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Epstein-Barr Virus (EBV)-Encoded RNA 2 (EBER2) but Not EBER1 Plays a Critical Role in EBV-Induced B-Cell Growth Transformation▽

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Epstein-Barr virus (EBV)-encoded RNA 1 (EBER1) and EBER2 are untranslated RNAs and the most abundant viral transcripts in latently EBV-infected cells. We previously reported that EBERs play a critical role in efficient EBV-induced growth transformation of primary B cells. To investigate whether EBER1 and EBER2 have distinct roles in B-cell growth transformation, recombinant EBVs carrying either EBER1 or EBER2 were generated. The transforming ability of recombinant EBVs expressing EBER2 was as high as that of EBVs expressing both EBER1 and EBER2. In contrast, the transforming ability of recombinant EBVs carrying EBER1 was impaired and was similar to that of EBV lacking both EBER1 and EBER2. Lymphoblastoid cell lines (LCLs) established with EBVs carrying EBER2 proliferated at low cell densities, while LCLs established with EBVs carrying EBER1 did not. Interleukin 6 (IL-6) production in LCLs expressing EBER2 was more abundant than in those lacking EBER2. The growth of LCLs lacking EBER2 was enhanced by the addition of recombinant IL-6 to the cell culture, while the growth of EBER2-expressing LCLs was inhibited by a neutralizing anti-IL-6 antibody. These results demonstrate that EBER2, but not EBER1, contributes to efficient B-cell growth transformation. We conclude that EBER1 and EBER2, despite their structural similarity, have different functions in latently infected lymphoblastoid cells.

Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus that establishes life-long latent infections in B lymphocytes following the primary infection (16, 23). EBV is associated with various malignancies, such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, gastric carcinoma, and lymphoma in immunosuppressed patients (23). EBV readily infects human resting B cells in vitro and converts them into indefinitely proliferating lymphoblastoid cell lines (LCLs). In LCLs, six EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP), three latent membrane proteins (LMP1, LMP2A, and LMP2B), BamHI A rightward transcripts, and EBV-encoded small RNAs 1 and 2 (EBER1 and EBER2, respectively) are expressed (16, 23). Among these transcripts, EBNA1, EBNA2, EBNA3A, EBNA3C, EBNA-LP, and LMP1 have been reported to be essential for growth transformation, whereas EBNA3B, LMP2A, LMP2B, BamHI A rightward transcripts, and EBERs are not essential (16).

EBER1 and EBER2 are the most abundant viral transcripts in cells that are latently infected with EBV. EBER1 and EBER2 are nonpolyadenylated, untranslated RNAs of 167 and 173 nucleotides, respectively (4, 21, 24), and are structurally highly conserved (2). They are known to form complexes with several cellular proteins, such as the RNA-activated protein kinase R (PKR) (3, 20), ribosomal protein L22 (8, 32, 33), La (18), and the retinoic acid-inducible gene I (RIG-I) (25). Thus, EBERs likely exert their biological effects through direct interactions with cellular proteins. We have previously shown that in Burkitt's lymphoma cells, EBERs confer resistance to alpha interferon (IFN-α)-induced apoptosis by binding PKR and inhibiting its phosphorylation (20, 22). We have also shown that EBERs induce the expression of various cellular cytokines, such as interleukin-10 (IL-10) in B lymphocytes (17), IL-9 in T-lymphocytes (37), and insulin-like growth factor 1 in epithelial cells (13, 14), each of which acts as an autocrine growth factor. More recently, we have shown that EBERs are recognized by RIG-I and activate signaling to induce type I IFN (25).

Although EBERs are not essential for the growth transformation (30), we previously reported that recombinant EBV lacking the EBER genes exhibited impaired transforming ability compared to the wild-type EBV in a quantitative transformation assay (36). The transforming titer of EBER-deleted EBV was restored by reconstituting both of the EBER genes (EBER1 and EBER2) in the context of the viral genome, indicating that the loss of EBERs is truly responsible for the impaired transforming ability of EBER-deleted EBV (36). Moreover, LCLs established with EBER-deleted EBV grew out less efficiently than those established with EBER-positive EBV when the cells were plated at low cell densities (36).

Whether EBER1 and EBER2 have distinct functions is still an open question. In a transient transfection assay, it was reported that both EBER1 and EBER2 are able to inhibit PKR phosphorylation and stimulate RIG-I to induce type I IFN (20, 25). However, the roles of EBER1 and EBER2 have never been evaluated separately in latently infected cells in which EBER is expressed at a physiological level.

In this study, we generated recombinant EBVs expressing either EBER1 alone or EBER2 alone and evaluated the individual roles of EBER1 and EBER2 in B-cell growth transformation. Our data demonstrate that EBER2 plays a critical role

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in efficient B-cell growth transformation, whereas EBER1 is dispensable.

MATERIALS AND METHODS

Cells. The EBV-negative Akata cell line (28), EBV-negative Daudi cell line (20), and LCLs were grown in complete RPMI 1640 medium, which was supplemented with 10% fetal calf serum (FCS), penicillin (40 U/ml), and streptomycin (50 μg/ml). The Akata cell lines harboring Neo^{r}EBV (29), EBER-knockout EBV, and EBER-reconstituted EBV (36) were maintained in complete medium containing 700 μg of G418 (Sigma-Aldrich Fine Chemicals, St. Louis, MO)/ml.

Plasmids. The EBER1 and EBER2 genes are located within the BamHI C fragment of Akata EBV DNA. pUC-A/C-PstI/EcoK contains part of the BamHI C fragment of the Akata EBV genome (corresponding to the EcoRI-PstI fragment and spanning nucleotides 4163 through 9699 of EBV strain B95-8). pUC-A/C-PstI/EcoKΔEBER2 was constructed by ligating two PCR products of 679 bp (corresponding to nucleotides 6281 through 6955 of EBV strain B95-8) and 187 bp (corresponding to nucleotides 7129 through 7315 of EBV strain B95-8) into SacI-EcoRI-digested pUC-A/C-PstI/EcoK. pUC-A/C-PstI/EcoKΔEBER1 was constructed by ligating two PCR products of 352 bp (corresponding to nucleotides 6281 through 6628 of EBV strain B95-8) and 521 bp (corresponding to nucleotides 6796 through 7315 of EBV strain B95-8) into SacI-EcoRI-digested pUC-A/C-PstI/EcoK. A NotI site was introduced downstream of the SacI site of pUC-A/C-PstI/EcoKΔEBER2 and pUC-A/C-PstI/EcoKΔEBER1. To generate pBS246/hyg, a hygromycin resistance gene driven by the simian virus 40 early enhancer and promoter was cloned into the BamHI site of pBS246 (Life Technologies), such that the hygromycin resistance gene was flanked by two *loxP* sites. The NotI fragment of pBS246/hyg was inserted into the NotI sites of pUC-A/C-PstI/EcoKΔEBER2 and pUC-A/C-PstI/EcoKΔEBER1 to make pEBER1-KI and pEBER2-KI, respectively. pEBER1-KI or pEBER2-KI was used as a targeting construct to generate EBER1-knockin EBV or EBER2-knockin EBV, respectively. Other known EBV open reading frames were not affected by this insertion. pSG-Cre was constructed by cloning the blunted XhoI-MluI fragment of pBS185 (Life Technologies), containing the *cre* recombinase gene, into the blunted BglII site of pSG5 (Stratagene).

Generation of recombinant EBVs. Twenty micrograms of targeting construct was digested with BstPI and EcoRV and introduced into EBV-negative Akata cells by an electroporation method, as described previously (40). Transfected cells were cultured for 2 days and then plated in 96-well plates at 10^4 cells per well in medium containing 150 μg of hygromycin B (Calbiochem)/ml for selection. Half of the culture medium was replaced with fresh medium containing hygromycin B every 5 days. To obtain cell clones harboring EBER1-knockin EBV, a total of 322 hygromycin-resistant cell clones from 1,152 wells were screened by Southern blotting for the existence of EBV episomes that had undergone homologous recombination. To obtain cell clones harboring EBER2-knockin EBV, a total of 231 hygromycin-resistant cell clones from 768 wells were screened. Screening for EBER1-knockin and EBER2-knockin EBVs resulted in the isolation of two clones and one clone, respectively, which harbored homologously recombined EBV episomes.

Virus infection and drug selection. Virus production was induced by cross-linking surface immunoglobulin G (IgG), as described previously (29). Briefly, Akata cells harboring EBV episomes (2×10^6 cells) were resuspended in 1 ml of fresh medium containing 0.5% rabbit anti-human IgG (DakoCytomation, Carpinteria, CA) and incubated for 6 h to induce lytic replication, and then the culture medium was replaced with fresh medium. Two days later, the culture supernatant was harvested and filtered through a 0.45-μm-pore-size membrane, and the filtrate was used as the virus solution. For infection, EBV-negative Akata cells (10^6) were resuspended in 1 ml of virus solution and incubated at 37°C for 90 min. The infected cells were washed and resuspended in fresh medium and then cultured for 2 days. The cells were then plated in 96-well plates at 10^4 cells per well in medium containing 150 μg of hygromycin/ml or 700 μg of G418/ml for selection. Half of the culture medium was replaced every 5 days. Drug-resistant clones were screened by Southern blotting to identify cell clones harboring recombinant EBVs.

Excision of the hygromycin resistance gene by *cre* expression. To excise the hygromycin resistance gene from the EBV genome, Akata cells, carrying EBV episomes containing the hygromycin resistance gene flanked by two *loxP* sites, were transfected with 20 μg of pSG-Cre via electroporation. Two days after transfection, virus production was induced in the cells, and the culture supernatants were harvested 2 days later. EBV-negative Akata cells were then infected with culture supernatants, and infected cells were selected in medium containing G418. Drug-resistant cell clones carrying Hyg^r-deleted EBV episomes were screened by Southern blotting.

Southern blotting. DNA was extracted using the AquaPure genomic DNA isolation kit (Bio-Rad). Extracted DNA was digested with BamHI, separated by electrophoresis on a 0.8% agarose gel, and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech). The HincII-SacI fragment (1,115 bp) of the BamHI C fragment of Akata EBV DNA (corresponding to nucleotides 5176 through 6285 of EBV strain B95-8) was used as a probe. Probe labeling was carried out using the AlkPhos direct labeling kit (Amersham), and signals were detected with the CDP-Star detection reagent (Amersham).

Northern blotting. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies). The RNAs (7.5 μg each sample) were electrophoresed on a 1% agarose gel containing 0.66 M formaldehyde and then transferred to a Hybond N^+ nylon membrane. 18S rRNAs were visualized by ethidium bromide staining. Probes were prepared by PCR amplification using primers 5′-AGGA CCTACGCTGCCCTAGA-3′ and 5′-AAAACATGCGGACCACCAGC-3′ for EBER1 (31) and primers 5′-AGGACAGCCGTTGCCCTAGTGGTTTCG-3′ and 5′-AAAAATAGCGGACAAGCCGAATACC-3′ for EBER2 (20). The PCR conditions were described previously (20). Probe labeling was carried out using the AlkPhos direct labeling kit, and signals were detected with the CDP-Star detection reagent.

Immunoblotting. Immunoblot analysis was performed as described previously (38). Whole-cell lysates prepared from the indicated cell clones were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were blotted onto a nitrocellulose membrane (Schleicher & Schuell). Membranes were incubated with EBV-immune human sera for EBNA detection and with mouse monoclonal antibody S12 for LMP1 detection. Membranes were then washed, incubated with horseradish peroxidase-conjugated species-specific secondary antibodies (Amersham), and developed with ECL enhanced chemiluminescence detection reagents (Amersham).

Immunofluorescence. EBV-negative Daudi cells (10^6) were infected with recombinant EBVs at 37°C for 90 min. The infected cells were washed and resuspended in fresh medium and then cultured for 2 days. Cells were smeared on a slide glass and fixed with a 1:1 mixture of acetone and methanol. EBNA proteins were visualized by the anti-complement immunofluorescence method. EBNA-reactive human serum was used as a primary antibody, and fluorescein isothiocyanate-conjugated anti-human C3C antibody (Dako) was used as a secondary antibody.

Quantification of virions in the culture supernatants. Virus production in Akata cells (7.5×10^7) was induced by surface IgG cross-linking, and virus solutions were prepared as described above. Thirty milliliters of the virus solution was ultracentrifuged using an SW28 rotor (Beckman) at 15,000 rpm ($30,000 \times g$) at 4°C for 2 h. The pelleted virions were resuspended in 400 μl of phosphate-buffered saline (without calcium and magnesium), and then 200 μl of lysis buffer (75 mM Tris-HCl [pH 8.0], 25 mM EDTA, 3% sodium dodecyl sulfate) and 3 μl proteinase K (20 mg/ml) were added. Lysates were incubated at 37°C for 1 h and then extracted twice with phenol and once with a 24:1 mixture of chloroform-isoamyl alcohol. The viral DNAs were then precipitated using ethanol and dissolved in 30 μl of H_2O. Aliquots (5 μl) of each viral DNA were digested with EcoRI and analyzed by 0.8% agarose gel electrophoresis. The amount of virus produced was estimated by the intensity of the ethidium bromide-stained DNA bands.

Growth transformation assay. Cord blood lymphocytes were plated at a density of 1.2×10^5 to 1.3×10^5 cells per well in 96-well plates and then infected with serial 10-fold dilutions of each recombinant virus. The infected lymphocytes were cultured, and half of the culture medium was replaced with fresh medium every 5 days. The number of wells with proliferating lymphocytes was counted at 6 weeks postinfection, and the titers for transformation (50% transforming doses [TD_{50}/ml]) were calculated by the Reed-Muench method (5). In some experiments, the infected cells were cultured in medium containing recombinant IL-6 (rIL-6) at a concentration of 500 pg/ml.

LCL growth assay. Serially diluted LCLs were plated in 96-well plates, and half of the culture medium was replaced with fresh medium every 5 days. The number of wells with proliferating lymphocytes was counted 4 weeks after the start of cell culture. To generate growth curves, LCLs (5×10^4) were cultured in 1 ml of medium with or without 100 pg of recombinant human IL-6 (PeproTech). LCLs (5×10^4) were also cultured in 1 ml of medium with 10 μg of goat anti-human IL-6 antibody (R&D Systems) or control normal goat IgG (R&D systems). The number of viable cells was determined every 2 days by trypan blue exclusion.

RT-PCR. Reverse transcription-PCR (RT-PCR) analysis was carried out as described previously (12, 17). Total RNA was isolated using TRIzol reagent and then treated with DNase I (Invitrogen), according to the manufacturer's instruc-

tions. To detect the expression of EBV latent genes, cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (MMLV RT [Invitrogen]) and 100 pmol of random primer (Takara) at 37°C for 60 min, followed by heat inactivation at 94°C for 10 min. The cDNA samples were then subjected to 30 cycles of PCR. The PCR products were electrophoresed on an agarose gel and transferred to a Hybond N^+ membrane, and amplified DNA was detected using the Gene Images 3′-oligolabeling module (Amersham) and ECL detection reagents. The quality of the isolated RNA was verified by PCR amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

To detect the expression of cytokine genes, cDNA synthesis was performed using MMLV RT and an oligo(dT) primer (Promega), and then the cDNA samples were subjected to PCR as follows. For the detection of tumor necrosis factor alpha (TNF-α) expression, primers 5′-CTTCTGCCTGCTGCACTTTGGA-3′ and 5′-TCCCAAAGTAGACCTGCCCAGA-3′ were used and the PCR conditions were 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. For the detection of TNF-β expression, primers 5′-AAGCTGCCAGAGGAGGAGCC-3′ and 5′-TCCCGCTCGTCAGAAACGCC-3′ were used, and the PCR conditions were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. For the detection of IL-6 expression, primers 5′-TACATCCTCGACGGCATCTC-3′ and 5′-GCTACATTTGCCGAAGAGCC-3′ were used and the PCR conditions were 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. For the detection of IL-10 expression, primers 5′-ATGCCCCAAGCTGAGAACCAAG-3′ and 5′-TCTCAAGGGGCTGGGTCAGCTA-3′ were used and the PCR conditions were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR products were electrophoresed on an agarose gel and visualized by staining with ethidium bromide.

Real-time RT-PCR analysis. Total RNA was isolated using TRIzol reagent and treated with DNase I, and then cDNA synthesis was performed using MMLV RT and an oligo(dT) primer. For real-time RT-PCR, the SYBR Premix Ex *Taq* kit (Takara) and the LightCycler (Roche) were used. The primers for IL-6 and GAPDH were the same as those used for RT-PCR analysis. Reactions were done in triplicate. The results were normalized to GAPDH. Analysis of the real-time RT-PCR data was done as described previously (17).

ELISA. For the enzyme-linked immunosorbent assay (ELISA), 1×10^6 LCL cells were cultured in 1 ml of complete medium for 48 h. The supernatants were harvested, and the amount of IL-6 in the culture supernatants was determined using the Endogen human IL-6 ELISA kit, according to the manufacturer's instructions.

Apoptosis assay. Apoptotic cells were measured by the appearance of cells with sub-G_1 DNA content. Cells (1×10^6) were cultured in 10 ml medium containing 5% FCS for 4 days. Then the cells were harvested and fixed, stained with propidium iodide, and analyzed by FACSCalibur (20).

RESULTS

Generation of recombinant EBVs carrying either the EBER1 or EBER2 gene. We previously generated two recombinant EBVs to study the biological activities of EBERs; one recombinant EBV has both EBER1 and EBER2 genes deleted [designated EBER(−)EBV], and the other recombinant EBV, which is derived from EBER(−)EBV, has both EBER1 and EBER2 genes reconstituted at the native EBER locus [designated EBERs(+)EBV] (36). EBER(−)EBV was derived from Neo^{r}EBV which carries wild-type EBER genes and a neomycin resistance gene (29). In EBER(−)EBV episomes, the *loxP* site (and short flanking sequences) that was inserted when making EBER(−)EBV remained at the native EBER locus (Fig. 1A). In the present study, we generated recombinant EBVs carrying either the EBER1 or EBER2 gene. To make the recombinants, instead of reconstituting both EBER1 and EBER2 genes, we knocked in either "EBER1 alone" or "EBER2 alone" to EBER(−)EBV. An EBV DNA fragment containing the EBER locus and flanking regions was used to make the targeting constructs. Targeting constructs were designed so that either EBER1 or EBER2 would be inserted back into the native EBER locus of EBER(−)EBV (Fig. 1A). For EBER1 knockin, a targeting construct in which the EBER2 gene had been deleted was generated; likewise, an EBER1-deleted targeting construct was used for EBER2 knockin. In addition, a hygromycin resistance gene flanked by two *loxP* sites was inserted upstream of the EBER locus in each targeting construct (Fig. 1A).

Akata cells harboring EBER(−)EBV episomes were transfected with targeting constructs, and transfected cells were subjected to selection in the presence of hygromycin. Hygromycin-resistant Akata cell clones were screened by Southern blot analysis for the presence of homologously recombined EBV episomes. Two cell clones were identified containing EBER1-reconstituted EBV episomes, designated EBER1(+)Hyg^{r}EBV, in addition to EBER(−)EBV episomes (Fig. 1B, lane 3 in the upper panel [data not shown]). One cell clone was identified containing EBER2-reconstituted EBV episomes, designated EBER2(+)Hyg^{r}EBV, in addition to EBER(−)EBV episomes (Fig. 1B, lane 3 in the lower panel). To segregate EBER1(+)Hyg^{r}EBV or EBER2(+)Hyg^{r}EBV from EBER(−)EBV, a mixture of the two viruses was produced by treating cells with anti-Ig and EBV-negative Akata cells were infected with the respective mixture virus. After selection in the presence of hygromycin, cell clones harboring only EBER1(+)Hyg^{r}EBV or EBER2(+)Hyg^{r}EBV were obtained (Fig. 1B, lane 4 in the upper and lower panels). Subsequently, the Hyg^r gene, which was flanked by two *loxP* sites, was removed from EBER1(+)Hyg^{r}EBV or EBER2(+)Hyg^{r}EBV by expression of the *cre* recombinase in the respective Akata cell clones. The DNA band representing episomes that had lost the Hyg^r gene, designated EBER1(+)EBV or EBER2(+)EBV, was detected in *cre*-transduced cells (Fig. 1B, lane 5 in the upper and lower panels). The *cre*-transduced cell clones were induced to produce virus, and virus mixtures containing EBER1(+)EBV and EBER1(+)Hyg^{r}EBV or EBER2(+)EBV and EBER2(+)Hyg^{r}EBV were used to infect EBV-negative Akata cells. After selection in the presence of G418 and screening by Southern blotting, Akata cell clones harboring EBER1(+)EBV or EBER2(+)EBV were obtained (Fig. 1B, lanes 6 through 9 in the upper and lower panels).

We next verified the expression of EBER1 and EBER2 in Akata cell clones infected with EBER1(+)EBV or EBER2(+)EBV. Total RNA was extracted from Akata cells harboring Neo^{r}EBV, EBER(−)EBV, EBER1(+)EBV, or EBER2(+)EBV, and the expression of EBERs was examined by Northern blot analysis. Akata cell clones harboring EBER1(+)EBV or EBER2(+)EBV expressed only EBER1 or EBER2, respectively, as expected (Fig. 1C). Importantly, the level of EBER1 expression in EBER1(+)EBV-infected Akata cell clones and EBER2 expression in EBER2(+)EBV-infected Akata cell clones was similar to that in Neo^{r}EBV-infected Akata cells (Fig. 1C). We also examined the expression of viral genes by immunoblotting and RT-PCR analysis and found that EBER1(+)EBV- and EBER2(+)EBV-infected Akata cell clones exhibited viral gene expression patterns identical to that of Neo^{r}EBV-infected Akata cells (Fig. 1D), with the exception of the expression of EBERs (Fig. 1C). Thus, the results indicated that we successfully generated recombinant EBVs carrying either EBER1 or EBER2, without affecting the expression of other viral genes.

Virus production and infectivity of EBER1(+)EBV and EBER2(+)EBV. We previously reported that EBERs have no

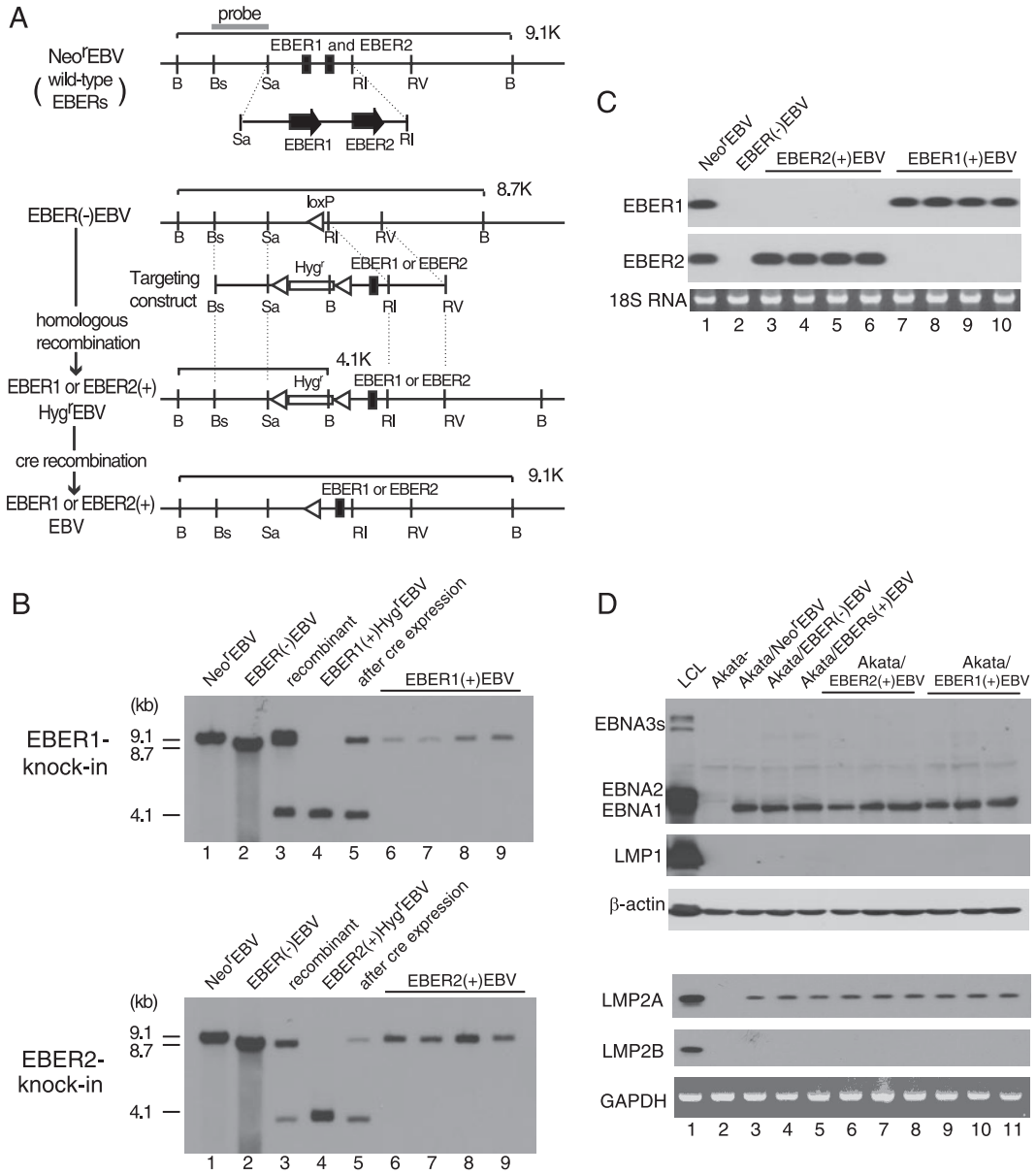

FIG. 1. (A) Schematic representation of the EBER locus in the genomes of Neo^{r}EBV, EBER(−)EBV, and EBER1- or EBER2-knockin EBVs. The targeting construct is also shown. Either EBER1 or EBER2 was reconstituted into the genome of EBER(−)EBV via homologous recombination, using the hygromycin resistance gene as a selection marker. Subsequently, the Hyg^r gene was removed by transient expression of the *cre* recombinase. The positions of restriction sites are indicated by B (BamHI), Bs (BstPI), Sa (SacI), RI (EcoRI), and RV (EcoRV). The gray line indicates the probe used for Southern blot analysis, and the sizes of the bands from each EBV that are detected by the probe are shown. White arrowheads indicate the *loxP* sites. The EBER1 and EBER2 genes (black boxes) and the hygromycin resistance gene (Hyg^r [open box]) are also indicated. (B) Southern blot analysis of Akata cell clones harboring various recombinant EBVs in EBER1-knockin experiments (upper panel) and EBER2-knockin experiments (lower panel). Shown are the results from a Neo^{r}EBV-infected cell clone (lane 1 in the upper and lower panels), an EBER(−)EBV-infected cell clone (lane 2 in the upper and lower panels), targeted cell clones (recombinant; lane 3 in the upper and lower panels), an EBER1(+)Hyg^{r}EBV- and an EBER2(+)Hyg^{r}EBV-infected cell clone (lane 4 in the upper and lower panels, respectively), the same cell clones upon transient expression of *cre* (lane 5 in the upper and lower panels, respectively), and EBER1(+)EBV- and EBER2(+)EBV-infected cell clones (lanes 6 through 9 in the upper and lower panels, respectively). (C) Northern blot analysis of EBER1 and EBER2 expression in Akata cell clones harboring recombinant EBVs. The results from a Neo^{r}EBV-infected Akata cell clone (lane 1), an EBER(−)EBV-infected Akata cell clone (lane 2), EBER2(+)EBV-infected Akata cell clones (lanes 3 through 6), and EBER1(+)EBV-infected Akata cell clones (lanes 7 through 10) are shown. (D) EBV gene expression in Akata cell clones harboring recombinant EBVs. Immunoblotting was used to examine the expression of EBNA and LMP1 (upper panel); RT-PCR analysis was used to examine the expression of other latent genes (lower panel). The results from an LCL (lane 1), an EBV-negative Akata cell clone (Akata−; lane 2), a Neo^{r}EBV-infected Akata cell clone (lane 3), an EBER(−)EBV-infected Akata cell clone (lane 4), an EBERs(+)EBV-infected Akata cell clone (lane 5), EBER2(+)EBV-infected Akata cell clones (lanes 6 through 8), and EBER1(+)EBV-infected Akata cell clones (lanes 9 through 11) are shown.

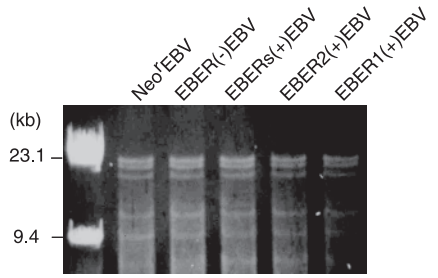

FIG. 2. Comparison of the amounts of recombinant virus produced by various cell clones. Akata cell clones harboring Neo^{r}EBV, EBER(−)EBV, EBERs(+)EBV, EBER2(+)EBV, or EBER1(+)EBV were treated with anti-IgG to induce virus production, and the culture supernatants were harvested. Viral DNAs were extracted from the pelleted virions, digested with EcoRI, and analyzed by agarose gel electrophoresis.

quantitative effect on viral late protein synthesis or on virion maturation. We also succeeded in producing large quantities of pure EBER(−)EBV and EBERs(+)EBV, which fully retained the infectivity of wild-type EBV (36). In the present study, we first examined whether Akata cell clones harboring EBV carrying either the EBER1 or EBER2 gene produced recombinant virus efficiently. Akata cell clones harboring Neo^{r}EBV, EBER(−)EBV, EBERs(+)EBV, EBER1(+)EBV, or EBER2(+)EBV were induced to produce virus with anti-IgG. Immunofluorescence analysis revealed that approximately 50% of cells harboring each type of EBV became positive for gp110 expression after anti-IgG treatment, indicating that EBER1(+)EBV and EBER2(+)EBV, as well as EBER(−)EBV, were as efficient in entering the lytic cycle of viral replication as Neo^{r}EBV and EBERs(+)EBV (data not shown). Culture supernatants containing virions were harvested, and viral DNAs were extracted from the pelleted virions, digested with EcoRI, and analyzed by agarose gel electrophoresis. The relative amounts of virions were estimated based on the band intensities of ethidium bromide-stained DNA. Based on this type of analysis, the amounts of Neo^{r}EBV, EBER(−)EBV, EBERs(+)EBV, EBER1(+)EBV, and EBER2(+)EBV were comparable, indicating that Akata cell clones harboring these recombinant EBVs released similar amounts of virions into the culture supernatants (Fig. 2).

Next, the infectivities of the recombinant EBVs were examined. Equivalent amounts of viral supernatants, assessed as for Fig. 2 and containing comparable amounts of virions, were serially diluted and used to infect EBV-negative Daudi cells. The expression of EBNA proteins in the recipient cells was examined by anti-complement immunofluorescence analysis 2 days after infection, and the EBNA-inducing titer of each type of EBV was estimated. We found that the EBNA-inducing titers of Neo^{r}EBV, EBER(−)EBV, EBERs(+)EBV, EBER1(+)EBV, and EBER2(+)EBV were comparable (Table 1). These results suggested that the reconstitution of either EBER1 or EBER2 did not affect virion maturation and the infectivity of the virus. Collectively, these results showed that purified EBV carrying either EBER1 or EBER2 was successfully produced and fully retained the infectivity of wild-type EBV.

TABLE 1. Comparison of infectivities of different types of recombinant EBVs

EBV type	No. of EBNA-inducing units/ml
Neo^r	6.0×10^5
EBER(−)	6.4×10^5
EBERs(+)	7.0×10^5
EBER2(+)	6.4×10^5
EBER1(+)	5.6×10^5

EBER2, but not EBER1, plays a critical role in efficient B-cell growth transformation. We next performed a B-cell growth transformation assay using pure EBV carrying either EBER1 or EBER2. Viral supernatants were harvested from cultures of lytically induced Akata cell clones harboring Neo^{r}EBV, EBER(−)EBV, EBERs(+)EBV, EBER1(+)EBV, or EBER2(+)EBV, and those with comparable EBNA-inducing titers were used for the following experiments. Cord blood lymphocytes were plated at a fixed cell density and then infected with serial 10-fold dilutions of virus. The transforming titer (TD_{50}/ml) of virus was determined by counting the number of wells with proliferating lymphocytes 6 weeks postinfection. The transforming titer of EBER(−)EBV was approximately 100-fold less than that of Neo^{r}EBV (Table 2), as reported previously (36). We also confirmed that the reconstitution of both the EBER1 and EBER2 genes into EBER(−)EBV restored the transforming ability of the virus (Table 2). Of note, the transforming ability of EBER2(+)EBV was as high as that of EBERs(+)EBV (Table 2). In contrast, the TD_{50}/ml of EBER1(+)EBV was clearly less than that of EBERs(+)EBV or EBER2(+)EBV, and similar to, or slightly less than, that of EBER(−)EBV (Table 2). In order to obtain compelling evidence that the loss of EBER2 gene is truly responsible for the impaired transformation efficiency, we utilized two independent EBER1(+)EBVs, which were obtained by independently performed EBER1 gene knockin. Importantly, both of the independent EBER1(+)EBV clones showed impaired transformation ability (Table 2). Therefore, it is highly unlikely that the defect in EBER1(+)EBVs is due to accidental mutations outside the EBER locus that affect its transformation efficiency. These results indicated that reconstitution of the EBER2 gene alone is sufficient to restore the impaired transformation ability of EBER(−)EBV, while reconstitution of EBER1 alone is not, demonstrating that EBER2, but not EBER1, has a critical role in efficient EBV-induced B-cell growth transformation.

TABLE 2. Comparison of TD_{50}s of different types of recombinant EBVs

EBV type[a]	TD_{50}/ml		
	Expt 1	Expt 2	Expt 3
Neo^r	$10^{5.4}$	$10^{5.6}$	$10^{5.0}$
EBER(−)	$10^{3.7}$	$10^{3.0}$	$10^{2.8}$
EBERs(+)	$10^{4.4}$	$10^{4.6}$	$10^{4.4}$
EBER2(+)	$10^{4.2}$	$10^{4.6}$	$10^{4.8}$
EBER1(+)	$10^{2.5}$	$10^{2.6}$	$10^{2.8}$

[a] Two independent EBER1(+)EBVs were used. One was used in experiments 1 and 2, and another was used in experiment 3.

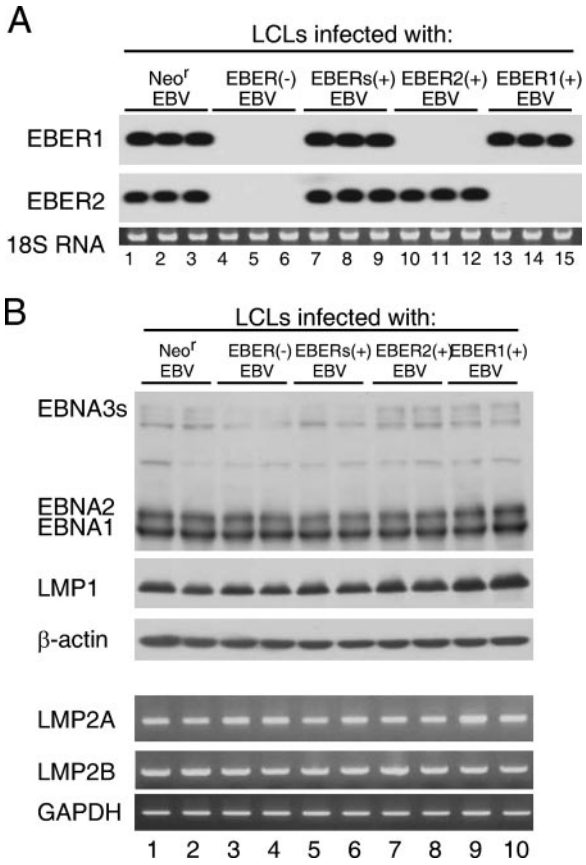

FIG. 3. (A) Northern blot analysis of EBER1 and EBER2 expression in LCLs infected with recombinant EBVs. The results from three independent Neo^rEBV-infected LCLs (lanes 1 through 3), EBER(−) EBV-infected LCLs (lanes 4 through 6), EBERs(+)EBV-infected LCLs (lanes 7 through 9), EBER2(+)EBV-infected LCLs (lanes 10 through 12), and EBER1(+)EBV-infected LCLs (lanes 13 through 15) are shown. (B) EBV gene expression in LCLs infected with recombinant EBVs. Immunoblotting was used to examine the expression of EBNA and LMP1 (upper panel); RT-PCR analysis was used to examine the expression of other latent genes (lower panel). The results from two independent Neo^rEBV-infected LCLs (lanes 1 and 2), EBER(−) EBV-infected LCLs (lanes 3 and 4), EBERs(+)EBV-infected LCLs (lanes 5 and 6), EBER2(+)EBV-infected LCLs (lanes 7 and 8), and EBER1(+)EBV-infected LCLs (lanes 9 and 10) are shown.

EBER2 is critical for the growth of LCLs at low cell density. We established LCLs infected with Neo^rEBV, EBER(−)EBV, EBERs(+)EBV, EBER1(+)EBV, or EBER2(+)EBV, using viruses with high EBNA-inducing titers, which enabled rapid outgrowth of LCLs. Thus, we avoided artifacts that might arise from long-term cultivation. When we examined viral gene expression in the LCLs using Northern blot analysis, we found that EBER1(+)EBV- or EBER2(+)EBV-infected LCLs expressed either EBER1 or EBER2, respectively, as expected (Fig. 3A). Moreover, the levels of EBER1 expression in EBER1(+)EBV-infected LCLs and EBER2 expression in EBER2(+)EBV-infected LCLs were similar to those in Neo^rEBV- or EBERs(+)EBV-infected LCLs. Immunoblot and RT-PCR analyses revealed that EBER1(+)EBV- and EBER2(+)EBV-infected LCLs were indistinguishable from Neo^rEBV-, EBER(−)EBV-, or EBERs(+)EBV-infected LCLs in their expression of other viral latent genes, indicating that reconstitution of either EBER1 or EBER2 did not affect the expression of other viral genes in LCLs (Fig. 3B).

Previously, we found that EBER(−)EBV-infected LCLs grew less efficiently than Neo^rEBV- or EBERs(+)EBV-infected LCLs when the cells were plated at low cell densities (36). Thus, we were interested in comparing the growth rates of LCLs harboring various recombinant EBVs at low cell densities. Serial two-fold dilutions of LCLs were plated in 96-well plates, and the number of wells with proliferating cells was determined 4 weeks after the start of culture. As reported previously, EBER(−)EBV-infected LCLs did not proliferate when plated at low cell densities, while Neo^rEBV-infected or EBERs(+)EBV-infected LCLs plated at low cell density proliferated well (Table 3). Significantly, EBER2(+)EBV-infected LCLs proliferated similarly to Neo^rEBV- or EBERs(+) EBV-infected LCLs when plated at low cell densities, while the proliferation of EBER1(+)EBV-infected LCLs plated at low cell densities was similar to that of EBER(−)EBV-infected LCLs (Table 3). Thus, LCLs expressing EBER2 proliferated at low cell densities, whereas LCLs lacking EBER2 did not. Similar results were obtained using LCLs that were derived from different donors (Table 3). Thus, the data indicated that EBER2 is critical for the growth of LCLs at low cell densities.

Higher IL-6 production in LCLs expressing EBER2 than in LCLs lacking EBER2. We have previously shown that EBERs stimulate cytokine production in a variety of cell types (13, 14, 17, 25, 37). Therefore, we were interested in the expression of cytokines, which have been reported to act as autocrine growth factors, in LCLs harboring recombinant EBVs. The results of RT-PCR analysis showed that IL-6 expression was higher in Neo^rEBV-, EBERs(+)EBV-, and EBER2(+)EBV-infected LCLs than in EBER(−)EBV- or EBER1(+)EBV-infected LCLs (Fig. 4A). The levels of TNF-α, TNF-β, and IL-10 expression were similar among the LCLs (Fig. 4A). There was no difference in the half-lives of IL-6 mRNA in Neo^rEBV-infected and EBER(−)EBV-infected LCLs, suggesting that the difference in the levels of IL-6 mRNA was at the level of transcription (Fig. 4B).

TABLE 3. Numbers of wells with proliferating cells after plating of various LCLs at low cell densities

EBV type of LCL[a]	No. of wells with indicated no. of plated cells/well[b]				
	1,000	500	250	125	62.5
Expt 1					
Neo^r	16	16	16	15	8
EBER(−)	15	4	1	0	0
EBERs(+)	16	16	14	8	3
EBER2(+)	16	16	16	9	4
EBER1(+)	8	1	0	0	0
Expt 2					
Neo^r	16	16	16	13	7
EBER(−)	12	2	0	0	0
EBERs(+)	16	16	12	7	4
EBER2(+)	16	16	10	5	1
EBER1(+)	4	1	0	0	0

[a] Different sets of LCLs derived from different donors were used in experiment 1 and in experiment 2.
[b] Numbers of wells are given out of a total of 16 wells.

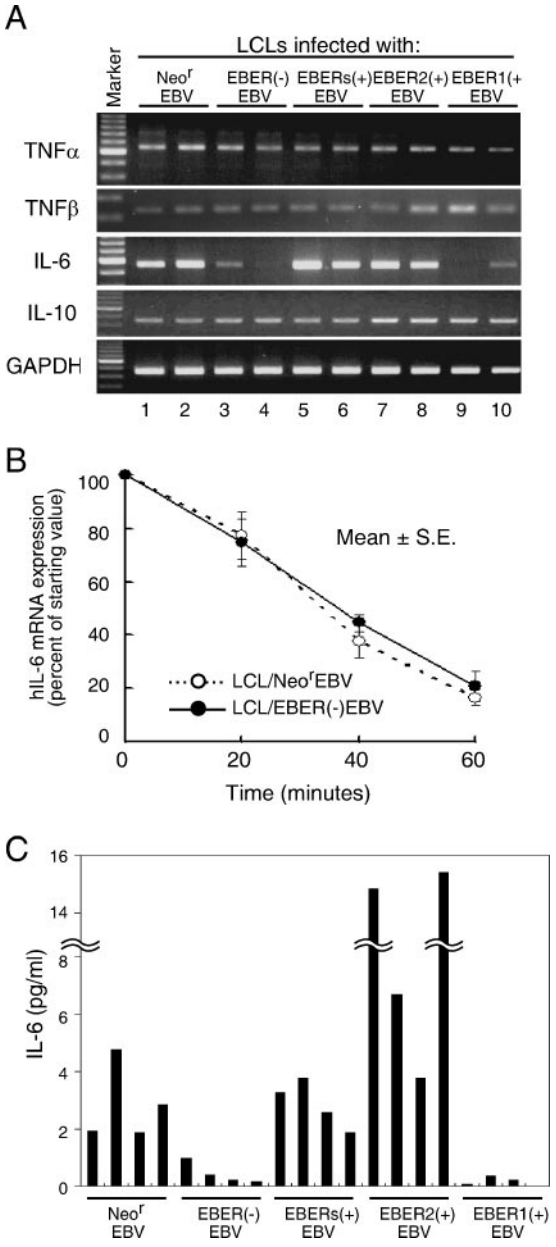

FIG. 4. (A) RT-PCR analysis of TNF-α, TNF-β, IL-6, and IL-10 expression in LCLs infected with recombinant EBVs. The results from two independent Neo^rEBV-infected LCLs (lanes 1 and 2), EBER(−) EBV-infected LCLs (lanes 3 and 4), EBERs(+)EBV-infected LCLs (lanes 5 and 6), EBER2(+)EBV-infected LCLs (lanes 7 and 8), and EBER1(+)EBV-infected LCLs (lanes 9 and 10) are shown. (B) IL-6 mRNA degradation in a Neo^rEBV-infected LCL and an EBER(−) EBV-infected LCL. Actinomycin D (5 μg/ml) was added to the cells to block mRNA synthesis. After cultivation for the indicated time, RNAs were isolated and the amount of IL-6 mRNA remaining was determined by real-time RT-PCR analysis. The data are representative of four experiments. S.E., standard error. In the Neo^rEBV-infected LCLs, the half-life is 32.5 ± 3.6 min; in the EBER(−)EBV-infected LCLs, the half-life is 34.0 ± 4.3 min. (C) IL-6 production by LCLs infected with recombinant EBVs. A total of 1×10^6 LCL cells were cultured in 1 ml of medium for 48 h. The amount of IL-6 in the culture supernatants was measured by ELISA. The results from four independent Neo^rEBV-infected LCLs, EBER(−)EBV-infected LCLs, EBERs(+)EBV-infected LCLs, EBER2(+)EBV-infected LCLs, and EBER1(+)EBV-infected LCLs are shown.

We then measured the amounts of IL-6 protein in culture supernatants of the various LCLs. The amount of IL-6 in the culture supernatants of Neo^rEBV-, EBERs(+)EBV-, and EBER2(+)EBV-infected LCLs was higher than in the culture supernatants of EBER(−)EBV- or EBER1(+)EBV-infected LCLs, which was consistent with the results of RT-PCR analysis (Fig. 4C). These results clearly showed that LCLs expressing EBER2 produced more IL-6 than LCLs lacking EBER2.

Growth-stimulatory as well as antiapoptotic effects of IL-6 on the LCLs. We examined whether IL-6 actually contributed to the growth of LCLs. First, EBER(−)EBV-infected LCLs and EBER1(+)EBV-infected LCLs, both of which secreted relatively low levels of IL-6, were examined for their growth in the absence or presence of rIL-6 added to the culture medium. We found that adding rIL-6 significantly enhanced the growth of EBER(−)EBV-infected as well as of EBER1(+)EBV-infected LCLs (Fig. 5A). The effect of adding rIL-6 was obscure when Neo^rEBV-infected or EBER2(+)EBV-infected LCLs, which secreted relatively high levels of IL-6, were subjected to the same assay (data not shown). However, when these LCLs were cultured in the medium supplemented with a reduced concentration (5%) of FCS, we observed that Neo^rEBV-infected LCLs and EBER2(+)EBV-infected LCLs grew faster in the presence of rIL-6 (Fig. 5B). Thus, growth-stimulatory effects of IL-6 were observed in all of the tested LCLs, regardless of the EBER status of the infected recombinant EBVs. Furthermore, we utilized neutralizing anti-IL-6 antibody to examine whether the antibody could negatively affect the LCL growth. As expected, adding neutralizing anti-IL-6 antibody to the culture medium significantly inhibited the growth of Neo^rEBV-infected or EBER2(+)EBV-infected LCLs (Fig. 5C). Thus, we conclude that IL-6 actually works as a growth factor and supports the LCL growth.

The poor growth rates of EBER(−)EBV-infected or EBER1(+)EBV-infected LCLs in the absence of IL-6 (Fig. 5A) prompted us to examine whether a significant number of EBER2-negative LCLs fell into apoptotic cell death in the absence of IL-6. The frequencies of apoptotic cells in EBER2-negative LCLs were up to 40%, which were higher than those of Neo^rEBV- or EBER2(+)EBV-infected LCLs (Fig. 5D). Addition of rIL-6 to the cell culture decreased the frequencies of apoptotic cells in EBER2-negative LCLs (Fig. 5D, left panel), and addition of anti-IL-6 antibody increased the apoptotic cells in Neo^rEBV- or EBER2(+)EBV-infected LCLs (Fig. 5D, right panel), indicating that IL-6 has an antiapoptotic effect on LCLs.

Based on the observed diverse effects of IL-6 on LCL growth, we examined whether adding IL-6 could complement the lack of EBER2 gene in the primary B-cell transformation assay. We found that addition of IL-6 slightly enhanced the transforming efficiency of EBER2-negative EBVs but did not completely complement the lack of the EBER2 gene in this assay (Table 4).

DISCUSSION

Both EBER1 and EBER2 are expressed at high levels in latently infected cells, and they are strikingly similar in their secondary structures (10, 24). Thus, it is conceivable that EBER1 and EBER2 share common biological activities. Sup-

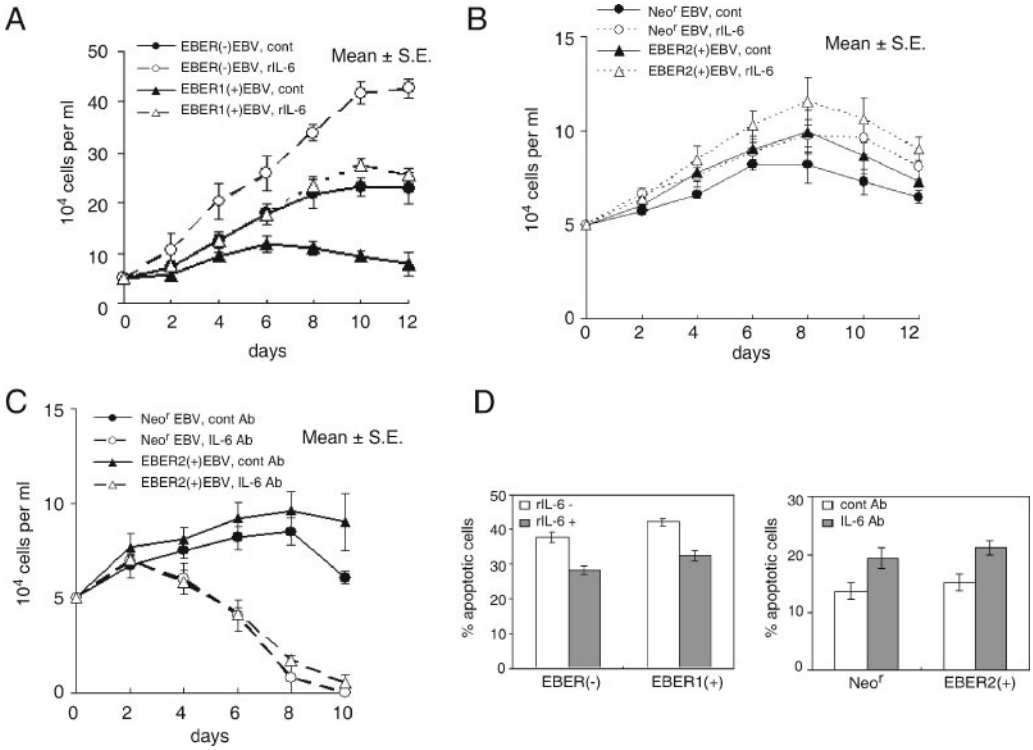

FIG. 5. (A) Effect of rIL-6 on the growth of EBER(-)EBV- and EBER1(+)EBV-infected LCLs. The indicated LCLs were cultured in medium containing 10% FCS in the absence (cont) or presence (rIL-6) of rIL-6 at a concentration of 100 pg/ml. The number of viable cells was determined by trypan blue exclusion. (B) Effect of rIL-6 on the growth of Neo^{r}EBV- and EBER2(+)EBV-infected LCLs. The indicated LCLs were cultured in medium containing 5% FCS in the absence (cont) or presence (rIL-6) of rIL-6 at a concentration of 500 pg/ml. The number of viable cells was determined by trypan blue exclusion. (C) Effect of neutralizing anti-IL-6 antibody on the growth of Neo^{r}EBV- and EBER2(+)EBV-infected LCLs. The LCLs were cultured in medium containing 5% FCS in the presence of goat anti-human IL-6 antibody (IL-6 Ab) or control normal goat IgG (cont Ab) at a concentration of 10 μg/ml. The number of viable cells was determined by trypan blue exclusion. (D) Effect of recombinant IL-6 or neutralizing anti-IL-6 antibody on the apoptotic cell death of the LCLs. The indicated LCLs were cultured in medium containing 5% FCS in the absence (rIL-6 −) or presence (rIL-6 +) of rIL-6 at a concentration of 500 pg/ml (left panel) or cultured in medium containing control antibody (cont Ab) or goat anti-human IL-6 antibody (IL-6 Ab) at a concentration of 10 μg/ml (right panel), for 4 days. The frequencies of apoptotic cells were determined by measuring the percentages of sub-G_1 cell populations by fluorescence-activated cell sorting. Bars represent the average of three independent experiments ± standard error (S.E.).

porting the idea, it was reported that both EBER1 and EBER2 inhibit PKR phosphorylation (20) and that both stimulate RIG-I to induce type I IFN (25). In the current study, we employed EBV genetic analysis to examine whether EBER1 and EBER2 play distinct roles in latently infected cells.

We generated recombinant EBVs carrying either EBER1 alone or EBER2 alone and evaluated the contributions of EBER1 and EBER2 to EBV-mediated B-cell growth transformation. We found that EBV expressing EBER2 alone transformed primary B cells as efficiently as EBV expressing both EBER1 and EBER2. In contrast, the transforming ability of EBV expressing EBER1 alone was significantly impaired, similar to the level of EBV lacking both EBER1 and EBER2. Furthermore, when we compared the growth rates of the LCLs that were established by the same set of the recombinant EBVs, LCLs expressing EBER2 proliferated at low cell densities, whereas LCLs lacking EBER2 did not. Thus, our data clearly show that EBER2 is actually important, while EBER1 is dispensable, for efficient B-cell growth transformation as well as for the enhancement of the LCL growth. In other words, the contribution of EBERs to the efficient growth transformation, which was reported previously (36), is attributable to EBER2 alone.

TABLE 4. Comparison of TD_{50}s of recombinant EBVs with or without rIL-6

EBV type and addition	TD_{50}/ml	
	Expt 1	Expt 2
Neo^r	$10^{4.8}$	$10^{5.0}$
EBER(−)	$10^{2.8}$	$10^{2.6}$
EBER(−) + rIL-6	$10^{3.0}$	$10^{3.0}$
EBER1(+)	$10^{2.3}$	$10^{2.5}$
EBER1(+) + rIL-6	$10^{2.8}$	$10^{2.8}$

Our data demonstrated that the level of IL-6 production in LCLs expressing EBER2 was higher than that in LCLs lacking EBER2. EBER2-negative LCLs grew faster by *trans*-supplying rIL-6 to the culture medium, while the growth of EBER2-expressing LCLs was inhibited by adding neutralizing antibody against IL-6. These results support the idea that EBER2 expression up-regulates IL-6 secretion and that the resultant IL-6 enhances LCL growth as an autocrine growth factor, as reported earlier (11, 27, 34, 39).

Our results also indicate that IL-6 can let the LCLs escape from apoptotic cell death. The results suggest that IL-6 perhaps contributes to LCL growth via two different mechanisms: one by working as an autocrine growth factor and the other by letting the LCLs escape from apoptotic cell death. We also tried to complement the lack of EBER2 gene by *trans*-supplying IL-6 in the B-cell transformation assay. The result revealed that *trans*-supplied IL-6 failed to completely complement the lack of EBER2 gene. One possible explanation is that exogenously added IL-6 may be less effective than endogenous autocrine IL-6. Alternatively, the result can be interpreted that IL-6 is not the only cellular factor that is induced by EBER2 and contributes to B-cell transformation. Presumably, IL-6 must cooperate with other EBER2-induced cellular factors to accelerate the initial phase of B-cell growth transformation.

The molecular mechanism by which EBER2 induces IL-6 expression remains unknown. Our data indicate that IL-6 mRNA expression is transcriptionally upregulated in LCLs expressing EBER2, raising the possibility that EBER2 stimulates the transcription of IL-6 in LCLs. Recently, we reported that EBERs are recognized by RIG-I and activate signaling to induce type I IFN (25). It is well known that activation of RIG-I results in the production of type I IFN as well as inflammatory cytokines such as IL-6, IL-8, and IL-12p40 (9, 15). We found that the expression of RIG-I in LCLs was much higher than in Burkitt's lymphoma cell lines. Thus, it is possible that EBER2 induces IL-6 transcription through activation of RIG-I. Our attempts to down regulate the expression of RIG-I in LCLs using small inhibitory RNA so far have been unsuccessful (data not shown). Studies are ongoing to clarify whether RIG-I is involved in the IL-6 induction by EBER2. Another possibility is that EBERs regulate gene expression by acting as microRNAs. Recently, it was reported that virus-associated (VA) RNAs of adenovirus are processed by Dicer and suppress gene expression in a sequence-dependent manner (1, 26). EBERs and VA RNAs resemble each other in that both are noncoding RNAs and transcribed by RNA polymerase III in virus-infected cells. Therefore, it is possible that EBERs act as microRNA, like VA RNAs, and have their own cellular targets.

EBER2 may cooperate with other latently-expressed viral proteins to induce IL-6 expression in the LCLs. We found that the expression level of IL-6 in LCLs, which express all of the EBV latent proteins (type III latency), was higher than in Burkitt's lymphoma cells, which express only EBNA1 and EBERs (type I latency) (data not shown). LMP1, a major transforming protein expressed in LCLs, is known to induce IL-6 in epithelial cell lines (6, 7). Thus, EBER2, but presumably not EBER1, may cooperate with LMP1 to induce IL-6 in LCLs. Further studies are required to identify the precise mechanism of EBER2-specific IL-6 induction in LCLs.

We reported previously that EBERs induce the expression of IL-10 in EBV-negative Burkitt's lymphoma cells (17). However, we did not see any significant difference in the mRNA levels of IL-10 in LCLs. Like IL-6, IL-10 can also be induced by the expression of LMP1 (19, 35). The reason why the expression of EBER2 had differential effects on IL-6 induction and IL-10 induction must be clarified. One possibility is that EBER2 expression is equally important as LMP1 expression in the case of IL-6 induction, while the effect of LMP1 overwhelms the effect of EBER2 in the case of IL-10 induction. If this is the case, the effect of EBERs on IL-10 expression could be masked by the effect of LMP1 in LCLs.

Our genetic analysis led to the somewhat unexpected conclusion that EBER1 and EBER2 have distinct biological function(s) in EBV-infected cells. Clearly, this study should be further extended to the analysis of other EBV-associated malignancies, such as Burkitt's lymphoma and nasopharyngeal carcinoma. Recombinant EBVs carrying either EBER1 alone or EBER2 alone would provide us with further information regarding to the individual functions of EBER1 and EBER2 in the EBV life cycle and in EBV-associated tumorigenesis.

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