Cell, Vol. 96, 35–45, January 8, 1999, Copyright ©1999 by Cell Press

Bcl10 Is Involved in t(1;14)(p22;q32) of MALT B Cell Lymphoma and Mutated in Multiple Tumor Types

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Summary

MALT B cell lymphomas with t(1;14)(p22;q32) showed a recurrent breakpoint upstream of the promoter of a novel gene, *Bcl10*. *Bcl10* is a cellular homolog of the equine herpesvirus-2 E10 gene: both contain an amino-terminal caspase recruitment domain (CARD) homologous to that found in several apoptotic molecules. *Bcl10* and E10 activated NF-κB but caused apoptosis of 293 cells. *Bcl10* expressed in a MALT lymphoma exhibited a frameshift mutation resulting in truncation distal to the CARD. Truncated *Bcl10* activated NF-κB but did not induce apoptosis. Wild-type *Bcl10* suppressed transformation, whereas mutant forms had lost this activity and displayed gain-of-function transforming activity. Similar mutations were detected in other tumor types, indicating that *Bcl10* may be commonly involved in the pathogenesis of human malignancy.

Introduction

The lymphomas of mature B cells are a heterogeneous group of disorders that frequently exhibit chromosomal translocations targeted to the immunoglobulin (*Ig*) loci. These translocations may be specific for histologically defined subtypes of disease and involve genes controlling cell proliferation and apoptosis. Thus, translocations involving the *Myc* gene with the *Ig* loci are seen in nearly all cases of Burkitt's lymphoma (Magrath, 1990). Similarly, *Bcl2*, which suppresses several forms of apoptosis, was cloned from the t(14;18)(q32;q21) found in follicular B cell lymphoma, whilst *Ig* translocations involving the *Bcl1/Cyclin D1* gene on chromosome 11q13 are seen in mantle cell lymphoma (Tsujimoto et al., 1984; Vaandrager et al., 1996). Translocation to the *Ig* loci may result not only in deregulated expression of the incoming oncogene, due in part to the proximity of potent B cell transcriptional enhancers within the *Ig* loci (Jain et al., 1993), but also in mutations due to the action of the *Ig* somatic hypermutation mechanism (Rabbitts et al., 1983; Migliazza et al., 1995; Matolcsy et al., 1996). This mechanism may also induce mutations in normal B cells in nontranslocated genes (Shen et al., 1998).

B cell lymphomas of mucosa-associated lymphoid tissue (MALT lymphomas) are the commonest form of lymphoma arising in extranodal sites, in most cases arising in the gastric mucosa (Isaacson and Spencer, 1995). They may be either high or low grade. It is likely that most high-grade cases represent transformation of undetected low-grade disease (Peng et al., 1997). Low-grade MALT lymphoma is an indolent disease and is often preceded by either chronic inflammation or autoimmune disease such as Hashimoto's thyroiditis in thyroid and chronic *Helicobacter pylori* infection in stomach. The etiological link between low-grade gastric MALT lymphoma and lymphoid reaction to *H. pylori* infection has been demonstrated by the regression of some cases with antibiotic therapy alone (Wotherspoon et al., 1993).

The molecular events leading to *H. pylori*–independent growth and high-grade transformation of MALT lymphoma are unknown. Cytogenetic studies of low-grade cases have identified abnormalities of chromosome 1p22, in particular translocation t(1;14)(p22;q32), as uncommon but recurrent events (Wotherspoon et al., 1992). Cases with 1p22 abnormalities were more aggressive than typical cases of low-grade disease and also grew in vitro without additional stimuli (Hussell et al., 1993 and M-Q. D. et al., unpublished observations).

We have cloned a t(1;14)(p22;q32) translocation breakpoint from a case of low-grade MALT lymphoma. This was a recurrent breakpoint adjacent to a novel gene, *Bcl10*, that exhibited an amino-terminal caspase recruitment domain, or CARD (Hofmann et al., 1997), similar to that found in apoptotic regulatory and effector proteins including RAIDD, (Duan and Dixit, 1997), RICK/RIP2/CARDIAK (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998), and ARC (Koseki et al., 1998). Unexpectedly, *Bcl10* was proapoptotic in 293 cells. However, a MALT lymphoma with the t(1;14)(p22;q32) showed a *Bcl10* frameshift mutation resulting in truncation beyond the CARD. Unlike the wild-type protein, truncated mutants failed to induce apoptosis and enhanced transformation by cooperating oncogenes. These data suggest that truncating mutations of *Bcl10* may confer a survival benefit to MALT B cell lymphomas and may allow progression to antigen-independent proliferation. Truncating *Bcl10* mutations similar or identical to those observed in the MALT lymphoma were seen in other tumor

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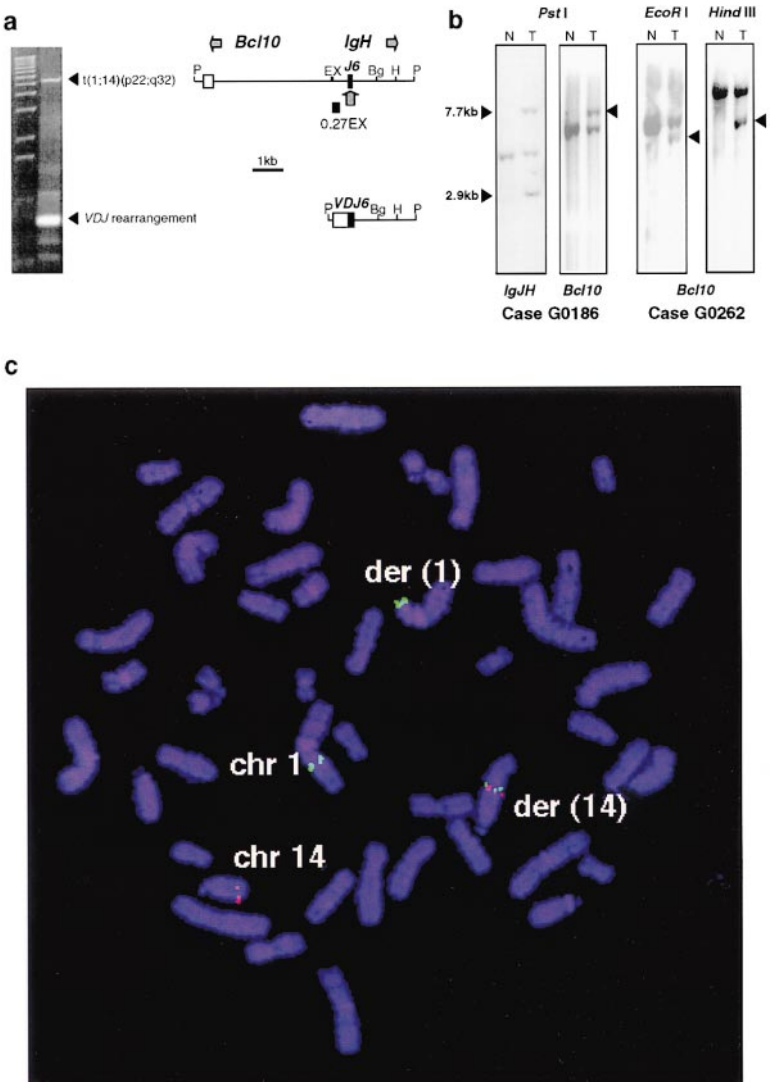

Figure 1. Molecular Cloning of t(1;14)(p22;q32) Translocation Breakpoint in a Case of Low-Grade MALT Lymphoma

(A) Gel electrophoresis of LDI-PCR products from PstI-digested DNA together with schematic representations of the two rearrangements. LDI-PCR yielded products of 5.5 and 0.7 kb. Sequence analysis of these revealed a productive *IgVDJ* rearrangement on the shorter allele and novel sequences upstream of *JH6* in the other. A single copy probe (0.27EX) was used to isolate cosmid clones that were mapped back to 1p22 by FISH, demonstrating that this allele represented the translocation breakpoint. Open box represents the region of identity with ESTs. Orientation of the open reading frame revealed a head-to-head configuration of *Bcl10* and *IgH*. Bg, BglII; E, EcoRI; H, HindIII; P, PstI; X, XbaI.

(B) Southern blot analysis of MALT lymphomas with t(1;14)(p22;q32). Left hand panel: Case G0186 (from which the breakpoint was cloned) showed biallelic *IgJH* rearrangement with one allele comigrating with rearranged *Bcl10* probe 0.27EX, indicating that no artifacts had been introduced during LDI-PCR. Right hand panel: Case G0262 showed one *IgJH* rearrangement. This represented a productive *VDJ* rearrangement (data not shown); we assume that this case had undergone translocation to a downstream switch region rather than to *IgJH*. This case showed rearrangements with the *Bcl10* probe in multiple digests, indicating a clustering of breakpoints within the same region of the *Bcl10* gene. "N" denotes normal and "T" tumor DNA samples. Arrowheads denote rearranged fragments.

(C) FISH image of MALT lymphoma case with t(1;14)(p22;q32). Cosmid *Ig3/64* containing sequences for *IgJH* and $C\mu$ was labeled red, and CEPH YAC 929e1 containing *Bcl10* was labeled green. The *Bcl10* YAC was split by the translocation and colocalized with *IgH* on the derivative chromosome 14.

types, indicating that *Bcl10* may be involved in the pathogenesis of several human malignancies.

Results

Cloning of the t(1;14)(p22;q32) Breakpoint and Identification of *Bcl10*

Three cases of low-grade MALT lymphoma with t(1;14)(p22;q32) were studied. On the assumption that the translocation breakpoint involved the *IgH* locus directly, both *IgJH* alleles from one case (G0186) were cloned using long-distance inverse PCR (LDI-PCR; Willis et al., 1997, 1998). Two products of 700 and 5500 base pairs (bp) were obtained (Figure 1A). The sequence of the 700 bp allele showed a productive and mutated *VH3-20* rearrangement (data not shown), while the other allele contained sequences with no homologies to any known sequences beyond *JH6*. To confirm that this represented the translocation breakpoint, cosmids were isolated using single copy probes from this region and mapped back to chromosome 1p22 on normal metaphases by fluorescent in situ hybridization (FISH; data not shown). In case G0186, *JH* and 1p22 probes showed comigration of rearranged bands, indicating that no major artifacts had been introduced during the LDI-PCR (Figure 1B). Further FISH experiments showed a break within a derived *Bcl10* YAC clone (Figure 1C), and Southern blots using 1p22 probes derived from the breakpoint showed rearrangement in all three cases, indicating that this was a recurrent breakpoint in MALT lymphoma (Figure 1B).

To identify the involved gene on chromosome 1p22, the 5.5 kb PstI fragment was sequenced and showed a 255 bp region 4.6 kb upstream of *JH6* with 100% identity to EST clone # 1184134. Genomic sequences immediately 5′ of this potential exon had features of a promoter site and contained many potential protein-binding sites (accession number AJ006290). These data indicated a head-to-head configuration of the translocation (Figure 1A).

By a combination of DNA–database searching using dbEST (www.ncbi.nlm.nih.gov/dbEST) and reverse transcription PCR methods, the full-length coding sequence of this gene was determined (accession number

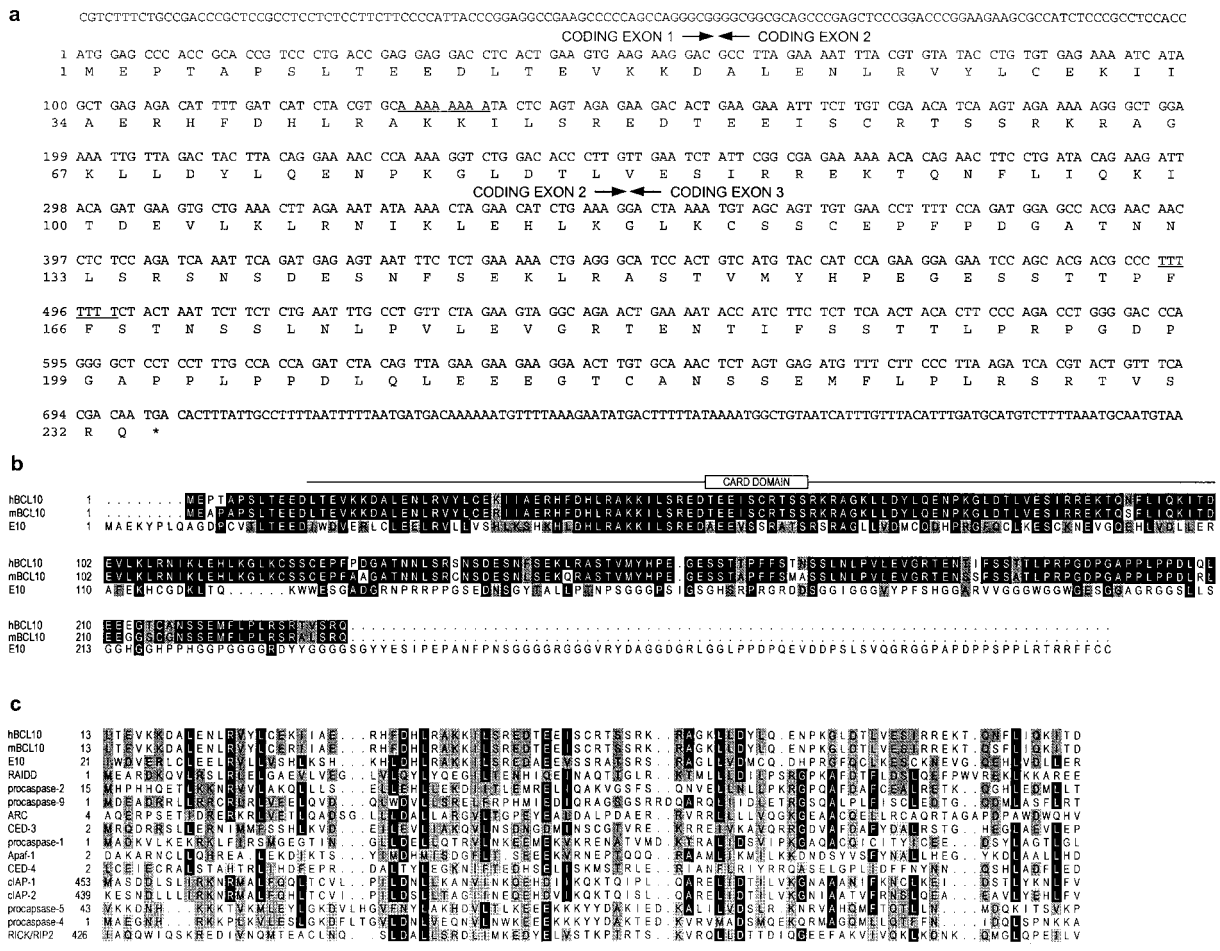

Figure 2. Sequence of *Bcl10*

(A) Nucleotide and predicted amino acid sequences of *Bcl10*. Exon/intron boundaries are indicated. Mononucleotide runs mutated in malignancies are underlined.

(B) Amino acid sequence alignment of human and mouse *Bcl10* and the EHV-2 open reading frame E10. *Bcl10* and E10 contain highly homologous CARDs but diverge thereafter. The published sequence of EHV-2 ORF E10 (accession U20824; Telford et al., 1995) contains a frameshift at codon 143, predicting a protein of 210 amino acids. The corrected sequence predicting a protein of 311 amino acids is shown. Black boxes indicate ≥50% amino acid identity; gray shading indicates ≥50% similarity through conservative amino acid substitutions.

(C) Amino acid alignment of CARD-containing proteins. The most homologous proteins to *Bcl10* and E10 were RAIDD and procaspase-2.

AJ006288; Figure 2A). This was predicted to encode a 233 amino acid protein with a molecular weight of 26.2 kDa. Due to the direct involvement of this gene in three cases of MALT lymphoma with t(1;14)(p22;q32), we have termed this gene *Bcl10* (B cell lymphoma/leukemia 10).

The mouse *Bcl10* gene was also cloned and sequenced (AJ006289); this was 91% identical to the human gene (Figure 2B). BLAST searches revealed that *Bcl10* contained a region with strong homology (47% identity and 24.7% similarity over 97 amino acids) to an EHV-2 open reading frame (ORF), E10; *Bcl10* therefore appeared to be a cellular homolog of E10. This homology was limited principally to the CARD (Figure 2B). *Bcl10* also had weaker homologies with the amino-terminal CARD of RAIDD (25% identity and 25% similarity); homologies with other molecules involved in apoptosis that also contain CARDs were lower (Figure 2C). Distal to the CARD, *Bcl10* showed no significant homologies with any other known proteins. Similarly, the homology with E10 was also much lower in this domain.

Bcl10 Expression in Normal and Malignant Tissues

Bcl10, expressed as a transcript of 4.2 kb, was observed in all normal and malignant tissues examined (data not shown). To examine the expression in mature B cells within normal lymphoid tissue, in situ hybridization (ISH) was performed. Levels of *Bcl10* expression were high within germinal centers but much lower within the mantle zones of normal lymphoid follicles (Figure 3). MALT lymphomas are thought to arise from marginal zone cells; marginal zone cells from normal Peyer's patches showed an intermediate level of *Bcl10* expression. Insufficient suitable material was available to study the expression of *Bcl10* in any of the three cases with t(1;14)(p22;q32) by Northern blot, but all three cases expressed *Bcl10* mRNA, as assessed by ISH (Figure 3). However, all cases of B cell malignancy examined by either Northern blot or by ISH expressed high amounts of *Bcl10* irrespective of whether they exhibited t(1;14)(p22;q32), indicating that, like *Bcl2* (Zutter et al., 1991) and *Bcl6* (Cattoretti et al., 1995), mechanisms other than

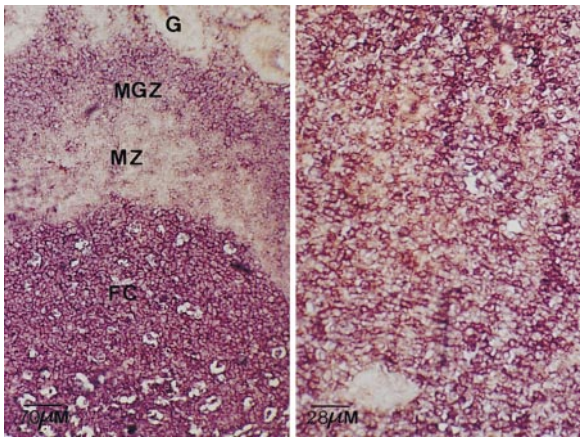

Figure 3. In Situ Hybridization for *Bcl10* mRNA

In reactive human Peyer's patch (left), high levels of *Bcl10* expression were seen in the follicle center cells (FC) but not in mantle zone cells (MZ). Intermediate levels of *Bcl10* expression were observed in the marginal zone cells (MGZ), the presumed cell of origin of MALT lymphoma. Glandular epithelial cells (G) were negative for *Bcl10*. In gastric MALT lymphoma with t(1;14)(p22;q32) (right), abundant *Bcl10* mRNA was detected in the tumor cells but not in the gastric gland epithelial cells.

translocation may result in high-level *Bcl10* expression (data not shown).

Bcl10 Induces Apoptosis and NF-κB Activation

By analogy with the t(14;18)(q32;q21) involving the *Bcl2* gene in follicular B cell non-Hodgkin lymphoma, and from the predicted functions of E10 (Hofmann et al., 1997), we anticipated that both Bcl10 and E10 proteins would be antiapoptotic. However, both induced apoptosis when transfected into 293 cells (Figure 4C) and induced activation of NF-κB (Figures 4D and 4E); comparable apoptosis was seen when *Bcl10* was transfected into other cell lines, including COS-7 and HeLa (data not shown). *Bcl10*-induced apoptosis was inhibited by both z-VAD-fmk and by cotransfection of CrmA (Figures 4C and 4G). Comparison with other apoptotic mediators, such as TNFR1, indicated that *Bcl10* and E10 were only weakly proapoptotic (Figure 4C). Furthermore, IL3-dependent BA/F3 B cell lines stably expressing exogenous *Bcl10* were isolated, and unlike *Bcl2*, expression of *Bcl10* did not promote survival in the absence of IL3 and did not protect significantly from apoptosis induced by a combination of TNFα and cycloheximide (data not shown).

Bcl10 Mutations in MALT Lymphoma with t(1;14)(p22;q32)

Given that wild-type *Bcl10* induced apoptosis, we examined case G0186 from which the translocation breakpoint was cloned for mutations within the *Bcl10* ORF. Using RT-PCR and DNA sequencing of cloned PCR products, 18 clones were sequenced. Two were normal, while 16 showed mutations within the coding region of *Bcl10*. Insertion of an additional thymidine into a mononucleotide run of seven consecutive thymidines (nucleotides 493 to 499) was observed in 10/16 mutated clones. In three clones (designated M106), this was the only mutation present. The consequence of this frameshift mutation was to induce premature termination of the ORF, resulting in a predicted protein of 168 amino acids (Figure 5A). In the most heavily mutated clone (M114), there were four other mutations, including a deletion of 11 amino acids (116–126) due to loss of a splice acceptor site at the boundary of the third coding exon (Figure 5B and see below), as well as two other point mutations within the CARD and another at codon 100. Most of the point mutations in this case of MALT lymphoma were A→G transitions consistent with *IgVH* gene somatic hypermutation. No mutations were detected in *Bax* and the *TGFβ-RII* genes that also contain mononucleotide repeats and are frequently mutated in diseases that exhibit microsatellite mutator phenotype (data not shown).

Truncated *Bcl10* Retains NF-κB Activation but Does Not Induce Apoptosis

To assess any possible differences between the wild-type and truncated *Bcl10* mutants, both M106 and M114 were transfected into 293 cells. Both M106 and M114 activated NF-κB to a level comparable to that seen with the wild-type protein (Figure 4E). However, neither mutant clone exhibited significant apoptosis in this assay (Figures 4C and 5C). These data indicate that the full-length *Bcl10* molecule was necessary for the apoptotic function but not NF-κB activation. Cotransfection of either M106 or M114 *Bcl10* mutants with wild-type *Bcl10* was also performed; coexpression of either truncated mutant did not significantly abrogate wild-type *Bcl10*-induced apoptosis (Figure 4G).

Mutation Activates a Transforming Function in *Bcl10*

Some proteins that induce apoptosis function as suppressors of transformation (Eliyahu et al., 1989; Yin et al., 1997). The observation that *Bcl10* induced apoptosis raised the possibility that it might also possess transformation suppressor properties. To address this issue, the effect of *Bcl10* expression was determined in the primary rat embryo fibroblast (REF) system. In this assay, nuclear oncoproteins such as adenovirus E1a, papillomavirus E7, and some mutant p53 proteins cooperate with an activated *ras* oncogene to induce morphological transformation (Crook et al., 1994). Such transformation is suppressed efficiently by some human tumor suppressor proteins such as wild-type p53. We therefore performed transfections in which primary REFs received a transforming combination of oncoproteins (e.g., E1a + *ras*, HPVE7 + *ras*, mutant p53 + *ras*) together with plasmids encoding either wild-type *Bcl10*, E10, or tumor-derived *Bcl10* mutants. Transfection of each cooperating pair of oncogenes, in the absence of exogenous *Bcl10* expression, generated numerous transformed colonies that could be readily propagated as cell lines. Cotransfection of wild-type *Bcl10* significantly reduced the numbers of transformed colonies generated by each pair of cooperating oncogenes (Table 1). In contrast, cotransfection with either of two tumor-derived mutants, M106 or M114, or with E10, markedly enhanced the

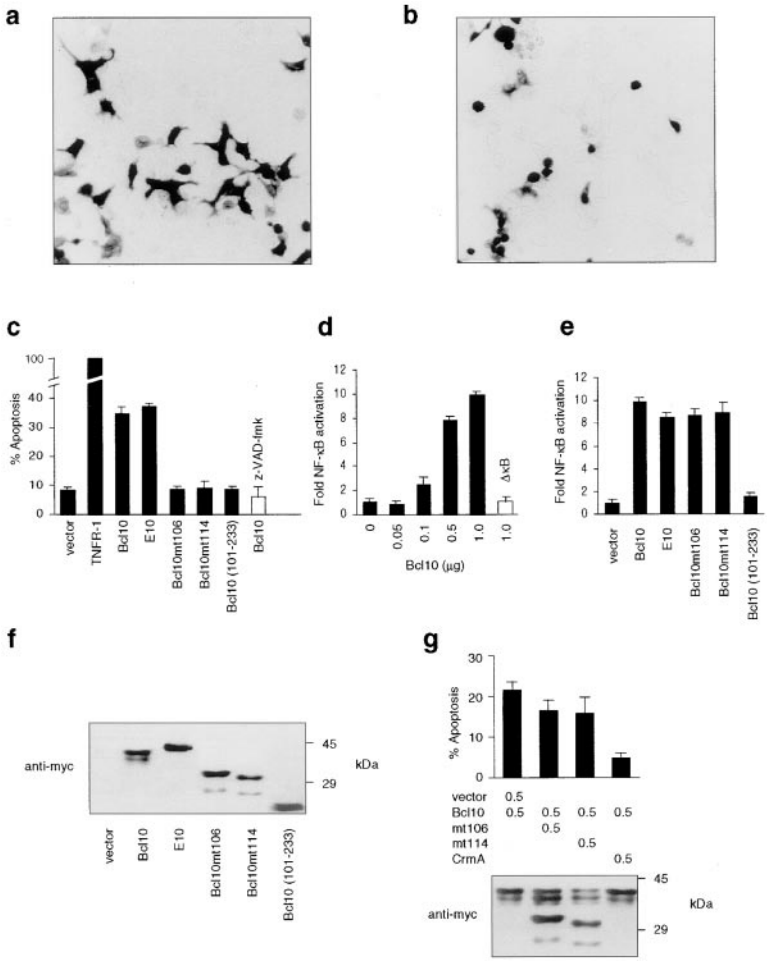

Figure 4. *Bcl10* and E10 Induce Apoptosis and Activate NF-κB

Apoptosis assay: 293 cells were transfected with 1 μg of the indicated expression constructs and 0.25 μg of pcDNA3.1 β-gal. Forty-eight hours after transfection, cells were stained with X-gal and at least 300 blue cells counted per well.

(A) Photomicrograph of cells transfected with pcDNA3.1.

(B) Photomicrograph of cells transfected with pcDNA3.1 *Bcl10* showing cells with apoptotic morphology.

(C) Apoptosis is induced by *Bcl10* and E10 but not by the carboxy-terminal region of *Bcl10* or by tumor-associated truncation mutants. Data (± SD) are shown as the percentage of morphologically apoptotic cells for three independent transfections. Where indicated, 25 μM z-VAD-fmk was added 6 hr post-transfection.

(D and E) NF-κB reporter assay: 293 cells were transfected with the indicated amounts of the expression construct, 0.25 μg pTK-cSPAP reporter with (filled boxes) or without (open boxes) the NF-κB response element, and 0.25 μg of pcDNA3.1 β-gal, to a total of 1.5 μg of DNA. Forty-eight hours after transfection, absorbance at 405 nm relative to vector-transfected cells was measured. Results ± SD for experiments performed in triplicate.

(F) Western blot analysis of transfected 293 cells. Cell lysates from cells transfected with the indicated plasmids were immunoblotted with monoclonal antibody to myc.

(G) Truncated *Bcl10* does not significantly inhibit wild-type *Bcl10*-induced apoptosis. 293 cells were transfected with wild-type *Bcl10* in the presence of either M106 or M114 mutant clones and apoptosis assessed. Comparable levels of expression of the wild-type and mutant proteins were observed as shown in the Western blot.

number of transformed colonies in each case and caused the transformed colonies to appear earlier. Moreover, cotransfection of either *Bcl10* mutant with *ras* alone revealed that each mutant had itself acquired a weak but reproducible ability to cooperate with *ras.*

Bcl10 Mutations Are Found in Multiple Human Tumor Types

No RNA samples were available from the other two cases of MALT lymphoma with t(1;14)(p22;q32). To search for *Bcl10* mutations in these cases, the genomic sequence and the exon/intron structure of the *Bcl10* coding region were determined and PCR primers designed to amplify the three coding exons from paraffin sections. Mutations in all three cases with t(1;14)(p22;q32) were sought by PCR-SSCP. All showed abnormal migrating bands consistent with mutation; different sections from different histological blocks from the same tumor showed different bands, indicating that in all three cases *Bcl10* mutation was ongoing (Figure 6A).

Chromosome 1p22 is also a common site of deletions and translocations in subtypes of lymphoma other than MALT, as well as a wide range of solid tumors (Tilly et al., 1994; Mertens et al., 1997; Thangavelu et al., 1997). We therefore sought *Bcl10* mutations in a panel of fresh lymphoma cases and in cell lines derived from various human malignancies. A panel of archival lymphoma cases were studied by PCR-SSCP, and in some cases, the abnormal SSCP bands were excised, reamplified, and sequenced.

PCR-SSCP analysis of a panel of 135 cases of B cell and 20 cases of T cell lineage lymphoma of various histological subtypes revealed abnormal migrating bands in 70 (45%); abnormal PCR-SSCP bands were observed in all lymphoma subtypes (M. J. S. D. et al., in preparation). A representative gel along with the derived sequences from one case of follicular B cell lymphoma that showed two deletions within the coding region of exon 3 is shown in Figure 6B. The sequence abnormalities in ten cases of MALT and follicular non-Hodgkin lymphoma in which the abnormal PCR-SSCP band was excised, successfully reamplified, and sequenced are shown in Table 2. Truncating *Bcl10* mutations were detected in both subtypes of lymphoma in the absence of 1p22 chromosomal translocation.

Subsequently, 87 cell lines derived from patients with other forms of malignancy were examined either by sequencing cDNA clones (n = 6) or by genomic PCR of

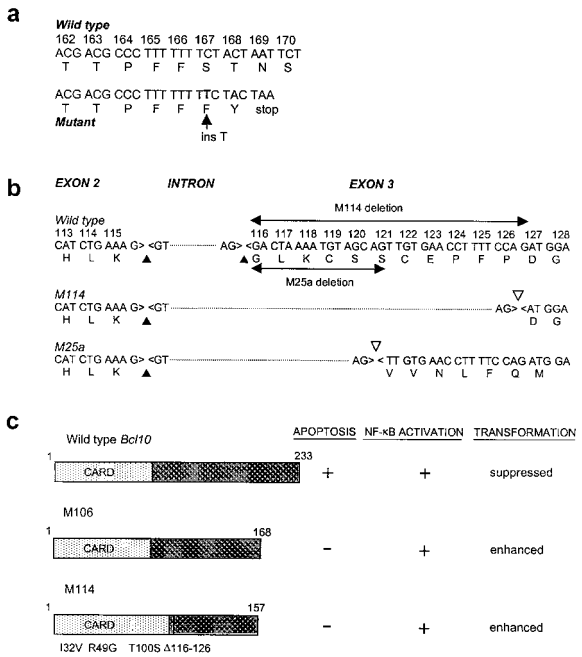

Figure 5. *Bcl10* Truncation Mutants

(A) An abnormality in MALT lymphoma mutants M106 and M114 in cell lines M25, Mero82 (mesothelioma), and Tera2 (teratoma) was the insertion of a thymidine in a mononucleotide run of seven thymidines at nucleotides 493–499, resulting in truncation at codon 168.

(B) *Bcl10* exon 2/3 splice site abnormalities. MALT mutant 114 had an 11 amino acid in-frame deletion at the start of exon 3. Mesothelioma line M25a had a 16 base pair deletion at the start of exon 3, causing a frameshift and a truncated protein of 141 amino acids. Closed triangles, normal splice sites; open triangles, new splice sites.

(C) Structure of wild-type *Bcl10* and the two tumor-derived truncated mutants studied, with phenotypes in apoptosis, NF-κB activation, and REF transformation assays.

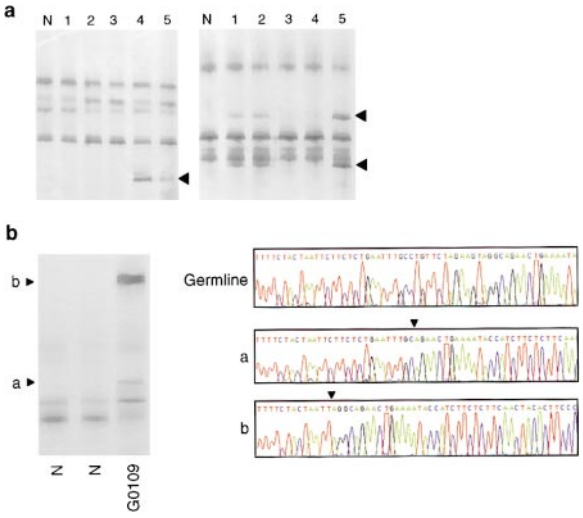

Figure 6. PCR-SSCP Analysis of *Bcl10* Mutations

(A) Ongoing mutations in a case of MALT lymphoma with t(1;14)(p22;q32). Exons 2.2 (left panel) and 3.2 (right panel) of a case of MALT lymphoma with t(1;14)(p22;q32) (case G0262) were amplified from different sections taken from different blocks of the same tumor and subject to SSCP. "N" denotes normal DNA, lanes 1–5 different samples from the same tumor. Arrowheads denote abnormal bands; note that these are only present in some tumor samples and not all.

(B) Two internal *Bcl10* deletions in a case of follicular B cell lymphoma (case G0109). A PCR-SSCP gel showing two normal controls and case G0109 with abnormally migrating bands is shown. Abnormal bands labeled "a" and "b" were excised, reamplified, and sequenced. The sequences are shown in the right-hand panel. Band "a" represented a 17 bp deletion, whereas band "b" represented a 28 bp deletion occurring within coding exon 3 and resulting in frameshifts within the *Bcl10* coding sequence with predicted protein products of 176 and 169 amino acids, respectively.

exons 2 and 3 and direct sequencing of the PCR product (n = 81). As summarized in Table 2, 10/87 cell lines showed truncating mutations. The frequency of *Bcl10* mutation depended on the origin of the derived cell line. All three mesothelioma and all three male germ cell tumor cell lines exhibited *Bcl10* mutation, whereas no mutations within coding exons 2 and 3 were seen in a panel of 15 breast carcinoma, 11 pancreatic adenocarcinoma, and 15 lung carcinoma cell lines. *Bcl10* mutations were seen in 2/25 colonic adenocarcinoma and 2/18 leukemia and lymphoma cell lines.

Of the 32 mutations shown in Table 2, 25 represented frameshift mutations that would result in truncated protein. Some of these mutations represented deletions resulting in loss of splice donor/acceptor sites at the exon 2/3 boundary or in the colon carcinoma cell line, LS513, at the exon 1/2 boundary (Figure 5B), while others represented internal deletions within exons 2 and 3 (Figure 5B). As with the MALT lymphoma with the t(1;14)(p22;q32), insertion of an additional thymidine into the mononucleotide run (insT499) was seen in two mesothelioma and one male germ cell tumor cell lines (Figure 5A). Similarly, insertion or deletion of a single adenine into a run of eight consecutive A's was observed in three cell lines of different cell types, including the T cell precursor ALL cell line MOLT-4, a colonic adenocarcinoma cell line, Lovo, and the mesothelioma cell line M41; these mutations fell within the region encoding the CARD and were predicted to result in proteins of 48 (insertion) and 69 (deletion) amino acids. Six of the 21 (28%) mutations in which the results of truncation could be predicted exhibited mutations that fell within the CARD; the remainder fell distal to the CARD and were similar to those seen in the MALT lymphoma with the

Table 1. Effect of *Bcl10* Expression on Transformation of Primary REFs

	Vector	*Wild-Type Bcl10*	*E10*	*M106*	*M114*
ras	0	0	0	6	8
ras + pLTRp53cG	12	1	>50	>50	>50
ras + E1a	16	1	>50	>50	>50
ras + p53 234C	6	0	>20	>20	>20
ras + E7	10	0	>50	>20	>20

Data shown are number of transformed colonies per plate following transfection with *ras* and the indicated cooperating oncogenes, with or without cotransfection with E10 or wild-type/mutant *Bcl10*. Data shown are typical of three transfections. Wild-type *Bcl10* suppressed transformation relative to vector, while E10 and both tumor-derived mutants, M106 and M114, enhanced transformation by cooperating oncogenes. The mutants showed weak transforming activity with *ras* alone.

Table 2. *Bcl10* Mutations in Primary NHL and in Cell Lines Derived from Lymphoid and Solid Tumors

Tumor	Case No. or *Cell Line*	Alteration	Coding Exon	Codon	Mutation Type	Predicted Protein Size (Full Length = 233 aa)
Lymphoid Tumors						
MALT lymphoma	G0186 M106[1]	499insT	3	167	Truncation	168 aa
	M114[1]	499insT	3	167	Truncation	
		94A → G	2	32	Ile → Val	
		145A → G	2	49	Arg → Gly	157 aa
		298A → T	2	100	Thr → Ser	
		346del33	3	116–126	Splice aberration[4]	
	G0193	163insA	2	55	Truncation	61 aa
	G0123	345delA	2	115	Truncation	116 aa
	G0524	345delA	2	115	Truncation	116 aa
Follicular lymphoma	G3018	428insTT	3	143	Truncation	147 aa
	G3009	231ins A	2	77	Truncation	96 aa
	G0109a[2]	525del17	3	175–181	Truncation	176 aa
	b[2]	508del28	3	170–180	Truncation	169 aa
	G3008	410delA	3	137	Truncation	146 aa
	G0206	398insT	3	133	Truncation	139 aa
	G3023	637del3	3	210	Del Glu	232 aa
T-ALL	*Molt-4*	136insA	2	46	Truncation	48 aa
Sezary syndrome	*Hut 78*	428delT	3	143	Truncation	146 aa
Solid Tumors						
Colonic carcinoma	*Lovo*	136insA	2	46	Truncation	48 aa
	LS513	delT	1/2	intron	Splice aberration	
Mesothelioma	*M25 a*[3]	346del16	3	116–120	Splice aberration[4]	141 aa
	b[3]	499insT	3	167	Truncation	168 aa
		238G → A	2	80	Asp → Asn	
	M41 a[3]	136delA	2	43	Truncation	69 aa
	b[3]	155C → T	2	52	Thr → Ile	Full length
	Mero82	499insT	3	167	Truncation	168 aa
Germ cell tumor	*Tera 1*	172C → G	2	58	Arg → Gly	Full length
		653C → T	3	218	Ser → Phe	
	Tera 2 a[3]	499insT	3	167	Truncation	168 aa
	b[3]	274C → T	2	91	Truncation	91 aa
		58G → A	2	20	Ala → Thr	
	GCT 44	172C → T	2	58	Truncation	57 aa

Cell lines denoted in italics.
[1] Mutants M106 and M114; see Figure 5C.
[2] Two independent deletions within coding exon 3 were identified in this tumor (see Figure 6B).
[3] cDNA clones containing independent mutations (designated "a" and "b") were identified from these cell lines.
[4] See Figure 5B.

t(1;14)(p22;q32). Otherwise, there was no apparent clustering of the point mutations.

Discussion

We report the cloning of an apoptotic regulatory gene, *Bcl10*, from its direct involvement in the t(1;14)(p22;q32) of low-grade MALT lymphoma. From cytogenetic, FISH, Southern blot, and PCR experiments, breakpoints within the 5′ region of *Bcl10* were recurrent but present in only a low percentage of cases (M-Q. D. et al., unpublished observations). The *Bcl10/IgH* translocations left the coding region of *Bcl10* intact. By analogy with *Ig* translocations that break either 5′ or within the promoter regions of other genes such as *Myc*, *Bcl2*, and *Bcl6*, it would be anticipated that the t(1;14)(p22;q32) would result in either deregulated overexpression of *Bcl10* due to the proximity of the *Ig* transcriptional enhancers and/or loss of normal regulatory controls. In situ hybridization showed high-level expression in all three MALT lymphomas with the t(1;14)(p22;q32), but comparable levels of expression were also seen in other lymphomas that lacked the translocation. In this regard, *Bcl10* may be similar to *Bcl2* and *Bcl6* where high-level expression may be seen in many B cell lymphomas irrespective of whether or not they exhibit *Ig* translocation (Zutter et al., 1991; Cattoretti et al., 1995).

Bcl10, like a number of proteins involved in the control and execution of apoptosis, contained a CARD (Hofmann et al., 1997). This domain has been shown to consist of six tightly packed antiparallel alpha helices and is similar in structure to the "Death Domain" (Chou et al., 1998). CARDs have been found in both pro- and antiapoptotic proteins and mediate homotypic CARD interactions between structurally related proteins. The CARD of RIP2/CARDIAK was sufficient for the apoptotic actions of this molecule (McCarthy et al., 1998) as well as NF-κB and JNK activation (Thome et al., 1998). However, CARD proteins may have functions other than apoptosis; the prodomain of caspase-2 contains a CARD that allows nuclear translocation (Colussi et al., 1998).

Bcl10 is the first CARD protein to be implicated in malignancy. Its involvement is unusual in two regards. First, there is at present no evidence to suggest that

overexpression or deregulated expression of the wild-type molecule might confer any survival benefit. Overexpression of *Bcl10* in 293 cells resulted in apoptosis, although the extent and rate at which apoptosis was induced was much less and slower than with death-signaling molecules such as TNFR1. Consistent with this was the efficient suppression of transformation of REFs by wild-type *Bcl10*. In contrast, although E10 induced a comparable degree of apoptosis as *Bcl10* in 293 cells, in the REF assay it behaved like the *Bcl10* mutants and markedly enhanced the number of transformed colonies. The biochemical reasons underlying this difference between wild-type *Bcl10* and E10 are not known, but B cell proliferation induced by E10 might allow EHV-2 viral persistence.

Second, *Bcl10* appears to be unusually susceptible to mutation. These mutations principally consisted of either nucleotide insertion or deletion resulting in protein truncation, in most cases distal to the CARD. Interestingly, the truncating mutation common to 10/16 mutated *Bcl10* clones observed in the MALT lymphoma with the t(1;14)(p22;q32), insertion T 499, was also detected in both mesothelioma and male germ cell tumor cell lines. All three cases of MALT lymphoma with t(1;14)(p22;q32) exhibited *Bcl10* mutation, and it appeared that this process was ongoing. Ongoing mutation is a feature of productively rearranged *IgVH* genes after a B cell has encountered antigen, resulting in affinity maturation of antibody, but has also been seen in other genes in B cell malignancies (Albert et al., 1994; Gutierrez et al., 1998). Many of the *Bcl10* mutations could be ascribed to the *Ig* somatic hypermutation mechanism resulting in nucleotide transitions, and the function of the t(1;14)(p22;q32) chromosomal translocation may be to place the *Bcl10* gene under the direct influence of this mechanism. Detection of *Bcl10* abnormalities was hindered by ongoing mutation, and we have found it necessary to sequence individual cDNA or genomic clones; the frequency of *Bcl10* mutations may be significantly underestimated by sequencing PCR reactions directly (T. G. W. et al., unpublished observations). The possible functional significance of the ongoing *Bcl10* mutations are not clear. Both *Bcl10* mutants cloned from the MALT lymphoma with t(1;14)(p22;q32) appeared to have similar properties in all assays.

The functional consequences of the *Bcl10* mutations truncating distal to the CARD were the loss of the proapoptotic functions with retention of NF-κB activation. Expression of NF-κB has been associated with suppression of apoptosis and prolongation of survival in many systems; NF-κB activation also appears to be an essential component of several transformation pathways (Wu et al., 1996; Mayo et al., 1997; Reuther et al., 1998; Van Antwerp et al., 1998). The dissociation of NF-κB activation and proapoptotic functions in the truncated forms of *Bcl10* was consistent with their marked enhancement of transformation by cooperating oncogenes. Whether other *Bcl10* truncating mutations that fall within the CARD have the same phenotype is not yet known.

Transdominant inhibition by tumor-associated mutants of wild-type *Bcl10*-induced apoptosis represents a basis for the selection of *Bcl10* mutants in cancer. However, coexpression of truncated mutants did not substantially affect the proapoptotic activity of wild-type *Bcl10* under the conditions of our assay. While the possibility of transdominant inhibition cannot be excluded, it seems likely that selection is for the loss of apoptotic activity and concomitant gain of proproliferative function in the truncated mutants rather than for abrogation of wild-type activity. In this regard, the mutants display some functional similarity to some p53 mutants, which, although unable to inhibit transdominantly the apoptotic activity of the wild-type protein, are nevertheless frequently selected in a wide range of human cancers, presumably as a result of their proproliferative activities (Gualberto et al., 1998).

Finally, *Bcl10* abnormalities were shown to be present in several subtypes of lymphoma other than MALT. In follicular lymphoma, abnormalities of chromosome 1p22 have been associated with rapid progression and a poor prognosis (Tilly et al., 1994). However, *Bcl10* mutations did not appear to be limited to malignancies of the lymphoid lineages. Chromosome 1p22 has been shown to be consistently deleted in several other tumor types, including 80% of mesothelioma (Lee et al., 1996) and 40% of male germ cell tumors (Mathew et al., 1994). So far, our analysis of these diseases has been restricted to derived cell lines. However, given the high frequency of *Bcl10* mutations in these, but not other cell lines, it seems unlikely that *Bcl10* is mutated solely as a consequence of prolonged in vitro culture. *Bcl10* may be the target gene for the chromosome 1p22 deletions in a wide range of human malignancy. Both the compromised proapoptotic activity and gain of transforming activity of human tumor–derived *Bcl10* mutants provide a mechanistic basis for their frequent selection in a wide range of cancers.

Experimental Procedures

Cloning of t(1;14)(p22;q32) Translocation Breakpoint

LDI-PCR was performed on high-molecular-weight DNA from a MALT lymphoma with t(1;14)(p22;q32) as described (Willis et al., 1997, 1998); 400 ng of DNA was digested with PstI (GIBCO-BRL, Gaithersburg, MD) in a volume of 30 μl and then ligated at 15°C overnight in a total volume of 500 μl with 5 U of T4 DNA ligase (GIBCO-BRL). Ligated DNA was purified using a Qiaquick column (Qiagen, Hilden, Germany), and 10 ng DNA was amplified with r*Tth* polymerase (GeneAmp, XL: Perkin-Elmer, Foster City, CA) using primers for *IgJH* and reamplified using nested primers. Following amplification, the PCR reaction was run on a 0.8% agarose gel and the two bands excised. These were purified and A-tailed with 5 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania), 200 μM dNTPs, and Taq buffer for 30 min at 72°C. The products were cloned into the Topo TA cloning vector pCR2.1 (Invitrogen, San Diego, CA). Sequencing was performed on an Applied Biosystems model 377 sequencer using AmpliTaq DNA polymerase, FS (Perkin-Elmer). Sequences were analyzed against the NCBI GenBank EST database using the BLAST program. cDNA clones corresponding to positive ESTs were obtained from the Human Genome Mapping Project Resource Centre (Cambridge, UK) and sequenced. Southern blot, fluorescence in situ hybidization, and Northern blot analysis were performed as described (Willis et al., 1997, 1998).

In Situ Hybridization

In situ hybridization for *Bcl10* mRNA was carried out as described (Pan et al., 1997). Briefly, fragments of paraffin sections were dewaxed in xylene, washed in ethanol, digested with proteinase K, fixed in 4% paraformaldehyde/PBS, and hybridized at 50°C for 16–18

hr with digoxigenin-11-UTP (Boehringer Mannheim, Lewes, UK) labeled *Bcl10* antisense RNA probe. The hybridized sections were washed, incubated twice in alkaline phosphatase–conjugated sheep antidigoxigenin for 1 hr, and visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium.

Expression Vectors

Expression clones were amplified by PCR, cloned into the TA cloning vector pCR2.1 TOPO, and then subcloned in frame into the expression vector pcDNA3.1/Myc-His (Invitrogen). The open reading frame of *Bcl10* was amplified from a *Bcl10* cDNA clone using primers 5′-CCATCTCAGATCTCCACCATG-3′ and 5′-AAAGTGTCTCGAGCGTG AACAGTA-3′, and the open reading frame for E10 was amplified from EHV-2 DNA using primers 5′-CCTCCAAGCAGATCTATGGCG-3′ and 5′CAGCAGATATCCCTCCT-3′. Both were subcloned into pcDNA3.1/Myc-His. The carboxy terminus of *Bcl10* (amino acids 101–233) and mutant MALT lymphoma cDNA clones 106 and 114 were amplified by PCR and subcloned into pcDNA3.1/Myc-His.

Cell Culture and Transfections

293, COS-7, and HeLa cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. For reporter and apoptosis assays, 2×10^5 cells/well were seeded on 6-well (35 mm) dishes. Cells were transfected the following day by calcium phosphate coprecipitation. Stable G418-resistant transfectants of the mouse IL3-dependent pro–B cell line BA/F3 were generated by electroporation of 10^7 cells in 400 ml of serum-free medium and mixed with 20 μg pcDNA3.1/V5-His plasmid with or without *Bcl10* insert at 250 V and 900 μF. Cells were selected with 1.2 mg/ml G418, seeded at 0.5 cells per well in 96 well plates, and screened for exogenous *Bcl10* expression.

Apoptosis Assays

293, COS-7, and HeLa cells were cotransfected with 1 μg of expression construct and 0.25 μg pcDNA3.1β-galactosidase. Where indicated, the caspase inhibitor z-VAD-fmk (25 μM) was added 6 hr posttransfection. After 48 hr, cells were washed with phosphate-buffered saline (PBS), fixed with 0.5% glutaraldehyde, washed again in PBS, and stained with X-gal (50 mM Tris HCl [pH 8], 2.5 mM potassium ferriferrocyanide, 15 mM NaCl, 1 mM $MgCl_2$, 0.5 mg/ml X-gal) for 4 hr. At least 300 β-galactosidase-positive cells were counted for each transfection (n = 3) and identified morphologically as apoptotic or nonapoptotic.

NF-κB Reporter Assay

293 cells were cotransfected in phenol-free DMEM with 1 μg (except where indicated) of expression construct and 0.25 μg of a secreted placental alkaline phosphatase reporter construct pTK-cSPAP containing a minimal HSV tyrosine kinase promoter with or without four copies of the NF-κB response element GGAAAGTCCC, and 48 hr posttransfection, 30 μl of medium was removed and heated at 65°C for 5 min. To this, 150 μl diethanolamine (DEA) buffer (1 M DEA, 0.28 M NaCl, 0.25 mM $MgCl_2$) containing 7 mM p-nitrophenyl phosphate (PNPP) was added and incubated at 37°C for 1 hr. The reaction was stopped by adding 75 μl 2 M NaOH, and absorbance was then measured at 405 nm for each transfection (n = 3).

Western Blotting Analysis

293 cells (10^6) were trypsinised, pelleted, and lysed by sonication in 1% SDS, 100 mM Tris (pH 6.8), 0.14 M β-mercaptoethanol, 20% glycerol, and 0.025% bromophenol blue. Proteins were separated using 12% polyacrylamide gel, electrotransferred onto nitrocellulose paper, and incubated with 1:4000 monoclonal mouse anti-myc antibody (Invitrogen) followed by 1:1000 sheep anti-mouse Ig horseradish peroxidase-linked whole antibody (Amersham). After washing, samples were developed using ECL Western blotting detection reagents (Amersham).

REF Transformation Assays

Primary REFs (BioWhittaker, Wokingham, UK) were maintained in DMEM plus 10% FCS. Subconfluent 90 mm dishes were transfected by the calcium phosphate method as described (Crook et al., 1994). Following transfection, cells were grown in DMEM/10% FCS in the presence of 500 μg/ml G418 sulphate (GIBCO). Morphologically transformed colonies were counted after 3 weeks. The following plasmids were used in the REF assays: pLTRp53cG (Eliyahu et al., 1985), $pCB6^+16E7$ (Parker et al., 1996), pEJ6.6 (encodes activated Ha-*ras*) (Shih and Weinberg, 1982), $pCB6^+p53$ 234C (Parker et al., 1996), and pCE (encodes adenovirus E1a) (Schneider et al., 1987).

Cloning and Sequencing of *Bcl10* Genomic Fragment

One hundred nanograms of normal human genomic DNA was amplified with r*Tth* polymerase using primers for the *Bcl10* open reading frame 5′-CCTCCTCTCCTTCTTCCCCATTACCC-3′ and 5′-CATTAAA AATTAAAAGGCAATAAAGTG-3′ in a 50 μl reaction at 95°C for 1 min followed by 35 cycles of 95°C for 15 s and 68°C for 10 min. A 10 kb product was subcloned into pCR2.1 TOPO, and automated DNA sequencing was performed using the primers 5′-AACATCAAGT AGAAAAAGGGCTGGAAA-3′, 5′-TTTCCAGCCCTTTTTCTACTTGAT GTT-3′, 5′-GAGGTTGTTCGTGGCTCCATC-3′, and those described above.

PCR-SSCP Studies

DNA samples from frozen and paraffin-embedded tissue blocks from 155 cases of lymphoma from the Department of Histopathology, University College were studied. The full coding sequence of the *Bcl10* gene was amplified by five different PCR reactions (two reactions for exons 2 and 3) using the following primer sets: *Exon 1*, 5′-GGACCCGGAAGAAGCGCCATCTCC-3′ and 5′-GATCCTCCTT GTCCTCGGACTC-3′ (anticipated product size = 187 bp); *Exon 2.1*, 5′-AAGACTGCCAACTAATAGTCACGT-3′ and 5′-AAGTAGTCTAAC AATTTTCCAGCCC-3′ (200 bp); *Exon 2.2*, 5′-CACTGAAGAAATTTCT TGTCGAACA-3′ and 5′-AAAAGCATTATTACATTTAAATTAGCTC-3′ (244 bp); *Exon 3.1*, 5′-TTAACAAGTCACAAGATGGACAGTG-3′ and 5′-CTAGAACAGGCAAATTCAGAGAAG-3′ (262 bp); *Exon 3.2*, 5′-GGAGAATCCAGCACGACGCCC-3′ and 5′-CATTAAAAATTAAAAG GCAATAAAGTG-3′ (257 bp). PCR was performed on a thermal cycler (Hybaid, Teddington, UK) using a "hot start" followed by a "touchdown" program. For SSCP analysis, PCR products (2 μl) were mixed with 4 μl sequencing loading buffer, denatured, and separated on Genphor electrophoresis system (Pharmacia-Amersham, Amersham, UK) under 15 W constant power for 2 to 3 hr at 5°C and then visualized by silver staining. In some cases, the abnormal bands were excised from the gel and reamplified prior to sequencing in both directions.

***Bcl10* cDNA and Genomic DNA Sequencing**

The *Bcl10* ORF was amplified from reverse-transcribed poly(A+) RNA using primers 5′-GGACCCGGAAGAAGCGCCATCTCC-3′ and 5′-CATTAAAAATTAAAAGGCAATAAAGTG-3′ and cloned into pCR2.1. In MALT lymphoma case G0186, two separate RT-PCR amplifications were performed and individual clones sequenced; both reactions contained wild-type and mutant *Bcl10* clones. Sequencing of the *Bcl10*-coding region from cell lines and cases with abnormally migrating SSCP bands was performed on amplified or cloned genomic PCR products using dRhodamine DNA polymerase (Perkin-Elmer) according to manufacturer instructions.

Acknowledgments

We acknowledge the assistance of Dr. Tim Diss and Rocio Hassan with the Southern blots. We thank Dr. A. J. Davison (Glasgow, Scotland) for providing EHV-2 DNA; Drs. Marjan Versnel (Dept. of Immunology, Erasmus University, Rotterdam, The Netherlands) and Sue Eccles (ICR) for kindly providing mesothelioma cell lines; Drs. A. Thiry and F. Andrien (Liege, Belgium) for kindly providing patient material; Dr. Stuart Farrow (Glaxo-Wellcome, Stevenage, UK) for kindly providing the NF-κB reporter constructs; Professor Mike Stratton (ICR) for tumor cell line DNA samples; Maurizio Valeri and Jacky Cordell (ICR) for their help with cell culture; and Rachel Jackson, Samantha Dibley, and Bina Desai (ICR) for their help with automated DNA sequencing. We thank the HGMP Resource Centre, Hinxton Hall, Cambridge for providing clones and computing facilities, and Dr. Gabriel Nunez (Dept. of Pathology, University of Michigan Medical School, Ann Arbor, MI) for helpful discussions and

for providing the TNFR-1 and CrmA constructs. This study was supported by grants from the Leukaemia Research Fund, the Kay Kendall Leukaemia Fund, and the Cancer Research Campaign. T. C. is a Leopold Muller Fellow, supported by Breakthrough Breast Cancer.

Received October 15, 1998; revised November 30, 1998.

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