Growth-Promoting Properties of Epstein-Barr Virus EBER-1 RNA Correlate with Ribosomal Protein L22 Binding

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The Epstein-Barr virus (EBV)-encoded RNAs, EBER-1 and EBER-2, are highly abundant noncoding nuclear RNAs expressed during all forms of EBV latency. The EBERs have been shown to impart significant tumorigenic potential upon EBV-negative Burkitt lymphoma (BL) cells and to contribute to the growth potential of other B-cell lymphoma-, gastric carcinoma-, and nasopharyngeal carcinoma-derived cell lines. However, the mechanisms underlying this EBER-dependent enhancement of cell growth potential remain to be elucidated. Here we focused on the known interaction between EBER-1 and the cellular ribosomal protein L22 and the consequences of this interaction with respect to the growth-promoting properties of the EBERs. L22, a component of 60S ribosomal subunits, binds three sites on EBER-1, and a substantial fraction of available L22 is relocalized from nucleolus to the nucleoplasm in EBV-infected cells. To investigate the hypothesis that EBER-1-mediated relocalization of L22 in EBV-infected cells is critical for EBER-dependent functions, we investigated whether EBER-1 expression is necessary and sufficient for nucleoplasmic retention of L22. Following demonstration of this, we utilized RNA-protein binding assays and fluorescence localization studies to demonstrate that mutation of the L22 binding sites on EBER-1 prevents L22 binding and inhibits EBER-1-dependent L22 relocalization. Finally, the in vivo consequence of preventing L22 relocalization in EBER-expressing cells was examined in soft agar colony formation assays. We demonstrate that BL cells expressing mutated EBER-1 RNAs rendered incapable of binding L22 have significantly reduced capacity to enhance cell growth potential relative to BL cells expressing wild-type EBERs.

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus that establishes a lifelong latent infection in B lymphocytes, is associated with a variety of human malignancies, including Burkitt lymphoma (BL) (14). Alternate programs of EBV latent gene expression, defined by the subset of viral genes which are expressed within a given cell type and by the human malignancies with which they are associated, have been described (28). Common to all latency programs is the expression of the Epstein-Barr virus-encoded RNAs, EBER-1 and EBER-2. The EBERs are highly abundant noncoding nuclear RNAs transcribed by cellular RNA polymerase III (3, 23, 29). While the precise role of the EBERs during EBV infection remains unclear, recombinant EBV engineered to lack the EBERs was recently shown to be impaired in its ability to transform cord blood lymphocytes (47). In agreement with this finding, the EBERs have been shown to impart significant tumorigenic potential upon EBV-negative BL cells. Unlike EBV-positive Akata BL cells, which readily establish tumors in SCID and nude mice, EBV-negative Akata BL cells derived from a parental EBV-positive Akata line are nontumorigenic (32, 35). However, when EBER expression is restored in EBV-negative Akata cells, these EBV-negative EBER-expressing BL cells give rise to tumors, clearly establishing a link between EBER expression and tumor formation (17, 31). The EBERs also contribute to the growth potential of other B-cell lymphoma-, gastric carcinoma-, and nasopharyngeal carcinoma-derived cell lines (12, 13, 48).

In addition to these growth-promoting activities, the EBERs have been shown to mediate resistance to alpha interferon-induced apoptosis and to confer autocrine growth capacity via upregulation of interferon-10 (16, 25, 30). However, the mechanisms underlying these EBER-associated effects have not been elucidated. Additionally, it is unknown whether EBER-1 and EBER-2 function cooperatively or whether they have distinct and separable functions. In support of individual roles for EBER-1 and EBER-2, it was recently reported that the transforming capacity of EBER-1-deleted virus is as high as that of wild-type EBV, whereas EBER-2-deleted virus is impaired in its transforming ability, resulting in transforming efficiencies comparable to that of EBV lacking both EBERs (46). However, the roles of EBER-1 and EBER-2 during established latency in vivo and their individual or distinct ability to promote the tumorigenic properties of BL cells, which have a more restricted pattern of latent gene expression than lymphoblastoid cell lines, have not yet been addressed.

The EBERs adopt significant secondary structure, resulting in formation of several stable stem-loops (9, 11) that facilitate interaction with a number of cellular proteins, including the autoantigen La (9, 11, 23, 29), which binds to the 3′ end of cellular polymerase III transcripts (43), and the double-stranded RNA-dependent protein kinase R (PKR) (4, 7, 25, 41). While both EBERs are capable of interacting with PKR and can inhibit PKR activation (autophosphorylation) in vitro (5, 25, 34), PKR activation in vivo is unaffected by EBER expression (30, 42). This difference is likely a result of distinct subcellular compartmentalization of these two molecules, with...
the EBERs being exclusively nuclear, while PKR is predominately found in the cytoplasm. Both EBERs have also been shown to activate the retinoic acid-inducible gene I (RIG-I) and stimulate type I interferon signaling (33). However, like PKR, RIG-I is a cytosolic protein, and studies that established the interaction between the EBERs and RIG-I utilized transiently overexpressed recombinant RIG-I. EBER-1 also interacts with the ribosomal protein L22, a component of the 60S eukaryotic ribosomal subunit unique to eukaryotes (6, 8, 24, 38, 39, 45). While L22 is known to interact with a number of cellular and viral proteins and RNAs, including stem-loop 7 of 28S rRNA, its function within the cell and during viral infection remains virtually undefined (6, 11, 15, 20–22, 26, 38, 39). With respect to viral infections, L22 is known to interact with viral components during EBV, herpes simplex virus (HSV), and hepatitis C virus (HCV) infections. Specifically, L22 relocates to viral replication compartments during late times after HSV infection (21, 22) and has been shown to bind the HCV 3'X region and stimulate internal ribosome entry site (IRES)-mediated translation of HCV RNA (44). Furthermore, in EBV-infected BL cells, roughly 50% of the cellular pool of EBER-1 is found in association with EBER-1 ribonucleoprotein (RNP) particles, and a substantial fraction of L22 is physically relocalized from nucleoli to the nucleoplasm (37). This relocalization of L22 may be mediated in part through interaction with EBER-1. While no direct evidence for this has been reported, recent data demonstrating that the subcellular localization of L22 is mediated by the specific RNA substrate bound (10) suggests that the relocalization of L22 in EBV-infected cells may be a result of its interaction with EBER-1. EBER-1, which contains three L22 binding sites located within stem-loops I, III, and IV (6, 8, 38), likely competes with 28S rRNA for L22 binding in the nuclei of infected cells (6). Binding to 28S rRNA likely serves to target L22 to nucleoli, while binding to EBER-1 RNA likely results in sequestration or retention of L22 in the nucleoplasm. Consequently, if the in vivo interaction of L22 with EBER-1 is key to the function of EBER-1 during EBV latency, it logically follows that one or more of the phenotypic effects associated with EBER expression may be mediated by or as a consequence of L22 relocalization.

To investigate the hypothesis that EBER-1-mediated relocalization of L22 in EBV-infected cells is critical for EBER-dependent functions, we tested whether EBER-1 expression is necessary and sufficient to mediate nucleoplasmic retention of L22. Following demonstration of this, we utilized RNA-protein binding assays and fluorescence localization studies to demonstrate that mutation of the L22 binding sites on EBER-1 prevents L22 binding and inhibits EBER-1-dependent L22 relocalization. Finally, the in vivo consequence of preventing L22 relocalization in EBER-expressing cells was examined in a soft agar colony formation assay. We demonstrate that BL cells expressing mutated EBER-1 RNAs incapable of binding to and relocalizing L22 have significantly reduced capacity to enhance cell growth potential relative to BL cells expressing wild-type EBERs.

**Materials and Methods**

**Cell culture.** Akata Burkitt lymphoma cells, which have been described previously (30), were maintained in RPMI 1640 medium supplemented with 2 mM t-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin. Akata cells stably expressing wild-type EBERs or mutated EBER-1 coexpressed with wild-type EBER-2 were generated by electroporation of Akata cells with pSG5-EBERs or pSG5-mSL1/3/4 and a puromycin expression vector followed by subsequent selection of transfectants in medium containing 0.35 μg of puromycin per ml. 293T and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 4.5 g of glucose per liter, 2 mM L-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin. HeLa-L22 cells, HeLa cells stably expressing GFP fused to L22 (GFP-L22), were generated by FuGENE6 (Roche) transfection of HeLa cells with pcDNA3.1GFP-L22 and subsequent selection of transfectants in medium containing 600 μg of G418 per ml.

**Plasmids and site-directed mutagenesis.** L22 cDNA was generated by reverse transcription-PCR using total RNA isolated from Akata cells. The primers used in amplification were 5'-ATATGGATCTCCATGTGCTCTGTG-3' and 5'-GAT CAGAATTCCTACGTAGGATACGG-3'. Amplified cDNA was digested with BamHI and EcoRI and cloned into digested pcDNA3 to generate pcDNA3-L22. PCR-amplified L22 DNA was then cloned into pcDNA3.1N-TP-GFP-TOPO (Invitrogen) to generate the N-terminal green fluorescent protein (GFP) fusion construct pcDNA3.1GFP-L22. To generate the base biotin acceptor peptide (BAP) vector (pCR3.1BAP), complementary oligonucleotides containing the BAP sequence and the tobacco etch virus protease cleavage sites were annealed (5'ACGTTATAGGCGCCACTAAGCAGATTTCCGAGGGCAGCCCAAGATCGAATGGCCACAGAATCTGGATCAGTACGG-3' and 5'-GATTCCCTGAAAGTGACATCCTGGTTGACCAAATCTTTGCGAGCTACATGTTTCTGGAATCTGTTTATGACGAAATGTTCTGTTGAACTGTAATGACG-3'). The L22 sequence was PCR amplified from pcDNA3-L22 using primers that incorporated 5'-AGCTTATGAGCGGACTCAACGACATTTCGAGGCCCAAAAGATCGAATGGCCACAGAATCTGGATCAGTACGG-3' and 5'-GATTCCCTGAAAGTGACATCCTGGTTGACCAAATCTTTGCGAGCTACATGTTTCTGGAATCTGTTTATGACGAAATGTTCTGTTGAACTGTAATGACG-3'). The amplified L22 sequence was inserted into the XbaI-HindIII sites of pCR3.1BAP to generate pcDNA3.1GFP-L22. To generate pSG5-EBERs, the entire EBV EcoRI-J genomic restriction fragment, containing both EBER-1 and EBER-2 and their transcriptional regulatory elements, was ligated into EcoRI-digested pSG5 (Stratagene). pTER-EBER-1 and pTER-EBER-2 were generated by PCR amplification of the EBER-1 or EBER-2 coding sequence using primers incorporating 5' HindIII sites and cloned into pBluescript II vector (pSG5-EBER-1) and pHSG474 (pSG5-EBER-2). pSG5-EBER-1 and pSG5-EBER-2 were generated by PCR amplification of the EBER-1 or EBER-2 coding sequence using a primer incorporating a 3' HindIII site and cloned into pBluescript II vector (pSG5-EBER-2). 12,000 107 cells per ml in ribosome lysis buffer (20 mM Tris [pH 8.0], 140 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 1% Triton X-100, 0.1 mg/ml cycloheximide). The lysates were homogenized on ice using a Dounce homogenizer and tight pestle. Following centrifugation for 10 min at 12,000 x g, the lysates were overlaid onto a 10 to 50% (wt/vol) sucrose gradient prepared by overlaying 10% buffered sucrose (20 mM Tris [pH 8.0], 140 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 1% Triton X-100, 0.1 mg/ml cycloheximide). The gradients were centrifuged at 35,000 rpm in an SW41 rotor for 160 min at 4°C. Fractions (0.5 ml) were collected using an Iso Fractionator and Foxy Jr. fraction collector. Each fraction was divided into two aliquots for subsequent RNA and protein analysis. Total RNA from gradient fractions was prepared by phenol-chloroform extraction and ethanol precipitation. RNA was fractionated by electrophoresis through a 1% agarose–2 M formaldehyde gel, followed by transfer to GeneScreen Plus hybridization transfer membrane (NEN Life Science). RNA blots were subjected to hybridization to 32P-labeled DNA probe (labeled by random priming), washed, and probed by autoradiography using standard techniques.

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Immobilon-P membrane (Millipore) and immunoblotted using an enhanced chemiluminescence detection system (HyGLO; Denville Scientific). The primary antibodies utilized were anti-L22 (BD Transduction Labs), anti-L23 (gift of H. Lu, Indiana University School of Medicine), and anti-glyceroldehyde-3-phosphate dehydrogenase (Imgenex). Immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies. For reprobing, membranes were stripped of antibody in stripping buffer (62.5 mM Tris-Cl [pH 6.8], 2% SDS, 100 mM 2-mercaptoethanol, 2% NDS) for 30 min at 50°C.

RNA binding assays. To generate in vivo-biotinylated BAP-L22 (BAP fused to L22) in the presence of EBERs for biotin-avidin affinity assays, 20% cells were cotransfected with 1 μg each of pBirA biotin ligase (provided by Adam Geballe, Fred Hutchinson Cancer Research Center), pCR3.1BAP-L22, and EBER expression constructs in the presence of 25 μM biotin. Forty-eight hours posttransfection, the coverslips were UV irradiated on ice for 4.5 min (254 nM) and then lysed in 500 μL NET-N (50 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40). Complete protease inhibitor cocktail (Roche). Following centrifugation to pellet cell debris, the lysates were mixed with 100 μL immobilized avidin beads (Pierce) and incubated at 4°C with rocking for 2 h. The beads were pelleted, separated from the supernatant, washed five times with NET-N, resuspended in 300 μL elution buffer (100 mM Tris [pH 8.0], 150 mM NaCl, 1% SDS, 12.5 mM EDTA), and then heated at 65°C for 15 min. RNA from the supernatant and bound fractions was extracted with phenol-chloroform and ethanol precipitated. Total RNA and 2.5 μg supernatant RNA were loaded onto a 1.2% agarose-2.2 M formaldehyde gel and processed by Northern hybridization using standard protocols.

Fluorescence in situ hybridization-immunofluorescence. HeLa-L22 cells were seeded into 60-mm tissue culture dishes containing coverslips and transiently transfected with EBER expression constructs (Invitrogen; Roche). Forty-eight hours posttransfection, the coverslips were washed in PBS and fixed for either fluorescence localization or combined in situ hybridization and immunofluorescence. For fluorescence localization, the coverslips were fixed in 4% paraformaldehyde for 10 min. For combined in situ hybridization and immunofluorescence, the coverslips were fixed for 10 min by the addition of fixative (3.7% formaldehyde and 10% acetic acid [final concentrations]) directly to the medium, followed by washing in PBS. The cells were permeabilized in 70% ethanol overnight at 4°C and then rehydrated in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min. The coverslips were incubated in hybridization solution (NorthernMax; Ambion) for 1 h at 37°C followed by incubation with biotinylated antisense EBER-1 RNA probe (1 ng per μL of hybridization solution) at 37°C overnight. Biotinylated antisense EBER-1 transcript was generated using the MEGAscript T7 kit and bio-16-UTP (Ambion) according to the manufacturer’s instructions. Unincorporated nucleotides were removed using NucAway columns (Ambion). Following incubation, the coverslips were washed twice in 2× SSC—50% formamide for 30 min and then with PBS for 15 min. The coverslips were blocked for 30 min in 3% bovine serum albumin, incubated in anti-GFP antibody (1:500) (ab290; Abcam), washed in PBS, and then incubated in Alexa Fluor 488-conjugated streptavidin (Roche). The coverslips were mounted in 4',6'-diamidino-2-phenylindole (DAPI) Vector Laboratories), and fluorescence was visualized by using a Zeiss Axioplan inverted fluorescence microscope.

Soft agar growth assays. A total of 10⁷ log-phase Akata cells were suspended in 0.33% soft agar medium (0.33% SeaPlaque agarose in standard RPMI 1640 growth medium minus phenol red) and plated onto a layer containing solidified 0.66% soft agar medium (0.66% SeaPlaque agarose in standard RPMI 1640 growth medium minus phenol red) in six-well plates and maintained at 37°C. The wells were fed weekly with 0.5 ml of 0.33% soft agar medium, and colony formation was monitored for 5 weeks. Colonies were stained overnight with 0.1% crystal violet and destained with 50% methanol prior to imaging.

RESULTS

L22 is relocalized in EBER-expressing cells. In EBV-infected cells, approximately 50% of L22 is associated with EBER RNP (37). Furthermore, L22 has been shown to be relocalized in EBV-infected cells such that nuclear L22 is sequestered in the nucleoplasm, whereas in uninfected cells it is found in nucleoli (37). To confirm these findings in our system and to determine whether EBER expression is sufficient to mediate L22 relocalization, sucrose density gradient analyses were performed on ribosome-containing lysates harvested from EBV-positive, EBV-negative, and EBV-negative EBER-expressing Akata cells. Following fractionation, the RNA and protein contents of each fraction were assessed by Northern and Western blot analysis, respectively. In EBV-negative cells, the majority of L22 was concentrated in fractions 12 to 14 (Fig. 1A, left panels) which, as illustrated by ethidium bromide staining of electrophoresed RNA and by the polysome profile (Fig. 1B), also contain 28S rRNA and correspond to 60S ribosomal subunits. For a control, we also examined an additional large-subunit protein, L23, and found an equivalent fractionation pattern. In contrast, glyceraldehyde-3-phosphate dehydrogenase, which is not a component of ribosomal subunits, was found to fractionate in a diffuse pattern throughout the top portion of the gradient. In EBV-positive cells, by contrast, L22 was primarily concentrated in fractions 2 to 5, where it cofractionated with the EBERs (Fig. 1A, middle panels). The fractionation pattern of L23, however, was unchanged relative to that seen in EBV-negative cells, indicating that EBV-dependent redistribution was specific for L22. To determine whether expression of the EBERs in the context of EBV-negative cells was sufficient to cause the observed change in fractionation, we evaluated EBV-negative Akata cells expressing EBERs. Again, while some L22 was found to cofractionate with 60S ribosomal subunits, a substantial portion of L22 was found to cofractionate with EBERs near the top of the gradient (Fig. 1A and B, right panels), supporting the hypothesis that EBER expression mediates this redistribution.

To confirm that EBER-mediated changes in the fractionation pattern of L22 correlated with alterations in the subcellular localization of L22 in vivo, HeLa cells stably expressing GFP-L22 (HeLa-L22) were transfected with an EBER expression construct and L22 localization was examined. GFP-L22 was localized, as expected, to the nucleoli and cytoplasm of untransfected cells and cells transfected with a control (vector) plasmid (Fig. 2A). In contrast, nuclear GFP-L22 was distinctly relocalized to the nucleoplasm and excluded from nucleoli in cells cotransfected with EBER-1 and EBER-2. As these were transiently transfected cells, we confirmed that the cells in which we observed relocalized L22 were in fact expressing EBER-1. To do so, we used anti-GFP antibody immunofluorescence (red) in combination with EBER-1 RNA in situ hybridization (green). Use of anti-GFP antibody for detection of GFP-L22 was necessary, as the GFP green fluorescence signal was quenched as a result of the fixation conditions required for in situ hybridization. As demonstrated in Fig. 2B, cells in which L22 was excluded from nucleoli expressed EBER-1, while cells displaying normal L22 localization patterns were EBER-1 negative.

Expression of EBER-1 is necessary and sufficient for L22 relocalization. L22 has been shown to interact with EBER-1 in vitro (8, 38, 39), leading to the hypothesis that the observed in vivo relocalization of L22 may be mediated by binding to EBER-1 without additional contribution from EBER-2. To investigate this, we examined the localization of GFP-L22 in HeLa-L22 cells transfected with EBER-1 or EBER-2 alone. When EBER-1 was expressed in the absence of EBER-2, GFP-L22 was relocalized to the nucleoplasm (Fig. 3A, left panel). Transfection of EBER-2 alone did not mediate any observable change in the localization of GFP-L22 (Fig. 3A,
Furthermore, when the EBER-1 equivalent (RhEBER-1) of the EBV-related lymphocryptovirus of rhesus monkey (cercopithecine herpesvirus 15) was expressed, relocation of L22 was also observed (Fig. 3A, right panel). As depicted in Fig. 3B, this RNA shares 72% nucleotide identity with EBER-1, with a completely conserved stem-loop III L22 binding site and a moderately well conserved stem-loop I site, and would be predicted to be bound by two molecules of L22 (27).

To further evaluate the contribution of EBER-1 to L22 relocation in vivo, we generated a panel of EBER-1 expression constructs in which one, two, or all three L22 binding sites (designated mSL3, mSL3/4 and mSL1/3/4, respectively) were mutated to disrupt L22 binding (8) (Fig. 4A). These mutations were generated based on the works of Toczyski and Steitz (38) and Dobbelstein and Shenk (6) and were designed to have as little impact on RNA structure, stability, and expression as possible. Specifically, mutational analyses of stem-loop III have shown that mutation of the conserved U residue at the 3′ end of the loop and the unpaired U residue in the stem reduce L22 binding to 15 to 17% of the wild type (38). Furthermore, we utilized the previously defined L22 binding consensus sequence consisting of a stem-loop structure with a G-C base pair at the base of a 5- to 7-nucleotide loop with a U residue at the 3′ end of the loop as a guide for our specific mutations in stem-loops I and IV (6). In addition to generating constructs in which mutated EBER-1 was expressed alone, we also constructed expression plasmids in which wild-type EBER-2 was coexpressed. Each construct was tested for expression by transient middle panel). Furthermore, when the EBER-1 equivalent (RhEBER-1) of the EBV-related lymphocryptovirus of rhesus monkey (cercopithecine herpesvirus 15) was expressed, relocation of L22 was also observed (Fig. 3A, right panel). As depicted in Fig. 3B, this RNA shares 72% nucleotide identity with EBER-1, with a completely conserved stem-loop III L22 binding site and a moderately well conserved stem-loop I site, and would be predicted to be bound by two molecules of L22 (27).

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transfection into 293T cells and subsequent Northern blot analysis (Fig. 4B). Mutation of EBER-1 did not substantially alter the expression level of EBER-1 and also had no negative downstream effect on EBER-2 expression. Additionally, the ability of La to bind these EBER-1 mutants was unaffected, suggesting that gross changes in RNA structure did not occur as a result of our mutations. To ensure that mutation of EBER-1 stem-loops successfully reduced or eliminated binding by L22, binding of L22 to mSL3, mSL3/4, and mSL1/3/4 was assessed by biotin-avidin affinity assay. In this assay, a 17-amino-acid biotin acceptor peptide was fused to the N terminus of L22 and biotinylation was accomplished in vivo using a coexpressed bacterial biotin ligase. 293T cells were transiently cotransfected with the relevant expression constructs, UV cross-linked, and lysed to generate whole-cell extracts. Biotinylated BAP-L22 was captured on avidin beads, and co-captured RNA was isolated and analyzed by Northern blotting (Fig. 4C). While EBER-1 and mSL3 RNAs were efficiently captured with L22, as with wild-type EBER-1 (Fig. 2A), the majority of endogenous L22 in mSL1/3/4-expressing cells was found in fractions 10 to 12, which also contained 28S rRNA and corresponded to 60S ribosomal subunits and no longer cofractionated with EBER-1 RNA (Fig. 5B and C). The finding that L22 was incorporated into ribosomal subunits in mSL1/3/4-expressing cells is in complete agreement with the finding that GFP-L22 is not relocalized in these cells (Fig. 5A).

**EBER-1-mediated enhancement of cell growth potential is dependent on interaction with L22.** The tumorigenic potential of EBV-negative BL cells expressing EBERs has been well-established (17, 31). However, the mechanism by which one or both EBERs confer tumorigenic potential is unknown. Given the high-affinity interaction of EBER-1 with L22 in vivo and the subsequent relocalization of L22 out of 60S ribosomal subunits, we hypothesized that EBER-1-mediated relocalization of L22 might contribute to tumorigenicity. To test the requirement for this interaction, we utilized a soft agar growth assay to assess the growth potential of A.2EBER cells or A.2mSL1/3/4 cells. EBV-negative (A.2) and EBV-positive (A.5) cells were used as a negative and positive control, respectively. As expected, A.5 cells were able to grow very efficiently in soft agar, producing numerous large colonies per well (Fig. 6A). In contrast, A.2 cells were unable to grow efficiently in soft agar, producing significantly fewer and substantially smaller colonies. A.2EBER cells expressing wild-type EBER-1 and EBER-2, although not as effective as A.5 cells, resulted in...
increased colony number and considerably larger colonies than the colonies formed by A.2 cells. In contrast, A.2mSL1/3/4 cells produced virtually no macroscopic colonies, demonstrating that mutation of EBER-1 to prevent L22 binding and relocalization altered the growth-promoting properties of EBER-1. Identical results were achieved using cell lines established in an independently derived EBV-negative Akata cell line 3F2 (Fig. 6B). Thus, inhibition of the EBER-1–L22 interaction, despite maintenance of EBER-2 expression, is sufficient to prevent enhancement of cell growth potential in BL cells.

**DISCUSSION**

Although the EBERs are known to interact with a number of cellular proteins in vitro, the majority of these interactions have not been substantiated in vivo and the functional consequences of these interactions have not been elucidated. Here we focused on the interaction between EBER-1 and L22 and the consequences thereof with respect to the demonstrated tumorigenic properties of the EBERs. L22 is known to bind three sites on EBER-1 (6, 8, 38). Given the high level of EBER expression in latently infected cells (greater than \(5 \times 10^6\) copies per cell) (23), this likely results in the sequestration of a substantial fraction of available L22 in EBER RNPs. In agreement with this, previous studies have demonstrated that \(~50\%\) of L22 is found in association with EBER RNPs in EBV-infected cells (37). Furthermore, L22 was previously shown to be relocalized from nucleoli to the nucleoplasm in EBV-infected cells (37). Taken together, these previously reported findings suggest that the L22–EBER-1 interaction does occur in vivo and likely has a functional role in latently infected cells. The data presented here substantiate this hypothesis.

In agreement with previous findings, we found that L22 was redistributed out of 60S ribosomal subunit-containing fractions when protein lysates generated from EBV-infected BL cells were analyzed by sucrose density gradient fractionation (Fig. 1). A substantial portion of L22 was instead found to cofractionate with the EBERs in these cell lysates. Similarly, in EBV-negative EBER-expressing BL cells, L22 was shown to be sufficient to alter the subcellular localization of L22, resulting in relocalization of L22 from nucleoli to the nucleoplasm (Fig. 2). Thus, the EBERs, in the absence of any additional EBV latency gene products, are sufficient to mediate in vivo L22 relocalization. Further, we

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demonstrate that expression of EBER-1 alone is sufficient to mediate this relocalization, while expression of EBER-2 alone does not alter L22 localization (Fig. 3). This is in agreement with the findings of Toczyski and Steitz in which L22 was originally observed to bind EBER-2 in vitro (39) but was later found to be incapable of binding EBER-2 (38). For further confirmation of the ability of EBER-1 to bind and relocalize L22 in vivo and to evaluate the sequence requirements for L22 relocalization, we analyzed the localization of GFP-L22 in cells expressing the EBER-1 equivalent from the EBV-related rhesus monkey lymphocryptovirus, RhEBER-1. This RNA shares 72% sequence identity with EBER-1, with complete conservation of stem-loop III and 85% identity with stem-loop I (Fig. 3B). The L22 binding site in stem-loop IV, however, is not well conserved. As shown in Fig. 3A, RhEBER-1 was capable of relocalizing GFP-L22 from nucleoli to the nucleoplasm, demonstrating that the presence of two of three L22 binding sites is sufficient to mediate relocalization. In agreement with this, EBER-1 RNA in which two of three L22 binding sites are intact (mSL3) was also found to be capable of binding and relocalizing L22, while EBER-1 RNAs in which two (mSL3/4) or all three (mSL1/3/4) L22 binding stem-loops are mutated were not able to bind or relocalize L22 (Fig. 4 and 5).

Having established that EBER-1 mediates the relocalization of L22, we next tested whether binding and relocalization of L22 are required to mediate functions attributable to EBER-1, particularly with respect to the ability of the EBER RNAs to promote tumorigenicity of Akata BL cells. As one measure of this, the growth capacity of EBV-negative Akata cells stably expressing either wild-type EBER-1 and EBER-2 or the triply mutated EBER-1 RNA (mSL1/3/4) in combination with wild-type EBER-2 was assessed using a soft agar colony formation assay. The results from this assay (Fig. 6) reveal that L22 binding to EBER-1 is a key event in enhancing the tumorigenic potential of these cells. Furthermore, the lack of growth enhancement in cells expressing mSL1/3/4, despite the presence of wild-type EBER-2, leads to the conclusion that EBER-1 is a critical contributor to tumorigenicity in EBV-infected BL cells. The significance of this is twofold. First, it was previously reported that EBER-2 and not EBER-1 is critical for the growth transformation of cord blood lymphocytes into lymphoblastoid cell lines (46). Our data, however, strongly support a role for EBER-1 in tumor formation in the context of type I latency (characteristic of BL), although it does not formally rule out a contributory role for EBER-2 as well. This apparent discrepancy with respect to the contribution of each EBER may be explained by considering that the EBERs may function differently during primary growth transformation of lymphoblastoid cell lines compared to established latency in BL cells, which have a more restricted pattern of latent gene expression and a different cellular differentiation status (28). Furthermore, expression of additional latency-associated gene products during growth transformation may mask a role for the EBERs which is exposed only during restricted latency. The difference is also exemplified by data from Laing et al. demonstrating that expression of EBER-1 alone in murine fibroblast 3T3 cells was sufficient to mediate the growth of these cells in soft agar, although no consistent growth-promoting
effect was observed in mouse tumor assays (18). Although these data clearly suggest that EBER-1 does play a role in enhancing cellular growth, use of murine fibroblast cells is not an ideal model to study the growth-promoting phenotype of the EBER RNAs, particularly when the contributions of additional genetic alterations (such as the c-MYC translocation characteristic of BL cells) are considered. Second, our data provide a direct demonstration of an in vivo interaction between EBER-1 and a cellular protein as well as the functional consequences of preventing this interaction. We cannot formally rule out the possibility that the decreased growth capacity of our mutant is the result of either substantial alterations in EBER-1 RNA structure or prevention of other currently undefined interactions. However, as we altered only 2 nucleotides per binding site and designed our mutations to conserve RNA structure (as predicted by RNAfold software) as much as possible, we believe we have minimized these possibilities. Furthermore, global changes in structure or stability are unlikely, given the maintenance of high levels of expression and the capacity of L22 to bind mSL3 (Fig. 4) and the capacity of La to bind mSL1/3/4 (data not shown). Subsequent studies will be needed to determine whether the loss of enhanced growth potential seen in soft agar assays will translate to a loss of enhancement of tumorigenic potential in tumor induction assays in mice. While tumorigenicity is a multifactorial process and EBER-1 alone may perhaps not be fully sufficient to enhance tumorigenic potential, the loss of the EBER-L22 interaction may well be enough to prevent tumor formation.

How binding and relocalization of L22 during EBV infection influence viral replication and maintenance of viral latency remains to be elucidated. As the cellular role of L22 remains essentially undefined, this is a difficult question to address. There are clearly multiple possibilities, including the following: the binding and relocalization of L22 may serve some direct functional role in EBV latency, or sequestration of L22 may prevent or modulate some cellular function which may have negative consequences with respect to viral replication or maintenance within the cell. Data demonstrating that L22 is relocated to viral replication compartments during HSV type 1 infection (21, 22) and that L22 interacts with the HCV genome and enhances IRES-mediated translation (44) suggest that L22 plays an active role during virus infection, perhaps by increasing the translation of a subset of viral and/or cellular RNAs. Alternatively, L22 may serve in a regulatory capacity and its relocalization during viral infection may be a way to remove a regulatory roadblock. The latter is supported by data demonstrating that L22 is not required for basal levels of translation (19) nor is the knockout of L22 lethal in mice (1), suggesting that L22 does serve a regulatory and nonessential role within cells. While tumor formation is obviously not a primary goal of these viruses, translation and cell growth are intimately intertwined processes, and perturbations in regulation, such as may occur during viral infection, combined with additional genetic alterations, such as the c-MYC translocation in BL cells, may ultimately combine to lead to an increased tumorigenic capacity.

This is the first report demonstrating a mechanism by which the EBERs may influence the tumorigenicity of Akata BL cells. This is significant, as the EBER RNAs are the only EBV latency gene products consistently expressed in BL tumors known to directly influence tumorigenicity in BL. While our data do not rule out a role for EBER-2, it does clearly dem-
onstrate a role for EBER-1 and its ability to interact with L22 in enhancing the growth potential of EBV-negative Akata BL cells.

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