

## Oncogenic Role of Epstein-Barr Virus-Encoded RNAs in Burkitt's Lymphoma Cell Line Akata

JUN KOMANO, SEIJI MARUO, KOICHI KUROZUMI, TAKANORI ODA, AND KENZO TAKADA\*

Department of Virology, Cancer Institute, Hokkaido University School of Medicine, Kita-ku, Sapporo 060-8638, Japan

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**Our previous reports indicated that Epstein-Barr virus (EBV) contributes to the malignant phenotype and resistance to apoptosis in Burkitt's lymphoma (BL) cell line Akata (N. Shimizu, A. Tanabe-Tochikura, Y. Kuroiwa, and K. Takada, *J. Virol.* 68:6069–6073, 1994; J. Komano, M. Sugiura, and K. Takada, *J. Virol.* 72:9150–9156, 1998). Here we report that the EBV-encoded small RNAs (EBERs) are responsible for these phenotypes. Transfection of the EBER genes into EBV-negative Akata clones restored the capacity for growth in soft agar, tumorigenicity in SCID mice, resistance to apoptotic inducers, and upregulated expression of bcl-2 oncoprotein that were originally retained in parental EBV-positive Akata cells and lost in EBV-negative subclones. This is the first report which provides evidence that virus-encoded RNAs (EBERs) have oncogenic functions in BL cells.**

Epstein-Barr virus (EBV) was demonstrated to be present in more than 90% of endemic Burkitt's lymphoma (BL) cases in equatorial Africa and New Guinea and in about 10% of sporadic BL cases in the western hemisphere (reviewed in reference 23). The important feature of the BL cell is the juxtaposition of the *c-myc* locus to the immunoglobulin H enhancer through a reciprocal translocation [t(8;14)] and, more rarely, to the Igk locus [t(2;8)] or the Igl locus [t(8;22)]. This feature led to the hypothesis that this translocation resulted in constitutive activation of the *c-myc* gene, which seemed to be a key step in the initiation of BL (12). However, the role of EBV in the genesis of BL is still an open question. To gain insight into this problem, we have investigated the pathogenic role of EBV in BL cell line Akata. The Akata cell line is an EBV-positive BL cell line derived from a Japanese patient and possessing chromosomal translocation t(8;14) (29). It retains type I latency even in long-term cultures, in which EBV nuclear antigen (EBNA) 1, EBV-encoded RNAs (EBERs, specifically EBER-1 and EBER-2), latent membrane protein (LMP) 2A, and BARF0 are expressed (13). In most BL cell lines, latency is converted from type I to type III in serial cultures, in which cells express all the EBNA (1, 2, 3a, 3b, 3c, and LP), LMPs 1, 2A, and 2B, EBERs, and BARF0 (reviewed in reference 1). EBV-immortalized B-cell lines (lymphoblastoid cell lines) also have type III latency (24).

Hitherto, it has been impossible to demonstrate that the presence of EBV with type I latency has any biological effects in BL cells. Akata cells enabled us to resolve this problem. EBV-negative cell clones can be isolated from parental Akata cells, since EBV DNA has been spontaneously lost during cultivation (27). Type I latency was restored by EBV reinfection in EBV-negative Akata cells. We successfully established a system to confirm whether any phenotypic differences in cells were due to EBV. This system compared two pairs of cell clones: (i) EBV-positive and -negative cell clones obtained from an EBV-positive Akata cell clone and (ii) neomycin resistance gene (*neoR*)-transfected and EBV-reinfected cell

clones obtained from an EBV-negative Akata cell clone by use of recombinant EBV carrying *neoR*. Using this system, we verified that, in Akata cells, EBV was necessary for the malignant phenotype, resistance to apoptosis, and upregulated expression of bcl-2 oncoprotein (13, 27). These were the first reports that provided direct evidence that the presence of EBV had an oncogenic role and that type I latency conferred resistance to apoptosis in vitro. We also demonstrated that EBNA-1 was not responsible for these phenotypes in Akata cells (13). Similar results were also reported by Ruf et al. (25).

In this report, we focused upon the possible involvement of EBERs in the malignant phenotype and resistance to apoptosis in Akata cells. EBER-1 and EBER-2 are small nuclear RNAs transcribed by the RNA polymerase III system; they are, respectively, 166 and 172 nucleotides long (7). Being abundant, EBER-1 has often been targeted as a probe for in situ hybridization studies to identify the presence of EBV (5). With an EBER deletion recombinant virus, it was demonstrated that EBERs were not necessary for the B-cell-immortalizing function of EBV (28). To date, the biological functions of EBERs remain unclear.

Here, we present evidence that EBERs play an oncogenic role in Akata cells. This is the first report which provides evidence that virus-encoded RNAs (EBERs) have oncogenic functions in BL cells.

### MATERIALS AND METHODS

**Cell culture.** The Akata cell line of BL origin was maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco), 40 U of penicillin per ml, and 50 µg of streptomycin per ml at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

**Plasmid construction.** *neoR* driven by the simian virus 40 promoter was derived from pcDNA3 (Invitrogen). EBER-1 and EBER-2 open reading frames (ORFs) are located at 6,628 to 6,796 bp and at 6,958 to 7,129 bp, respectively, on the *EcoRI* K fragment of Akata EBV DNA, which corresponds to the *EcoRI* J fragment of B95-8 EBV DNA. pEK contained the *EcoRI* K fragment of Akata EBV DNA and *neoR*. pEKS10 contained 10 tandem repeats of the *SacI-EcoRI* subfragment (6,297 to 7,325 bp) from the *EcoRI* K fragment of Akata EBV DNA and *neoR*. At each end of the *SacI-EcoRI* subfragment from the *EcoRI* K fragment, *BglII* or *BamHI* sites were added and concatenated in a direction-restricted fashion.

**Transfection and cloning.** Plasmids were introduced into Akata cells by the electroporation method as described previously (13). For cloning cells, 10<sup>4</sup> cells were transferred to each well of a flat-bottom 96-well plate (Falcon) with 200 µl

\* Corresponding author. Mailing address: Department of Virology, Cancer Institute, Hokkaido University School of Medicine, N15 W7, Kita-ku, Sapporo 060-8638, Japan. Phone: 81-11-706-5071. Fax: 81-11-717-1128. E-mail: kentaka@med.hokudai.ac.jp.

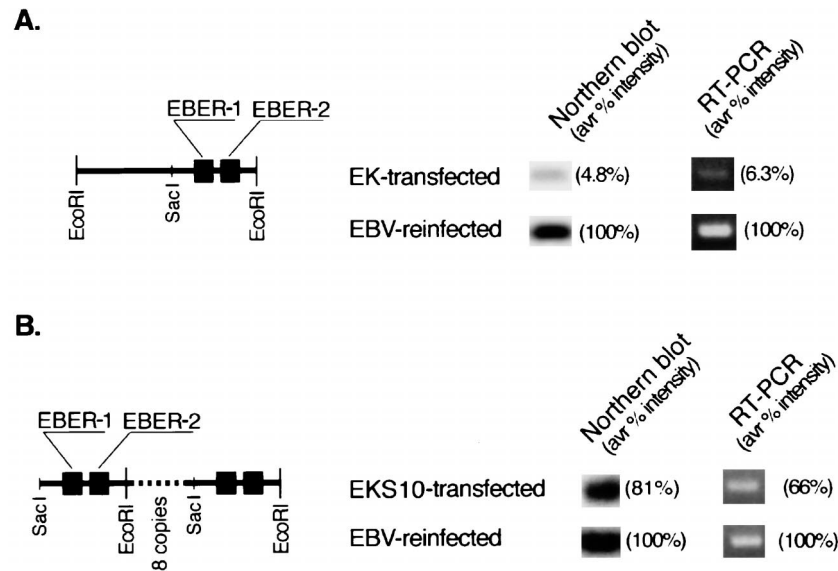


FIG. 1. Relative EBER expression in EK- and EKS10-transfected Akata cells compared with EBV-reinfected Akata cells. For each EBER transfectant derived from different EBV-negative clones, more than 50 cell clones were examined for EBER expression, and 4 to 6 clones with the highest levels of EBER expression were chosen for further studies. Representative results are shown. (A) Schematic drawing of the *EcoRI* K fragment of Akata EBV DNA (left) and relative EBER expression in EK-transfected cells (right). (B) Schematic drawing of EKS10 (left) and relative EBER expression in EKS10-transfected cells (right). avr, average.

of medium containing 700  $\mu\text{g}$  of G418 (Gibco) per ml 2 days after transfection. Half of the medium was changed every 5 days until colonies emerged.

**EBV infection.** Preparation of the virus solution was described previously (10). Cells ( $5 \times 10^6$ ) were suspended in 2 ml of 1:2-diluted EBV solution (Akata strain with the *neoR* gene) for 1 h at room temperature with continuous mild mixing. Then, they were washed three times and cultured for 2 days. Selection and cloning procedures are described above.

**Northern blot analysis.** Total RNA was extracted from Akata cells with Trizol reagent (Gibco) according to the manufacturer's instructions. Samples (1 to 2  $\mu\text{g}$  per lane) were electrophoresed in 2% agarose-formaldehyde gels, transferred to Hybond N+ nylon membranes (Amersham), fixed with alkali, prehybridized, hybridized with a probe, and washed under stringent conditions (15 mM NaCl, 1.5 mM sodium citrate, 0.1% sodium dodecyl sulfate). The probe for EBERs was an *EcoRI* K fragment, so that both EBER-1 and EBER-2 transcripts were detected. Fifty nanograms of DNA was radiolabeled with a Ready-Prime labeling kit (Amersham).

**RT-PCR.** Reverse transcriptase PCR (RT-PCR) was performed as described previously (13). Briefly, total RNA was extracted from Akata cells with Trizol reagent (Gibco). RNA was solubilized in distilled water and treated with DNase I (Gibco). The primers used for EBER-1 were AGG ACC TAC GCT GCC CTA GA (5' sequence) and AAA ACA TGC GGA CCA CCA GC (3' sequence); the product was 166 bp (30). After reverse transcription, 100 ng of the cDNA was subjected to PCR in the presence of 5 U of *Taq* polymerase (Gibco) in a total volume of 50  $\mu\text{l}$  with a model 2400 Thermal Cycler (Perkin-Elmer) for 12 cycles. The amplified products were electrophoresed in 2% agarose gels and stained with ethidium bromide.

**Soft-agar colony assay.** Wells of a six-well plate (Falcon) were covered with 0.5% SeaPlaque agarose (FMC, Rockland, Maine) containing RPMI 1640 medium and 12% FBS. Onto this base,  $10^4$  cells in 0.4% SeaPlaque agarose containing RPMI 1640 medium and 12% FBS were embedded. After 2 to 3 weeks of incubation, colonies that contained more than 100 cells were counted.

**EBER-1 in situ hybridization.** EBER-1 was detected with a digoxigenin-labeled oligonucleotide probe complementary to the EBER-1 sequence (5'-AGA CAC CGT CCT CAC CAC CCG GGA CTT GTA-3') (5). A sense probe against EBER-1 was used for negative control studies.

**Apoptosis assay.** Cells in the log phase were incubated for 16 h in the presence of 20  $\mu\text{g}$  of cycloheximide (CHX) (Wako, Osaka, Japan) per ml, for 40 h with 1 mM glucocorticoid (Pharmacia or Upjohn), or for 65 h in a 100%  $\text{CO}_2$ -saturated humidified atmosphere (hypoxic stress). The viability of cells was quantified by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Cell Titer 96; Promega).

**Western blot analysis.** Western blot analysis was performed as described previously (27). For immunostaining, blots were incubated with mouse anti-human bcl-2 monoclonal antibody bcl-2/100 (Pharmingen).

## RESULTS

**EBER expression in EK- and EKS10-transfected Akata cells.** We constructed a plasmid, pEK, that carried a single copy of EBER and *neoR*. We isolated G418-resistant clones by transfecting pEK into an EBV-negative Akata cell clone. Compared with the level of EBERs in EBV-reinfected cell clones, the average signal intensities for EBERs in four EK-transfected cell clones were 4.8% by Northern blot analysis and 6.3% by RT-PCR, even though clones expressing the highest levels were analyzed (Fig. 1A). To obtain higher levels of EBER expression in transfected cells, we constructed a plasmid, pEKS10, that contained 10 tandem repeats of the *SacI-EcoRI* subfragment from the *EcoRI* K fragment of Akata EBV DNA. We isolated G418-resistant clones by transfecting pEKS10 into an EBV-negative Akata cell clone. Compared with the level in EBV-reinfected cell clones, the average levels of EBER expression in four EKS10-transfected cell clones were 81% by Northern blot analysis and 66% by RT-PCR (Fig. 1B). It was difficult to obtain levels of EBER expression in transfected cells equivalent to those in EBV-reinfected cells. The amount of the EBER-1 transcript was approximately two-fold higher than that of the EBER-2 transcript in EBV-positive, EBV-reinfected, and EBER-transfected Akata cell clones by a semiquantitative RT-PCR assay.

**Growth characteristics of EK- and EKS10-transfected Akata cells.** The growth characteristics of EK- and EKS10-transfected Akata cell clones were compared with those of EBV-reinfected cell clones and *neoR*-transfected cell clones. First, we performed a soft-agar colony assay using cell clones derived from three independent EBV-negative clones (Fig. 2). EBV-reinfected cell clones formed colonies, but *neoR*-transfected cell clones scarcely did. The absolute number of colonies differed among experiments, presumably due to clonal variation. EK-transfected cell clones formed a few colonies, whereas EKS10-transfected cell clones formed significantly

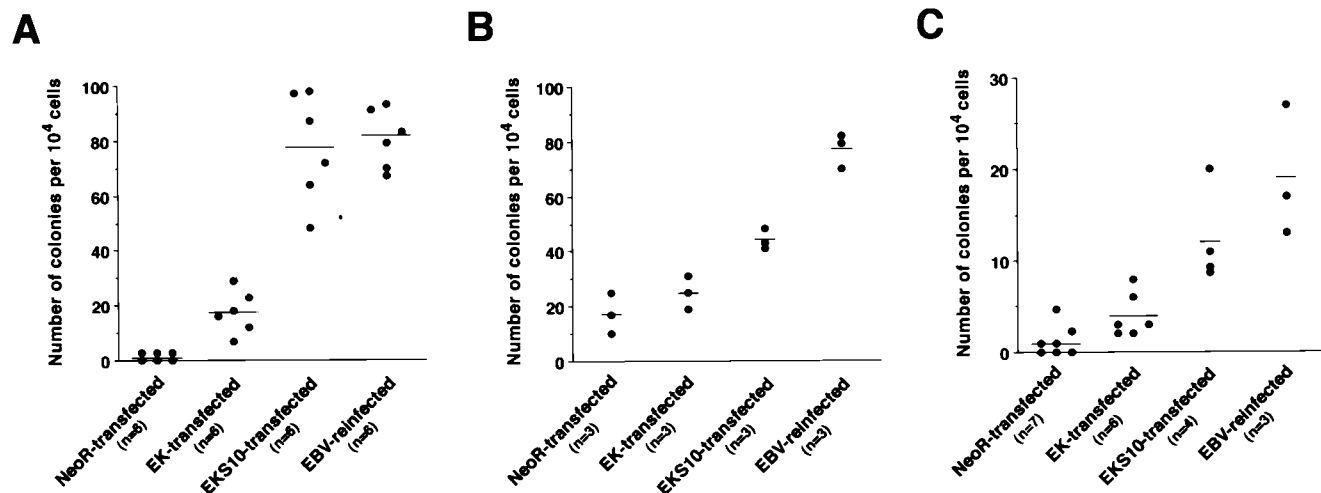


FIG. 2. Colony production in soft agar for *neoR*-transfected, EK-transfected, EKS10-transfected, and EBV-reinfected cells. The individual panels show independent experiments with different EBV-negative Akata cell clones. Each dot represents the number of visible colonies emerging per 10<sup>4</sup> cells. Horizontal bars represent the mean values for each group. The differences between *neoR*-transfected and EKS10-transfected cell clones were significant (*P*, <0.01), as determined by the Mann-Whitney U test.

more colonies than *neoR*-transfected cell clones but formed fewer colonies than EBV-reinfected cell clones (Fig. 2). Compared with data for EBV-reinfected cell clones, the restoration rates for colony production in soft agar in three independent experiments were 21, 13, and 17% (average, 17%) for EK-transfected cell clones and 95, 57, and 63% (average, 72%) for EKS10-transfected cell clones. As the level of EBER expression went up, more cells formed colonies in soft agar.

Second, we examined whether EKS10-transfected cells were tumorigenic in SCID mice. In three separate experiments, tumors developed from EKS10-transfected cells and from EBV-reinfected cells but not from *neoR*-transfected cells (Table 1). The tumors derived from EKS10-transfected Akata cells showed a typical lymphoma pattern with intensive blood vessel infiltration (Fig. 3). In an in situ hybridization study, almost all the tumor cells were positive for EBER-1 expression.

**Resistance to apoptosis of EK- and EKS10-transfected Akata cells.** To test whether the expression of EBERS restored resistance to apoptosis, we performed an apoptosis assay using a set of cell clones. Apoptosis was induced by exposing cells to inducers such as CHX, glucocorticoid, and hypoxic stress, and

surviving cells were measured by an MTT assay (Fig. 4). The survival rate (%SR) was the highest in EBV-reinfected cells and the lowest in *neoR*-transfected cells. The difference in %SR between *neoR*-transfected cells and EK-transfected cells was not significant. In contrast, the %SR of EKS10-transfected cells was significantly higher than that of *neoR*-transfected cells for all stimuli. Compared with data for EBV-reinfected cells, the restoration rates for EK-transfected cells were 16, 18, and 2.2% (average, 12%) against CHX, glucocorticoid, and hypoxic stress, respectively, and those for EKS10-transfected cell were 50, 48, and 70% (average, 56%), respectively. These results illustrated that the more that EBERS were expressed in cells, the greater the resistance to apoptosis of the cells.

**EBERs upregulate the expression of bcl-2 oncoprotein.** We next examined whether the upregulation of bcl-2 oncoprotein had been restored in EKS10-transfected cells. The level of bcl-2 protein expression in EBV-reinfected cells was found to be higher than that in *neoR*-transfected cells (Fig. 5). bcl-2 expression in EKS10-transfected cells was higher than that in *neoR*-transfected cells. We could not find significant upregulation of bcl-2 protein in EK-transfected cells. Finally, the average restoration rate for bcl-2 expression in EKS10-transfected

TABLE 1. Tumorigenicity of EKS10-transfected Akata cells in SCID mice

| Cells                           | No. of mice with tumors in expt <sup>c</sup> : |                |                                |
|---------------------------------|--|----------------|--------------------------------|
|                                 | 1 <sup>a</sup>                                 | 2 <sup>a</sup> | 3 <sup>b</sup>                 |
| EBV-positive clones             | 3/4  | Not done       | 3/3                            |
| EBV-negative clones             | 0/4  | Not done       | 0/3                            |
| EBV-reinfected clones           | 2/4  | 2/4            | 8/9 (2/3, 3/3, 3/3)            |
| EKS10-transfected clones        | 1/5  | 1/5            | 7/15 (1/3, 2/3, 2/3, 1/3, 1/3) |
| <i>neoR</i> -transfected clones | 0/4  | 0/4            | 0/15 (0/3, 0/3, 0/3, 0/3, 0/3) |

<sup>a</sup> Six cell clones transfected with *neoR* or EKS10 or six EBV-reinfected clones derived from an EBV-negative Akata cell clone were pooled, and 10<sup>7</sup> cells were inoculated into the thigh subcutis of 6-week-old male SCID mice (FOX CHASE C.B-17/1cr-scld Jel; Clea, Tokyo, Japan).

<sup>b</sup> Five cell clones transfected with *neoR* or EKS10 or three EBV-reinfected clones derived from an EBV-negative Akata cell clone (1.5 × 10<sup>7</sup> cells each) were individually inoculated into 4-week-old male SCID mice.

<sup>c</sup> Mice were sacrificed 8 weeks after inoculation, and the developed tumors were measured. The tumors ranged in size from 0.8 to 4.5 cm.

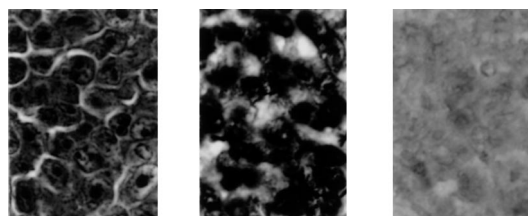


FIG. 3. Histological examination of tumors derived from EKS10-transfected Akata cells in SCID mice. Adjacent sections from paraffin-embedded blocks were stained by hematoxylin-eosin or subjected to an in situ hybridization study. Typical lymphoma histology was observed (left panel; magnification, ×400). The EBER-1 in situ hybridization study revealed that almost all the tumor cells were positive for EBER-1 expression (middle panel; magnification, ×400). Dark signals representing the presence of EBER-1 transcripts were specifically present in the nuclei of tumor cells. A negative control for the EBER-1 in situ hybridization study is also shown (right panel; magnification, ×400).



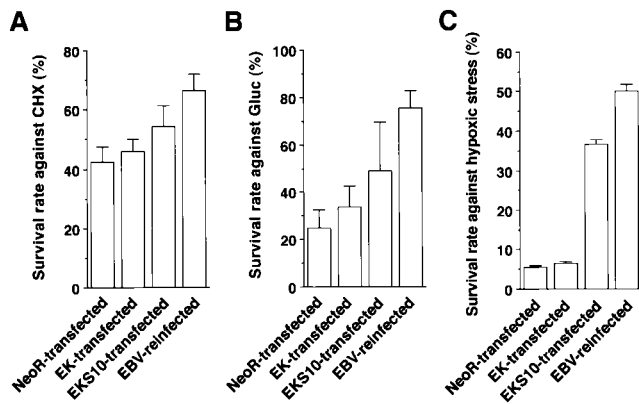


FIG. 4. Resistance to apoptosis of *neoR*-transfected, EK-transfected, EKS10-transfected, and EBV-reinfected cells tested with CHX (A), glucocorticoid (Gluc) (B), and hypoxic stress (C). The bars show the means  $\pm$  standard deviations for six clones. The data are typical results from three independent experiments. As determined by a *t* test analysis, the differences between mean values for *neoR*- and EKS10-transfected cells were significant at *P* values of  $<0.01$  (CHX),  $<0.05$  (Gluc), and  $<0.001$  (hypoxic stress).

cells was estimated to be 65% by densitometric analysis in several experiments.

## DISCUSSION

In the transfection study, we demonstrated that EBER-expressing Akata cell clones restored the malignant phenotype, resistance to apoptosis, and upregulated expression of *bcl-2* protein to levels comparable to the restoration rates for EBER expression in EBV-reinfected cell clones. Thus, it was clear that EBERs were responsible for the malignant phenotype and resistance to apoptosis in Akata cells. Many RNAs are known to have catalytic functions; however, there has been no report describing an oncogenic RNA. This is the first paper that provides evidence that RNA polymerase III-transcribed virus-encoded small RNAs affect the malignant phenotype and resistance to apoptosis.

There was a good correlation between the rate of restoration of EBER expression and the biological activity of EBV-reinfected cells. This result clearly illustrates that EBERs contribute to the malignant phenotype and resistance to apoptosis. All the restoration rates for biological phenotypes estimated from individual experiments were somewhat lower than the levels of expression of EBERs in EKS10-transfected cells. The reason for this result is probably that there was a gradual loss of EBER expression in EKS10-transfected cells during cultivation. RT-PCR revealed that EBER-1 expression in EKS10-transfected cells at 14 weeks after transfection was 60% that at 6 weeks (data not shown). The reason for this result is largely unknown; however, it is often seen in stable transformants.

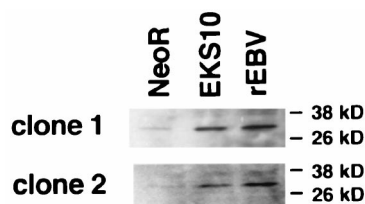


FIG. 5. Expression of *bcl-2* protein in *neoR*-transfected, EKS10-transfected, and EBV-reinfected (rEBV) cells derived from two independent EBV-negative Akata cell clones. The data are typical results from several experiments.

More interestingly, how could EBERs (RNAs) contribute to malignant phenotypes and apoptosis resistance? EBERs were reported to bind to some cellular proteins, La (16), EAP/L22 (31, 32), and PKR (6). Above all, the association of EBERs with PKR is noteworthy. PKR, previously described as an interferon-induced protein kinase, is induced by interferons and activated by double-stranded RNAs to phosphorylate eIF-2 $\alpha$ , a translation initiation factor (18). This phosphorylation results in the blockage of protein synthesis, through which interferons are thought to exert their antiviral effects. EBERs were shown to bind to PKR and block the activation of PKR as well as adenovirus VAI and VAII RNAs, which might result in the enhancement of protein synthesis of general mRNAs (26). The dominant negative form of PKR has been shown to predispose cells to malignant transformation or even initiate it (14, 19). In addition, GCN4, a yeast homologue of PKR, is known to regulate specific gene expression via translational control (1). Since the levels of expression of  $\beta$ -actin, *c-myc*, *bax*, and PKR in EBV-positive Akata cells were equal to those in EBV-negative Akata cells (data not shown), whereas the expression of *bcl-2* protein was specifically upregulated in EBV-positive Akata cells, it is possible that the inactivation of PKR by EBERs enhances the translation of *bcl-2* mRNA more efficiently than that of other mRNAs to confer the malignant phenotype and resistance to apoptosis.

However, it remains unclear whether the effects of EBERs are mediated via upregulation of *bcl-2* protein. *bcl-2* acts in synergy with the *c-myc* oncogene in tumor progression, this idea was suggested by clinical investigations indicating that the activation of both *c-myc* and *bcl-2* may have conferred an aggressive clinical outcome in lymphoma cases (4, 11, 21). This idea was clearly demonstrated in a transgenic mouse study in which *bcl-2 c-myc* transgenic mice exhibited accelerated lymphomagenesis (9, 17). In mammalian cells, deregulated expression of *c-myc* has been shown to contribute not only to tumorigenesis (15) but also to the induction of apoptosis in various cell lines, including BL cell lines (2, 8, 20). The mechanism of synergy between *bcl-2* and *c-myc* seems to be that *bcl-2* protects cells from *c-myc*-induced apoptosis (3, 22, 33). Like Akata cells (29), all BL cells possess a chromosomal translocation involving the *c-myc* locus and resulting in constitutive activation of the *c-myc* gene (12). Therefore, BL cells were thought to be predisposed to *c-myc*-induced apoptosis. Our data suggest that EBV infection would upregulate the expression of *bcl-2* protein to protect cells from *c-myc*-induced apoptosis and to allow *c-myc* to exert its oncogenic functions. In this way, *bcl-2* might cooperate with *c-myc* in the development of BL.

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