Epstein-Barr virus noncoding RNAs are confined to the nucleus, whereas their partner, the human La protein, undergoes nucleocytoplasmic shuttling

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The Epstein-Barr virus (EBV) noncoding RNAs, EBV-encoded RNA 1 (EBER1) and EBER2, are the most abundant viral transcripts in all types of latently infected human B cells, but their function remains unknown. We carried out heterokaryon assays using cells that endogenously produce EBERs to address their trafficking, as well as that of the La protein, because EBERs are quantitatively bound by La in vivo. Both in this assay and in oocyte microinjection assays, EBERs are confined to the nucleus, suggesting that their contribution to viral latency is purely nuclear. EBER1 does not bind exportin 5; therefore, it is unlikely to act by interfering with microRNA biogenesis. In contrast, La, which is a nuclear phosphoprotein, undergoes nucleocytoplasmic shuttling independent of the nuclear export protein Crm1. To ensure that small RNA shuttling can be detected in cells that are negative for EBER shuttling, we demonstrate the shuttling of U1 small nuclear RNA.

Introduction

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is associated with several human malignancies (Kieff and Rickinson, 2002). The most abundant of the few viral genes (4–11) expressed during EBV latency are the noncoding RNAs, EBV-encoded RNA 1 (EBER1) and EBER2, which are expressed at $\sim 5 \times 10^6$ per cell (Lerner et al., 1981). The EBERs, which are ~170 nts in length, are transcribed by RNA polymerase III and assembled into nuclear ribonucleoprotein particles containing the La protein (Lerner et al., 1981). EBER1 also binds the ribosomal protein L22 and relocalizes a large fraction of the free cellular L22 to the nucleoplasm in EBV-positive cell lines (Toczyski et al., 1994).

The physiological function of EBERs has remained elusive. Although not necessary for EBV-mediated immortalization of B cells in vitro, EBERs promote cellular transformation in various systems (Takada and Nanbo, 2001; Yajima et al., 2005) and inhibit apoptosis that is induced by α interferon (Nanbo et al., 2002; Ruf et al., 2005). These activities have been attributed to the binding and inhibition of the double-stranded RNA–dependent protein kinase R (PKR; Sharp et al., 1993; Takada and Nanbo, 2001; Nanbo et al., 2002), despite multiple studies that have found that EBERs are nucleoplasmic (Howe and Steitz, 1986; Barletta et al., 1993), whereas PKR and its well documented effect on translation initiation are cytoplasmic (Takizawa et al., 2000). Recent results (Ruf et al., 2005; Wang et al., 2005) indicate that EBERs do not inhibit PKR activity in vivo when cells are challenged with various PKR stimuli.

The La protein is an abundant nuclear phosphoprotein that facilitates the correct folding and maturation of RNA polymerase III transcripts through its specific association with the short polyU sequence at their 3′ ends (Wolin and Cedervall, 2002). The human La protein has also been reported to play a role in the translational regulation of some messages (Costa-Mattioli et al., 2004), including those that harbor unique terminal oligopyrimidine–rich motifs at their 5′ ends. Indeed, an unphosphorylated form of La has been detected that is specifically bound to terminal oligopyrimidine–containing mRNAs (Intine et al., 2003). Previously, the idea that La actively shuttles between the nucleus and cytoplasm was supported only by observations of its localization in drug-treated cells (Bachmann et al., 1989).

We used heterokaryon and other assays to define the cellular trafficking of the EBERs and the La protein. We find that the EBERs are confined to the cell nucleus, whereas the endogenous La protein undergoes nucleocytoplasmic shuttling. As a control for the shuttling of small RNAs, we report that

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Abbreviations used in this paper: DIG, digoxigenin; EBER, EBV-encoded RNA; EBV, Epstein-Barr virus; Exp5, exportin 5; hnRNP, heterogeneous nuclear ribonucleoprotein; HEK, human embryonic kidney; LMB, leptomycin B; PKR, protein kinase R; snRNA, small nuclear RNA.

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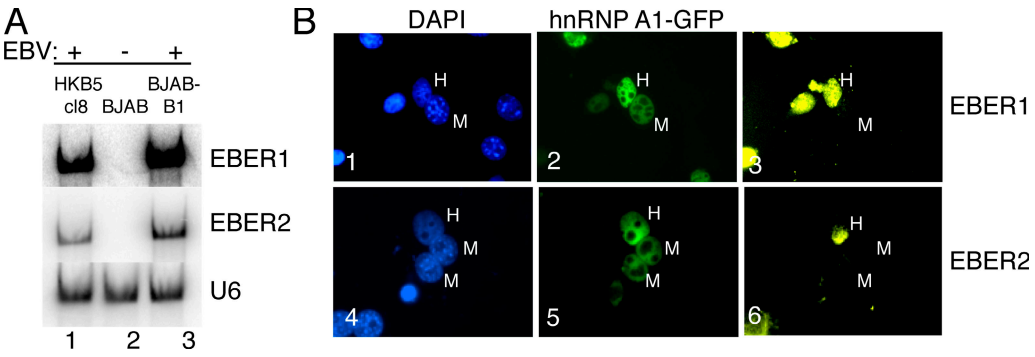

Figure 1. **EBER1 and EBER2 do not shuttle in HKB5cl8 cells.** (A) EBER1 and EBER2 expression in HKB5cl8 cells. A Northern blot of 5 μg total RNA from HKB5cl8 (lane 1), BJAB (lane 2), and BJAB-B1 (lane 3) was sequentially probed for EBER1, the U6 loading control, and EBER2. (B) Lack of EBER nucleocytoplasmic shuttling. Heterokaryons were prepared by fusing HKB5cl8 cells transfected with a plasmid producing hnRNP A1-GFP and mouse NIH3T3 cells for 6–7 h in the presence of cycloheximide. Heterokaryons were identified by the shuttling of hnRNP A1-GFP (2 and 5, green) into mouse nuclei, identified by punctate DAPI staining (1 and 4). Human (H) and mouse (M) nuclei of the heterokaryons are labeled. A total of 14 heterokaryons were analyzed. Endogenous EBER1 (3) and EBER2 (6) were detected using DIG-labeled probes (yellow).

spliceosomal U1 small nuclear RNA (snRNA) does traffic to the other nucleus in human/mouse heterokaryons that are negative for EBER shuttling.

Results and discussion

We initially undertook heterokaryon-shuttling experiments (Borer et al., 1989) with the well characterized EBV-transformed suspension cell line, BJAB-B1. Because these cells did not adhere well to glass slides, we switched to the human HKB5cl8 cell line, which is a hybrid between human embryonic kidney 293S (HEK293S) cells and 2B8 cells, which are an EBV-positive Burkitt's lymphoma B-cell line (Cho et al., 2002; El-Guindy et al., 2002). HKB5cl8 cells not only attach to the glass slides but are morphologically superior in that the nucleus and cytoplasm can be readily distinguished. By RT-PCR analyses (unpublished data), HKB5cl8 cells establish type I latency (Kieff and Rickinson, 2002) that is characteristic of Burkitt's lymphoma cells. We also performed Northern blot analyses and found that EBER1 and EBER2 are expressed in HKB5cl8 (Fig. 1 A, lane 1) at levels only two- to threefold lower than in BJAB-B1 cells (Fig. 1 A, lane 3).

To test whether the endogenously expressed EBERs shuttle in and out of the nucleus, heterokaryons were formed by

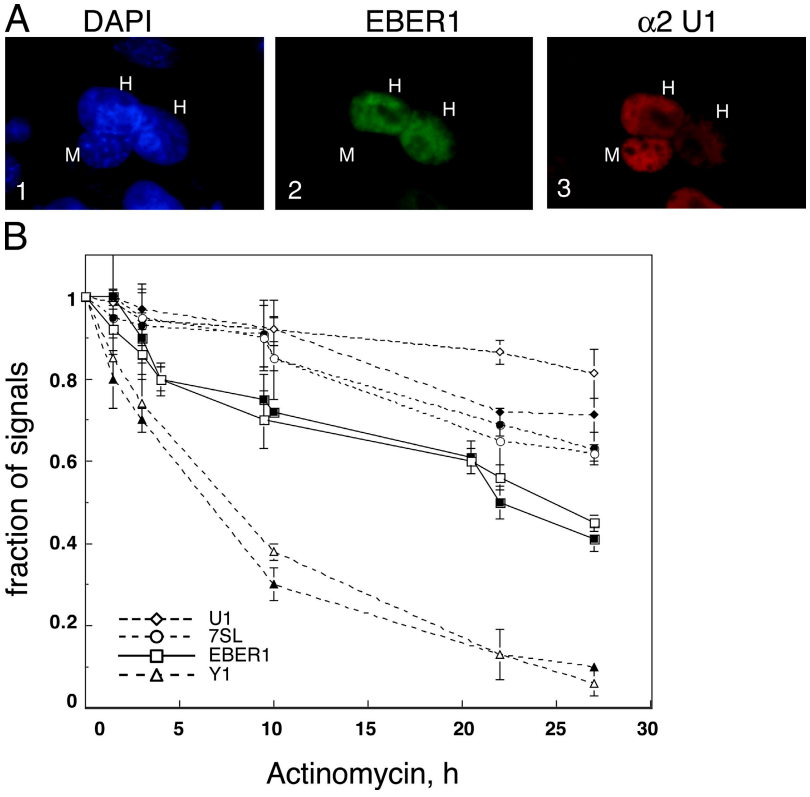

Figure 2. **Detection of α2 U1 RNA shuttling.** (A) Heterokaryons were prepared as in Fig.1, except that an α2 U1–expressing plasmid, rather than an hnRNP A1-GFP–expressing plasmid, was transfected into HKB5cl8 cells and no cycloheximide was added. The two human (H) and one mouse (M) nuclei in the heterokaryon are labeled as identified by DAPI (1). The α2 U1 RNA (red) shuttled into the mouse nucleus (3), whereas the endogenous EBER1 (green) did not (2). A total of seven heterokaryons were analyzed. (B) Turnover rates of EBER1 compared with U1, 7SL, and Y1 RNAs. HKB5cl8 (open symbols) and BJAB-B1 (closed symbols) cells were treated with actinomycin D, and the indicated RNAs were detected by Northern blotting. Each time point is the mean of three independent experiments, with error bars indicating the SD.

fusing human HKB5cl8 cells with mouse NIH3T3 cells (Borer et al., 1989). The human cells had previously been transfected with plasmids expressing the shuttling heterogeneous nuclear ribonucleoprotein (hnRNP) A1-GFP protein (Pinol-Roma and Dreyfuss, 1991); heterokaryons were identified by the appearance of hnRNP A1-GFP in both the human and the mouse nuclei (Fig. 1 B, 2 and 5). Mouse nuclei were readily distinguished by punctate DAPI staining, which replicates the species-specific nuclear staining difference previously reported for Hoechst dye (Moser et al., 1975).

EBER1 and EBER2 were detected by in situ hybridization using DIG-labeled antisense DNA oligonucleotides. These probes were complementary to the 3′ half of the EBERs, but not to regions including conserved polymerase III promoter elements A and B (which may explain the unique report of cytoplasmic localization of EBERs [Schwemmle et al., 1992]). As shown in Fig. 1 B (3–6), EBERs remained in the human nuclei and did not shuttle into the mouse nuclei during the 6-h incubation. HEK293 cells transiently expressing EBERs also did not exhibit shuttling (unpublished data); titration of the EBER-expressing plasmids showed that in situ hybridization signals would have been detected even with RNA levels $<10\%$ (as observed by Northern blotting; unpublished data).

To ensure that the nucleocytoplasmic shuttling of RNA, as well as of protein molecules, could be observed in our assays, we examined U1 snRNA. We used a modified human U1 RNA, α2 U1 RNA, in which the first 20 nts are significantly different from either the human or mouse U1 snRNA (Yuo and Weiner, 1989). This U1 RNA is functional in vivo (Yuo and Weiner, 1989) and, therefore, is expected to follow the wild-type maturation pathway, which involves export to the cytoplasm before assembly with Sm proteins and reimport into the nucleus (Feeney et al., 1989; Mattaj et al., 1993). For heterokaryon assays, we transfected an α2 U1 RNA–expressing plasmid into HKB5cl8 cells and visualized the RNA with probes that hybridize specifically to the modified region. We observed α2 U1 RNA in both the human and the mouse nuclei (Fig. 2 A, 3), indicating that α2 U1 moves out of and back into the nuclei of somatic human cells. Importantly, in the same heterokaryons where U1 shuttling was observed, endogenous EBER1 was confined to the human nuclei (Fig. 2 A, 2); the same result was obtained with a longer 12-h incubation (not depicted), as opposed to a 6-h incubation. In the RNA-shuttling assays, cycloheximide was omitted, ruling out the possibility that the lack of EBER1 shuttling is protein synthesis-dependent. EBER2 was also tested, but we were unable to find a hybridization temperature that would allow simultaneous detection of EBER2 and α2 U1 RNAs (unpublished data).

The absence of EBER signals from mouse nuclei in heterokaryons could be attributable to the rapid cytoplasmic degradation of RNA once it is exported from the human nucleus. Therefore, we compared the turnover rates of EBER1 and other small RNAs; 7SL and Y1 RNAs are both cytoplasmic and transcribed (like EBERs) by RNA polymerase III, whereas U1 RNA is a nuclear RNA polymerase II product. After the addition of actinomycin D to HKB5cl8 or BJAB-B1 cells, EBER1 exhibited an apparent half-life of 25–30 h (Fig. 2 B), which is

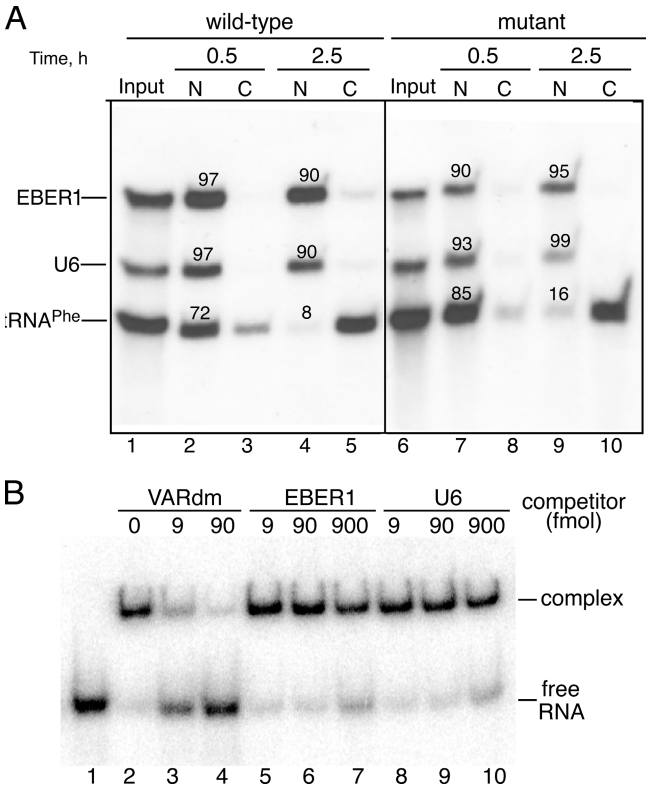

Figure 3. **Lack of oocyte nuclear export and Exp5 binding by EBER1.** (A) Oocyte microinjections. A mixture of T7-transcribed, α-[^{32}P]UTP–labeled U6, tRNA^{Phe}, and either wild-type EBER1 or mutant EBER1 lacking its 3′ polyU terminus (0.5–1 fmol per oocyte) was microinjected into the geminal vesicles of whole *X. laevis* oocytes. After either a 0.5-h (wild-type, lanes 2 and 3; mutant, lanes 7 and 8) or 2.5-h (wild-type, lanes 4 and 5; mutant, lanes 9 and 10) incubation at RT, 5–6 oocytes were fractionated. RNAs extracted from the nuclear (N), cytoplasmic (C), or total fractions were resolved on a urea polyacrylamide gel and visualized by autoradiography. The percentages of RNA in the nucleus are indicated. (B) Electrophoretic mobility shift assays performed on binding reactions containing 4.5 fmol of labeled VARdm RNA, 1 pmol of recombinant Exp5 (lanes 2–10) and RanQ69LGTP (see Materials and methods), and the indicated amounts of unlabeled competitor RNA: VARdm (lanes 3 and 4), EBER1 (lanes 5–7), or U6 (lanes 8–10). Lane 1 contained no protein.

significantly greater than Y1 (apparent half-life of 7 h; Rutjes et al., 1999) and only slightly less than 7SL and U1 (Fury and Zieve, 1996). Because shuttling was observed for U1, but not for EBER1 (Fig. 2 A), and they are both extremely stable RNAs, rapid cytoplasmic degradation cannot explain the lack of EBER1 shuttling.

To confirm nuclear retention in another system, we performed *X. laevis* oocyte microinjection assays using in vitro–transcribed EBER1, U6, and tRNA^{Phe}. 2.5 h after injection, almost all of the positive nuclear export control, tRNA^{Phe}, was detected in the cytoplasmic fraction (Fig. 3 A, lanes 1, 4, and 5). In contrast, EBER1 remained in the nucleus, as did the negative export control, U6 RNA (Fig. 3 A, lanes 1, 4, and 5). To address whether La is responsible for the nuclear retention of EBER1, we repeated the microinjection assays using an EBER1 mutant lacking its 3′ polyU tail (required for stable La binding); the terminal nts were changed from $UGUUUU_{OH}$ to $GAACAC_{OH}$. As expected, this EBER1 mutant exhibits eightfold reduced binding to La, based on immunoprecipitation using BJAB

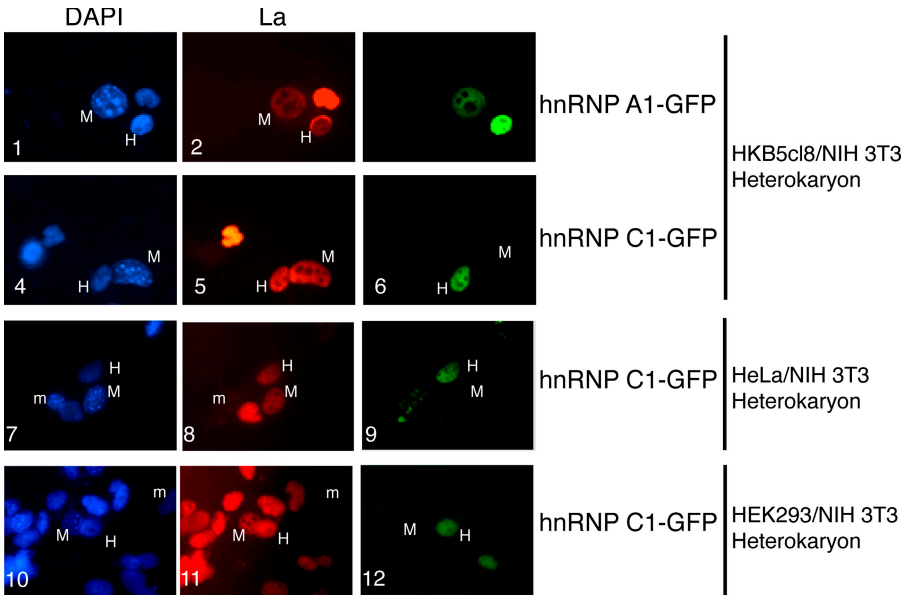

Figure 4. **Human La protein undergoes nucleocytoplasmic shuttling in multiple cell lines.** Heterokaryons were made by fusing HKB5cl8 (1–6), HeLa (7–9), or HEK293 (10–12) cells, which were transfected with a plasmid producing either the shuttling hnRNP A1-GFP (1–3) or the nonshuttling hnRNP C1-GFP (4–12), with mouse NIH3T3 cells. After 4 h, human La protein (red) was detected with a monoclonal anti-La antibody specific for human La, which is demonstrated by the absence of signal in the unfused mouse cells labeled m (7, 8, 10, and 11). Human (H) and mouse (M) nuclei are labeled as in Fig. 1. More than 20 heterokaryons were analyzed.

cell extracts (unpublished data). 2.5 h after injection, mutant EBER1 remained in the oocyte nucleus, whereas most $tRNA^{Phe}$ was in the cytoplasm (Fig. 3 A, lanes 6, 9, and 10). Therefore, it is unlikely that La is responsible for the nuclear retention of EBER1.

Finally, to probe why EBERs are not exported, we performed in vitro exportin 5 (Exp5)–binding assays. Exp5 mediates nuclear export of premicroRNAs and adenovirus noncoding RNA VAI by binding to a terminal stem (Gwizdek et al., 2001, 2003; Brownawell and Macara, 2002; Yi et al., 2003; Lund et al., 2004), which is also proposed to exist in EBER1 (Gwizdek et al., 2001). Using an electrophoretic mobility shift assay, we performed competition experiments to ask if EBER1 can displace the VARdm RNA (Gwizdek et al., 2003) from recombinant Exp5. Although unlabeled VARdm efficiently competed with the Exp5-bound substrate (Fig. 3 B, lanes 2–4), neither EBER1 (Fig. 3 B, lanes 5–7) nor the negative control U6 RNA (Fig. 3 B, lanes 8–10) significantly displaced the probe, even at 200-fold excess. The same EBER1 preparation was active in binding its protein ligand L22 (Fok et al., 2006). Thus, lack of binding to an export receptor may explain why EBER1 is not exported from the nucleus. Moreover, it is unlikely that EBERs function by interfering with host cell microRNA biogenesis, which is consistent with observations (unpublished data) that the level of let-7 microRNA is not altered by the presence of EBERs.

Our strategy in investigating the cellular trafficking of EBERs included testing if its obligatory protein partner La undergoes nucleocytoplasmic shuttling. A typical EBV-infected cell harbors $\sim 5 \times 10^6$ copies of each EBER (Lerner et al., 1981), whereas most human cells express $\sim 2 \times 10^7$ molecules of La protein (Wolin and Cedervall, 2002). Thus, even though EBERs do not shuttle, the La protein could. To examine La protein shuttling, HKB5cl8 cells were transfected with plasmids expressing either the shuttling hnRNP A1-GFP or the nonshuttling hnRNP C1-GFP as controls. After fusion with mouse NIH3T3 cells for 4 h, endogenous human La protein was detected using a monoclonal anti-La antibody that does not cross

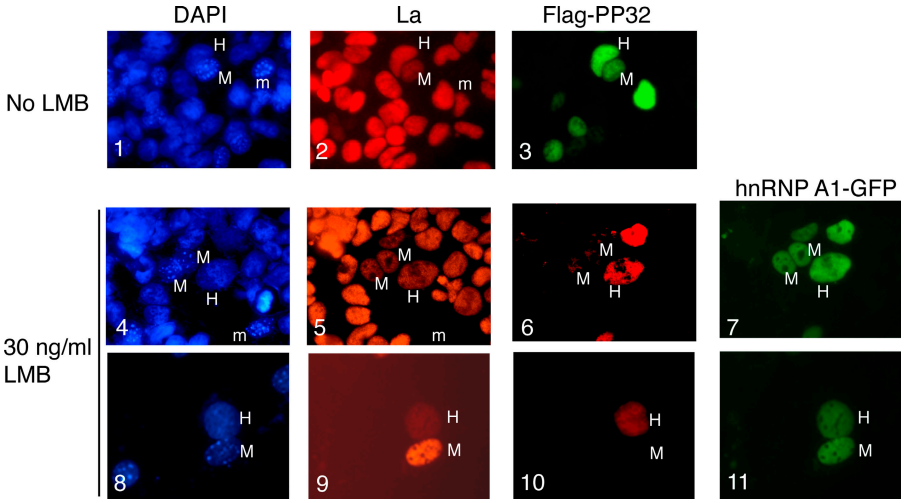

Figure 5. **Shuttling of the human La protein is not blocked by LMB.** Heterokaryons were made by fusing HEK293 cells transfected with plasmids producing Flag-PP32 (1–3) or Flag-PP32 and hnRNP A1-GFP (4–11) with mouse NIH3T3 cells as described in Fig. 4, except that no LMB (1–3) or 30 ng/ml LMB (4–11) was included during fusion. Detection of the human La protein and labeling of the nuclei are as described in Fig. 4. La is shown in red (2) or in pseudocolor orange (5 and 9). Flag-PP32 is in green (3) or in pseudocolor deep red for infrared (6 and 10). 2 of 11 heterokaryons analyzed are shown.

react with mouse La protein (unpublished data; Wolin, S., personal communication), demonstrated by the lack of nuclear staining of unfused mouse cells, labeled m in Fig. 4 (panels 7, 8, 10, and 11) and Fig. 5 (panels 1, 2, 4, and 5).

Fig. 4 clearly shows that La shuttled from the human nucleus into the mouse nucleus (Fig. 4, panel 2), mimicking the shuttling of hnRNP A1-GFP in the same heterokaryon (Fig. 4, panel 3). Inclusion of cycloheximide during the fusion period ruled out the possibility that newly synthesized human La protein was imported into mouse nuclei. Although the nonshuttling hnRNP C1-GFP remained in the human nucleus (Fig. 4, panel 6), the human La protein moved into the mouse nucleus (Fig. 4, panel 5). We then confirmed that La nucleocytoplasmic shuttling is not cell-type specific by repeating the experiments with nonvirally infected human cells, HeLa or HEK293. Again, the nonshuttling hnRNP C1-GFP remained in the human nuclei and the human La protein shuttled into the mouse nucleus in both kinds of heterokaryons (Fig. 4, panels 8 and 9 and 11 and 12, respectively). We conclude that La, which is predominantly nuclear in multiple types of mammalian cells (Wolin and Cedervall, 2002), has the capacity to exit and return to the nucleus.

Next, we asked whether La protein is exported via the Crm1 nuclear export receptor because a human La protein lacking its putative nuclear retention element had been reported to accumulate in the cytoplasm, but to be retained in the nucleus in the presence of the Crm1 inhibitor leptomycin B (LMB; Intine et al., 2002). To ensure that LMB inhibits Crm1 in heterokaryons of HEK293 cells and NIH3T3 cells, we included as a control PP32, which is a known shuttling protein whose nuclear export is Crm1-dependent (Brennan et al., 2000). We transfected HEK293 cells with a plasmid-expressing Flag-PP32 and, as expected, observed that both La and Flag-PP32 shuttled from the human to the mouse nucleus (Fig. 5, panels 2 and 3, respectively). In the presence of 30 ng/ml LMB, Flag-PP32, but not La, movement was inhibited (Fig. 5, panels 5, 6, 9, and 10). In this experiment, hnRNP A1-GFP, which does not require Crm1 for nuclear export (Brennan et al., 2000), was coexpressed to identify the hybrid cells (Fig. 5, panels 7 and 11). Because inhibition of Crm1 blocked the shuttling of Flag-PP32, but not of intact La protein, we conclude that the nuclear export of full-length La is either Crm1 independent or that La is exported by more than one pathway. Further studies are needed to resolve the pathways and whether the phosphorylation state of La regulates its shuttling activity (Intine et al., 2003).

Because EBERs do not exit the nucleus of either human cells (Fig. 1) or *Xenopus laevis* oocytes (Fig. 3; even in the absence of a La binding site), it is not the La protein, but rather some other feature of their RNA structure, that retains the EBERs in the nucleus of EBV-infected cells. We tested the prediction, based on the presence of a terminal stem, that EBERs might bind and interfere with the activity of Exp5 (Gwizdek et al., 2001), which is limiting in the case of pre-microRNA export (Yi et al., 2003). Our findings suggest that EBERs do not function in this way, but instead participate in some other exclusively nuclear process that enhances the expression of several growth factors, including insulin-like growth factor I, interleukin-9, and interleukin-10 (Kitagawa et al., 2000; Iwakiri et al., 2003; Yang et al., 2004) in EBV-transformed cells. Whether these consequences represent an active function of the EBER particles or arise through partial sequestration of La, ribosomal protein L22, or some other protein partner remains to be determined.

Materials and methods

Cell culture and heterokaryon assays

HKB5cl8, BJAB, and BJAB-B1 cells were grown in RPMI 1640 medium (Invitrogen) containing 10% FBS. HEK293 cells were grown in DME (Invitrogen) containing 10% FBS. NIH3T3 cells were grown in DME containing 10% calf serum.

10^5 HKB5cl8 cells were transfected with 2 μg hnRNP A1-GFP, hnRNP C1-GFP (both gifts from G. Dreyfuss, University of Pennsylvania School of Medicine, Philadelphia, PA), or pα2U1 (Yuo and Weiner, 1989) plasmid using 6 μl TransIT reagent (Mirus) for ∼40 h on coverslips. 10^5 HeLa cells were transfected with 2 μg hnRNP C1-GFP plasmid using 6 μl Lipofectamine reagent (Invitrogen) for ∼40 h on coverslips. 10^5 HEK293 cells were transfected with 2 μg hnRNP C1-GFP plasmid, 2 μg Flag-PP32 plasmid, or 1 μg each of hnRNP A1-GFP and Flag-PP32 plasmids using 6 μl TransIT reagent (Invitrogen) for ∼40 h on coverslips.

For heterokaryon assays, 10^5 mouse NIH3T3 cells were added to the transfected human cells described in the previous paragraph and allowed to seed on coverslips for 3 h. 100 μg/ml cycloheximide and 30 ng/ml LMB, as indicated in the figure legends, were added to the medium to block protein synthesis for 30 min, and the cells were fused using 50% PEG 3350/PBS for 2 min at RT. Cells were then washed in PBS three times and incubated in medium containing 100 μg/ml cycloheximide and 30 ng/ml LMB, as indicated in the figure legends, for 4–7 h to allow shuttling. The lack of signals in mouse nuclei for hnRNP C1-GFP and for Flag-PP32 when LMB was added indicates that cycloheximide effectively shut down translation. Cells were fixed in 4% formaldehyde/PBS and were processed for either in situ hybridization or immunofluorescence, as described in the following sections. Light microscopy and the appearance of shuttling proteins in the mouse nuclei were used to identify heterokaryons. Fluorescence images were photographed using a digital charge-coupled device camera (model C4742-95-12; Hamamatsu) through a microscope (Axioplan II; Carl Zeiss MicroImaging, Inc.) with a 40×, 1.3 NA, oil objective (Plan-Neofluar; Carl Zeiss MicroImaging, Inc.). Images were captured using Openlab imaging software (Improvision) and incorporated into figures using Photoshop CS and Illustrator CS software (both Adobe).

In situ hybridization of EBER and α2 U1 RNAs

Fixed cells were washed with PBS twice for 5 min, permeabilized with 0.5% Triton X-100/PBS on ice for 10 min, and washed with PBS once and 2× SSC twice at RT. Cells were prehybridized with Phil's hybridization solution at 37°C for 1 h and hybridized with 2 ng/ml EBER1R152 or EBER2R134 probe (complementary to EBER1 nts 130–152 or EBER2 nts 106–134; see Northern blot analysis section for sequences) in Phil's hybridization solution (Forrester et al., 1992) overnight at 37°C. These probes were conjugated with DIG label using the 3′-DIG labeling kit (Roche) and were detected by incubation with a 1:200 dilution of rhodamine-conjugated anti-DIG antibody (Invitrogen) in PBS at RT for 1 h. Cells were washed three times with PBS at RT for 10 min each and once with 0.2 μg/ml DAPI/PBS solution at RT for 10 min, and then mounted for fluorescence microscopy. Alternatively, when EBER1 and α2 U1 were simultaneously probed at RT, the following oligonucleotides replaced the DIG-labeled probes and anti-DIG antibody: for EBER1, NEB1R148, 5′-XCTGGTACTTGACCGAAGACGGCAGAAA-3′; for α2 U1, NHA2U1A, 5′-XCTGCTTGTGTTAGATTATGTGGAT-3′; and for α2 U1, NHA2U1B, 5′-XCCCCTGCTTGTGTTAGATTATGTGGAT-3′. X denotes the 5′-amino group attached to a six-carbon linker. The 5′-amino group allowed conjugation of the Alexa Fluor 488 dye onto NEB1R148 and of the Alexa Fluor 594 dye onto NHA2U1A and NHA2U1B, using Alexa Fluor Oligonucleotide Amine labeling kits (Invitrogen).

Immunofluorescence detection of human La and Flag-PP32

Fixed cells on coverslips were washed with PBS twice for 5 min, permeabilized with 0.4% Triton X-100/1% normal goat serum (Invitrogen) in PBS on ice for 10 min, and washed with 1% normal goat serum/PBS three times at RT for 10 min each. The cells were then incubated with primary antibodies in 1% normal goat serum/PBS at RT for 1 h. A mouse monoclonal

anti–human La (a gift from M. Bachmann, Technical University Dresden, Dresden, Germany) and rabbit polyclonal anti-Flag (Sigma-Aldrich) antibodies were used at 1:100 dilutions. The coverslips were washed three times with 1% normal goat serum/PBS at RT for 10 min each, incubated with Alexa Fluor 594–conjugated (red) goat anti–mouse (for La) or Alexa Fluor 488– (green) or 680–conjugated (infrared) goat anti–rabbit (for Flag-PP32) antibodies for 1 h, washed three times with PBS at RT for 10 min each and once with 0.2 μg/ml DAPI/PBS solution at RT for 10 min, and mounted for fluorescence microscopy.

Turnover rate measurements

Actively growing HKB5cl8 and BJAB-B1 cells at 4×10^5 cells/ml were treated with 10 μg/ml actinomycin D. At indicated time points, 2×10^5 cells were removed and pelleted by centrifugation. Total RNAs were analyzed by Northern blotting.

Northern blot analysis

Total cellular RNA was purified using Trizol reagent (Life Technologies) and 5 μg of RNA (Fig. 1), subjected to 7 M urea gel electrophoresis, transferred to Zeta-blot (Bio-Rad Laboratories), and cross-linked to the membrane by UV irradiation. The immobilized RNA was hybridized with the indicated probe, and the signal detected and quantified with a PhosphorImager (Molecular Dynamics). In Fig.1, EBER levels were normalized to the signal obtained for cellular U6 snRNA; the probe was produced from plasmid pT7U6 (Wassarman and Steitz, 1993) that was linearized with EcoRI and transcribed in the presence of α-[^{32}P]UTP. Other RNA sequences were detected by Northern blotting using the following γ-[^{32}P]–labeled DNA oligonucleotide probes: EBER1R152, 5′-CCAGCTGGTACTTGACCGAAGAC-3′; EBER2R134, 5′-ATTAGAGAATCCTGACTTGCAAATGCTCT-3′; U1R96, 5′-AATCGCAGGGGTCAGCACATCCGGAG-3′; HY1R60, 5′-GTTCGATCTGTAACTGACTGTGA-3′; and 7SLR99, 5′-GCATAGCGCACTACAGCCCAGAA-3′.

Plasmid construction

The wild-type EBER1 coding sequence was cloned into the pUC19 vector (Fok et al., 2006). Using this plasmid as template, EBER1 3′ polyU mutant was generated by PCR amplification with the primers ECORIT7, 5′-CGCGAATTCTAATACGACTCACTATAG-3′ and EBER1PML, 5′-GCCGGATCCCACGTGTTCTGCGGACCACCAGCTGGTACTTGA-3′. To generate tRNA^{Phe} plasmid, oligonucleotides PHE5P, 5′-CGCGAATTCTAATACGACTCACTCTAGGCGAAATAGCTCAGTTGGGAGAGCGTTAGACTGAAGATCTAAAGG-3′, which contains a T7 promoter, and PHE3P, 5′-GCCGGATCCCAGCTGGTGCCGAAACCCGGGATGGAACCAGGGACCTTTAGATCTTCAGTCTAACGCTCTCCC-3′ were hybridized and filled in with Klenow polymerase. The construct for producing the VARdm substrate (Gwizdek et al., 2003) was generated by PCR using pAdEasy (American Type Culture Collection) as a template with primers TopVA, 5′-GACCGAATTCTCGGGACGCTCTGGCCGGTCAGG-3′, which contains a T7 promoter, and VA D4M, 5′-CGCGGATCCAGTACTAGGAGCACTCCCCCGTTGTCTGACGTCGCACACCTGGGTTATCACGGCGGACGGCCGGATACGG-3′. All DNA fragments were inserted into the pUC19 vector using the EcoRI and BamHI restriction sites.

***X. laevis* oocyte microinjection and RNA isolation**

The EBER1 plasmid and pT7U6 were linearized with DraI, the mutant EBER1 plasmid with PmlI, and the tRNA^{Phe} plasmid with PvuII. RNAs were in vitro transcribed in the presence of α-[^{32}P]UTP (GE Healthcare), gel purified, and injected into the germinal vesicles of whole *X. laevis* oocytes. 0.5–1 fmol each of U6 RNA, tRNA^{Phe}, and EBER1 were injected in a volume of 9.2 nl with 20 mg/ml blue dextran as a marker. Oocytes were incubated at RT in OR2 buffer (5 mM Hepes, pH 7.8 with KOH, 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) for 0.5 or 2.5 h and manually dissected in cold isolation buffer (80 mM KCl, 17 mM NaCl, 6 mM Na_2HPO_4, 3.5 mM KH_2PO_4, and 10 mM $MgCl_2$). Five to six oocytes were fractionated into nucleus and cytoplasm (Huang and Steitz, 2001) and pooled for each time point. Proteinase K digestion and phenol/choloform extraction were performed, and 0.5 oocyte equivalents were run on an 8% urea polyacrylamide gel.

Immunoprecipitation of wild-type and mutant EBER1

The La-specific antiserum used was ON, which was provided by J. Hardin (Yale University, New Haven, CT). Whole-cell sonicates (16 μl) from 2×10^5 BJAB cells (Lerner et al., 1981) were incubated with 40 fmol ($\sim 2 \times 10^5$ cpm) in vitro α-[^{32}P]UTP–labeled wild-type or mutant EBER1 and 2 μg *Escherichia coli* tRNA as a nonspecific competitor in 20 μl for 30 min at RT. The reactions were immunoprecipitated with either anti-La or anti-L22 (Toczyski et al., 1994) attached to protein A–Sepharose beads (GE Healthcare), or beads alone at 4°C for 1–2 h. The beads were then washed five times with NET-2 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% NP-40) at 4°C. RNA was extracted with phenol/chloroform/isoamyl alcohol (25:25:1), ethanol-precipitated, and electrophoresed in an 8% urea polyacrylamide gel. Equal amounts of wild-type and mutant EBER1 were immunoprecipitated in control reactions with anti-L22, suggesting proper folding of the EBER1 mutant.

Recombinant proteins

Expression and purification of Exp5 was performed as previously described (Brownawell and Macara, 2002), except that the purified protein was dialyzed against buffer A (20 mM TrisHCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, and 10% glycerol). RanQ69LGTP was prepared as previously described (plasmid provided by I. Macara, University of Virginia, Charlottesville, VA; Brownawell and Macara, 2002; Rebane et al., 2004).

Electrophoretic mobility shift assays

The VARdm substrate (Gwizdek et al., 2003) was generated from the VARdm plasmid linearized with ScaI by in vitro transcription in the presence of α-[^{32}P]UTP. Binding reactions (10 μl) containing 4.5 fmol VARdm RNA, 0.1 μM Exp5, 0.5 μM RanQ69LGTP, and the indicated amounts of competitor RNAs were incubated for 40 min at 30°C in RNA-binding buffer containing 20 mM TrisHCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 10% glycerol, and 2 pmol of the T7 terminator DNA oligonucleotide 5′-GCTAGTTATTGCTCAGCGG-3′ to reduce nonspecific binding. Before loading, 1 μl of a 0.6 mg/ml heparin and 0.2 mg/ml Bromophenol blue mixture was added to each sample. The samples were loaded on a preelectrophoresed (30 min) 6% native gel in 0.5× TBE buffer (45 mM Tris borate and 1 mM EDTA). Electrophoresis was performed at 12 V cm^{-1} for 1 h at RT.

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