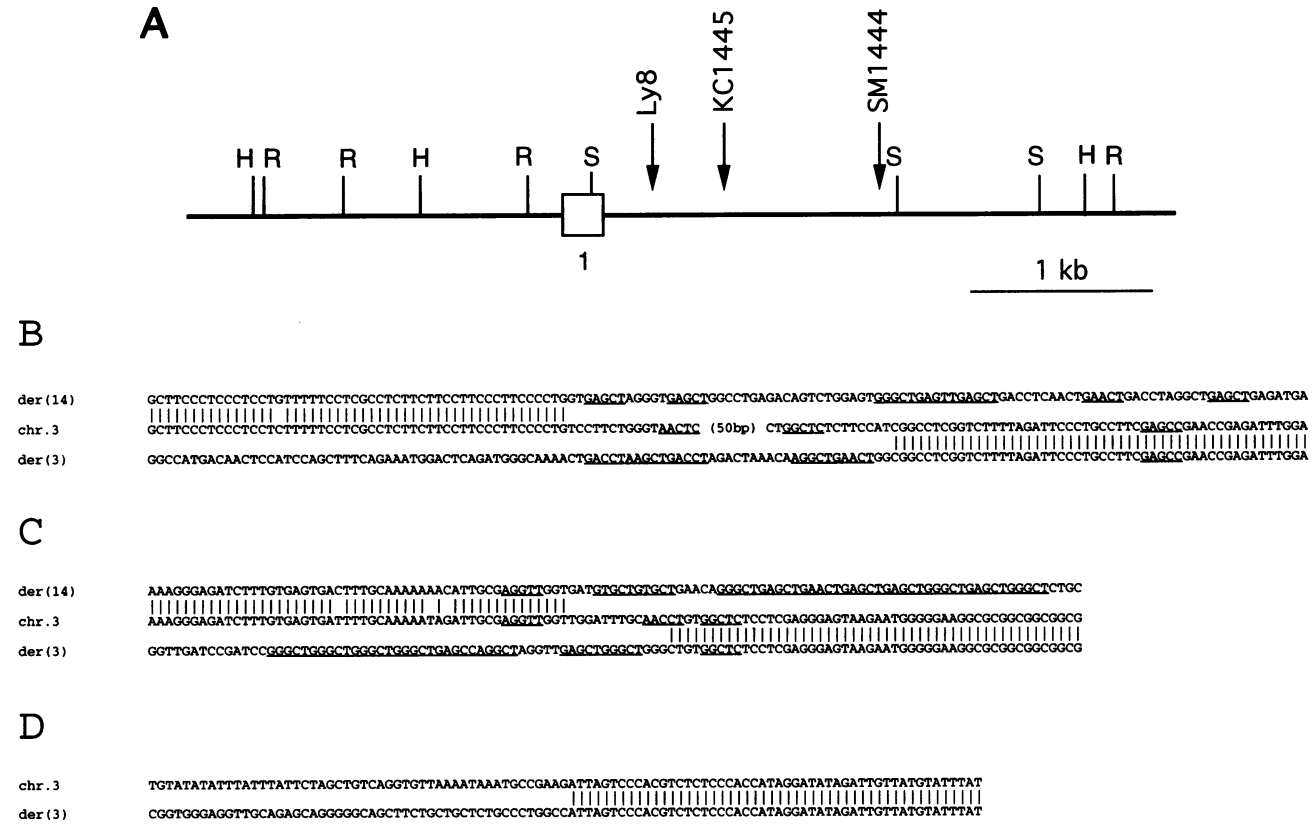

Fig. 2. Nucleotide sequence analysis of cloned t(3;14) breakpoint junctions from NHL cases SM1444, KC1445 and Ly8, and alignment to the corresponding germline regions. (**A**) Schematic representation of the genomic region spanning *BCL6* exon 1 showing the position of the chromosomal breakpoints (arrows) in the three tumor cases. Restriction enzyme symbols are as described in the legend to Figure 1. Shown below are the sequences spanning the breakpoints and their alignment to chromosome 3 sequences for SM1444 (**B**), KC1445 (**C**) and Ly8 (**D**). Sequence identity is shown by vertical bars. A point mutation(s) is seen in the der(14) portion shown for KC1445 and SM1444. The sequences present in germline chromosome 3 but absent in der(3) and der(14) are a result of deletion during translocation. S_μ motifs are underlined.

identified, a termination codon in *BCL6* exon 2 in phase with the downstream *BCL6* initiation codon was retained just 3′ to the fusion junction. This indicated that these transcripts could encode a normal full-length BCL6 protein whose translation would be initiated from its normal initiation codon (50 nucleotides 3′ to the IgH–*BCL6* junction in exon 3).

In summary, our analysis of the 5′ terminus of *BCL6* RNA indicated that in all three NHL cases the der(3) chromosome expressed Ig–*BCL6* chimeric transcripts initiated within the IgH locus, most commonly at the I promoters and spliced to *BCL6* exon 2 sequences. All these transcripts retained an intact *BCL6* coding domain.

The chimeric, but not the normal, BCL6 allele is transcribed in Ly8 cells

To gain further insight into the structure and pattern of expression of the various *BCL6* mRNA species in NHL with t(3;14), we analyzed *BCL6* mRNA in Ly8 cells using RNase protection assays. First, we tested whether Ly8 cells expressed a *BCL6* RNA lacking exon 1 sequences, as predicted by the RACE product shown in Figure 3. Previous RNase protection and cDNA cloning studies have shown that *BCL6* transcription is normally initiated from a major (Pb) promoter, active in all cells tested, and an upstream minor (Pa) promoter, active in only some B cell lines (data not shown). Representative results in Figure 4A demonstrate that with a ribo probe spanning exons 1–3 and diagnostic for Pa versus Pb transcripts, abundant Pb transcripts are detectable in B cell lymphoma lines carrying unrearranged *BCL6* genes (Bjab, Daudi, Ramos and Ly1), while Pa transcripts are detectable at low abundance only in the Bjab and Ly1 cell lines. However, while Ly8 cells do not contain any intact Pa or Pb transcript, they do contain a novel species (see 69 nucleotide protected band) consistent with a transcript retaining exon 2–3 sequences but lacking exon 1 sequences. In addition, the absence of Pb and Pa transcripts indicates that the normal *BCL6* allele is silent in Ly8 cells. The failure to detect a truncated Pb transcript corresponding to *BCL6* exon 1 also implies that the reciprocal *BCL6*–IgH transcript is either not produced or unstable.

To determine whether the 5′ terminus of this abnormal transcript contained $I_\gamma 3$ sequences, the same Ly8 RNA was analyzed with a ribo probe derived from the $I_\gamma 3$–*BCL6* cDNA junction (Figure 4B). To determine whether $I_\gamma 3$–*BCL6* transcripts represented the major species of *BCL6* transcripts in Ly8 cells, a second ribo probe corresponding to *BCL6* exons 8–9, and thus detecting all *BCL6* transcripts, was also tested in parallel. The result showed that Ly8 cells, but not control cells carrying normal *BCL6* alleles, contain transcripts compatible with an $I_\gamma 3$–*BCL6* chimeric RNA, and that these transcripts account for the majority, if not all, of the *BCL6* species in these cells (compare the intensities of the 340 versus the 221 nucleotide fragments, noting the smaller size of the $I_\gamma 3$–*BCL6* fragments).

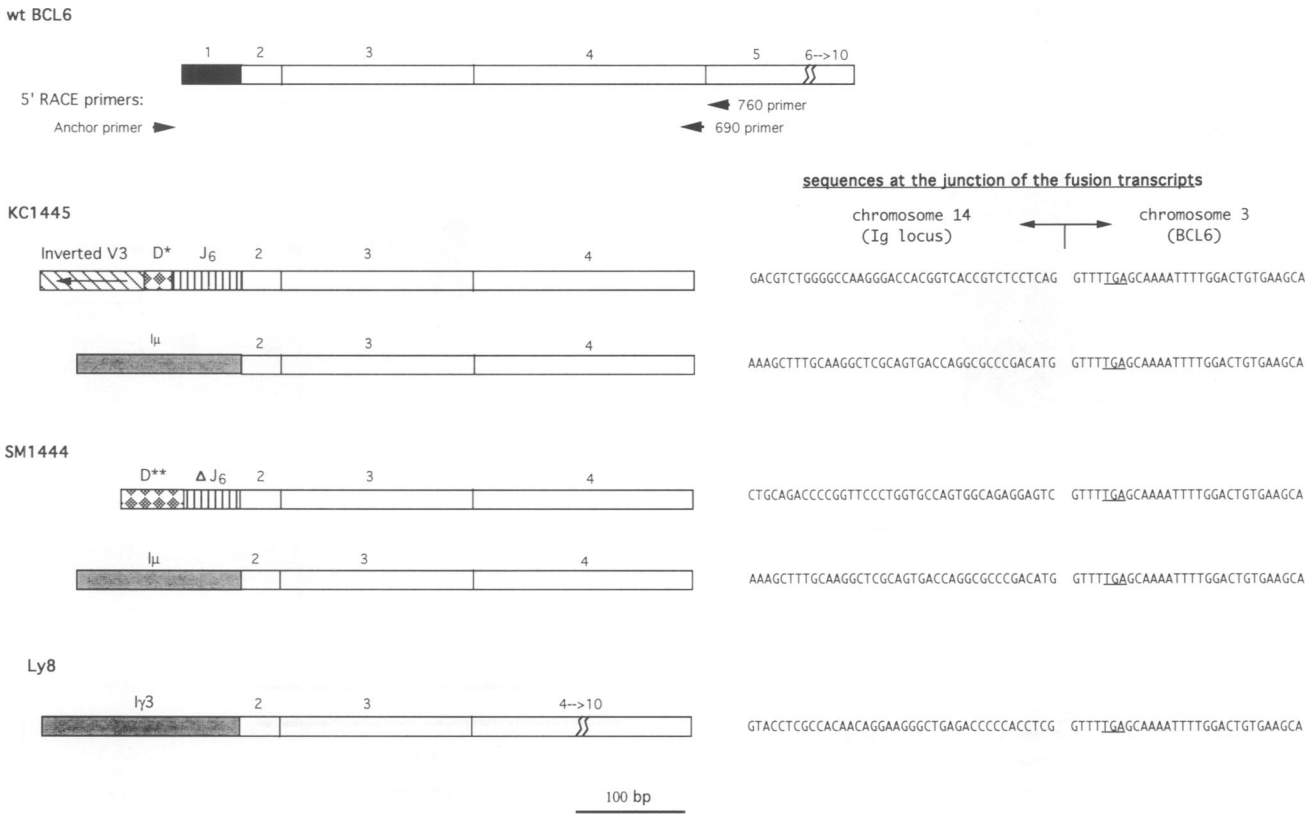

Fig. 3. Cloning of the 5′ termini of IgH–*BCL6* fusion transcripts and a nucleotide sequence analysis of the IgH–*BCL6* cDNA junctions. At the top of the figure, a schematic representation of the normal *BCL6* cDNA (wild-type *BCL6*; exons are numbered on top) shows the approximate position of the *BCL6*-specific (760, 690) and 5′ anchor primers used for the RACE analysis. Aligned below are the structures of the 5′ RACE products from SM1444, KC1445 and the Ly8 cDNA clone. Sequences at the junction of each fusion transcript are also given. A TGA termination codon located 3′ to the IgH–*BCL6* junctions and in-frame with the downstream *BCL6* initiation codon (in exon 3, results not shown) is underlined.

Taken together, these results indicate that the chimeric IgH–*BCL6* gene on the der(3) chromosome produces a fusion $I_\gamma 3$–*BCL6* mRNA species in which the $I_\gamma 3$ exons are spliced to the *BCL6* exons 2–10 (Figure 4C). Because the rearranged *BCL6* gene is expressed under the control of the $I_\gamma 3$ promoter(s) when the normal allele is silent, these results also suggest that *BCL6* expression is deregulated in these cells (see below).

Chimeric IgH–BCL6 transcripts produce a normal BCL6 protein

To determine whether the $I_\gamma 3$–*BCL6* transcript detected in Ly8 could produce a normal BCL6 protein, as predicted by a nucleotide sequence analysis (Figure 3), whole-cell lysate from Ly8 cells were examined for BCL6 expression by a Western blot analysis using a rabbit antiserum against the N-terminal portion of the BCL6 protein (N-70-6; Cattoretti *et al.*, 1995). Figure 5 shows that a normal 95 kDa protein was detected in Ly8 cells indistinguishable from that detected in B cell lines that express BCL6 from non-rearranged *BCL6* genes (Ramos) or from a transfected *BCL6* expression vector [EB3(+); Cattoretti *et al.*, 1995]. Because we have shown that the normal *BCL6* allele is silent in Ly8 cells, the BCL6 protein must be coded by the rearranged allele, confirming its intact BCL6 coding capacity.

Discussion

Rearrangements involving the *BCL6* gene occur in 30–40% of DLCL and in a small fraction (6–14%) of FL, representing the second most frequent lesion in human lymphoid malignancies after *BCL2* rearrangements (reviewed by Dalla-Favera *et al.*, 1994). Despite this frequency and the clinical relevance of *BCL6* alterations (Offit *et al.*, 1994), their mechanism of occurrence and functional consequences on *BCL6* function are not known. By focusing on a subset of *BCL6* rearrangements (~10% of cases) involving the IgH locus, this study has elucidated one mechanism of *BCL6* alteration with implications for the mechanism of chromosomal translocation, the consequences of other types of translocation involving *BCL6* and the role of these alterations in lymphomagenesis.

Implications for the mechanism of chromosomal translocation

At the structural level, the IgH–*BCL6* recombinations observed in DLCL are typical examples of reciprocal translocations involving the IgH switch region in lymphoma, similar to those involving the *MYC* gene in t(8;14)(q24;q32) in sporadic and AIDS-associated Burkitt's lymphoma (BL; Neri *et al.*, 1988). Analogous to BL-associated translocations, t(3;14) represents non-homologous recombinations because the S sequences on

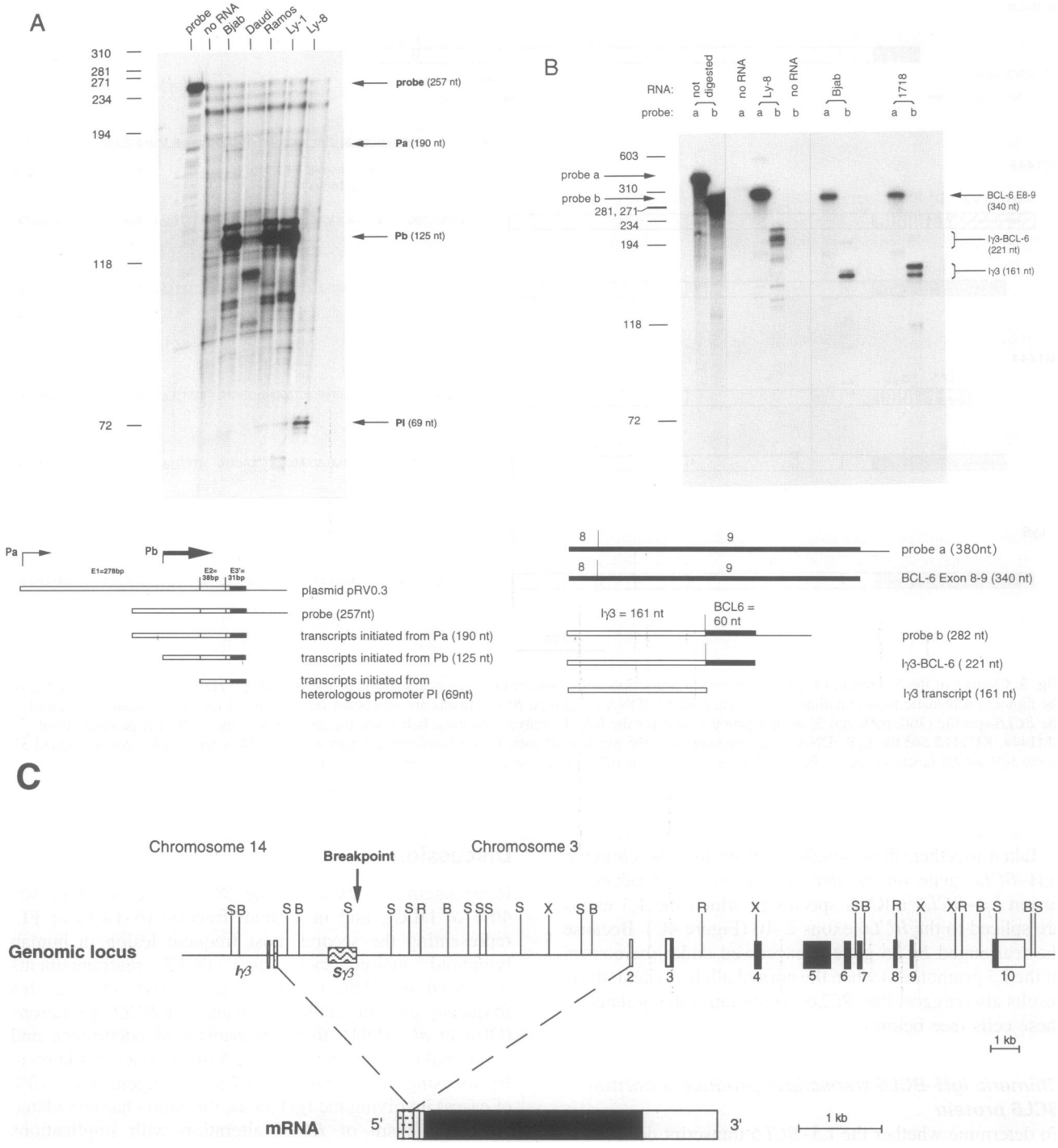

Fig. 4. RNase protection analysis of the IgH–*BCL6* fusion transcripts in Ly8 cells. (**A**) Detection of transcripts lacking 5′ *BCL6* sequences in Ly8 cells using a ribo probe (257 nucleotides) spanning *BCL6* exons 1–3 (E1–3) and diagnostic for Pa versus Pb transcripts. Schematic representations of the plasmid insert used to generate the ribo probe, of the probe itself and of the protected RNA fragments are shown below the autoradiogram. The shaded area represents the coding region. In the autoradiogram, arrows point to the protected fragments. The PI (69 nucleotides) band, corresponding to the transcripts lacking *BCL6* exon 1 sequences, is protected only in Ly8, but not in RNAs from the NHL cell lines Bjab, Daudi, Ramos and Ly1. Bands corresponding to normal Pa- and Pb-initiated transcripts are detectable in all RNAs except for Ly8. Additional protected bands below the Pb product in Daudi might be caused by mutation(s) within exon 1 in these cells. (**B**) Identification of $I_\gamma3$–*BCL6* sequences in Ly8 cells. Two ribo probes derived from cDNAs spanning *BCL6* exons 8–9 (probe a) or the $I_\gamma3$–*BCL6* junction in Ly8 cDNA (probe b) were used to confirm the presence and to estimate the relative abundance of $I_\gamma3$–*BCL6* transcripts in Ly8 RNA. Multiple bands corresponding to $I_\gamma3$–*BCL6* transcripts from multiple initiation sites within the $I_\gamma3$ promoter region are detectable in Ly8 RNA, but not in RNAs from Bjab or 1718 (a DLCL biopsy carrying the unrearranged *BCL6* gene). The absence of normal $I_\gamma3$ transcripts in Ly8 cells may be due to either the use of an alternative $I_\gamma3$ promoter sequence located 3′ to those represented in the probe (Neale and Kitchingman, 1991) or the deletion of the $I_\gamma3$ region. (**C**) Altered *BCL6* genomic locus in Ly8 and the structure of the $I_\gamma3$–*BCL6* fusion transcript. Translocation fused the truncated $_\gamma3$ locus in a head-to-tail fashion to *BCL6* intron 1. Transcription was initiated from the $I_\gamma3$ promoter. The 177 bp $I_\gamma3$ sequences were spliced to *BCL6* exon 2, leaving the *BCL6* coding domain (solid areas) intact. The restriction enzyme symbols are as described in the legend to Figure 1.

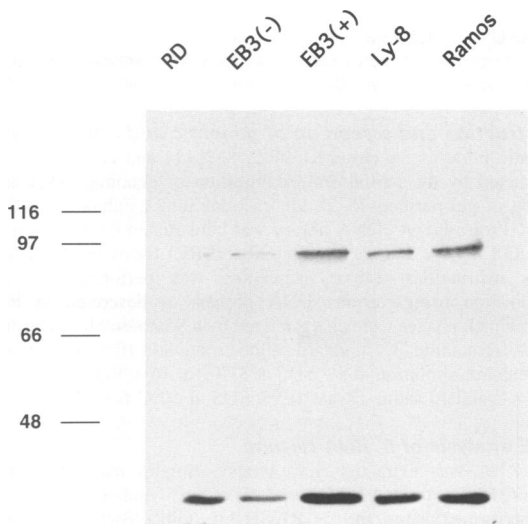

Fig. 5. Western blot analysis of BCL6 protein expression in Ly8 cells. Cell lysates from the RD, Ramos or Ly8 cells, or from the EB3 cell line stably transfected with a control vector [EB3(–)] or with a vector expressing a full-length *BCL6* cDNA coding domain [EB3(+)], were analyzed using the N-70-6 anti-BCL6 antiserum (Cattoretti *et al.*, 1995). A 95 kDa band corresponding to the BCL6 protein was detectable in lysates from Ly8, Ramos and BCL6-transfected EB3 cells, while only trace amounts were detectable in RD and EB3 cells, two Epstein–Barr virus-immortalized lymphoblastoid cell lines which contain trace amounts of *BCL6* RNA (Cattoretti *et al.*, 1995). The same 95 kDa protein is recognized by additional antiserum and monoclonal antibodies generated against distinct epitopes of the *BCL6* protein (Cattoretti *et al.*, 1995; Flenghi *et al.*, 1995).

14q32 are joined to sequences on 3q27 which lack recognizable S motifs or any apparent homology in the proximity of the breakpoints. In the two DLCL cases in which both reciprocal junctions could be analyzed, the recombination involved deletions of chromosome 3 sequences. This finding, in further analogy to BL-associated translocations, is consistent with the recombination being preceded by double-strand breaks, followed by the deletion of the staggered DNA strands and blunt-end ligation (Neri *et al.*, 1988).

Translocations involving Ig S sequences have been proposed to occur at the stage of B cell differentiation associated with Ig isotype switching and to represent mistakes of this mechanism (Lieber, 1993). This model is consistent with the invariant association between this type of recombination and NHL displaying a mature B cell phenotype, corresponding to the stage of B cell differentiation during which the Ig isotype switch normally occurs (Dalla-Favera *et al.*, 1987; Haluska *et al.*, 1991). Our findings, that some translocations involving *BCL6* are mediated by Ig S sequences, provide further support to this model because DLCL also appear to derive from mature B cells within germinal centers, where Ig switching normally occurs. Because it is apparently not based on sequence homology, this 'false switch' mechanism may also underlie the recombinations between *BCL6* and other non-homologous chromosomal regions in 3q27 translocations which do not involve Ig loci.

Promoter substitution: a novel role for Ig genes in chromosomal translocation

The juxtaposition of the IgH locus with the *BCL6* gene and the expression of IgH–*BCL6* fusion transcripts initiated from IgH promoters define a novel role for IgH genes in chromosomal translocation. In other types of NHL-associated translocation, IgH sequences are juxtaposed to the *MYC*, *BCL-2* or *BCL1* genes, and lead to the deregulated expression of these genes by contributing enhancer or other distantly acting regulatory sequences (e.g. in the case of *MYC* and *BCL1*, see Dalla-Favera, 1993 for a review) or by participating in fusion transcripts in which the 3′ untranslated sequences derive from Ig genes (*BCL-2*; Korsmeyer, 1992). Conversely, in the t(3;14) translocations, the IgH genes provide promoter and 5′ untranslated sequences which substitute for the corresponding *BCL6* sequences, a mechanism that we have called promoter substitution. While translocations involving promoter substitutions have not been observed before in B cell malignancies (for a review see Rabbitts, 1994), one example exists in T cell-derived acute lymphoblastic leukemia in which the chromosome 1p33 deletion can join *SIL* exon 1 to *TAL-1* exon 3 in a head-to-tail fashion (Aplan *et al.*, 1992; Baer, 1993)

In all three cases studied here, IgH promoter regions (I_μ or $I_\gamma 3$) were found fused to *BCL6* transcripts, while in two DLCL biopsies (KC1445 and SM1444; Figure 3), additional fusion transcripts initiated from further upstream D or V–D sequences could also be identified. Although the 5′ termini and the abundance of the latter transcripts could not be characterized, the recurrent selection of I promoters, together with the fact that $I_\gamma 3$–*BCL6* fusion transcripts represent the most abundant *BCL6* RNA species in Ly8 cells (Figure 4B), suggest that the recruitment of I promoters may be the most common and therefore the most biologically significant consequence of the Ig–*BCL6* recombination. It remains to be determined whether the I promoters are selected because of their physical proximity to the breakpoint junction or for some specific regulatory property (see below).

Although demonstrated in three cases with t(3;14) translocations, the promoter substitution mechanism may also apply to 3q27 translocations which do not involve band 14q32 and the IgH locus. In most NHL cases carrying rearranged *BCL6* genes, the chromosomal breakpoint lies within the 5′ portion of the first intron, similar to the three cases studied here. This implies that in these cases the *BCL6* gene also loses its promoter region and is most probably transcribed as a fusion transcript driven by a heterologous promoter. This hypothesis is consistent with the recent report that a DLCL case carrying a t(3;4)(q27;p11) translocation produces chimeric transcripts in which *BCL6* sequences are fused to 5′ untranslated sequences of a gene, *TTF*, located on chromosome 4 and normally expressed in B cells (Dallery *et al.*, 1995). In a minority of cases carrying *BCL6* rearrangements, the breakpoints are located immediately 5′ to the promoter region and therefore remove only the putative 5′ regulatory sequences. In these cases, it is conceivable that the consequence of the translocation may be the substitution of these sequences with enhancers or distantly acting regulatory elements from other chromosomes.

In Ly8 cells, no transcripts were detected which accounted for the *BCL6*–IgH fusion, and therefore the reciprocal *BCL6*–IgH allele was not transcriptionally active. A similar phenomenon was also observed in the t(3;4)(q27;p11) translocation, whereas the reciprocal

BCL6–TTF fusion was not transcribed (Dallery *et al.*, 1995). These results strongly suggest that the altered expression of the *BCL6* gene is the critical event selected during lymphomagenesis and that the reciprocal translocation junction may not have biological significance.

Consequences of BCL6 promoter substitution on BCL6 expression

The finding that in some NHL the normal *BCL6* promoter is substituted by heterologous regulatory sequences, such as I_μ or $I_\gamma 3$, implies that *BCL6* expression is deregulated in these tumors. This notion is supported directly by the finding that the chimeric Ig–*BCL6* gene, but not the normal *BCL6* allele or the *BCL6*–IgH allele, is transcribed in Ly8 cells (Figure 3), indicating that the translocation causes the expression of *BCL6* in cells where the normal *BCL6* promoter is silent. Although the pattern of regulation of I_μ and $I_\gamma 3$ promoters has not been characterized extensively in human cells, several observations, including studies in murine cell lines, suggest that these promoters are regulated differently from those of *BCL6*. For instance, while the *BCL6* gene is not regulated by mitogenic signals and is downregulated during the differentiation of mature B cells into immunoblasts and plasma cells (Cattoretti *et al.*, 1995; Flenghi *et al.*, 1995), the murine I_μ promoter is induced by mitogens (Li *et al.*, 1993) and is expressed in plasma cells (Su and Kadesch, 1990). Similarly, the $I_\gamma 3$ promoter contains an interleukin (IL)-4-responsive element and is upregulated by IL-4 (Kuze *et al.*, 1991), while the *BCL6* promoter region lacks such an element and is downregulated by IL-4 in some B cell lines tested (J.Zhang, unpublished data). One common consequence of these alterations may be the prevention of the physiological downregulation of *BCL6* subsequent to the differentiation of B cells into plasma cells (Cattoretti *et al.*, 1995; Flenghi *et al.*, 1995).

The *BCL6* gene encodes a zinc-finger transcription factor with potent transcriptional repression activity (C.-C.Chang and B.H.Ye, unpublished results). Although the BCL6 DNA binding site has been found (Kawamata *et al.*, 1994; B.H.Ye, unpublished results), the genes regulated by *BCL6 in vivo* have not been identified. Therefore, the biological consequences of *BCL6* deregulation and its contribution to lymphomagenesis await further studies. The Ig–*BCL6* chimeric genes identified in NHL will serve as tools to address these questions in transgenic mice.

Materials and methods

Tumor biopsies and cell lines

Lymph node biopsies from NHL patients were collected at the time of diagnosis at the Memorial Sloan-Kettering Cancer Center in New York. Diagnoses were based on the results of histopathological and immunophenotypical analyses (Knowles, 1992). The fraction of malignant cells in each biopsy was at least 70%, as determined by a cytofluorimetric analysis of cell-surface markers and/or a rearrangement analysis of antigen receptor genes. Cell lines Ly1 and Ly8 were first established at the Ontario Cancer Institute, Toronto, Canada, from two patients with DLCL and immunoblastic lymphoma, respectively (Tweeddale *et al.*, 1987). RD is an Epstein–Barr virus-immortalized lymphoblastoid cell line. EB3, Ramos, Daudi and Bjab are BL cell lines with a normal *BCL6* gene. Using a Northern blot analysis, RD and EB3 were shown to express barely detectable levels of *BCL6* RNA, whereas Ramos, Daudi and Bjab had abundant *BCL6* messages. The EB3 cell line was stably transfected with either an episomally replicating plasmid pHeBo–CMV–BCL6, which has the full-length *BCL6* coding region under the control of the cytomegalovirus (CMV) enhancer/promoter, or a control plasmid lacking *BCL6* sequences (Cattoretti *et al.*, 1995).

Construction and screening of genomic and cDNA libraries

Genomic libraries for cases KC1445, SM1444 and cell line Ly8 were constructed by the partial *Sau*3A digestion of genomic DNA and the ligation of gel-purified 15–20 kb fractions into Lambda-Gem11 phage vector (Promega). A cDNA library was constructed for Ly8 cells in the λZIPLOX cDNA cloning vector (Gibco/BRL) following the manufacturer's instructions. Library screening was performed by plaque hybridization using various DNA probes, as described in Results. Recombinant phages were characterized by a Southern blot hybridization in 50% formamide, 3× standard saline citrate and 10× dextran sulfate–5× Denhardt's solution–0.5% SDS at 37°C for 16 h. Filters were washed in 0.2× standard saline citrate–0.5% SDS at 60°C for 2 h.

RACE analysis of 5′ RNA termini

Total RNA was extracted from biopsy samples using the TRISOL (Gibco/BRL) method. A RACE analysis of the 5′ end of *BCL6* transcripts was performed using the 5′ RACE kit (Gibco/BRL) following the manufacturer's instructions. The first strand cDNA was primed with the 760 primer (5′-GTTGAGGAACTCTTCAC-3′) located at the 5′ end of exon 5, and amplified using a nested PCR using the anchor primer and the 690 primer (5′-CAAGTGTCCACAACATGC-3′) located in exon 4. The final PCR products were cloned into the pCR vector (Invitrogen) and sequenced on the ABI 373A DNA sequencer (Perkin-Elmer, Applied Biosystems Division).

RNase protection assays

Plasmids containing the probe templates were linearized by an appropriate restriction digestion at the 5′ ends of the inserts. The antisense ribo probes were transcribed from the downstream viral promoter in the presence of [α-^{32}P]UTP. Purified probe (5×10^5 c.p.m.) was annealed to 20 μg total RNA in hybridization solution [80% (v/v) formamide, 400 mM NaCl, 1 mM EDTA, 40 mM PIPES] overnight at 57°C, and then digested with a mixture of RNase A (40 μg/ml final concentration) and RNase T1 (2 μg/ml final concentration) at 37°C for 10–15 min. After terminating the reaction by phenol–chloroform extraction, the protected ribo probes were purified by ethanol precipitation and resuspended in 80% (v/v) formamide-containing dye, before being electrophoresed on a 6% polyacrylamide gel containing 7 M urea. End-labeled ØX174/*Hae*III fragments were also loaded on the gel to serve as single-strand molecular weight markers.

Western blot analysis

Proteins were extracted from exponentially growing cells, subjected to denaturing gel electrophoresis, transferred to nitrocellulose membranes and immunostained according to methods published previously (Towbin *et al.*, 1979). The characterization of the rabbit anti-BCL6 antiserum (N-70-6) has been described elsewhere (Cattoretti *et al.*, 1995).

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