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RESEARCH ARTICLE

High Expression of B-Cell Receptor Inducible Gene *BIC* in all Subtypes of Hodgkin Lymphoma

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In a search for genes specifically expressed in Reed–Sternberg (RS) cells of Hodgkin lymphoma (HL), we applied the serial analysis of gene expression (SAGE) technique on the HL-derived cell line DEV. Genes highly expressed in DEV were subjected to an RT-PCR analysis to confirm the SAGE results. For one of the genes, a high expression was observed in DEV and other HL-derived cell lines but not in non-Hodgkin lymphoma (NHL)–derived cell lines and normal controls, suggesting an HL-specific expression. This gene corresponds to the human *BIC* gene, a member of the noncoding mRNA-like molecules. RNA in situ hybridization (ISH) indicated an exclusive nucleolar localization of *BIC* transcripts in all RS cells in 91% of HL cases, including nodular lymphocyte predominance (NLP) HL and classical HL. Analyses of normal human tissues revealed *BIC* transcripts in only a small number of CD20-positive B-cells in lymph node and tonsil tissue, albeit at a much lower level compared to that of RS cells. *BIC* RT-PCR in the Burkitt lymphoma–derived cell line Ramos demonstrated a significant up-regulation upon cross-linking of the B-cell receptor (BcR). IκBα-mediated blocking of NF-κB translocation in Ramos did not effect the up-regulation of *BIC* expression upon BcR triggering, suggesting that activation of NF-κB is not involved in regulation of *BIC* expression. In summary, our data show that expression of *BIC* is specific for RS cells of HL. In normal tissue, *BIC* is expressed weakly in a minority of germinal center B cells. Expression of *BIC* can be modified/influenced by BcR triggering, indicating that *BIC* might play a role in the selection of B cells.

INTRODUCTION

Hodgkin lymphoma (HL) is characterized by the presence of neoplastic cells, the so-called Reed–Sternberg (RS) cells and their mononuclear variants. RS cells have clonal rearrangements of the immunoglobulin genes with numerous somatic mutations of the variable regions, suggesting a germinal center B-cell origin (Kanzler et al., 1996). The presence of otherwise crippling mutations such as stop codons in the variable regions suggests rescue of the RS precursors from apoptosis by a transforming event such as the Epstein–Barr virus (EBV), which indeed is present in a high proportion of cases of classical HL.

To gain insight into the genes that are expressed in RS cells, we have applied serial analysis of gene expression (SAGE) (Velculescu et al., 1995). By this method, we previously identified a high expression of TARC (thymus and activation regulated chemokine) in RS cells of classical Hodgkin lymphoma and also demonstrated expression of HLA class I, β2-microglobulin, and CD1a mRNA in these cells (Van den Berg et al., 1999, 2000a). In this study, we performed SAGE analysis on a third Hodgkin lymphoma–derived cell line (DEV) (Poppema et al., 1985). We identified a SAGE tag occurring frequently in DEV that corresponds to the human homolog of the gallus gallus *Bic* gene identified previously in virally induced lymphomas (Tam et al., 1997). Tam et al. (1997) reported an association with integration of viral sequences in intron 1 of the gallus gallus *Bic* gene and a strong up-regulation of *Bic* expression upon viral infection. Gallus gallus *Bic* does not contain a functional open reading frame (ORF) and is most likely a member of the family of noncoding mRNA-like molecules, suggesting that *Bic* functions through its RNA transcripts. Although the function of the *Bic* gene remains unknown, many genes belonging to this family of noncoding genes have been demonstrated to have important regulatory functions (Askew and Xu, 1999; Sleutels et al., 2002; Kelley and Kuroda, 2000; Lottin et al., 2002; Wutz et al., 2002). In a recent study, chicken oncogenicity assays indicated that *Bic* cooperates with *Myc* in lymphomagenesis (Tam et al., 2002). In this study, we

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report a high level of *BIC* expression in HL, whereas no expression was observed in NHL tissues. In addition, we show that *BIC* expression is regulated through BcR activation.

MATERIALS AND METHODS

Cell Lines

Dr. Volker Diehl and co-workers (Cologne, Germany) kindly provided the L428, L540, L591, and L1236 HL-derived cell lines. The DEV cell line was established in our own laboratory from a case that originally was thought to be nodular sclerosis HL, but subsequently was retyped as nodular lymphocyte predominance (NLP) HL. Large B-cell NHL cell lines Ver and Rose were established in our laboratory (unpublished data). Ver is derived from a large cell B-cell lymphoma with t(8;14). Rose is a large B-cell lymphoma with t(14;18) derived from a transformed follicular lymphoma. Anaplastic large cell lymphoma cell line Karpas299, leukemic T-cell line Jurkat, and Burkitt lymphoma cell lines Raji and Ramos were obtained from the American Type Culture Collection (Rockville, MD).

Tissues

Frozen and formalin-fixed paraffin-embedded tissue samples of lymph nodes involved with HL and NHL were randomly selected from the tissue bank available at the Department of Pathology from the University Medical Center Groningen. Normal lymph node and tonsil tissue specimens were included as control tissues.

SAGE

A detailed protocol for the SAGE procedure and the SAGE2000 computer program (version 3.03) used for the analysis of the tags were kindly provided by Dr. Kinzler (Johns Hopkins Oncology Center, Baltimore, MD) (Velculescu et al., 1995). The SAGE protocol used for the analysis was performed as described previously (Van den Berg et al., 1999). SAGE clones were sequenced on a MegaBace automated sequencer (Amersham Biosciences, Piscataway, NJ), and resulting sequence files were analyzed with the SAGE2000 computer program (Velculescu et al., 1995). The tag sequences were used to screen for homologies with genes present in the GenBank and Unigene databases. In case a tag corresponded to a known gene or expressed sequence tag (EST), the 15th base that usually is present in the ditag sequence was used to confirm the homology.

RT-PCR

Total RNA was isolated with Trizol (GibcoBRL, Paisley, UK) from cell lines and from cryostat tissue sections. DNaseI treatment was performed to purify the RNA samples from contaminating DNA. Total RNA (5 μg) was primed with 1.0 μg oligo$(dT)_{18}$ and used for the cDNA syntheses reaction in a total volume of 50 μl by use of the buffer provided by the manufacturer for 1 hr at 37°C (GibcoBRL). The PCR was performed with 1 μl of the cDNA synthesis reaction by use of 1 unit of *Taq*-polymerase (Amersham Pharmacia Biotech, Piscataway, NJ) and the reaction buffer provided by the manufacturer. A negative control was included in each PCR experiment. The PCR program consisted of 30 cycles with a denaturation step of 30 sec at 94°C, annealing step of 45 sec at 57°C (60°C for BIC-FR), and an extension step of 30–45 sec at 72°C. The first denaturation step lasted for 5 min, and the final extension step lasted for 7 min. Primer sequences used for the amplification of *BIC* mRNA were selected from ESTs belonging to Unigene cluster Hs.89104 and to the genomic sequence (AP000085) (Table 1).

RNA In Situ Hybridization (ISH)

The BIC-F new R PCR product was subcloned in the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). DIG-labeled RNA probes were made with the DIG RNA labeling Kit (Sp6/T7) (Roche, Mannheim, Germany). ISH was performed on routinely fixed paraffin-embedded tissue sections by use of standard laboratory protocols. Briefly, paraffin tissue sections were deparaffinized and air-dried for 10 min. Slides were treated with proteinase K (Roche) at 37°C for 1 hr. Generally, four different concentrations of proteinase K were used (5, 10, 15, and 20 μg/ml in TBS) for each tissue sample. After being washed with PBS, slides were incubated with 1 ng/μl DIG-labeled probe (anti-sense or sense) in a hybridization solution consisting of 5× Denhardt's solution, 2× SSC, 10% dextran, 30% formamide, 1 mg/ml t-RNA, and 2 mg/ml fish sperm DNA overnight at 55°C. After being washed, slides were treated with 10 mg/ml RNase (Sigma-Aldrich, Steinheim, Germany) at 37°C for 30 min. Positive cells were visualized with anti-DIG–labeled alkaline phosphatase (Roche) for 1 hr in 0.1 M maleic acid buffer containing 0.15 M NaCl, 2% blocking buffer, and 1% Triton X-100. The staining reaction was performed overnight with NBT and BCIP (Roche) in TBS buffer (pH = 9.0) containing 50 mM $MgCl_2$ and 0.01 M levami-

TABLE 1. Overview of All Primer Sets Used for the Analysis of the *BIC* Gene

Forward primer 5′–3′	Reversed primer 5′–3′	Primer set	Size (bp)	Template	Location
taatccaagaactagtcaccc	ggttacagtgaataacctggac	F/R	208	RNA	Exon 3
gattcaactgttagaaatgtggg	ggttacagtgaataacctggac	Fnew/R	755	RNA	Exon 3
gagacgctcctggcactgc	taaaccacagatttccccttcctgg	Ex1F/Ex3R	132	RNA	Exons 1–3
ggtgaggactctcttcctgg	tctactctctccttgcaggg	15/16	1868	DNA	Promoter
ggtccaggaggaaaaacctcc	aggcgacaccacagagtccc	5/6	786	DNA	Promoter
gagacgctcctggcactgc	ggtcatttgacagaaatcaggg	Ex1F/27	1457	DNA	Exon 1
aggtacggatgtgggatggg	gatgaggtatttaaagatgaggg	28/29	659	DNA	Intron 1
ggacgctatgctagacactg	atctacaagcaggtaaaagcc	7/10	2501	DNA	Intron 1
ggataaggtgaaacagtgccc	atctacaagcaggtaaaagcc	9/10	706	DNA	Intron 1
cagagaccacactgacatgg	cccagggaacagaaaatagggg	19/20	1897	DNA	Intron 1
ggaagggtgacaaaacactcc	tgagtcaacttctctgctacc	21/22	1238	DNA	Intron 1
gaagatgtgacgccagttgg	atgcagacaagttatctgggg	11/12	1446	DNA	Intron 1
tctctcctcagcccttcacc	acttccagtcccctcactcc	23/24	1931	DNA	Exon 2
ctgacaatggatatttcatgtgg	aaacacaaccttgtcatacagg	13/14	689	DNA	Intron 2
catcatgcctgtatgacaagg	acagaaaatcgttacctgggg	25/26	2270	DNA	Exon 3

sole. For all negative cases, an additional ISH analysis was performed, starting with a pretreatment consisting of a 7-min incubation in 10 mM citrate buffer (pH = 6.0) in a microwave oven. The same ISH protocol was applied with the only exception that the proteinase K treatment was performed at a concentration of 0.1 μg/ml for 30 min. For *BIC*, this approach yielded a higher sensitivity than that of the standard protocol.

RNA ISH Combined With Immunostaining

For immunostaining with BB4 (CD138) (IQ Products, Groningen, The Netherlands) and CD56 (Novacastra, Newcastle upon Tyne, UK), a heat-induced antigen retrieval was performed for 7 min in 10 mM citrate buffer (pH = 6.0) in a microwave oven. Antibodies were incubated for 1 hr at room temperature in PBS. The second incubation was with rabbit anti-mouse for 30 min and the third with peroxidase-labeled goat anti-rabbit for 30 min. All incubations and washes were performed with DEPC-treated solutions. Antibody incubations were performed in the presence of RNase inhibitor (GibcoBRL). Peroxidase enzyme staining was performed with AEC and H_2O_2. RNA ISH was performed as described above with a proteinase K treatment at a concentration of 0.1 μg/ml for 30 min. For CD3 (Dako, Glostrup, Denmark), CD20 (L26, Dako), and immunoglobulin light chains (kappa and lambda) (Dako), RNA ISH was performed first as described above (standard protocol). For CD3 immunostaining, no further antigen retrieval procedure was performed. CD20 was stained after a heat-induced antigen retrieval (microwave) procedure. For immunoglobulin kappa and lambda light chains, a pretreatment with 0.1% protease for 30 min was followed by a three-step streptavidin-biotin procedure. Peroxidase enzyme staining was performed with DAB and H_2O_2.

Regulation of *BIC* Expression

PCR and Southern blot analyses. Genomic DNA was isolated from cell lines and from cryostat-cut frozen tissue sections by use of standard laboratory techniques. Briefly, the method consisted of proteinase K treatment (100 μg/ml) in a buffer consisting of 25 mM EDTA, 75 mM NaCl, and 1% SDS for 18 hr at 50°C. After adding 0.33 volume of a 6 M NaCl solution, DNA was extracted with one volume chloroform and precipitated with one volume iso-propanol. The DNA was dissolved in 10 mM Tris–HCl and 0.1 mM EDTA. For PCR amplification, 100–300 ng DNA and the PCR protocol as described above (RT-PCR procedure) were used. Primer sequences used for the amplifications are listed in Table 1. For Southern blot analysis, DNA was digested with *Eco*RI, *Hin*dIII, *Spe*I, *Sma*I, and *Cfr*9L in the buffer provided by the manufacturer (GibcoBRL). DNA was separated on a 0.7% agarose gel and transferred to a Hybond N^+ filter (Amersham, Arlington Heights, IL) by use of an alkaline blotting protocol. Blots were hybridized with ^{32}P-labeled probes and exposed to X-ray film (Fuji, Tokyo, Japan).

Methylation of the* BIC *genomic region. The Rose cell line was cultured in RPMI 1640 (Bio-Whittaker, Verviers, Belgium) containing 10% heat-inactivated FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mmol/L L-glutamine in a 37°C humidified atmosphere (5% CO_2). The demethylating agent 5-azacytidine (Sigma-Aldrich) was added in a concentration of 1×10^{-5} M. Every

other day, cultures were split and the medium was replenished with 5-azacytidine. Treated and untreated cells were harvested and washed once with sterile PBS. Total RNA was isolated by use of Trizol reagent followed by RT-PCR (see above) and real-time PCR (see below) analyses for *BIC*. RT-PCR for *CDKN2A* (P16) was used as a positive control for effective demethylation. The PCR conditions and primers used for the RT-PCR were as reported previously (Van der Velden et al., 2001).

Up-regulation of BIC expression upon cross-linking of the B-cell receptor (BcR) in Ramos cells. Ramos cells were cultured in RPMI 1640 medium containing 14% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were diluted to a concentration of 0.5×10^6 cells/ml in 2 ml RPMI medium containing 2% FCS. After 30 min, 5 μg/ml goat anti-human IgM (109-005-129; Jackson Immunoresearch Laboratories, West Grove, PA) was added (T = 0) and cells were harvested at T = 0.5, 1.5, 3, and 24 hr. RNA isolation and cDNA syntheses were performed as described above, and quantification of BIC transcripts was performed by real-time PCR (see below).

Generation of Ramos-IκBα$_{ND}$ (R-M-SPII) subline. An HA-tagged non-degradable IκBα (IκBα$_{ND}$), kindly provided and previously described by Dr. D. Brenner (UNC, Chapel Hill, NC) (Iimuro et al., 1998), was cloned into the retroviral LZRS-pBMN-IRES-EGFP (kindly provided by H. Spits, NKI, The Netherlands), which was constructed from the LZRS-LacZ(A) originally described by Kinsella and Nolan (1996) by replacing the LacZ by the IRES-EGFP sequence, allowing easy selection of transduced cells. LZRS-pBMN-IRES-EGFP-IκBα$_{ND}$ plasmid DNA was used to transduce the retroviral packaging cell line FNX. Ramos cells were transduced by co-culture of Ramos cells with FNX-IκBα$_{ND}$, after which transduced Ramos cells were selected flow-cytometrically, by gating for EGFP by use of excitation and emission wavelengths of 495 and 525 nm, respectively. Cellular expression of IκBα$_{ND}$ was confirmed by Western blotting by use of antibodies against IκBα and HA.

Real-Time PCR

Real-time quantitative RT-PCR for *BIC* and *HPRT* was performed by use of the ABI PRISM 7900 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA). Primers and probes used for *HPRT* were as reported previously (Specht et al., 2001). The *BIC* primers (forward primer 5′-TCAAGAACAACCTACCAGAGACCTT-3′ and reverse primer 5′-TCCTGGTTTGTGCCACCAT-3′) and probe (5′-6-FAM-ACCTTGGCTCTCCCACCCAATGGA-TAMRA-3′) used for the quantitative RT-PCR were selected from the published sequence data (accession number AF402776) by use of Primer express software (PE Applied Biosystems). The amount of *BIC* target was normalized relative to the amount of *HPRT* target (ΔCt). The up-regulation of *BIC* expression was measured by determining the ΔΔCt values of treated vs. untreated samples. PCR was performed with the qPCR Core kit (Eurogentec, Seraing, Belgium) by use of 5 μl of diluted cDNA, 200 nmol/L of the probe, and 900 nmol/L primers by use of a pre-amplification incubation of 2 min at 50°C and 10 min at 95°C, followed by a two-step amplification (15 sec at 95°C and 60 sec at 60°C) procedure. For each experiment, the samples were analyzed in triplicate.

RESULTS

SAGE Analysis of HL-Derived Cell Lines

SAGE analysis of the HL-derived cell line DEV resulted in the identification of 3,575 tags corresponding to 1,911 genes. The frequency at which these 1,911 independent SAGE tags were detected varied from 1 to 97. This SAGE library was compared to the previously constructed SAGE libraries of the HL-derived cell line L428 and of the EBV-transformed lymphoblastoid cell line RAY (Van den Berg et al., 1999). The three SAGE libraries consisted of a total of 5,734 tags representing 2,912 genes. Comparison of the tags to all known genes revealed the expression of a number of genes known to be expressed in HL, like CD30, Fascin, Restin, and NF-κB. Analysis of differentially expressed tags resulted in high frequencies of tags corresponding to RNase genes in DEV as compared to L428 and RAY. A total of 15 tags detected more than 5 times in DEV and not in the RAY SAGE library were subjected to a further analysis. RT-PCR analysis for one of these tags, corresponding to the human *BIC* gene, revealed a very high expression in DEV as well as in the other HL-derived cell lines. Either no or a low level of expression was detected in the NHL-derived cell lines Rose, Raji, Karpas299, Ver, and Jurkat, and in the EBV-transformed lymphoblastoid B-cell lines RAY and POP (Fig. 1).

RNA ISH for the Detection of *BIC* Transcripts

RNA ISH on paraffin tissue sections of randomly selected cases diagnosed with classical and NLP HL revealed a very strong signal with the anti-

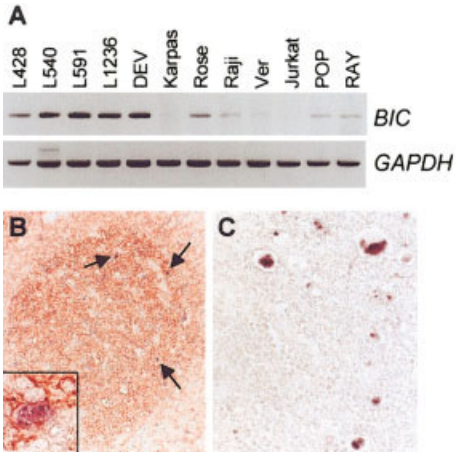

Figure 1. *BIC* expression in HL, NHL, and normal tissues. **A:** RT-PCR for *BIC* and *GAPDH* for HL- and NHL–derived cell lines. The inverted image of a 2% agarose gel is shown. **B:** *BIC* RNA ISH (black) by use of anti-sense *BIC* probe on a tonsil in combination with CD20 staining (red) (amplification 200×). Double positive cells can be seen in the germinal center (arrows). An enlargement of a double positive cell is shown in the left corner. **C:** RNA ISH by use of anti-sense *BIC* probe on a NHL-involved tissue sample. RS cells stain strongly positive.

sense probe in the nuclei of RS cells. *BIC* transcripts were detected in all or at least the vast majority of RS cells (Fig. 2). In total, we screened 63 cases of HL, including 55 classical EBV-positive (24) and -negative (31) cases and 8 EBV-negative NLP HL cases. *BIC* transcripts were detected in 53 cases, including NLP and EBV-positive and -negative classical HL cases. No signal was obtained for the sense probe. A control RNA in situ hybridization experiment by use of an anti-sense β-actin probe revealed no or weak signals in five *BIC*-negative cases. Exclusion of these five cases with poor-quality RNA resulted in *BIC* positivity in 91% (53 out of 58) of HL cases (Table 2).

Analysis of 43 cases of NHL (including 15 follicular lymphomas, 7 diffuse large B-cell lymphomas, 5 T-cell–rich B-cell lymphomas, 9 Burkitt lymphomas, and 7 anaplastic large cell lymphomas) showed positive *BIC* signals in only a minority of cells. These resembled the minority of positive cells seen in normal lymph node tissue. A few cases of diffuse large B-cell lymphoma and one case of Burkitt lymphoma showed weak expression of *BIC* in a minority of tumor cells. A higher percentage of positive tumor cells (i.e., 1–2%) was observed in most diffuse large B-cell lymphomas. In general, the vast majority of tumor cells of NHL cases did not stain positively for *BIC*.

Analysis of a large number of adult and fetal normal tissues revealed a positive signal only in lymph node and tonsil tissue samples from adult patients. In these tissues, a moderate signal was detected in a limited number of cells located predominantly in the germinal centers. Immunohistochemical staining in combination with an RNA ISH for *BIC* did not reveal double positive cells for CD3 (T cells), CD56 (NK cells), CD138 (plasma cells), and immunoglobulin light chains (kappa and lambda). Analysis of CD20 (B-cells) revealed that a proportion of the *BIC*-positive cells also stained positively for this B-cell marker, indicating that at least part of the *BIC*-positive cells in normal lymph node or tonsil tissue are CD20+ B-cells.

Regulation of *BIC* Expression

To determine whether genomic aberrations might be responsible for the high expression of the human *BIC* gene in HL, we applied PCR with overlapping primer sets and Southern blotting by use of different probes and restriction enzymes (*Eco*RI, *Hin*dIII, *Spe*I, and *Sma*I) to screen the whole *BIC* gene region. Twelve overlapping primer sets were selected from the 15-kb region containing the promoter region and the complete coding region of *BIC* (Table 1). Genomic DNA of five different HL-derived cell lines and a number of NHL-derived cell lines and controls were amplified with all primer sets. No aberrant PCR products were detected, indicating that there are no structural aberrations in the genomic DNA samples that can be detected with PCR. To exclude the presence of large deletions or insertions, which cannot be detected by PCR, Southern blot analysis was performed by use of purified PCR products as probes. Most of the probes failed to give a specific signal attributed to repetitive sequences present in the amplicon. For two of the amplicons (F/R and 25/26), good hybridization results were obtained, and these two PCR products were used as probes to screen genomic DNA for the presence of aberrations in the *BIC* gene region. Southern blots were made by use of four different restriction enzymes, resulting in DNA fragments covering the *BIC* gene and upstream region. For the F/R probe, no aberrant fragments were detected. For the 25/26 probe, DNA fragments larger than expected, based on the genomic sequence, were detected in the NHL-derived cell lines and in the normal controls for the *Sma*I digestions. Normal-size DNA fragments were seen in the HL-derived cell lines (Fig. 2). No aberrant bands were detected for the other three restriction enzymes (*Eco*RI, *Hin*dIII, and *Spe*I). Because *Sma*I is a methylation-sensitive enzyme, we repeated the experiment with *Cfr*9L, a restriction enzyme that recognizes the same sequence but is

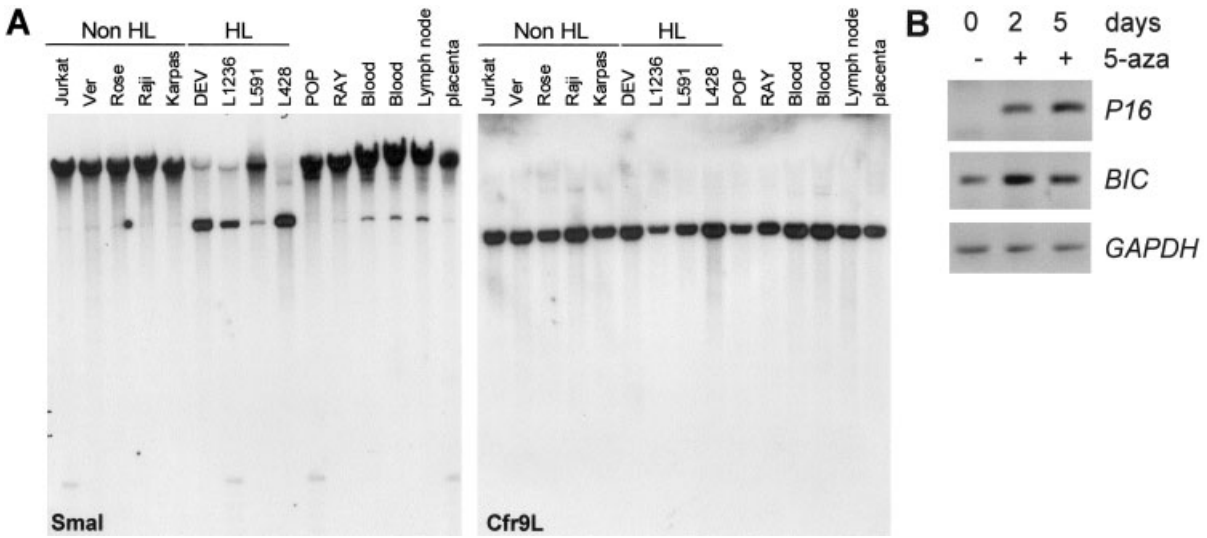

Figure 2. Methylation of the *BIC* promoter region. **A:** Southern blot with *SmaI* and *Cfr*9L on HL cell lines, NHL–derived cell lines, and normal tissue samples. **B:** RT-PCR for *BIC* and *CDKN2A* (P16) after treatment of the B-cell NHL–derived cell line Rose with 5-aza-cytidin (5-aza) for different periods (0, 2, and 5 days). The inverted image of a 2% agarose gel is shown. Expression of *BIC* and *P16* is up-regulated in the treated samples.

TABLE 2. Summary of the *BIC* RNA ISH Results

	NS		MC			HL	NHL
	EBV+	EBV−	EBV+	EBV−	NLP	(total)	(total)
BIC+	9	22	14	1	7	53	0
BIC−	1	3	0	1	0	5	43
Total	10	25	14	2	7	58	43

not methylation-sensitive. This revealed normal-size DNA fragments for all samples analyzed, indicating that methylation of the *BIC* gene region was responsible for the generation of the aberrant DNA fragments.

To test further a possible involvement of methylation in the regulation of *BIC* expression, we treated the B-cell NHL-derived cell line Rose with 5-aza-cytidin, which resulted in an overall demethylation of the DNA. RT-PCR of treated and untreated cells revealed a 16-fold up-regulation of *BIC* in the Rose sample treated for 5 days with 5-aza-cytidin (Fig. 2B and Table 3). For the *CDKN2A* gene that was used as a positive control for an effective treatment, we also observed up-regulation of expression in the treated samples.

To gain insight into the regulation of *BIC* expression in B cells, we analyzed Ramos cells upon cross-linking of the BcR. To this end, Ramos cells were cultured in the presence of polyclonal anti-IgM–specific antibodies for different times followed by RT-PCR for *BIC*. BcR cross-linking revealed 10- to 4,290-fold up-regulation of *BIC* expression, which was first detectable 1.5 hr after BcR cross-linking (Fig. 3 and Table 3). To determine a possible involvement of NF-κB in the regulation of BIC expression, we also analyzed the *BIC* expression in the Ramos subline with non-degradable IκBα upon cross-linking of the BcR for different times. RT-PCR revealed a *BIC* target ratio of 81- to 708-fold in treated vs. untreated samples, which is similar to the *BIC* target ratio observed in the original Ramos cell line (Table 3).

DISCUSSION

The *Bic* locus was originally detected as a common site of integration of viral sequences in 10 out of 34 virally induced lymphomas in gallus gallus (Clurman and Hayward, 1989). Screening of the *Bic* locus resulted in the identification of a novel gene called *Bic* (B-cell Integration cluster), which lacked a functional ORF and was suggested to function through its noncoding RNA (Tam et al., 1997). More recently, the human (Van den Berg et al.,

TABLE 3. Real-Time PCR Data for the Quantitative Expression Analyses of *BIC**

Sample	Treatment	Time	ΔCt	ΔΔCt	Target ratio treated/untreated
Rose	nt	0 d	1.2		
Rose	5-aza	2 d	0	1.2	2,3
Rose	5-aza	5 d	−2.7	3.9	16
Ramos	nt	0.5 h	13.5	0.2	1,1
Ramos	Anti-IgM	0.5 h	13.7		
Ramos	nt	1.5 h	17.5	3.3	9,7
Ramos	Anti-IgM	1.5 h	14.2		
Ramos	nt	2 h	19.9	6.5	86
Ramos	Anti-IgM	2 h	13.4[a]		
Ramos	nt	3 h	19.7	7.7	218
Ramos	Anti-IgM	3 h	12.0[a]		
Ramos	nt	6 h	19.1	8.9	478
Ramos	Anti-IgM	6 h	10.2[a]		
Ramos	nt	24 h	20.9	12.0	4,290
Ramos	Anti-IgM	24 h	8.9[a]		
Ramos	nt	2 h	19.8	6.3	81
Ramos IκBα$_{ND}$	Anti-IgM	2 h	13.5[a]		
Ramos IκBα$_{ND}$	nt	6 h	20.4	9.3	691
Ramos IκBα$_{ND}$	Anti-IgM	6 h	12.1[a]		
Ramos IκBα$_{ND}$	nt	24 h	20.6	9.6	708
Ramos IκBα$_{ND}$	Anti-IgM	24 h	11.2[a]		

*ΔCt, HPRT Ct value minus BIC Ct value. ΔΔCt relative up-regulation of *BIC* expression as determined by comparison of the ΔCt values of treated vs. untreated samples. nt, no treatment; 5-aza, 5-azacytidine treatment; d, days; h, hours.
[a]No BIC signal was obtained after 40 cycles of amplification; the Ct value used for the calculations is 40; the ΔCt, ΔΔCt, and target ratio (treated/untreated) given in the table should be interpreted as more than the calculated values.

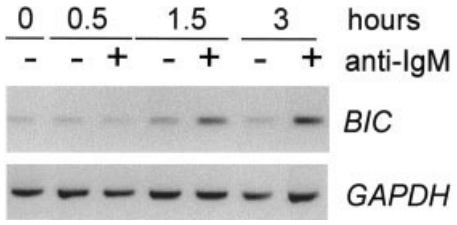

Figure 3. Up-regulation of *BIC* expression upon cross-linking of the BcR in Ramos cells. RT-PCR results are shown for *BIC* after BcR triggering of the Ramos cells. *GAPDH* was used as a control for RNA quality and cDNA synthesis. The inverted image of a 2% agarose gel is shown. Up-regulation of *BIC* can be seen in the samples treated with anti-IgM for 1, 5, and 3 hr.

2000b; Tam, 2001) and mouse (Tam, 2001) *BIC* homologs were identified based on a high percentage of identity to the gallus gallus *Bic* sequence.

Our results demonstrate a consistent and high expression of *BIC* in 91% of HL cases, including classical EBV+ and EBV− HL and NLP HL cases. *BIC* transcripts are located in the nuclei of all or the majority of RS cells of positive HL cases. Five of the *BIC*-negative HL cases with good-quality RNA most likely represent *BIC*-negative HL cases. Re-evaluation of the histological tissue slides indeed confirmed the HL diagnosis. Screening of normal tissues revealed *BIC* expression in only a minority of cells predominantly located in the germinal centers of follicles in tonsil and lymph node. The high percentage of strongly *BIC*-positive HL cases, compared with the lack of *BIC* staining in NHL cases and the weak staining in normal tissue, indicates that a high expression of *BIC* is specific for HL. Whether this is caused by overexpression of the *BIC* gene or by an increased stability of the *BIC* transcripts is not known. Combination of RNA ISH with immunohistochemical staining for several antibodies revealed double positive cells only for CD20, suggesting a B-cell origin for the *BIC*-positive cells in normal lymph node and tonsil tissue.

The characteristics of the human *BIC* gene (spliced, polyadenylated, no ORF) indicate that this gene is a member of the family of noncoding mRNA-like transcripts. Besides *BIC*, several other members belonging to the family of noncoding mRNA like molecules have been identified in man and also in several other species (http://www.man.poznan.pl/5SData/). On the basis of their genomic organization, they can be divided into three subgroups: noncoding RNA transcripts derived from intron sequences from both (1) coding or (2) noncoding host genes and (3) noncoding mRNA-like molecules consisting of exon sequences. On the

TABLE 4. Overview of the Transcription Factor Binding Sites Detected in the *BIC* Promoter Sequence*

Transcription factor	Consensus	Position	Sequence
TBP	TATAAA	−30 to −24	TATAAAA
AP1	TGASTMA	−47 to −39	TGAGTCA
AP2	CCCMNSSS	−12 to −3	CGCAGGCG
		35 to 45	CTGCAGCC
SP1	KRGGCKRRK	−83 to −75	GGGGGGGGG
NF-κB	GGGAMTTYCC	−371 to −362	GCCACTTCCC

*Bases were numbered according to the first putative transcription start site. S = G/C; M = A/C; N = A/G/T/C; K = G/T; R = A/G; Y = C/T.

basis of the high percentage of identity between the exon sequences of the human, mouse, and gallus gallus *BIC* genes, it can be concluded that *BIC* belongs to the third group of noncoding mRNA-like genes, suggesting a functional role for the spliced RNA transcripts.

Although only little is known about the cellular location of noncoding mRNA-like RNA transcripts, a nuclear location, as shown for *BIC*, may indicate a regulatory function for *BIC* in the nuclei of RS cells and germinal center B cells. Analyses of other noncoding mRNA-like molecules have demonstrated that these genes can play a role in carcinogenesis (Askew et al., 1991; Lottin et al., 2002; Wutz et al., 2002). A possible cooperation of *Bic* with *Myc* has been demonstrated upon co-transfection experiments in chicken embryo fibroblasts showing an increased growth rate. In addition, a higher incidence of lymphomas and erythroblastosis was observed upon co-transfection of *Bic* and *Myc* viral constructs in chicken embryos (Tam et al., 2002). In a recent study, Haasch et al. (2002) demonstrated a strong up-regulation of *BIC* expression in CD3/CD28-activated T cells, suggesting a possible role for *BIC* in T-cell activation.

To gain insight into the mechanism leading to up-regulation of *BIC* expression in HL, a search for genomic aberrations was performed of the *BIC* genomic and upstream region. These analyses indicated that a high level of methylation is associated with either a low level or the absence of *BIC* expression in NHL cell lines. Involvement of methylation was supported by the finding that demethylation of the B-cell NHL-derived cell line Rose resulted in up-regulation of *BIC* expression. In general, DNA methylation is associated with the global inactive state of chromatin and turning off of transcription from a large number of genes that are not required in a particular differentiated cell (Bird, 1995). Although gene activation can be achieved by chromatin remodeling and demethylation (Bergman and Mostoslavsky, 1998), the up-regulation of *BIC* expression detected upon cross-linking of the BcR in Ramos cells suggests a role for the BcR by one of its downstream signaling targets.

Cross-linking of the BcR can result in the activation of different signaling pathways, including both pro- and anti-apoptotic signals (DeFranco, 1997; Yankee and Clark, 2000). In normal B cells, BcR cross-linking results in activation of members of the *SRC* and *Syk* kinase family, which induce various signal transduction pathways. One of the pathways includes activation of B-cell linker protein (BLNK) and formation of Bruton's tyrosine kinase (Btk)–phospholipase-gamma-2 (PLG-γ2) complex, which regulates NF-κB activation (Tan et al., 2001). NF-κB is a known transcription factor that plays an important role in B-cell activation and survival. It is known that activation of NF-κB is a very early step upon cross-linking of the BcR (Francis et al., 1998). Analysis of the *BIC* upstream region by use of TESS, TSSG, and MatInspector software resulted in the identification of an Sp1, an AP-1, and two AP-2 binding sites directly upstream of exon 1. In addition, a putative NF-κB binding site was identified 370 bp upstream of the transcription start site (Table 4). Therefore, it may be speculated that NF-κB is involved in up-regulation of *BIC* expression upon triggering of the BcR in Ramos cells. Analysis of *BIC* expression in Ramos cells overexpressing a non-degradable mutant of IκBα revealed normal up-regulation of *BIC* expression after BcR triggering. This suggests that NF-κB is not involved in the up-regulation of *BIC* upon BcR triggering of the Ramos cell line. Most likely, other components of the BcR pathway are involved in up-regulation of *BIC* expression upon cross-linking of the BcR in Ramos cells.

In summary, we demonstrated that *BIC* is highly expressed in over 90% of all HL cases, including EBV-positive and -negative classical HL and NLP HL. *BIC* transcripts are located exclusively in the nuclei of RS cells. In normal lymph node and tonsil tissue, a small minority of cells are *BIC*-positive cells, of B-cell origin, and cluster predominantly in germinal centers. In addition, we found up-regula-

tion of *BIC* expression upon triggering of the BcR, which is not mediated by NF-κB. On the basis of these findings, it can be speculated that *BIC* plays a physiological role in normal germinal center B-cell development as well as in the pathogenesis of HL.

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