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EBNA2 Interferes with the Germinal Center Phenotype by Downregulating BCL6 and TCL1 in Non-Hodgkin's Lymphoma Cells[▽]

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Epstein-Barr virus (EBV)-negative diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma-derived cell lines infected in vitro with a recombinant EBV expressed type II/III latency. High expression of EBNA2 inversely correlated with expression of germinal center (GC)-associated genes, BCL6 and TCL1. The decreased expression of BCL6 appeared to be dose dependent, with almost complete abrogation in highly EBNA2-expressing clones. The role of EBNA2 in negative regulation of these genes was confirmed by transfection and in a hormone-inducible EBNA2 cell system. LMP1 transfection reduced expression of TCL1, but not of BCL6, in DLBCLs. The GC-associated gene repression was at the transcriptional level and CBF1 independent. A decrease in HLA-DR, surface immunoglobulin M, and class II transactivator expression and an increase in CCL3, a BCL6 repression target, was observed in EBNA2-expressing clones. Since BCL6 is indispensable for GC formation and somatic hypermutations (SHM), we suggest that the previously reported lack of SHM seen in EBNA2-expressing GC cells from infectious mononucleosis tonsils could be due to negative regulation of BCL6 by EBNA2. These findings suggest that EBNA2 interferes with the GC phenotype.

Among non-Hodgkin's lymphomas (NHLs), the noted association of Epstein-Barr virus (EBV) is with endemic Burkitt lymphomas (BLs). Diffuse large B-cell lymphomas (DLBCLs) are a heterogeneous group of tumors, about 10% of which are EBV associated. Its high frequency (30 to 40% of all NHLs) makes DLBCL one of the most common cancers in adults (13). The most common genetic abnormality observed in DLBCL is the chromosomal translocation involving 3q27. The BCL6 gene was identified due to this chromosomal anomaly (51). Based on gene expression profiling, DLBCLs can be subdivided into two broad categories, namely, germinal center (GC) B-like DLBCLs and activated DLBCLs (1). This division is mainly based on the expression of the BCL6 proto-oncogene. While the former are BCL6 expressers, the latter are BCL6 negative, indicating a post-GC origin. The association of EBV is more frequent with the BCL6-negative variants (23). It is not clear, however, if EBV preferentially infects BCL6-negative DLBCL cells or if any virally encoded proteins are responsible for its downregulation.

The BCL6 gene encodes a protein that belongs to the BTB/POZ family of transcription factors. The amino terminus of the protein contains several BTB domains, and the C terminus contains six zinc finger domains (31). One of the main well-characterized functions of this protein is its indispensability for GC formation and, consequently, for somatic hypermutations (SHM) (52). The GCs are structures generated within the follicles of secondary lymphoid tissues containing antigen-driven, extensively proliferating B cells. BCL6$^{-/-}$ mice lack such structures (10). Furthermore, through its BTB/POZ domain, BCL6 binds to the silencing mediator of retinoid and thyroid hormone receptor and other corepressors, like NCoR, and BCoR, and recruits them to various target genes (31).

TCL1 belongs to a family of proteins whose expression is correlated with B-cell differentiation, with prominent expression during the GC phase, which gradually decreases with a plasmacytoid phenotype (42). TCL1 transgenic mice develop Burkitt-like lymphomas (15). Its capacity to activate AKT serine/threonine kinase seems to be responsible for its pathogenic role in the development of such tumors (25).

Human tumors, other than endemic BLs, with consistent EBV association include nasopharyngeal carcinoma and Hodgkin's disease. The virus establishes three forms of latency in infected cells. The expression of EBNA1 and EBERs represents type I latency. EBV-infected normal B lymphocytes express type I latency in vivo. Under pathological conditions, the viral latent-gene expression varies in different tumors. The phenotypically representative BL and corresponding cell lines express EBNA-1 and LMP2A. A drift in these lines toward an immunoblastic phenotype is accompanied by expression of all latency-associated viral proteins, EBNA1 to -6 and LMP1, -2A, and -2B, known as a type III program. The viral latent-gene expression observed in nasopharyngeal carcinoma and Hodgkin's disease represents intermediate type II latency (LMP$^+$ EBNA2$^-$) (44).

EBNA2 is critically required for B-cell transformation, as the P3HR1-derived EBV strain, which lacks EBNA2, is defi-

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cient in this capacity and the transforming potential is reconstituted with EBNA2 expression (8, 35). It activates transcription by binding to recombination signal-binding protein for J-kappa (RBPJK) and activating the downstream target genes, such as CD23 (47). In this, EBNA2 is considered a functional homologue of NotchIc (14, 22). The transactivating functions of EBNA2 are suppressed when it is phosphorylated by cdc2/cyclin B1 during mitosis (53). Furthermore, EBNA2 plays a major role as an adapter protein that targets the SWI/SNF complex to a specific region in chromatin (49). The SWI/SNF chromatin-remodeling complex is important for both transcription activation and repression (40). The EBNA2-SWI/SNF association thus might play an important role in regulating gene expression from both viral and cellular chromatins.

About 10% of non-AIDS-associated DLBCLs in the general population are associated with EBV (12, 16). The viral latent-gene expression in such tumors was characterized as a type II (EBNA1$^+$ LMP1$^+$ EBNA2$^-$) or III (LMP1$^+$ EBNA2$^+$) pattern (23). In order to investigate the role of the virus in the pathogenesis of this tumor, an EBV-negative DLBCL and a BL cell line were infected in vitro with a recombinant EBV. The latent viral-gene expression was studied in these EBV convertants. A recent study reported that EBNA2-expressing cells in GC from infectious mononucleosis (IM) tonsils do not show ongoing SHM (21). We therefore investigated whether type III latency, and in particular EBNA2 expression, might have any influence on GC-associated gene expression.

MATERIALS AND METHODS

Cells. U2932 is a previously characterized EBV-negative DLBCL (3). Oma BL cl-4 is an EBV-negative variant derived from an originally EBV-positive BL (45). BJAB is an EBV-negative B-cell lymphoma. BJAB K3 expressing hormone-inducible EBNA2 has been previously characterized (19). EBV-negative BL DG75 transfected with LMP1 has been described elsewhere (9). DG75 carrying a somatical knockout RBPJK and its EBNA2-transfected derivative have been described in detail (26). Akata BL, lymphoblastoid cell line (LCL), BC3 primary effusion lymphoma (PEL), and RPMI 8226 cells were used as controls. The cells were grown in RPMI supplemented with 10% fetal calf serum, 100 IU of penicillin, and 100 μg/ml streptomycin.

Infection with a recombinant EBV strain. Akata recombinant EBV producer cells were treated with anti-immunoglobulin G (IgG) as previously described to obtain infectious virus particles (28, 41). The supernatant containing recombinant EBV was used to infect U2932 and Oma cl-4 cells. The viral infection was carried out for 2 hours at 37°C, with intermittent shaking. The cells were washed and resuspended in complete medium for 48 h before selection in 1 mg/ml of G418. Three weeks later, the GFP-positive and G418-resistant clones were propagated. Five EBV-infected clones of U2932 and three EBV-infected clones of Oma cl-4 were used for further studies.

Transfection of EBNA2 and LMP1 into U2932. Five million cells were transfected with an EBNA2 expression vector, pSVgpt-EBNA2; LMP1 expression vector J132-G5 (a kind gift of Lars Rymo, Gothenburg, Sweden); and the corresponding vector control pSVgpt by electroporation (230 V, 960 μF in 200 μl phosphate-buffered saline [PBS]). Two days later, the cells were placed in a selection medium containing 1.5 μg/ml mycophenolic acid, 160 μg/ml xanthine, and 10 μg/ml hypoxanthine. Drug-resistant clones were isolated after 4 weeks and used to verify EBNA2 and LMP1 expression.

Immunoblotting. The expression of EBNA1 to -6 and LMP1 was verified by immunoblotting. The cells (10^7) were lysed in 1 ml of lysis buffer. Thirty micrograms of protein extracts was separated on 7.5% discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to Protran (Amersham) filters. The EBNAs were detected by using a previously characterized polyclonal human serum derived from a seropositive healthy donor. The expression of LMP1 was verified with S12 monoclonal antibody (27). For detection of EBNA2, PE2 (Dako) monoclonal antibody was used. A chemiluminescence kit (Amersham) was employed to visualize the proteins. A 15% SDS-polyacrylamide gel was utilized for verification of TCL1 expression using the anti-TCL monoclonal antibody (MBL International). The anti-BCL6 and anti-β-actin antibodies were purchased from Santa Cruz and Sigma, respectively. The working conditions for all antibodies were those indicated by the respective manufacturer. Densitometric analysis was performed with Quantity-one software (Bio-Rad). The change in expression was calculated relative to the β-actin signal.

TABLE 1. PCR primers and conditions

Transcript	Product size (bp)	Type	Sequence
EBER1[a]	167	5′ Primer	AGGACCTACGCTGCCCTAGA
		3′ Primer	AAAACATGCGGACCACCAGC
LMP2A[b]	411	5′ Primer	TCCATCTGCTTCTGGCTCTT
		3′ Primer	AAACAGAAGAGAATTAAAA
		5′ Nested	CGAACGATGAGGAACGTGAA
		3′ Nested	TTGTGCAGCGGCATATGAG
LMP2B[b]	210	5′ Primer	CAGTGTAATCTGCACAAAGA
		3′ Primer	AAACAGAAGAGAATTAAAA
		5′ Nested	CAACGTTGGGAGGTCGTTGG
		3′ Nested	TTTGTGCAGCGGCATATGAG
BCL6[c]	143	5′ Primer	GTGCTTATCCACACTGGTGA
		3′ Primer	ACGGAAATGCAGGTTACACT
TCL1[d]	386	5′ Primer	CTGGCTCTTGCTTCTTAGGCGG
		3′ Primer	GTGCTGCCAAGACCATACAT CAGT
CIITA[c]	205	5′ Primer	CTGAAGGATGGTGGAAGACCT GGGAAAGC
		3′ Primer	GTGCCCGATCTTGTTCTCACTC
CCL3[c]	184	5′ Primer	GCAACCAGTTCTCTGCATCA
		3′ Primer	ACTCCTCACTGGGGTCAGC

[a] Denaturing was done at 94° for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. Cycles, 12.
[b] Denaturing, 94°C for 30 s; annealing, 45°C for 90 s; extension, 72°C for 2 min. Cycles, 30. Nested: denaturing, 94°C for 30 s; annealing, 58°C for 1 min; extension, 72°C for 2 min. Cycles, 25.
[c] Denaturing, 95°C for 3 min; denaturing, 95°C for 1 min; annealing, 60°C for 30 s; extension, 72°C for 45 s. Cycles, 30.
[d] Denaturing, 95°C for 1 min; annealing, 55°C for 1 min; extension, 72°C for 2 min. Cycles, 30.

RT-PCR. The expression of EBER1 and LMP2A and -2B was verified by reverse transcription (RT) PCR. Similarly, RT-PCR was also employed for detection of BCL6, TCL1, class II transactivator (CIITA), and CCL3. RNA was extracted by the TRIzol method according to the manufacturer's instructions, followed by DNase treatment. The first-strand cDNA was generated from 1 μg of RNA by using Moloney murine leukemia virus reverse transcriptase. The RNA was incubated in 19 μl reaction mixture containing first-strand buffer, 10 mM deoxynucleoside triphosphate mixture, 0.1 mM dithiothreitol, 20 U RNasin, and 15 U of reverse transcriptase for 60 min at 37°C and 10 min at 95°C to inactivate the enzyme. The reactions were performed in an automated thermal cycler (Techne PHC-2); 2 μl of template cDNA was used for the PCR amplifications. The reactions were carried out in a 50-μl volume containing 20 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM primers, and 2.5 mM deoxynucleotide triphosphate mixture. One unit of *Taq* polymerase was added to the reaction mixture. The amplification conditions and primer sequences are shown in Table 1.

Flow cytometry. One million cells were washed with PBS. The pellets were incubated with HLA-DR monoclonal antibody L243 and anti-IgM antibodies at recommended dilutions on ice for 30 min. R-phycoerythrin-conjugated anti-mouse antibodies were used as secondary antibodies. The cells were subsequently washed twice with PBS. The HLA-DR and surface IgM (sIgM) expression was analyzed with Becton Dickinson Cellquest software.

RESULTS

Viral latent-gene expression in EBV-infected cell lines. Five drug-resistant clones from the EBV-infected DLBCLs were tested for EBV latent-gene expression. Clones (cl.) 1 to 3 and

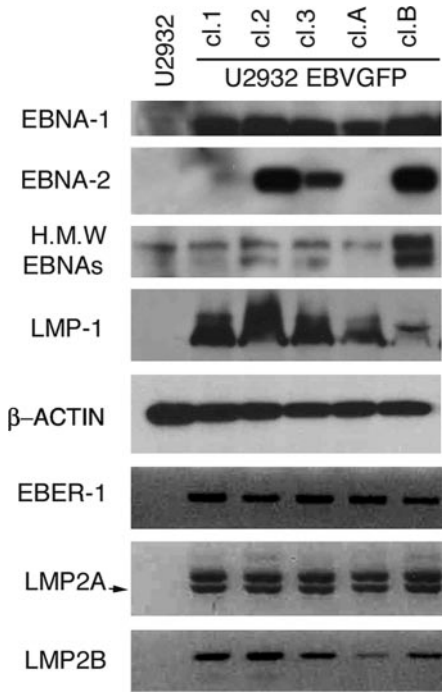

FIG. 1. EBV latent-gene expression in DLBCLs infected with recombinant EBV. EBNAs were detected with a polyclonal serum. EBNA2 and LMP1 were visualized by PE2 and S12 monoclonal antibodies, respectively. EBER-1 and LMP2A and -2B were detected by RT-PCR. The inverted images of 2% agarose gels are shown. The arrow indicates the correct amplified product in LMP2A RT-PCR. The primers and amplification conditions for RT-PCR are described in Table 1. H.M.W., high-molecular-weight.

cl. A and B were derived from two separate infections. Figure 1 shows EBV latent-gene expression in EBV-infected clones of U2932 cells. Expression of EBNA2, as indicated by PE2 antibodies, was variable in different clones. Furthermore, all three latent membrane proteins were expressed in EBV-infected DLBCLs. The lower band in the LMP2A RT-PCR represents the correct amplified product. EBV infection of DLBCLs in vitro thus leads to type II/III latency. The Oma cl-4 convertants expressed a type III latency profile with variable EBNA2 levels (Fig. 2B).

Expression of BCL6 and TCL1 in EBV-infected DLBCLs and BLs. All EBV-infected clones of U2932 were further investigated for BCL6 and TCL1 expression. The most remarkable pattern of expression was that of BCL6 (Fig. 2A). The expression levels of EBNA2 and BCL6 were inversely correlated. In the top row, the EBNA2 expression blot shown in Fig. 1 is shown again to facilitate comparison. The highly EBNA2-expressing clones, cl. 2 and cl. B, of U2932 showed severe reduction (cl. 2) or almost complete abrogation (cl. B) of BCL6 expression. The intermediate EBNA2 expresser (U2932 EBVGFP cl. 3) had intermediate levels of BCL6. The EBNA2-negative clones 1 and A had conserved BCL6 expression at levels similar to those seen in the uninfected parental line. This suggests a dose-dependent inverse correlation between the two proteins. The lack of downregulation of BCL6 in EBNA2-negative clones 1 and A further suggests that other latent EBV proteins and selection markers (G418 and green fluorescent protein [GFP]) expressed in these clones do not participate in BCL6 regulation.

Since TCL1 has been reported to be associated with the GC phenotype, we analyzed its expression in EBV-infected U2932 cells. The highly EBNA2-expressing clones showed reduced TCL1 expression (Fig. 2A, top, row 3). In vitro-EBV-infected clones of Oma cl-4 similarly indicated that high EBNA2 expression was associated with reduced BCL6 and TCL1 expression (Fig. 2B). The relative change in BCL6 and TCL1 expression versus β-actin signal in U2932 and Oma EBV convertants is shown below the corresponding immunoblots.

BCL6 and TCL1 expression in EBNA2- and LMP1-transfected cells. Next, we investigated BCL6 and TCL1 expression in EBNA2-transfected U2932 and BJAB cells. Figure 3A, top, top row shows EBNA2 expression in two transfected clones of U2932 cells. As seen in Fig. 3A, top, row 2, the BCL6 expression in these two clones was either severely reduced (U2932 EBNA2 cl. 1) or completely abrogated (U2932 EBNA2 cl. 2). The same clones showed a reduction in TCL1 expression (Fig. 3A, top, row 3). β-actin expression was verified as a protein load control (Fig. 3A, top, row 4).

The effects of EBNA2 on BCL6 and TCL1 expression were further investigated in BJAB K3 EBNA2 transfectants, in which EBNA2 expression was induced by treatment with estrogen. BJAB K3 cells were treated with 1 μM β-estradiol for 2 days. Figure 3B, top, top row shows a strong induction of EBNA2 and estrogen receptor (ER)-EBNA2 after the hormone treatment. The EBNA2 induction was accompanied by reduced BCL6 and TCL1 expression in these cells (Fig. 3B, top, rows 2 and 3).

LMP1-transfected U2932 cells showed no alteration in BCL6 but had decreased TCL1 expression. The same viral protein, however, did not effect the expression of these two genes in DG75 cells (Fig. 3C). The relative change in expression of the two genes in EBNA2 and LMP1 transfectants is shown below the corresponding immunoblots.

These data suggest that EBNA2 alone is sufficient to downregulate both BCL6 and TCL1 in cells derived from different tumor types, whereas LMP1 has no effect on BCL6 expression in both U2932 DLBCLs and DG75; however, it did negatively modulate TCL1 in U2932 cells.

Transcriptional repression of BCL6 and TCL1. In order to delineate the mechanisms behind the observed downregulation of BCL6 and TCL1, RT-PCR was performed. Figure 4 demonstrates that in the highly EBNA2-expressing clones 2 and B, BCL6 transcription was significantly decreased. Similarly, TCL1 transcription was adversely affected in U2932 EBVGFP cl. 2 and cl. B. An extremely weak signal corresponding to TCL1 transcripts seen in these clones might account for the low protein expression observed (Fig. 2A, top, row 3). U2932 EBVGFP cl. 1 without EBNA2 had increased BCL6 transcription in comparison with the parental cells. A corresponding rise in BCL6 protein, however, was not observed in this clone (Fig. 2A, top, row 2). This might be due to the already high BCL6 protein expression levels in the clone. A faint band corresponding to TCL1 transcripts was observed in this EBNA2-negative clone. Akata BL served as a positive control, while BC3 PEL cells, which do not express BCL6 protein, had a weak cDNA signal. β-Actin RT-PCR served as an RNA

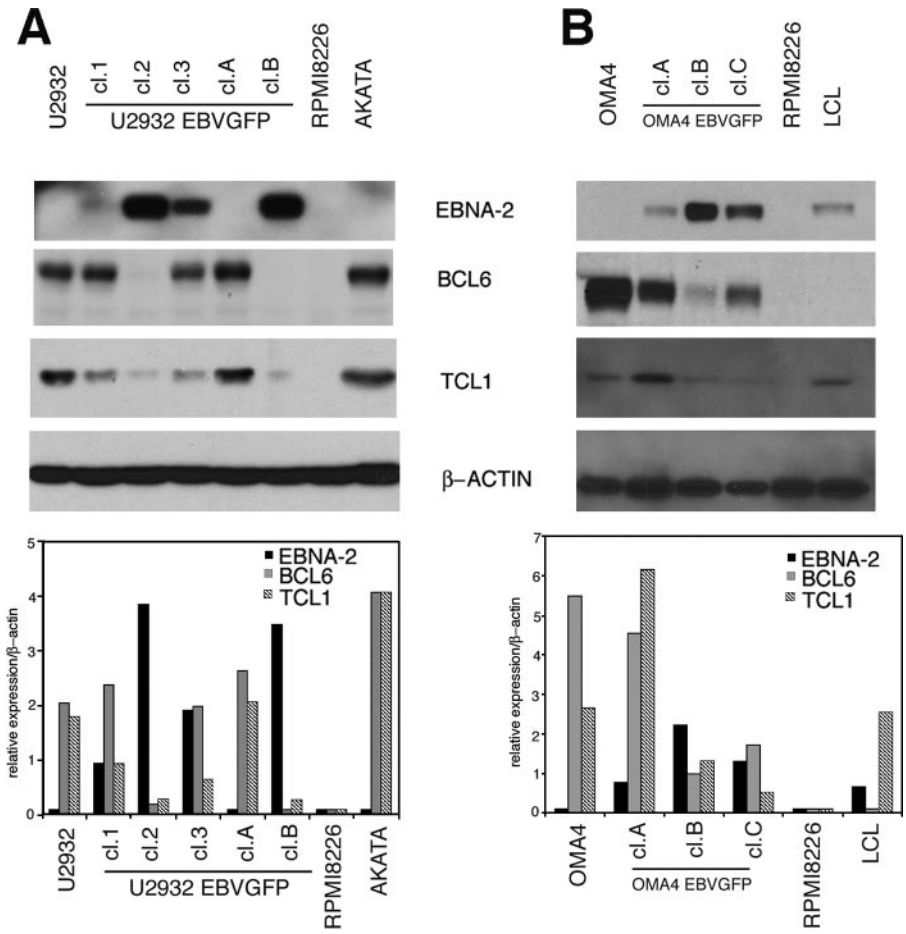

FIG. 2. BCL6 and TCL1 expression in EBV-infected NHLs. (A) U2932. (B) Oma cl-4. Protein extracts were separated on 7.5 and 15% SDS-polyacrylamide gels for BCL6 and TCL1, respectively. A polyclonal anti-BCL6 antibody was employed. The same EBNA2 filter as in Fig. 1 is presented in Fig. 2A (top, top row) to facilitate comparison. Akata, LCL, and RPMI 8226 cell lines were used as controls. β-Actin expression was verified as a protein load control. The graph below each immunoblot shows the relative change in expression normalized to the β-actin signal.

quality and loading control. The RT-PCR was repeated at least five times.

CBF-1 independent regulation of BCL6 and TCL1 by EBNA2. Since EBNA2 regulates several cellular genes through activation of CBF-1 (RBPJK), we sought to determine if this transcription factor plays any role in the EBNA2-mediated BCL6 and TCL1 repression. To this end, EBV-negative DG75 cells carrying somatically knocked out CBF1 and subsequently transfected with estrogen-regulated EBNA2 were analyzed. As seen in Fig. 5, expression of EBNA2 was barely detected in the control DG75 CBF1$^{-/-}$ ER-EBNA2-transfected cells, which were not treated with estrogen. The same cells when treated with the hormone for 48 h expressed EBNA2 and the fusion protein ER-EBNA2. BCL6 expression remained unchanged in the untreated DG75 CBF$^{-/-}$ ER-EBNA2 cells but was downregulated in the hormone-treated, highly EBNA2-expressing DG75 cells, which lacked CBF1 expression (Fig. 5, top, row 2). TCL1 expression in the hormone-treated DG75 CBF$^{-/-}$ ER/EBNA2 cells was marginally downregulated. EBNA2 thus represses expression of these GC-derived genes in a CBF1-independent manner.

HLA-DR, sIgM, and CIITA expression in EBV-infected DLBCLs. We next investigated B-cell surface marker expression in EBV-infected U2932 cells. HLA-DR is generally expressed on B cells derived from the GC stage of differentiation. The numbers of HLA-DR- and sIgM-positive cells were unaltered in U2932 cl.1, which expresses no EBNA2 (Fig. 6 A and D). The GFP fluorescence (FL1) crossover into FL2 readout occasionally resulted in quenching of the peak; however, the percentage of positive cells was not influenced by this phenomenon. The highly EBNA2-expressing U2932 clones, on the other hand, showed a pronounced reduction in HLA-DR and sIgM (Fig. 6B, C, E, and F). Since CIITA regulates HLA class II expression, we asked if the observed HLA-DR downregulation was correlated with expression of CIITA. Indeed, the U2932 EBVGFP cl. 2 and cl. B, with high EBNA2 expression, had relatively lower levels of CIITA transcripts (Fig. 6G, lanes 3 and 4). In comparison, parental U2932 and the U2932 EBVGFP cl. 1, which lacks EBNA2 expression, showed higher CIITA transcription, as judged by RT-PCR. The β-actin expression was analyzed as a control. The flow cytometry analysis was repeated three times.

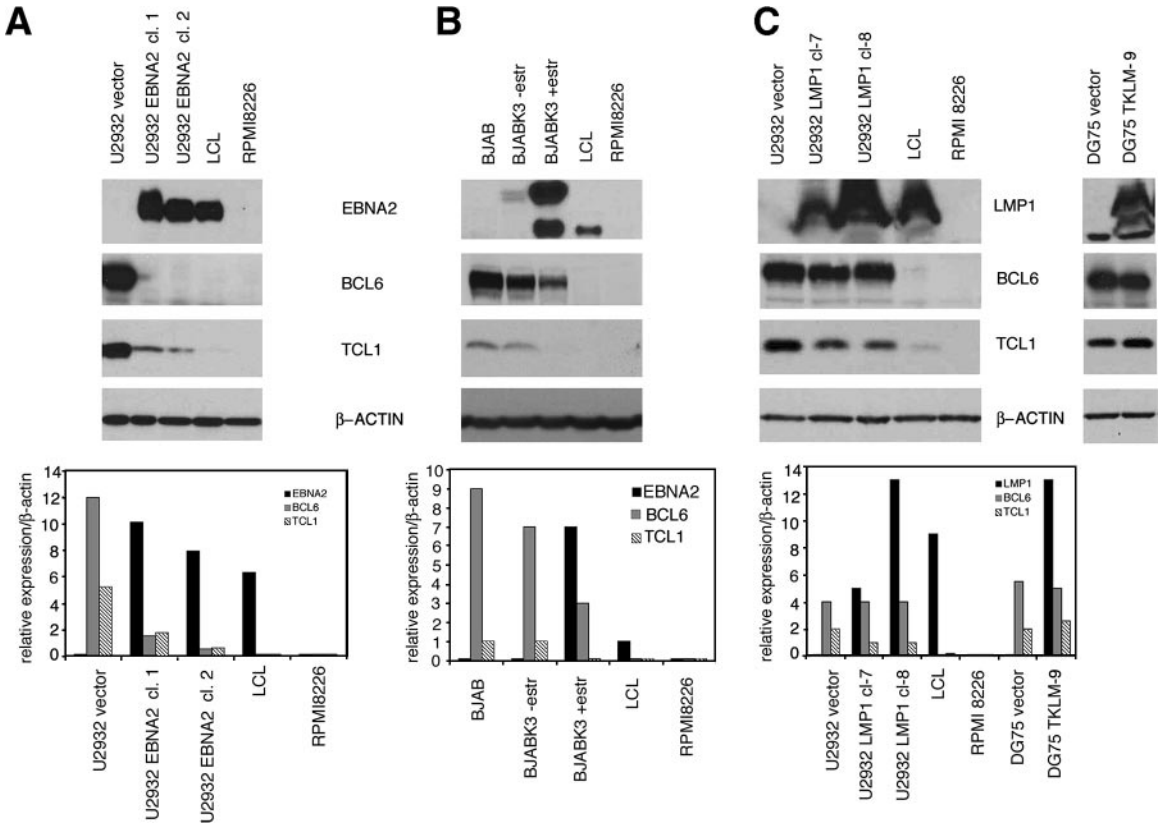

FIG. 3. BCL6 and TCL1 expression in EBNA2 and LMP1 transfectants. (A) U2932 EBNA2 transfectants. (B) BJAB EBNA2 transfectants. The top rows show EBNA2 expression as detected by PE2 monoclonal antibody. BJAB K3 cells were treated with 1 μM estradiol for 2 days for EBNA2 induction. The higher molecular band of approximately 120 kDa represents ER-EBNA2 fusion protein. BCL6 and TCL1 expression in the EBNA2 transfectants of both lines was detected by the respective antibodies. (C) LMP1-transfected U2932 and DG75 cells. The relative expression change normalized against β-actin expression is shown below the corresponding blot.

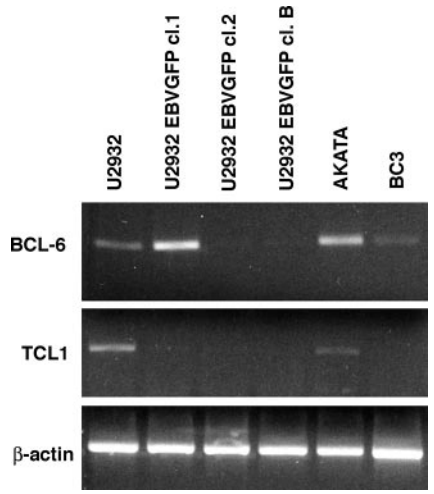

FIG. 4. Transcriptional repression of BCL6 and TCL1 in U2932 DLBCLs after EBV infection. BCL6 transcription is affected in highly EBNA2-expressing cl. 2 and cl. B of U2932 DLBCLs, as seen by RT-PCR. U2932 EBVGFP cl. 1, with undetectable EBNA2, maintained BCL6 transcription. TCL1 transcription is also repressed in high EBNA2 expressers. A weak band corresponding to TCL1 cDNA was barely visible. β-Actin expression was analyzed as a control. One representative experiment out of five is shown.

CCL3 expression in U2932 EBV-infected clones and EBNA2-transfected BJAB cells. CCL3 expression is normally repressed by BCL6 (37). We investigated if the downregulation of BCL6 in U2932 EBV-infected clones altered CCL3 transcription. The experiment shown in Fig. 7A demonstrates that CCL3 expression was increased as a consequence of BCL6 repression in U2932 EBVGFP cl. 2 and cl. B. The role of EBNA2 in the increase of CCL3 transcription was confirmed in estrogen-treated BJAB cells, which expressed only EBNA2. The untreated cells lacked CCL3 expression; however, the upregulation of EBNA2 (Fig. 3B) was accompanied by an increase in CCL3 transcription in the hormone-induced EBNA2-positive BJAB cells (Fig. 7B). These experiments were repeated with at least three different extractions of RNA. The figure shows one experiment out of five RT-PCRs.

DISCUSSION

Here, we infected the most common adult NHL tumor type, the DLBCL, and a BL with a recombinant EBV. EBNA2 expression was associated with downregulation of the GC hallmark genes, particularly BCL6. The experiments with EBNA2-transfected U2932 and the estrogen-inducible EBNA2-carrying BJAB cells directly implicate this virally encoded nuclear protein in the interference with the GC phenotype. Further-

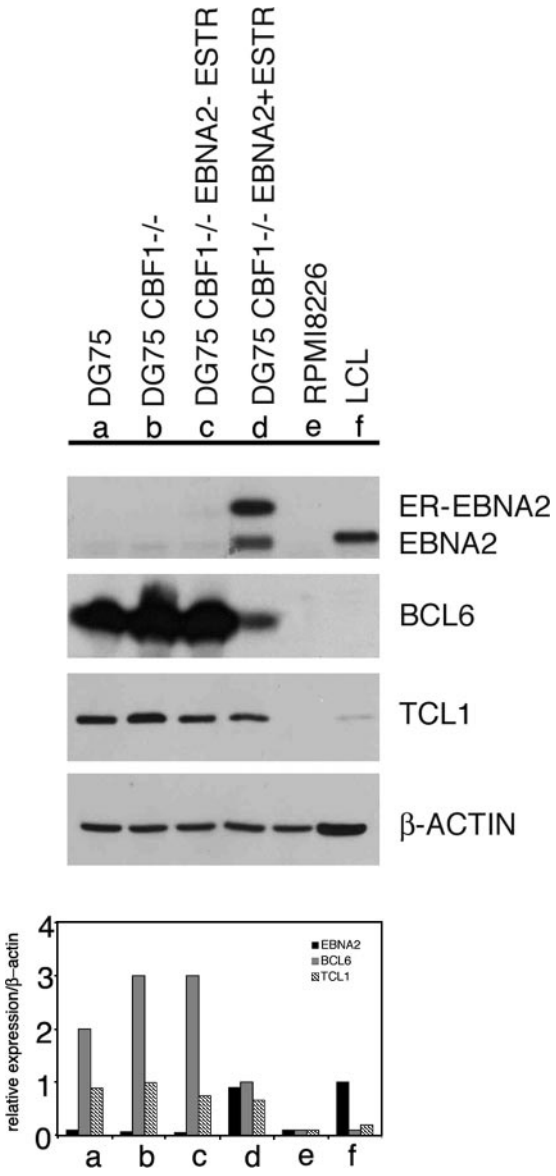

FIG. 5. CBF1-independent repression of BCL6 and TCL1 by EBNA2. (A) DG75 cells carrying a somatically knocked out RBPJK and its EBNA2-transfected derivative were used. EBNA2 and ER-EBNA2 were induced in the transfectants by treating the EBNA2-transfected $CBF^{-/-}$ DG75 cells with estrogen (ESTR) for 2 days. The relative expression changes in BCL6 and TCL1 measured against β-actin expression are shown below.

more, the U2932 EBV-infected clones, which express other latent viral proteins but not EBNA2, do not show downregulation of BCL6. This underscores the key role played by EBNA2 in GC interference.

EBNA2 regulates LMP1 (11), and consequently, this membrane protein may negatively influence BCL6. Previously, it was noted that LMP1 and BCL6 expression levels are inversely correlated in DLBCL biopsy specimens (7, 23). Furthermore, two studies of LMP1 transgenic mice suggest that highly LMP1-expressing animals fail to form GCs (30, 46). This was correlated with the lack of BCL6 expression (30). The indispensability of BCL6 for GC formation has been well documented (10, 52). The effect of LMP1 on BCL6 and TCL1 expression in our study was variable between the two cell lines tested. While TCL1 was more prominently downmodulated, BCL6 expression was not influenced in LMP1 U2932 transfectants. DG75 LMP1 expressers, on the other hand, showed no change in the expression of these two GC-associated genes. In contrast, the negative regulation exercised by EBNA2 on these GC-derived genes was more uniform and was observed across the three cell lines derived from different tumor types.

In a wider context, the EBNA2-mediated downregulation of BCL6 might have interesting implications. Recent series of elegant studies have suggested a model (44) that shows that when resting naive B lymphocytes are infected in vivo, the outcome is proliferating EBNA2-positive B blasts. EBV-infected cells traverse the GC reaction and differentiate into a memory cell. EBNA2 is not expressed during the GC reaction and in EBV-carrying IgD^- memory B cells among healthy carriers (44). If the virus has to reach its destination for long-term persistence, the memory B cells, EBNA2-mediated downregulation of the master regulator of GC, BCL6, might be a biological disadvantage in this process. The lack of EBNA2 expression during this phase ensures that the virus traverses the GC reaction and reaches memory B cells. It was remarkable, therefore, that Kurth et al. (21) found EBNA2 expression in EBV-infected cells from GCs of IM tonsils. Interestingly, when intraclonal V gene diversity, a hallmark of GC reaction, was examined in the virus-negative and -positive cells from a GC, all EBV-negative cells showed ongoing SHM. In contrast, the EBV- and EBNA2-positive cells showed no ongoing SHM. These findings prompted the authors to suggest that EBV-carrying B cells in GC do not participate in the GC reaction in IM tonsils (21). A similar lack of intraclonal V gene diversity has also been reported in posttransplantation lymphoproliferative-disease cases, which expressed EBNA2 (5). Since BCL6 is essential for SHM (10, 52), we suggest that the lack of SHM seen in EBNA2-expressing GC cells may be a direct result of negative regulation of BCL6 by EBNA2.

The surface phenotype of U2932 EBV-infected clones revealed downregulation of HLA-DR and sIgM. It is known that B-cell differentiation toward a plasma cell phenotype is accompanied by a decrease in class II expression. This is believed to be due to lack of CIITA in plasma cells (33). Indeed, the highly EBNA2-expressing clones of U2932 had reduced CIITA transcription. The previously described role of EBNA2 in inducing differentiation of B cells is in line with this observation (14). While this is the first report describing the role of EBNA2 in CIITA transcription regulation, its negative effect on IgM expression has been reported (17).

CCL3, or MIP-α, is a known BCL6 repression target, and it is not expressed in the GC phase (37). The highly EBNA2-expressing clones showed increased CCL3 expression. This chemokine is implicated in inflammatory responses and chemotaxis (29). The increased recruitment of lymphocytes provoked by EBNA2-mediated CCL3 increase might be important for new infection. This suggestion is consistent with the observation that plasma cell differentiation is linked with EBV replication (24). Thus, the repression of BCL6 transcription and consequent CCL3 upregulation by EBNA2 might also have functional relevance for viral spread. Our findings thus suggest

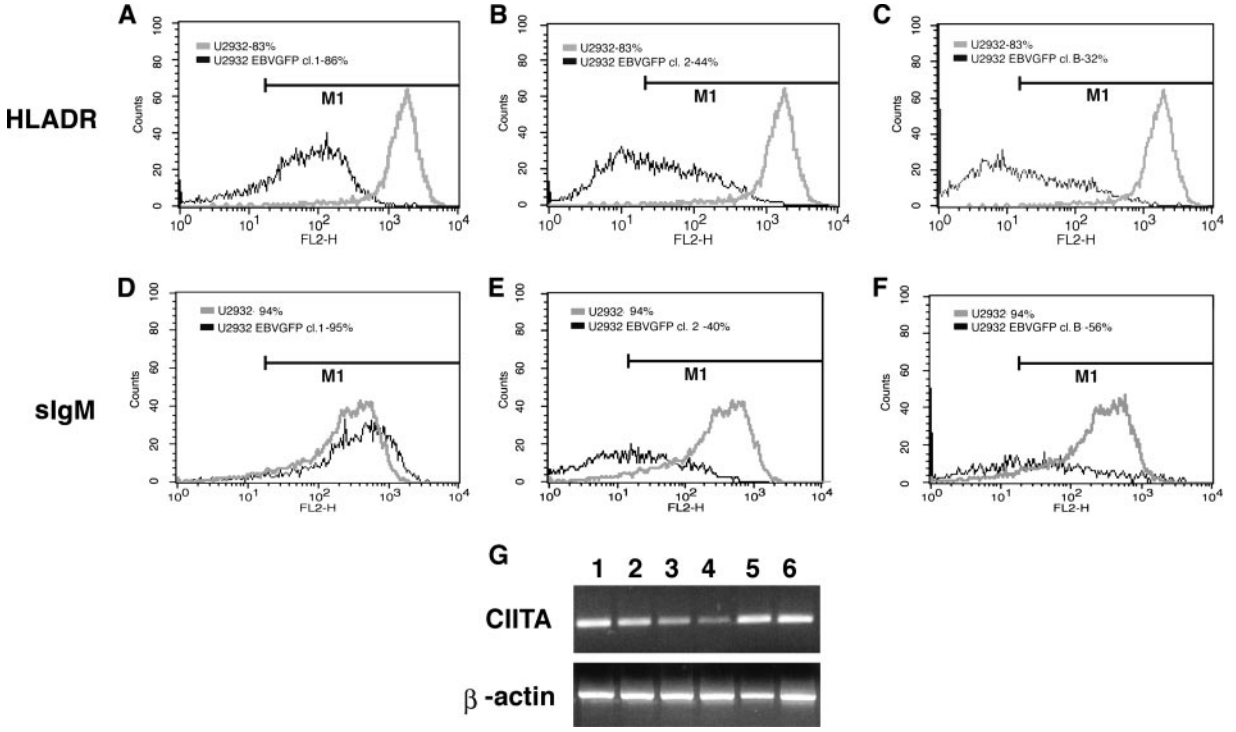

FIG. 6. HLA-DR and sIgM downregulation in EBV-infected U2932 DLBCLs. Exponentially growing cells were analyzed for HLA-DR and sIgM expression by flow cytometry using a monoclonal antibody followed by phycoerythrin-conjugated anti-mouse secondary antibodies. (A and D) EBNA2-negative U2932 EBVGFP cl. 1. (B and E) EBNA2-positive U2932 EBVGFP cl. 2. (C and F) EBNA2-positive U2932 EBVGFP cl. B. (G) RT-PCR for CIITA expression. Lanes: 1, U2932; 2, U2932 EBVGFP cl. 1; 3, U2932 EBVGFP cl. 2; 4, U2932 EBVGFP cl. B; 5, Akata; 6, BC3.

that EBNA2 may play an important role in the replication cycle.

Expression of EBNA2 under normal and pathological conditions is a rarity. Most EBV-associated tumors, except AIDS-associated immunoblastic lymphomas, do not express EBNA2.

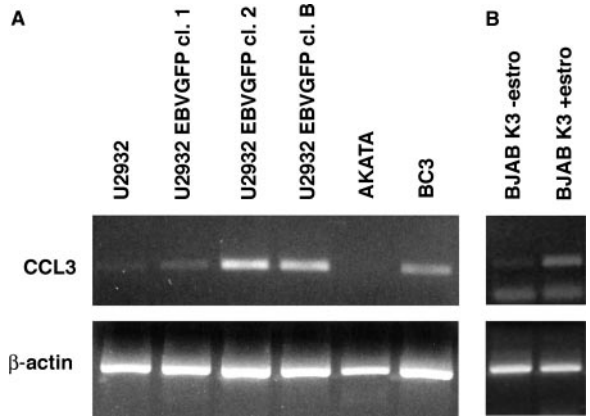

FIG. 7. CCL3 expression as shown by RT-PCR in U2932 EBV-infected clones and EBNA2-transfected BJAB cells. (A) Increased CCL3 expression in U2932 EBVGFP cl. 2 and cl. B, which are high EBNA2 expressers. Cl. 1, with no detectable EBNA2, and the parental U2932 cells are only weakly positive for CCL3 expression. (B) Estrogen-induced EBNA2 expression results in CCL3 upregulation. The lower, nonspecific bands are unincorporated primers. One representative experiment out of five is shown.

Conversely, the EBNA2-positive AIDS immunoblastic lymphomas are BCL6 negative (7). Furthermore, Jijoye, an EBNA2-expressing BL, does not express BCL6, whereas its clonal derivative, EBNA2-deleted P3HR1, is a high BCL6 expresser (54). This mutually exclusive pattern of expression is consistent with the present findings. In most EBV-associated tumors, EBNA2 expression is silenced either through methylation (2) or deletion (18). Based on these observations, it is suggested that in BL pathogenesis, EBNA2- and c-*myc*-driven cellular gene activation programs are not compatible. Furthermore, c-*myc* overexpression renders LCL proliferation independent of EBNA2 and imposes a nonimmunogenic, group I BL-like phenotype on an LCL when it is overexpressed in the absence of EBNA2 (34, 39). There are several similarities between c-*myc* and BCL6 functions in BLs and DLBCLs, respectively. Both are expressed in GC-derived tumors, and their respective deregulated expression by means of translocation seems to be one of the principal events in the development of BLs and DLBCLs. Both c-*myc* and BCL6 silence the CDKN1A gene through the transcription factor Miz-1 (32, 36), and both repress transcription through recruitment of corepressors (6, 31). From the present data, it can be surmised that, akin to the EBNA2/c-*myc* program incompatibility in BLs, EBNA2-driven activation programs and BCL6-driven cellular programs are not compatible in BLs and DLBCLs.

The data in the present paper suggest that the mechanism through which EBNA2 represses BCL6 and TCL1 is inhibition

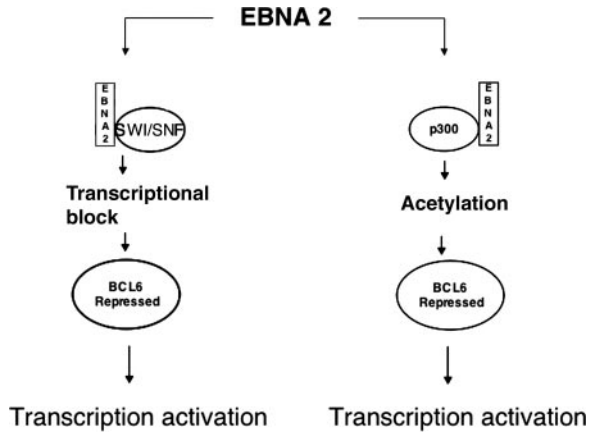

FIG. 8. Repression of the repressor. Suggested mechanisms through which EBNA2 might activate transcription are shown.

of the transcription of these two genes. In other cell systems, translational or posttranslational modifications might be involved as well. Acetylation through the histone acetyltransferase p300 renders BCL6 inactive (4). Interestingly, EBNA2 has been shown to be associated with the histone acetyltransferase domain of p300 (48). It thus might facilitate increased recruitment of p300 to BCL6 and consequently acetylate and inactivate the GC master regulator (Fig. 8). It is known that CBF1 (RBPJK), prior to being activated by EBNA2, functions as a repressor due to its association with histone deactylases. RBPJK converts from a repressor to an activator after EBNA2 displaces histone deactylase binding (14). However, we found that CBF1 does not participate in BCL6 repression. The SWI/SNF nucleosomal remodeling complex is involved in both transcriptional activation and repression. EBNA2 has been shown to bind to one of its components, namely, hSNF5/Ini1 (50). It is conceivable that EBNA2 might facilitate SWI/SNF complex recruitment to BCL6 regulatory regions and bring about chromatin remodeling, eventually resulting in transcriptional repression of this repressor (Fig. 8).

It has been shown that TCL1 expression is also associated with GC (37). A recent study also showed that TCL1 transgenic mice have increased expression of activation-induced deaminase (AID) (38). EBNA2, on the other hand, downregulates AID (43). In view of the data in the present paper, we suggest that EBNA2 decreases AID by turning off TCL1 expression. In a previous study, the loss of EBV genomes was associated with the loss of TCL1 expression in three BLs. Reinfection of the EBV-negative variants of these BLs resulted in reactivation of TCL1 expression (20). These findings are not in disagreement with the data in this paper, principally because the three BL lines in the previous study, when reinfected, expressed a type I viral latent-gene expression program ($LMP1^-$ $EBNA2^-$). The EBV-infected cell lines described here show latency types II/III. Based on these data, we suggest that viral genes expressed during a type I latency program enhance GC-associated gene expression but the type III latency pattern, and in particular EBNA2 expression, has the opposite effect.

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