4932–4943 *Nucleic Acids Research, 2009, Vol. 37, No. 15* *Published online 15 June 2009*
doi:10.1093/nar/gkp497

Translation of intronless RNAs is strongly stimulated by the Epstein–Barr virus mRNA export factor EB2

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Received November 28, 2008; Revised May 18, 2009; Accepted May 21, 2009

ABSTRACT

The Epstein–Barr virus protein (EB2) allows the nuclear export of a particular subset of early and late viral RNAs derived from intronless genes. EB2 is conserved among most herpesvirus members and its presence is essential for the production of infectious particles. Here we show that, besides its role as a nuclear export factor, EB2 strongly stimulates translation of unspliced mRNAs without affecting overall cellular translation. Interestingly, this effect can be reversed by the addition of an intron within the gene. The spliced mRNA is then efficiently exported and translated even in the absence of EB2. Moreover, we show that EB2 associates with translating ribosomes and increases the proportion of its target RNA in the polyribosomal fraction. Finally, testing of EB2 homolog proteins derived from EBV-related herpesviruses, shows that, even if they play similar roles within the replication cycle of their respective virus, their mechanisms of action are different.

INTRODUCTION

In eukaryotic cells, gene expression is tightly controlled from the biogenesis of messenger RNAs (mRNAs) within the cell nucleus, until their export and translation in the cytoplasm (1). In particular, the control of mRNA translation is a multi-step complex mechanism mediated by a large number of factors. Translation initiation appears to be the rate-limiting and most regulated step of the overall translation mechanism (2,3). Regulation of translation initiation is mediated mostly by initiation factors, which recruit the 40S ribosomal subunit to the 5′ cap of the mRNA, allow scanning to the initiation codon and then the recruitment of the 60S ribosomal subunit (4).

Even though maturation of pre-mRNAs occurs in a different cellular compartment than translation, proteins that participate in the former process can also play a role in the latter. Indeed, translation stimulation of intron-containing genes has been observed in several systems and is linked to proteins that participate either in splicing or in the export of spliced mRNAs (5–12). Among these, the exon junction complex (EJC), which is deposited during splicing and plays an important role in mRNA surveillance, is able to modulate translation of spliced mRNAs through the mTOR pathway (7). Other proteins involved in translation stimulation of spliced mRNAs comprise the Ser-Arg-rich (SR) proteins that play a role not only in pre-mRNA splicing and spliceosome assembly but also in splice-site recognition and selection (13,14). Conversely, recent data have also shown that some of the SR proteins, which shuttle from the nucleus to the cytoplasm together with the spliced mRNA, can be associated with translating ribosomes to stimulate the translation of spliced mRNAs (11,15). This would allow the cell to ensure that only fully spliced RNAs are expressed as opposed to unspliced or incompletely spliced RNAs that could result in translation of aberrant proteins.

Viruses have evolved different mechanisms to efficiently export and translate unspliced RNAs. One example is the constitutive transport element (CTE) present in simple retroviruses, such as the Mason–Pfizner monkey virus (MPMV), which interacts with the TAP/NXF1 export protein and the cellular protein NXT1/p15 to promote

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export and translation of unspliced genomic RNA (15–18). Again, translation stimulation of unspliced RNAs containing the CTE seems to rely on SR proteins such as 9G8 (15). For complex retroviruses, such as lentiviruses, the unspliced genomic RNA is exported by the viral protein Rev which interacts with *cis*-acting sequences located within the envelope coding region of the RNA, allowing export and translation stimulation (19–22).

The Epstein–Barr virus (EBV) early protein EB2 (also known as BMFL1, Mta or SM) shares several features with mRNA export factors. It is able to interact with RNA both *in vitro* and *in vivo* (23–25), it shuttles between the nucleus and the cytoplasm and it allows the cytoplasmic accumulation of unspliced RNAs generated from intronless and intron-containing genes, probably by the recruitment of REF and TAP/NXF1 (24,26–28). EB2 is essential for the production of viral particles and promotes the nuclear export of some early and most late viral mRNAs generated from EBV intronless genes (28). Moreover, like EBV many other herpesviruses code for a protein similar to EB2, i.e. ICP27 from herpes simplex virus type 1 (HSV1) (29–31), UL69 from cytomegalovirus (CMV) (32) ORF57 from Kaposi's sarcoma-associated herpesvirus (KSHV) (33) and ORF57 from herpesvirus saimiri (HVS) (34). All these proteins act as nuclear mRNA export factors but surprisingly their function cannot be *trans*-complemented between each other (27,35).

As cellular mRNA export factors, EB2 shuttles from the nucleus to the cytoplasm, probably associated with its target mRNAs, suggesting that it could also affect their translation. We thus tested the effect of EB2 on translation from an intronless reporter construct coding for the Renilla luciferase. In this system, translation stimulation was measured by analyzing the expression levels of Renilla luciferase normalized to the amount of cytoplasmic mRNAs determined by quantitative PCR. Our results show that EB2 strongly and specifically stimulates translation of intronless mRNAs without affecting overall cellular translation or protein stability. Introduction of an intron in this construct stimulates the efficiency of export and translation of the luciferase mRNA and at the same time abrogates the effect of EB2. Interestingly, the increase in translation of the luciferase mRNA generated from the intronless construct in the presence of EB2, correlates with an increase in the proportion of luciferase mRNA associated with polyribosomes. Moreover, we show that EB2 itself is associated with polyribosomes. Finally, a comparison between EB2 homologs from EBV-related viruses (HSV-1, CMV, KSHV) led us to conclude that their mechanisms of action are different.

MATERIALS AND METHODS

Cell lines and transfections

HeLa and HEK293T cells were grown in Dulbecco's modified Eagle medium supplemented with penicillin, streptomycin and 5% fetal calf serum (Invitrogen). NIH3T3 PKR-deficient cells (36) and NIH3T3 wild-type cells were grown as described for HeLa cells. For experiments using EBV gene-derived constructs, transfections were performed in 100 mm plates using calcium phosphate with 15 μg of total DNA (0.5 μg of pTRE2-Flag.BDLF1, 0.25 μg of pCI-Flag.EB2 and 0.5 μg of pTet-On or pTet-Off expression vectors (Clontech) and pUC18 up to 15 μg). When necessary, doxycyclin was added at a concentration of 1 μg/ml. For experiments using the luciferase reporter plasmids, transfections were carried out in 60 mm plates using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. For the metabolic labeling experiments, transfection of HeLa cells with the EB2 expression plasmid was carried out in six-well plates using Lipofectamine 2000 (Invitrogen). The efficiency of transfection was evaluated by transfecting the pEGFP-C1 plasmid (Clontech) in the same conditions and counting the number of green fluorescent cells by FACS. Over 60% of the cells expressed GFP. For polysomal fractionation, HEK293T cells were seeded at 2×10^6 on a 150 mm culture dish 3 days before polysomal fractionation. Forty-eight hours before harvesting, cells were transfected with 1.25 μg of luciferase-coding plasmid together or not with 6.25 μg of pCI-Flag.EB2, using PEI reagent.

Plasmids

The pCI-Flag.EB2 construct has been previously described (28). For pTRE2-Flag.BDLF1, the EBV BDLF1 open reading frame tagged with the Flag epitope was amplified by PCR and cloned into the BamHI and XbaI sites of the pTRE2 expression plasmid (Clontech). The pTet-On vector was supplied by Clontech (Tet-Off and Tet-On gene expression systems). The pcDNA GlobinRen reporter plasmid was constructed by cloning the human β-globin 5′UTR (with the authentic initiation codon) followed by the Renilla luciferase reporter gene, amplified by PCR from the p-Globin Renilla vector (37) into the double digested (XbaI/AflII) pcDNA3.1 expression vector. For the pcDNAIntron-GlobinRen, the sequence corresponding to the intron of the human β-globin gene was amplified by PCR and cloned into the pcDNAGlobinRen vector previously digested by XbaI. pCI-mycORF57 contained the complete ORF57 coding sequence (first exon included) cloned in frame with the myc epitope, in pCI (Promega). The expression plasmid for UL69 (pCMV-UL69) was kindly provided by T. Stamminger (38). The expression plasmid for ICP27 (pCI-FlagICP27) has been previously described (27).

RNA extraction and real-time quantitative PCR (RT-PCR) from cytoplasmic RNAs

Cells were first scraped from the dish and then resuspended in 200 μl of cold RLNa buffer (10 mM Tris–HCl (pH 8), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5% NP40 and 10 U/ml of RNaseOUT (Invitrogen). After 5 min incubation on ice, lysed cells were centrifuged for 2 min at 400*g* at 4°C and the supernatant was then recovered. One milliliter of Trizol (Invitrogen) was then added to the supernatant and RNAs were extracted following the Trizol protocol provided by the manufacturer. Cytoplasmic RNAs (1 μg) were treated with RQ1 DNAse (Promega) to avoid DNA contamination and reversed

Table 1. PCR primers used in this study[a]

Primer name	Primer sequence
BDLF1 forward	CAGATTTGAAAGTGGTAGTGTC
BDLF1 reverse	TTATCTTAACCAGCAAGTGGCCG
β-actin forward (Human)	GCTGCGTGTGGCTCCCGAGGAG
β-actin reverse (Human)	ATCTTCATTGTGCTGGGTGCCAG
GAPDH forward (Human)	TCCACCACCCTGTTGCTGTAG
GAPDH reverse (Human)	ACCCACTCCTCCACCTTTGAC
HPRT forward (Mouse)	TCATTATGCCGAGGATTTGGA
HPRT reverse (Mouse)	CAGAGGGCCACAATGTGATG
Renilla forward	AGGTGAAGTTCGTCGTCCAACATTATC
Renilla reverse	GAAACTTCTTGGCACCTTCAACAATAGC
18S rRNA forward (Human)	GTGGAGCGATTTGTCTGGTT
18S rRNA reverse (Human)	CGCTGAGCCAGTCAGTGTAG
28S rRNA forward (Human)	TGGGTTTTAAGCAGGAGGTG
28S rRNA reverse (Human)	AACCTGTCTCACGACGGTCT
U6 forward (Human)	CGCTTCGGCAGCACATATAC
U6 reverse (Human)	AAAATATGGAACGCTTCACGA

[a]Primers for real-time PCR were designed using Beacon designer software (from PREMIER Biosoft).

transcribed with $(dT)_{16}$ and 1 µl of Superscript II enzyme (Invitrogen) in a 20 µl reaction mix at 42°C for 1 h.

PCRs were performed using a *Taq* core kit (Q-Biogen) with a set of specific primer pairs (BDLF1 forward/BDLF1 reverse: Table 1) on various amounts of the RT reaction mixtures (0.05, 0.4, 1 or 2 µl) to have a linearly increasing signal after 25 PCR cycles. The PCR-amplified fragments were then analyzed on 2% agarose gels. We evaluated the endogenous expression of β-actin mRNA by RT-PCR (β-actin forward/β-actin reverse: Table 1). Amplification of a 690-bp DNA fragment corresponding to the β-actin mature mRNA showed that no DNA contamination was present in our RNA preparations. We tested for the presence of U6 snRNA using RT-PCR (U6 forward/U6 reverse: Table 1).

For RT-qPCR, a 20 µl reaction was prepared with 5 µl of template cDNA (1/20 diluted), 10 µl of MESA green SYBR premix (Eurogentec), 0.2 µM of each primer and subjected to amplification using a fluorescence thermocycler (Applied Biosystems 7000 RT-PCR, Foster City, CA) under the following conditions: 10 min at 94°C for initial denaturation, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and elongation at 72°C for 30 s. This program was followed by a melting curve analysis in order to verify the specificity of the PCR product. Renilla luciferase was amplified in parallel with the housekeeping gene GAPDH (for HeLa cells) or HPRT (for mouse cells) and relative copy numbers of Renilla cDNAs were compared to GAPDH using $x^{-\Delta Ct}$ (where x corresponds to the experimentally calculated amplification efficiency of each primer couple). The primer sequences used in this study (presented in Table 1) were designed using Beacon designer software (from PREMIER Biosoft).

Western blotting analysis

Cells were collected by centrifugation, lysed on ice for 30 min in 100 µl of HNTG buffer (50 mM HEPES pH 7.5; 150 mM NaCl; 1% Triton X-100; 10% glycerol; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride). Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels and then transferred to a nitrocellulose membrane by electroblotting (Hybond-ECL; Amersham Biosciences). Membranes were incubated with, respectively, anti-Flag M2 (Sigma) or anti α-tubulin (T5168, Sigma) monoclonal antibodies or an anti-PABP (kind gift from S. Morley) rabbit polyclonal antibody (39). Goat anti-mouse horseradish peroxidase conjugate or goat anti-rabbit horseradish peroxidase conjugate (Amersham) were used at a dilution of 1:5000 as secondary antibodies. For immunoblot detection, the ECL system (Amersham) was used.

Luciferase assays

Renilla activity from transfected cells was measured in a Veritas™ Luminometer (Turner Biosystems) using the Renilla luciferase assay system (Promega Madison Co). Luciferase activity was measured for identical amounts of total protein as evaluated by Bradford assay.

Polyribosome fractionation

Polyribosome fractionation was performed essentially as described previously (40,41). Forty-eight hours after transfection, HEK293T cells were treated with 100 µg/ml cycloheximide at 37°C for 5 min and harvested by scraping from the plate. In some experiments, EDTA was added to the cell lysate at a final concentration of 15 mM to disrupt the polysomes. The gradient was collected from the top using a Piston Gradient Fractionator (BioComp, New Brunswick, Canada) with concomitant measurement of the absorbance at 254 nm using an AKTA purifier (GE Healthcare, Amersham Biosciences, Piscataway, NJ) coupled to a fraction collector. For western blotting, 20 µl of each fraction were separated on 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gels and then transferred to a PVDF membrane (Amersham biosciences) by electroblotting. The membrane was then incubated with various antibodies as described above in the 'western blotting analysis' section. RNA extraction was performed as described (15). RNAs were then reverse transcribed and analyzed by quantitative PCR as described above.

RESULTS

EB2 stimulates protein expression from EBV-derived mRNAs

It has been shown that the EBV-encoded viral protein EB2 acts as a nuclear export factor for a particular subset of mRNAs generated from intronless genes and for some unspliced mRNAs derived from intron-containing genes (24,26–28). However, although EB2 probably shuttles from the nucleus to the cytoplasm together with the exported RNA, the effect of EB2 on mRNA translation has never been reported. This prompted us to test whether expression of EB2 could have an influence on translation of its target viral mRNAs. We thus tested the effect of EB2 on translation of an EBV-encoded mRNA whose cytoplasmic accumulation depends on EB2. In order to control

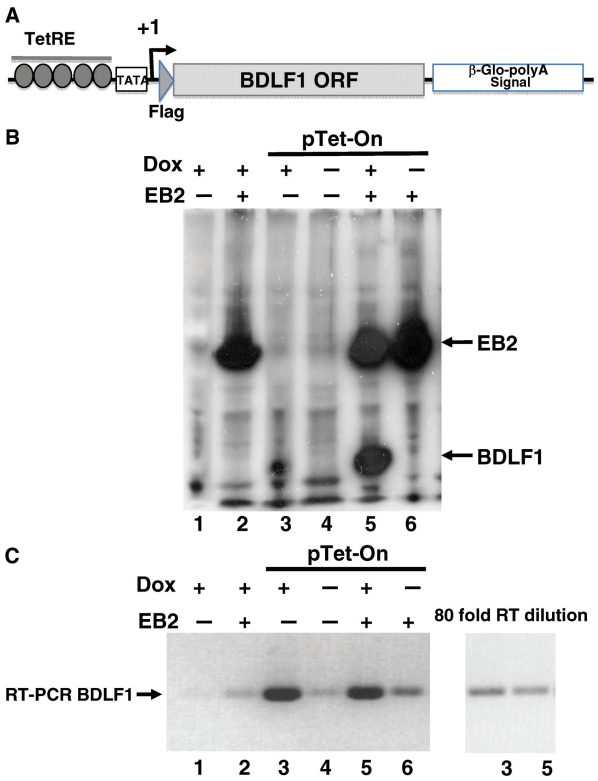

Figure 1. EB2 stimulates expression of the late EBV viral protein BDLF1. (**A**) Schematic representation of the BDLFI-encoding construct pTRE2-Flag-BDLF1. (**B**) Western analysis of Flag-BDLF1 expression in HeLa cells transfected with pTRE2-Flag-BDLF1, together with pTet-On and an expression plasmid for Flag-EB2 (pCI-FEB2) as indicated in the figure. Doxycycline was added as indicated. The M2 anti-Flag MAb was used to visualize both Flag-BDLF1 and Flag-EB2 proteins. (**C**) Quantification of the BDLF1 cytoplasmic RNA by semi-quantitative RT-PCR in HeLa cells transfected as described above.

transcription of the target mRNA, we used the Tet-On gene expression system (Clontech) to express the EBV BDLF1 late mRNA (Figure 1A). For this, the pTRE2-Flag-BDLF1 plasmid was transfected into HeLa cells together with the pTet-On vector (Clontech) and with or without the EB2 expression plasmid. Expression of EB2 and the BDLF1 protein was then monitored by western blotting using an anti-Flag antibody (Figure 1B). The amount of cytoplasmic BDLF1 mRNAs was quantified by semi-quantitative RT-PCR (Figure 1C). In cells cotransfected with the BDLF1 reporter plasmid and the pTet-On vector, in the absence of doxycycline, no BDLF1 protein was detected (Figure 1B, lane 4) and only a low amount of BDLF1 mRNAs were detected by RT-PCR (Figure 1C, lane 4). In these conditions, the expression of EB2 enhanced the cytoplasmic accumulation of the corresponding BDLF1 mRNA (Figure 1C, lane 6) but again no BDLF1 protein was detected (Figure 1B, lane 6). When doxycycline was added, the transcription of BDLF1 mRNAs was strongly stimulated (Figure 1C, lane 3) although only low levels of BDLF1 protein were detected by western blot in the absence of EB2 (Figure 1B, lane 3). However, in the presence of EB2 there was a large increase in the amount of BDLF1 protein expressed (Figure 1B, lane 5) although the amount of BDLF1 mRNA detected in the cytoplasm was comparable to that in the absence of EB2 (Figure 1C, compare lanes 3 and 5). Indeed, in these conditions, transcription of BDLF1 triggered by doxycycline was so strong that it appeared to compensate for the otherwise poor cytoplasmic accumulation observed in the absence of EB2. This allowed us to focus just on the effect of EB2 on BDLF1 translation without introducing a bias with the amount of mRNA. Finally, upon addition of doxycycline, only a weak signal corresponding to the BDLF1 mRNA expression was detected by RT-PCR in the absence or presence of EB2 (Figure 1C, lanes 1 and 2) and no BDLF1 protein expression was detected (Figure 1B, lanes 1 and 2) thus ruling out any non-specific effect of doxyxycline on BDLF1 expression in the absence of the pTet-On vector. Essentially similar results were obtained using the Tet-Off system for which transcription of BDLF1 was triggered by the absence of doxycycline (data not shown).

These results strongly suggest that EB2 is able to stimulate protein expression from EBV-derived mRNAs.

EB2 stimulates both export and translation independently of an EBV-specific sequence without affecting global mRNA translation

In order to quantify the relative effects of EB2 on mRNA export and translation we designed a reporter construct containing the 5′UTR of the human β-Globin gene followed by the Renilla luciferase coding region under the control of the cytomegalovirus (CMV) immediate early promoter (Figure 2A). It is important to note that this construct does not contain any EBV-related sequence. This construct was cotransfected together with the EB2 expression vector in HeLa cells and total luciferase activity was analyzed 24 h after transfection (Figure 2D, *top panel*), while expression of EB2 was measured by western blotting (Figure 2B). Cytoplasmic RNAs were extracted and we first quantified the amount of U6 snRNA in order to make sure that our cytoplasmic fractions were not contaminated by nuclear material (Figure 2C). Then cytoplasmic luciferase RNAs were quantified by relative quantitative RT-PCR using the housekeeping gene GAPDH as an internal control (Supplementary Figure 1). Interestingly, EB2 had no effect on GAPDH mRNA export thus ruling out any bias in the relative quantification of luciferase-coding mRNAs. As expected, expression of EB2 promoted the cytoplasmic accumulation of luciferase-coding RNAs in a dose-dependent manner (Figure 2D, *middle panel*) thus leading to a stimulation of luciferase expression (Figure 2D, *top panel*). The luciferase activity measured in each assay was then normalized to the amount of luciferase cytoplasmic RNAs. This gives an exact measure of the luciferase activity per RNA molecule, and thus can be considered as a quantitative representation of the translation rate. Interestingly, using this quantification we found that an average 7-fold stimulation of luciferase mRNA translation in the presence of EB2 was observed (Figure 2D, *bottom panel*).

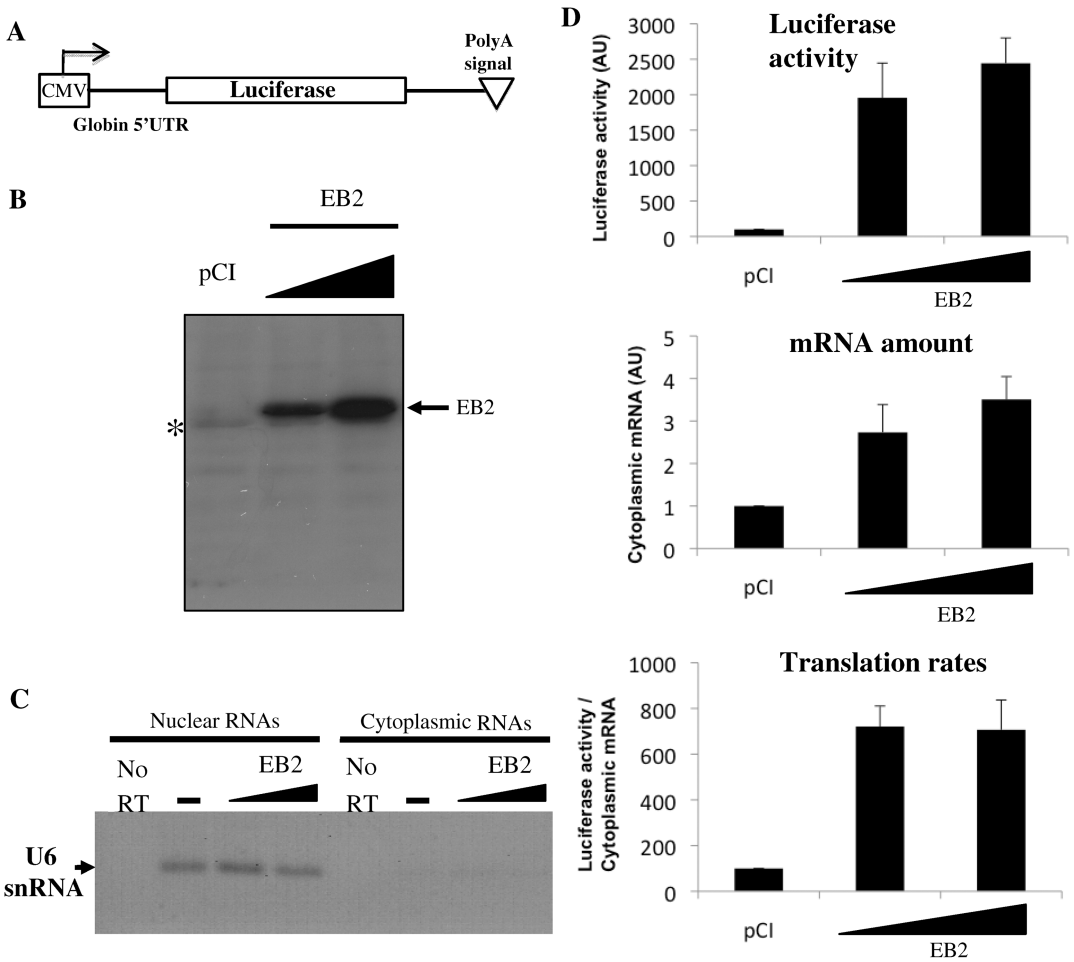

Figure 2. EB2 stimulates expression of a Renilla luciferase intronless gene at the translational level, independently of any viral-derived sequence. (**A**) Schematic representation of the luciferase intronless coding vector used in this study (pcDNAGlobinRen) showing positions of the CMV promoter and BGH polyadenylation signal. (**B**) Immunoblot of HeLa cells cotransfected with pcDNAGlobinRen together with the empty pCI vector or increasing amounts of the FlagEB2-encoding plasmid, pCI-FlagEB2 (250 and 500 ng). The M2 anti-Flag MAb was used to visualize Flag-EB2. Asterisk denotes an unspecific band detected by the M2 anti-Flag antibody. (**C**) Quantification of the amount of U6 snRNA present respectively in the nuclear and cytoplasmic fractions of cellular extracts used in D. U6 snRNA was amplified by RT-PCR using the specific primer set indicated in Table 1, and analyzed on a 2% agarose gel. (**D**) Measure of luciferase activity and quantification of cytoplasmic luciferase-encoding mRNAs by quantitative RT-PCR using GAPDH as an internal control. Total luciferase activity was measured 24 h post-transfection (top panel) and the amount of cytoplasmic luciferase coding mRNAs was quantified (middle panel). Translational efficiency (bottom panel) was calculated by normalizing the total luciferase activity by reference to the amount of cytoplasmic luciferase mRNA. AU: arbitrary units.

To test any impact EB2 could have on the stability of the neosynthesized luciferase protein we analyzed the stability of the luciferase protein in the absence and presence of EB2. For this, we blocked cellular translation by adding cycloheximide and measured luciferase decay activity over time on cells expressing, or not, EB2. As shown in Figure 3A, EB2 did not affect the stability of the Renilla luciferase protein, which had a 30-min half-life both in the absence and presence of EB2.

Furthermore, the lack of EBV-derived sequences on the luciferase reporter construct prompted us to test the effect of EB2 on global cellular mRNA translation. We thus performed a metabolic labeling of cells expressing (or not) EB2 (Figure 3B). For this, cells were pulsed in the presence of radiolabeled methionine for 30 min. Cells were then lysed and proteins resolved on SDS–PAGE to quantify the overall cellular translation rates. As shown in Figure 3B, we did not detect any significant difference in translation rates between cells expressing EB2 and those not expressing EB2.

We also wanted to exclude the possibility that translation stimulation driven by EB2 depends on protein kinase R (PKR). PKR is the principal cellular factor involved in the interferon-mediated inhibition of viral translation. This kinase is activated by double-stranded RNA and this activation leads to the phosphorylation of initiation factor eIF2α, thus inhibiting translation initiation of all cellular and viral RNAs (42,43). We thus monitored luciferase translation both in NIH3T3 PKR-deficient cells (36) and wt NIH3T3 in the presence or absence of EB2. We found that EB2 was able to stimulate translation of luciferase coding mRNAs even in the absence of PKR (Supplementary Data Figure 2), which argues for a mechanism of translation stimulation independent of the PKR pathway.

Finally, to rule out the possibility that translation rates were affected by the amount of cytoplasmic mRNAs, thus

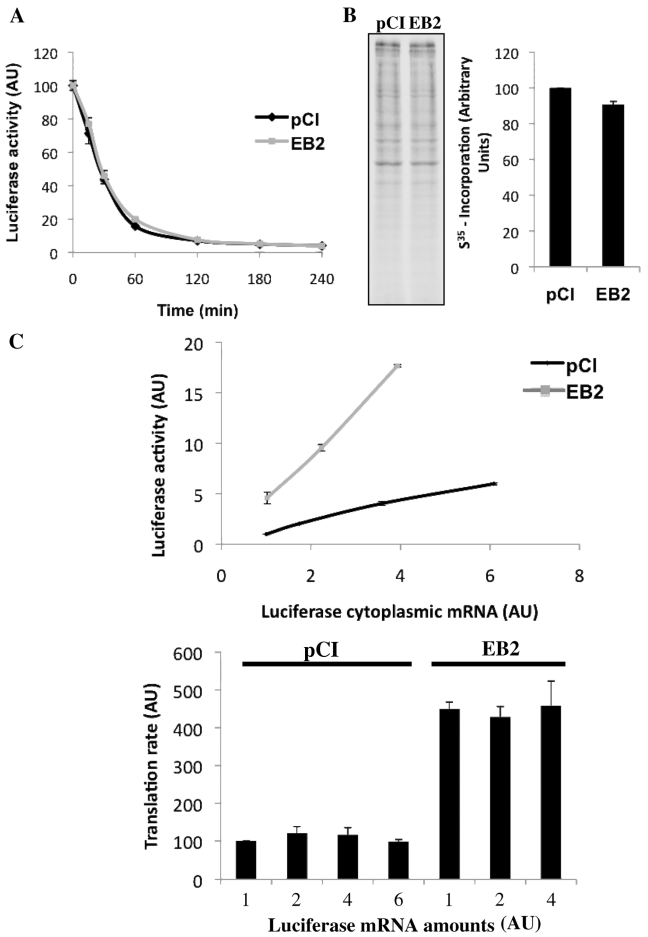

Figure 3. EB2 does not affect protein stability or global cellular mRNA translation and its effect on translation is independent of the amount of cytoplasmic luciferase coding mRNA. (**A**) Time-lapse measure of total luciferase activity from HeLa cells mock transfected (pCI) or transfected with an EB2 expression vector (pCI-FlagEB2) (250 ng) after addition of cycloheximide to block translation. Luciferase activity was measured 0, 15, 30, 60, 120, 180 and 240 min after addition of cycloheximide to the cell medium. (**B**) Metabolic labeling of HeLa cells mock transfected (pCI) or transfected with the EB2-encoding plasmid (250 ng), using ^{35}S-labeled methionine. After a 30-min pulse labeling, cells were lyzed and total cellular proteins resolved on 12% SDS-PAGE. Total translation was quantified by phosphorimaging using a Fujifilm FLA5100. (**C**) Top panel: Luciferase activity was plotted against the amount of cytoplasmic luciferase coding mRNAs in HeLa cells transfected with increasing amounts of luciferase-encoding plasmid in the absence (pCI) or presence (pCI-FlagEB2) of EB2 (250 and 500 ng). Bottom panel: Translation rates per unit of luciferase-encoding mRNAs (calculated by normalizing luciferase activity by reference to the amount of cytoplasmic luciferase-encoding mRNAs) in HeLa cells transfected with increasing amounts of luciferase-encoding plasmid in the absence or presence of EB2 expression plasmid. AU: arbitrary units.

leading to a stimulation of translation which would be independent of EB2, we transfected increasing amounts of luciferase expression vector in HeLa cells expressing, or not, EB2 (Figure 3C). As EB2 expression leads to a 4–5-fold increase of mRNA levels in the cytoplasm, it was of interest to test if increasing the amount of luciferase mRNAs in the absence of EB2 could lead to a stimulation of translation. For this, we measured luciferase translation rates (luciferase activity/amount of luciferase mRNA) for increasing amounts of cytoplasmic mRNAs either in the presence or absence of EB2 (Figure 3C). As presented in the *top panel*, luciferase expression increased proportionally with the amount of cytoplasmic mRNAs, both in the presence and absence of EB2. However, when luciferase activity was normalized to the amount of cytoplasmic mRNAs (which corresponds to the translation rate per arbitrary unit of mRNA) there was no significant change in luciferase translation rates for increasing amounts of cytoplasmic luciferase mRNAs in control (pCI) and EB2-expressing cells (*bottom panel*). Nevertheless, translation rates in the presence of EB2 were systematically 4–5-fold more than those in the absence of EB2, and this for identical mRNA amounts measured in the cytoplasm of the cells.

Taken together, these results show that EB2 stimulates translation of mRNAs without affecting the stability of the neosynthesized protein. Interestingly, EB2 expression does not affect global mRNA translation. Moreover, translation stimulation does not depend on the amount of cytoplasmic mRNA available for translation.

Addition of an intron within the reporter construct impairs translation stimulation driven by EB2

Our results show that EB2 is able to stimulate translation of viral genes and that of a non-related reporter gene without affecting global cellular translation. Consequently, we focused on understanding the lack of an effect of EB2 on cellular mRNA translation. Interestingly, EB2 has been shown to specifically stimulate the nuclear export of mRNA generated from intronless genes (23). This prompted us to test the translation of an mRNA generated from an intron-containing gene in the presence of EB2. We have thus introduced the β-globin intron within the 5′UTR of the luciferase reporter gene (Figure 4A). In order to test the efficiency of splicing of the corresponding mRNA, an RT-PCR from cytoplasmic mRNAs was performed using a forward and reverse primer flanking the intron (Figure 4B). We observed that transfection of the intronless construct in HeLa cells led to the expression of an unspliced mRNA which, upon RT-PCR, yielded a band of the same size to that from the control PCR, performed directly using the DNA plasmid as a template (Figure 4B, lanes 2, 3 and 4). On the contrary, transfection of the intron-containing construct led to the expression of an mRNA which, upon RT-PCR, yielded a band slightly longer than that of the intronless mRNA, but shorter than that of the control PCR obtained from the intron-containing DNA plasmid (Figure 4B, lanes 5–7). This band corresponds to the spliced form of the luciferase-coding mRNA. Thus the mRNA transcribed from this intron-containing reporter gene was efficiently spliced both in the presence or absence of EB2.

We then quantified the exact amount of cytoplasmic luciferase mRNA by quantitative RT-PCR and measured the corresponding luciferase activities (Supplementary Figure 3). When translation rates were calculated by normalizing the luciferase activity to the amount of

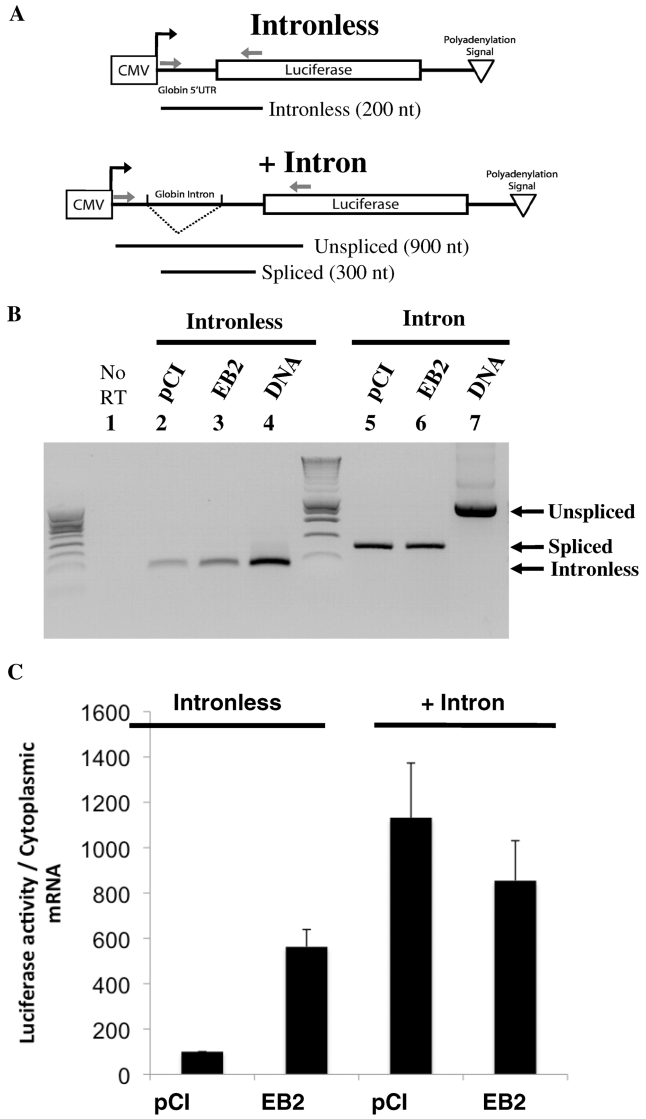

Figure 4. Translation stimulation does not occur with spliced mRNAs. (**A**) Schematic representation of the intronless (pcDNAGlobinRen) and the intron-containing (pcDNAIntronGlobinRen) vectors encoding the Renilla luciferase showing positions of the human β-globin intron within the 5′UTR of the luciferase construct (gray arrows correspond to positions of the PCR primers used to test efficient splicing of the intron). (**B**) RT-PCR (using primers shown in A) from cells cotransfected with pcDNAGlobinRen and pCI or the EB2-encoding plasmid pCI-FlagEB2 (lanes 2 and 3) or from cells cotransfected with pcDNAIntronGlobinRen and pCI or pCI-FlagEB2 (lanes 5 and 6), or directly from the purified DNA vector (lanes 4 and 7). (**C**) Luciferase activity normalized by reference to the amount of cytoplasmic luciferase-encoding mRNAs from HeLa cells cotransfected with pcDNAGlobinRen and pCI or pCI-FlagEB2, or pcDNAIntronGlobinRen and pCI or pCI-FlagEB2. The amount of luciferase-encoding mRNAs was monitored by quantitative RT-PCR.

luciferase-coding mRNA (Figure 4C), we observed a 10-fold more efficient translation of the spliced mRNA compared to unspliced in the absence of EB2. As expected, in the presence of EB2 the translation rate of the unspliced mRNA was increased by a factor 6. However, expression of EB2 did not further stimulate translation of the spliced luciferase mRNA but rather led to a mild inhibition. It is noteworthy, that we also observed an inhibitory effect of EB2 on the accumulation of luciferase mRNA generated from the intron-containing construct (Supplementary Figure 3) which corroborates previously published data from Ruvolo *et al.* (44).

These results indicate that EB2 specifically stimulates translation of intronless mRNAs without significantly affecting translation of spliced mRNAs.

EB2 co-sediments with polyribosomes and increases the utilization of reporter mRNA by the translation machinery

To determine whether EB2 is associated with the translation machinery and to find out whether it increases the association of its target mRNAs with polyribosomes, we performed sucrose gradient analysis to separate polyribosomes from monoribosomes and uncomplexed ribosomal subunits. HEK293T cells were transfected with pcDNAIntron-GlobinRen alone, pcDNAGlobinRen alone or pcDNAGlobinRen together with pCI-Flag.EB2. Cell extracts were prepared and fractionated on 10–50% sucrose gradients and fractions were first analyzed by western blotting. An example of the UV absorbance profile of the gradients is shown in Figure 5A and the corresponding 18S and 28S RNA profile, determined by RT-PCR, in Figure 5B. UV absorbance and the 18S/28S RNA profiles from all gradients were very similar. The polyribosome distribution of EB2 was compared with that of PABP, a general translation factor for polyA+ mRNA (45,46) (Figure 5A). As expected, PABP was present across the gradient from mRNPs to polyribosomal fractions. Interestingly, EB2 cosedimented with the 80S ribosome but was also found in the lighter polysomal fractions. As a control, α-tubulin was only found associated with the uncomplexed ribosomal subunit fractions. Furthermore, treatment of cytoplasmic extracts with EDTA, which is known to induce a dissociation of mono- and polyribosomes into ribosomal subunits, induced a redistribution of EB2 to the top of the gradient (Figure 5D and E).

We then analyzed the distribution of the reporter mRNAs throughout our sucrose gradients by quantitative RT-PCR (Figure 5C). Interestingly, the proportion of the Renilla luciferase mRNA generated from the intron-less construct, which is found associated with the polyribosomal fractions, is greatly increased in the presence of EB2 (compare the *middle* and *top panels*). Furthermore, in the latter case, the profile of repartition of the Renilla luciferase mRNA throughout the gradient is very similar to that obtained with the luciferase mRNA generated from the intron-containing construct (*bottom panel*). As expected, Renilla luciferase mRNA moved to the lighter fractions of the gradient following EDTA treatment (Figure 5F). Taken together, these data suggest that EB2 directly increases the utilization of its target reporter mRNA by the translation machinery.

EB2 viral homolog proteins exhibit different effects on mRNA generated from intronless genes

Herpesviruses code for EB2 homolog proteins that also serve as viral mRNA export factors. Interestingly, despite

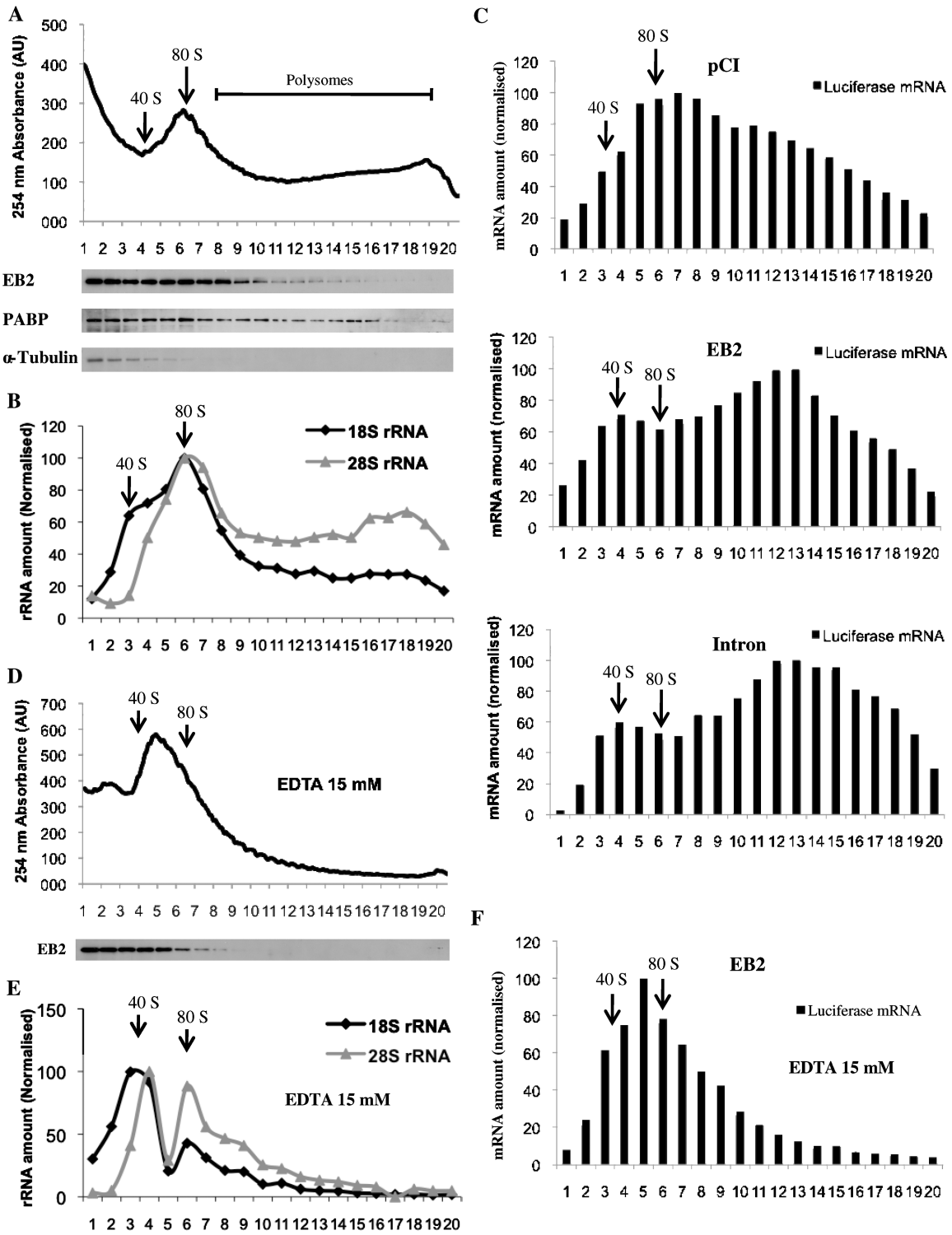

Figure 5. EB2 cosediments with polyribosomes and enhances utilization of its target mRNA by the translation machinery. (**A**, **B** and **C**) Cell cytosolic extracts of HEK293T transfected with pcDNAGlobinRen alone or pcDNAGlobinRen together with pCI-Flag.EB2 or pcDNAIntron-GlobinRen alone, were fractionated across 10–50% sucrose gradients. (A) Fractions from the gradient corresponding to HEK293T transfected with pcDNAGlobinRen together with pCI-Flag.EB2 were analyzed by western blotting with antibodies against the Flag epitope to detect Flag.EB2, poly(A)-binding protein (PABP) or α-tubulin. (Top) UV absorbance (254 nm) profile of cytoplasmic ribonucleoprotein complexes. (B) 18S and 28S RNA profile determined by quantitative RT-PCR. (C) Quantification of the Renilla luciferase reporter mRNA fractionated across 10–50% sucrose gradients by quantitative RT-PCR. (Top panel) HEK293T transfected with pcDNAGlobinRen. (Middle panel) HEK293T transfected with pcDNAGlobinRen together with pCI-Flag.EB2. (Bottom panel) HEK293T transfected with pcDNAIntron-GlobinRen. (**D**, **E** and **F**) An EDTA-treated cytoplasmic extract of HEK 293T cells transfected with pcDNAGlobinRen together with pCIF.EB2 was fractionated across a 10–50% sucrose gradients. (D) EB2 is relocalized to the top of the gradient. (Top) UV absorbance (254 nm) profile of cytoplasmic ribonucleoprotein complexes. (E) 18S and 28S RNA profile determined by quantitative RT-PCR. (F) Quantification of the Renilla luciferase reporter mRNA fractionated across a 10–50% sucrose gradient by quantitative RT-PCR.

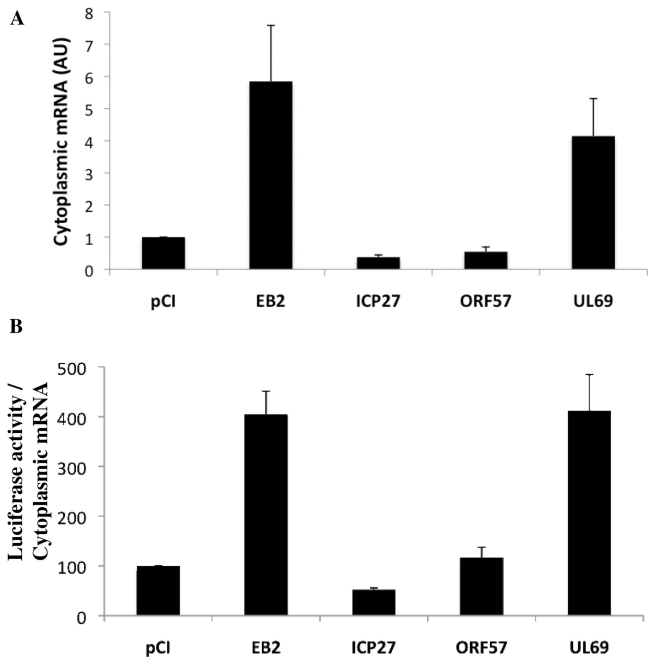

Figure 6. Differential effects on translation from EB2-related proteins derived from different herpesviruses. (**A**) Cytoplasmic luciferase mRNA levels monitored by quantitative PCR in HeLa cells mock transfected (pCI) or transfected with 500 ng of EBV EB2, HSV-1 ICP27, HKSV ORF57 and HCMV UL69-encoding plasmids. (**B**) Translation rate. Luciferase activity was normalized by the amount of cytoplasmic mRNAs from cells mock transfected (pCI) or transfected with EBV EB2, HSV-1 ICP27, HKSV ORF57 and HCMV UL69-encoding vectors, together with the reporter plasmid pcDNAGlobinRen. AU: arbitrary units.

their similarities, EB2 homolog proteins cannot *trans*-complement each other for viral production (35,47). Thus, it was of interest to test the effect of EB2-related proteins in our system. For this, we used proteins from each herpesvirus sub-family: ICP27 from HSV1 (herpes simplex virus 1), an α-herpesvirus, ORF57 from KSHV (Kaposi's sarcoma-associated herpesvirus), like EBV a γ-herpesvirus and UL69 from CMV (cytomegalovirus), a β-herpesvirus. Among these proteins, only ICP27 has been previously shown to stimulate translation of specific viral mRNAs (48,49). In order to monitor the effect of these proteins in our luciferase reporter system, we cotransfected HeLa cells with the intronless luciferase construct and expression plasmids for the different EB2-related herpesvirus proteins and we quantified both cytoplasmic mRNA accumulation (Figure 6A) and luciferase activity (not shown). Luciferase expression was again normalized to the amount of cytoplasmic luciferase-encoding mRNAs in order to specifically measure the impact of each viral protein on translation (Figure 6B). Expression of each of the viral proteins was verified by western blotting (data not shown). Unexpectedly, the effect observed with EB2 was not conserved for all of the homolog proteins. Indeed, only UL69 led to a strong luciferase translation stimulation similar to that of EB2 (i.e. 4–5-fold stimulation of translation), whereas ICP27 and ORF57 did not have a significant effect (Figure 6B). This was probably due to the fact that neither ICP27 nor ORF57 appear to export the luciferase mRNA (expression of ICP27 led in fact to a 40% reduction of the amount of luciferase cytoplasmic mRNA) contrary to EB2 and UL69, which provoked a 4–5-fold increase of luciferase cytoplasmic mRNA levels (Figure 6A).

This result shows that EB2 homologs derived from related viruses have differential effects on a heterologous mRNA and suggests that export of the mRNA and stimulation of its translation are strongly linked.

DISCUSSION

Although the role of EB2 in the nuclear export of unspliced RNAs has been extensively studied, its effect on translation has never been evaluated. With the growing evidence that cellular mRNA splicing and export factors are also able to modulate translation of spliced mRNAs, we decided to test whether the viral protein EB2 could also affect translation. Indeed, most of the EBV early and late genes do not contain any intron, suggesting that both export and translation of the corresponding mRNAs should be very inefficient. However, this defect is overcome by expression of the early viral protein EB2, which interacts with the viral mRNAs to facilitate their cytoplasmic accumulation. This tight interaction of EB2 with the exported mRNA and its transit to the cytoplasm strongly suggested that EB2 could also affect translation. In fact, expression of EB2 in cells coding for an EBV-derived unspliced RNA (BDLF1) led to a strong stimulation of BDLF1 accumulation that did not depend on an increase in cytoplasmic levels of the corresponding mRNA. This result indicates that, besides its role as a nuclear export factor, EB2 can also stimulate translation of EBV unspliced RNAs. Interestingly, the effect of EB2 on protein accumulation from unspliced mRNAs did not depend on any EBV-derived *cis*-acting sequence since expression of an artificial unspliced mRNA encoding for the Renilla luciferase was also strongly stimulated without affecting protein stability. Surprisingly, even though EB2 lacked a requirement for a specific RNA sequence, its expression did not affect global cellular mRNA translation suggesting a role for EB2 on translation of only a specific subset of mRNAs.

Since EB2 has been shown to export mostly mRNAs generated from intronless genes (23) including its specific EBV-encoded target genes we tested the effect of the introduction of intronic sequences within the 5′UTR of our reporter gene. Interestingly, we found that after addition of an intron within the luciferase reporter construct, EB2 was no longer able to stimulate translation, whereas it had a strong effect on the same RNA transcribed from an intronless construct.

These results together with previous results showing that EB2 can interact with mRNA independently of any specific sequence (50) suggest that EB2 can bind to both spliced and unspliced mRNAs. However, in the case of unspliced mRNAs, which do not recruit the normal set of splicing factors, EB2 would allow their export and stimulate their translation to levels similar to those of spliced mRNAs. In the case of intron-containing genes, EB2

could also bind to the mRNA but we suggest that either it is excluded from the mRNA by a cellular splicing or export factor, or it is exported to the cytoplasm with the spliced mRNA. In the latter case, its effect on translation would be redundant in the presence of splicing proteins. Indeed, it is possible that EB2 recruited cotranscriptionally to nascent mRNAs is able to interact with an as yet unknown cellular factor necessary for translation stimulation of spliced mRNAs. This would explain the stimulation of the translation of intronless mRNAs in the presence of EB2. On the contrary, if EB2 also interacts with spliced mRNAs, it could interfere with the cellular factors that normally stimulate their export and translation. This would explain the mild inhibition of export and translation that we observed upon addition of an intron in our luciferase reporter construct.

In accordance with the fact that EB2 specifically stimulates export and translation of mRNAs generated from intronless genes we have shown that cellular mRNAs, which are, in the majority, generated from intron-containing genes, are not globally affected by expression of EB2. There are however few cellular genes which are known for their absence of introns. It would be interesting to look at the effect of EB2 on the export and translation of mRNAs generated from such genes. In the case of EBV, most of the viral mRNAs of the productive cycle are intronless. We have previously shown that EB2 is necessary for the efficient export of the majority of these, but it is interesting to note that some are efficiently exported even in the absence of EB2, suggesting that they use an alternative export pathway, independent of splicing. Such an alternative pathway has been previously reported with the SR proteins, 9G8 and ASF/SF2, which have been found to promote the recruitment of TAP to mRNPs (51). Another interesting example of mRNA generated from an intronless gene and which is not affected by EB2 is the firefly luciferase mRNA expressed from an intronless construct (25). However, although there is no effect of EB2 on firefly luciferase expression, EB2 bound efficiently to its mRNA *in vivo* (25). Again, it is likely that this mRNA uses an alternative pathway for its export, independent of splicing. Thus, even if EB2 is associated with these mRNAs *in vivo*, its effect is probably redundant as discussed above in the case of spliced mRNAs.

In order to definitively conclude on a direct effect of EB2 on translation efficiency we studied the association of Renilla luciferase mRNAs with polyribosomes. The data clearly showed that the proportion of Renilla luciferase mRNAs associated with polyribosomes is largely increased in the presence of EB2. Moreover, we found that there is also an association of EB2 with polyribosomes, suggesting that EB2 binds the mRNPs in the nucleus, where it stimulates their export and then remains associated with the mRNPs as far as the polyribosomes. Taken together, these results argue for a role of EB2 in *cis* similar to that of the EJC proteins responsible for the translation stimulation of cellular mRNAs. One proposed mechanism involves an interaction between the EJC and the 48S preinitiation complex mediated by an interaction between Y14:Magoh and the protein PYM (52). Another mechanism involves the EJC-dependent recruitment of the 40S ribosomal protein S6 kinase 1 (S6K1) which is a central player in the TOR signaling cascade (7). When activated by the TOR pathway, S6K1 enhances translation initiation both by activating stimulatory factors and by inactivating inhibitory factors bound at the 5′ cap of mRNAs. The SR protein ASF/SF2 has also recently been reported to enhance translation initiation via recruitment of mTOR (8).

Finally, we tested several EB2 homologous proteins from other herpesviruses for their ability to stimulate translation of the intronless luciferase gene. Among the proteins tested, only UL69 from human cytomegalovirus (hCMV) behaves like EB2, while ICP27 and ORF57 were unable to stimulate luciferase translation. For UL69, this is the first report suggesting that this protein plays a role in stimulation of translation. On the contrary, it has been previously shown that ICP27 (from the herpes simplex virus type 1) plays a role in regulating translation of a subset of late viral mRNAs (48,49,53). However, the interaction between ICP27 and the viral mRNAs has been shown to depend on specific RNA sequences distributed along the viral genome (54). In addition, ICP27 has been shown to be able to stimulate translation of a luciferase mRNA only if it was previously tethered to it (53). Thus, it is not surprising that in our system, ICP27 does not stimulate Renilla luciferase mRNA translation nor cytoplasmic Renilla luciferase mRNA accumulation, suggesting that translation stimulation is probably dependent on the binding of the herpes simplex virus proteins to the mRNA. Since all the herpesvirus EB2 homologous proteins have been shown to shuttle from the nucleus to the cytoplasm (31,38,55) it is tempting to speculate that they first interact with their unspliced mRNA targets inside the nucleus, and then shuttle to the cytoplasm bound to these mRNAs where they play a role in translation. Accordingly, it is interesting to notice that ICP27 has also been shown to be associated with polyribosomes (53). Taken together, these results suggest firstly that both EB2 and the herpesvirus EB2 homologous proteins have a direct role on translation of the mRNA they interact with, and secondly that protein–mRNA interaction, mRNA export and translation stimulation are strongly linked.

EB2 expression is essential for viral particle production and its absence leads to very poor viral DNA replication probably because of the low expression of early viral mRNAs that depend on EB2 for their export (i.e. BALF5 and BALF2) (27). An even more drastic effect was seen on most of the late viral mRNAs (28,56). We have shown here that EB2 expression leads to a 25-fold stimulation of renilla luciferase reporter gene expression with a cytoplasmic accumulation of the corresponding mRNA stimulated 3.5-fold and the translation itself stimulated from 5- to 7-fold. This suggests that the essential role of EB2 during the EBV productive cycle could be explained by a combined role on mRNA export and translation stimulation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank B. Blanquier from the 'Plateau d'analyse génétique' of IFR128 for advice on quantitative PCR. We thank A. Chaboud and Y. Tauran from the 'Plateau de production et d'analyse des protéines' of IFR 128 for use of the AKTA purifier. We thank Dr S. Morley for providing anti-human PABP antibodies and Dr D. Levy and S. Delpeut for providing PKR-deficient cells. We also thank the Réseau Herpesvirus and Cancer for its support. Finally, we thank Dr R. Buckland for reading the manuscript.

FUNDING

'Institut National de la Santé et de la Recherche Médicale' (INSERM), the 'Agence Nationale pour la Recherche' (ANR) [ANR MIME: grant number RPV06120CSA (to E.M.) and ANR blanche: grant number 06-0290-01 (to T.O.)] and the pole of competitivity Lyon Biopole. Fellowship from the 'Association pour la Recherche contre le cancer' (ARC) (to C.M.-P.); fellowship from the 'Ministère de la Recherche et de la Technologie' (MRT to E.R.). E.M. is a CNRS scientist. Funding for open access charge: INSERM.

Conflict of interest statement. None declared.

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