Epstein-Barr Virus EB2 Protein Exports Unspliced RNA via a Crm-1-Independent Pathway

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Human herpesviruses encode posttranscriptional activators that are believed to up-regulate viral replication by facilitating early and late gene expression. We have reported previously that the Epstein-Barr virus protein EB2 (also called M or SM) promotes nuclear export of RNAs that are poor substrates for spliceosome assembly, an effect that closely resembles the human immunodeficiency virus type 1 Rev-dependent nuclear export of unspliced viral RNA. Here we present experimental data showing that EB2 efficiently promotes the nuclear export of unspliced RNA expressed from a Rev reporter construct. Site-directed mutagenesis as well as domain swapping experiments indicate that a leucine-rich region found in the EB2 protein, which matches the consensus sequence for the leucine-rich nuclear export signal, is not a nuclear export signal per se. Accordingly, leptomycin B (LMB), a specific Crm-1 inhibitor, impairs Rev but not EB2-dependent nuclear export of unspliced RNA. Moreover, EB2 nucleocytoplasmic shuttling visualized by a heterokaryon assay is, unlike Rev shuttling, not affected by LMB. We also show that overexpression of an N-terminal deletion mutant of Nup214/can, a major nucleoporin of the nuclear pore complex involved in several aspects of nuclear transport, blocks both Rev- and EB2-dependent nuclear export of RNA. These results strongly suggest that EB2 nuclear export of unspliced RNA is mediated by a Crm-1-independent pathway.

Epstein-Barr virus (EBV) is a human gamma-herpesvirus widely spread in the adult human population. This virus is associated with several malignancies such as Burkitt’s lymphoma, nasopharyngeal carcinoma, Hodgkin’s disease, gastric carcinoma, breast carcinoma, and B- and T-cell lymphomas and induces the permanent proliferation (immortalization) of quiescent B lymphocytes in vitro. In EBV-associated tumors in vivo as well as in EBV-infected B cells proliferating ex vivo, entry into a productive cycle is a rare event and the transcription of the EBV genome is usually restricted to a few genes at a late stage of the viral cycle (for reviews, see references 25 and 36). Although the molecular events occurring during the switch from latency to the productive cycle are now partially understood for in vitro-immortalized B cells (42), the functions of many EBV genes products expressed during the lytic cycle have only partially been characterized, and very little is known about certain others. Among these, the early nuclear protein EB2 (7), which is also called M or SM (8), was originally described as a promiscuous transcription factor, as it activates transient expression of the chloramphenicol acetyltransferase (CAT) gene placed under the control of many different promoters (26). We reported recently that EB2 could activate cytoplastic accumulation of unspliced RNAs, particularly when they are poor substrates for spliceosome assembly, which suggested an effect of EB2 on either splicing or RNA export or both (4). A recent report, using heterokaryon assays, has revealed that EB2, like its herpes simplex virus type 1 (HSV-1) homologue ICP27, has properties of an RNA export protein, i.e., nuclear-cytoplasmic shuttling and RNA binding activities, although no specific responsive RNA sequences have been identified so far on EB2 target RNA (38, 39). This was very reminiscent of the lentivirus Rev protein that mediates active nuclear export of intron-containing viral mRNA after binding to a cis-acting sequence on the RNA called the Rev response element (RRE) (for a review, see reference 34). Rev-dependent nuclear export of RNA requires at least two domains of the human immunodeficiency virus type 1 (HIV-1) protein: a highly basic N-terminal domain that also specifies nuclear-nuclear localization and a short C-terminal leucine-rich nuclear export signal (NES). Similar leucine-rich NESs have now been found in many other viral and cellular proteins (for a review, see reference 17). These NESs are transferable to heterologous proteins, and mutations in the Rev NES impair both Rev shuttling and Rev-dependent export of RNA (12, 27, 46). Furthermore, it is now well documented that nuclear export of Rev is mediated by a complex trimolecular interaction involving the NES, the importin b-like protein Crm-1 (also called exportin 1) and ranGTP, a small GTPase in its GTP-bound state (13). The elucidation of the role of Crm-1 in this process comes from experiments performed by Wolff and coworkers (47) showing that the antibiotic leptomycin B (LMB), which was reported to interact specifically with Crm-1, was a potent inhibitor of Rev nuclear export. However, the Crm-1-dependent pathway is not unique, and other export routes that are not sensitive to LMB exist. Indeed, NESs that do not belong to the growing family of leucine-rich NESs have now been described for different proteins such as TAP (1), hnRNPK (30), hnRNP A1 (29), and HuR (10).

It was recently published that the EBV protein EB2 contains a leucine-rich region (LRR) that could fit the consensus for leucine-rich NES (39). It was reported subsequently that the EB2-mediated export of intronless RNA and the intracellular localization of EB2 could be mediated by a direct association with Crm-1 (3). Our current observations lead to different conclusions. In this report, we have used a functional assay

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originaly devised to assess the effect of Rev in exporting intron-containing RNAs. In this Rev-controlled assay, EB2 efficiently stimulated the nuclear export of intron-containing RNAs. Our results clearly demonstrate that, under conditions where Rev-mediated export of intron-containing RNA is inhibited by inactivating the Crm-1-dependent pathway using LMB, the EB2-mediated export of intron-containing RNA is unaffected. Moreover, although a leucine-rich NES-like sequence has been identified in EB2, we show here by both site-directed mutagenesis and domain swapping that this domain is not required for nuclear export of intronless or intron-containing RNA and is therefore not a leucine-rich NES. Finally, in cell fusion experiments, we report that EB2 nucleocytoplasmic shuttling is LMB resistant. Therefore, our results suggest that the herpesvirus protein EB2 is involved in nuclear export of RNA through a mechanism distinct from what is used by the lentivirus Rev protein.

**RESULTS**

EB2 induces the cytoplasmatic accumulation of intron-containing RNA expressed from an HIV-1 Rev reporter gene. EB2 EB2 and HIV-1 Rev both have been shown previously to increase the nuclear export of incompletely spliced RNAs which are poor substrates for splicing (4, 6, 43). However, these observations were made in different systems, and in the case of Rev, an RNA cis-acting element, the RRE, was found to be necessary for activity. In order to further understand the molecular mechanisms of EB2 function, we took advantage of an assay which has been extensively used to study Rev activity. The reporter plasmid that we used, pCAT.RRE, is a derivative of pDM128 (Fig. 1A). When transfected into HeLa cells, pCAT.RRE expressed a two-exon, one-intron pre-mRNA which is mostly spliced, resulting in the excision of the CAT gene and very low levels of CAT protein expressed (Fig. 1B, lane 1). As described previously, Rev expression induced the cytoplasmatic accumulation of unspliced RNA and, therefore, an increase in the amount of CAT protein detected (Fig. 1B and D, lane 2). Conversely, the M10 Rev mutant, in which a mutation inactivates the NES, has no effect on the basal level of CAT protein synthesis (Fig. 1B, lane 3). When a plasmid expressing EB2 was cotransfected with pCAT.RRE, the amount of CAT protein detected dramatically increased and reached a level similar to what could be seen in the presence of Rev (Fig. 1B, lane 4). EB2Cter, an EB2 N-terminal deletion mutant, appeared to be incompetent in this reporter gene, suggesting that the amino terminus of EB2 is required for full activity (Fig. 1B, lane 5). It is noteworthy that maximum transactivation by Rev and EB2 was obtained with different amounts of transfected plasmids, 100 ng of the Rev-expressing construct and 250 ng of the EB2 expression construct (data not shown).

We and others have previously reported that EB2 activates
CAT mRNA expression from different constructs lacking the RRE (5, 26, 37). Consequently, EB2-mediated transactivation in the Rev system was likely to be RRE independent. However, recent studies have suggested that RRE-containing mRNA could be the target of proteins other than Rev, which, like sam68, could activate their nuclear export (35). To eliminate the possibility that CAT induction by EB2 was indirect and dependent on the RRE, we investigated the effect of EB2 on CAT expression from plasmid pCAT.XRE in which the HIV-1 RRE sequence has been changed to the human T-cell leukemia virus type 1 XRE. Using this reporter construct, we show that Rev only had a slight effect whereas CAT induction upon EB2 expression was strong and similar in value to what we have obtained with pCAT.RRE (Fig. 1B, lanes 6 to 10).

To confirm that the effect of EB2 on CAT protein expression was due to elevated levels of CAT mRNA in the cytoplasm, we analyzed, by RT-PCR, the cytoplasmic RNA coming from the transfection experiment depicted in Fig. 1B. As shown in Fig. 1D, the level of CAT mRNA increased upon addition of both Rev and EB2 but remained very low with mutants M10 and EB2Cter, suggesting that, like Rev, EB2 induces nuclear export of CAT mRNA in this system. Activation by Rev and EB2 was shown to be specific for the CAT reporter gene since expression of the endogenous β-actin mRNA was not affected by expression of these proteins as visualized by RT-PCR analysis (Fig. 1D). Since it has been published that EB2 does not activate transcription but exports EBV intronless RNAs, the observation that EB2 also increases the cytoplasmic accumulation of intron-containing RNAs reinforces the idea that it is an RNA export factor.

The LRR in EB2 is not a leucine-rich NES. Having shown that EB2 is able to induce cytoplasmic accumulation of intron-containing RNA expressed from pCAT.RRE, we decided to take advantage of this Rev-controlled system to study the mechanism of EB2-mediated nuclear export of RNA. In a recent publication, Semmes and coworkers reported, using the heterokaryon assay, that EB2 is able to shuttle between the nucleus and the cytoplasm (39). Furthermore, they have pointed out that an LRR showing strong homology with various leucine-rich NESs could specify EB2 nuclear export (Fig. 2A). An extensive mutagenesis in the Rev protein has revealed that changing any one of the three leucines of the NES (L78, L81, and L83) to alanines resulted in a complete loss of activity (27). In order to test whether the putative NES was important for EB2 function, we generated a mutant, EB2L/A, in which the last two leucines (L234 and L236) were changed to alanines (Fig. 2A). If the LRR is indeed the EB2 NES, this mutation should completely abolish the effect of EB2 on CAT mRNA export. On the other hand, if the LRR includes the EB2 NES, it should be able to substitute for the Rev NES in the context of the Rev protein. Consequently, we have also constructed a Rev mutant, called Rev/EB2, by replacing the Rev NES with EB2 amino acids 225 to 237 encompassing the LRR (Fig. 2A). An extensive mutagenesis in the Rev protein has revealed that changing any one of the three leucines of the NES (L78, L81, and L83) to alanines resulted in a complete loss of activity (27). In order to test whether the putative NES was important for EB2 function, we generated a mutant, EB2L/A, in which the last two leucines (L234 and L236) were changed to alanines (Fig. 2A). If the LRR is indeed the EB2 NES, this mutation should completely abolish the effect of EB2 on CAT mRNA export. On the other hand, if the LRR includes the EB2 NES, it should be able to substitute for the Rev NES in the context of the Rev protein. Consequently, we have also constructed a Rev mutant, called Rev/EB2, by replacing the Rev NES with EB2 amino acids 225 to 237 encompassing the LRR (Fig. 2A). These mutants were tested for their ability to transactivate the reporter plasmid pCAT.RRE. As demonstrated in Fig. 2B, Rev efficiently increased the nuclear export of unspliced RNA (Fig. 2B, compare lanes 1 and 2), but the RevM10 NES mutant (lane 3) and the Rev/EB2 hybrid protein (lane 4) failed to do so, indicating that the EB2 LRR is unable to substitute for the Rev NES. Using the same assay, we show that both EB2 (lane 6) and the LRR mutant EB2L/A (lane 7) efficiently exported unspliced RNA. Another Rev/EB2 mutant that included an extended version of the EB2 LRR (amino acids 221 to 240) similarly failed to activate our reporter gene (data not shown). As shown by Western blotting (Fig. 2B, lower panel), the amount of proteins expressed validated the results drawn from
the CAT experiment. However, it is possible that EB2 activates gene expression by different mechanisms depending on the substrate RNA; thus, the EB2L/A mutant could be fully active in one system and inactive in another. To test this hypothesis, we compared the effects of EB2 and of the LRR mutant using two other EB2 reporter constructs, pUCbD128SV and pRcCMV-Na (4). pUCbD128SV contains a thalassemic allele of the human β-globin gene (Fig. 2C). The β-thalassemic gene contains a G-to-A transition at position 1 in the first intron (IVS1), which causes the activation of three cryptic 5′ splice sites, otherwise completely silent in the wild-type precursor RNA (Fig. 2C). Upon transient transfection of plasmid pUCbD128SV in HeLa cells, RT-PCR analysis showed that the three cryptic 5′ splice sites were used (Fig. 2D, lane 1). EB2 and the EB2L/A mutant (Fig. 2D, lanes 2 and 3) strongly increased the cytoplasmic accumulation of unspliced β-thalassemic RNA and decreased the amount of spliced RNA as previously reported (4). We also used a construct called pRcCMV-Na from which the naturally occurring intronless BRRF1 EBV early RNA is expressed. As shown in Fig. 2D, lane 4, the intronless BRRF1 RNA could be detected by RT-PCR in the cytoplasm of transfected cells. Coexpression of EB2 resulted in an increase of the cytoplasmic accumulation of BRRF1 RNA, and this effect was clearly not affected by mutation of the EB2 LRR (Fig. 2D, lane 6). Collectively, our results strongly suggest that EB2 leucines 234 and 236 do not participate in EB2 function and indicate that the LRR is not a leucine-rich NES.

**EB2-associated nuclear export of unspliced RNA is LMB resistant.** Having shown that the EB2 LRR was not a NES, we next asked whether another leucine-rich NES differing from the consensus could be present in the EB2 protein. Leucine-rich NESs are found in a variety of proteins. They are known to function via a direct interaction with an importin β-like protein called Crm-1 in a RanGTP-dependent manner. The fungal metabolite LMB is known to specifically target Crm-1 and to block its interaction with both RanGTP and the NES. This in turn results in an inhibition of the protein nuclear export (13, 16). To test whether EB2-mediated nuclear export of intron-containing RNA was dependent on the Crm-1 export factor or not, transient transfections of HeLa cells using plasmid pCAT.RRE and Rev or EB2 expression vectors were repeated. Twelve hours after transfection, cells were washed and incubated for a further 6 h without or with 10 nM LMB. A 6-h incubation time was chosen because (i) the effect of LMB...
on Rev activation was strong at that time and (ii) we noticed that an incubation time of 16 h resulted in a dramatic reduction in the amount of EB2 and Rev proteins expressed (data not shown). As expected, Rev activation of CAT expression was dramatically reduced when LMB was added to the medium (Fig. 3B, lanes 4 and 5) and with EB2Cter (Fig. 3B, lanes 8 and 9), which are inactive in RNA export. As shown by Western blotting (Fig. 3C), the amounts of proteins expressed in this experiment were not affected by LMB. Therefore, it appears that EB2 induces the nuclear export of intronless RNA by a Crm-1-independent mechanism.

EB2 nucleocytoplasmic shuttling is not inhibited by LMB. It has been previously published that, although EB2 localizes predominantly to the nucleoplasm, it shuttles continuously between the nucleus and the cytoplasm (39). To confirm and extend these observations, we have evaluated whether EB2 nucleocytoplasmic shuttling was sensitive to LMB. HeLa cells were transfected with plasmids allowing the expression of EB2 and Rev and then fused to mouse NIH 3T3 cells by the polyethylene glycol method. As shown in Fig. 4A, in HeLa/NH 3T3 heterokaryons, EB2 was detected in the mouse nucleus after a 30-min incubation period (panel a). At 2 h postfusion, EB2 appeared to equilibrate between the mouse and human nucleus (panel c), as did Rev (panel e), indicating that both proteins are shuttling. As reported previously (47), Rev shuttling was strongly inhibited by LMB (Fig. 4A, panel f). However, we did not notice any effect of LMB on the ability of EB2 to relocate in the mouse nucleus (panels b and d), demonstrating that EB2 nuclear export was Crm-1 independent. As a control, we also looked at the endogenous human hnRNPC protein localization in the fused cells. As expected, hnRNPC, which carries a nuclear retention signal, was found restricted to the HeLa nucleus after a 0.5- or 2-h incubation time, as revealed by indirect immunofluorescence using a human-specific anti-hnRNPC antibody (panels a’, c’, b’, and d’). Shuttling of the EB2L/A mutant was also tested in the heterokaryon assay. Cell fusions between NIH 3T3 cells expressing EB2L/A and HeLa cells were performed. As shown in Fig. 4B (panel h), EB2L/A shuttled between the mouse and human nuclei similarly to wild-type EB2.

Furthermore, we noticed that both Flag.Rev (Fig. 5a) and Flag.EB2Cter proteins (Fig. 5d) localized in the nucleus as well as in the cytoplasm of transfected cells. Although we cannot explain at the moment the reasons underlying their subcellular localization, Flag.EB2Cter appeared to be a useful tool to evaluate the Crm-1 dependence of EB2 shuttling. Indeed, Flag.EB2Cter contains the LRR identified by Semmes and coworkers (39) and Flag.Rev contains a functional leucine-rich NES. We then reasoned that if EB2 shuttling was mediated by a direct interaction between Crm-1 and the LRR, LMB treatment would completely relocate Flag.EB2Cter to the cell nucleus. However, as demonstrated in Fig. 5, the intracellular distribution of EB2Cter protein was not affected by addition of various concentrations of LMB to the cell culture medium (Fig. 5e and f), whereas Flag.Rev was found to be exclusively nuclear upon LMB treatment (Fig. 5b and c). These results further strengthen our findings that the EB2 LRR is not a Crm-1-dependent leucine-rich NES.

Δcan, a transdominant negative mutant of Nup214, inhibits EB2 export pathway. Nuclear export of proteins and RNA occurs through the nuclear pore complex, a huge macromolecular structure of 125 MDa composed of about 100 proteins called nucleoporins. One of them, Nup214/Δcan, is found in a multiprotein complex including Crm-1 (14). Nup214 has recently been implicated in the Crm-1-dependent pathway as well as in other nucleocytoplasmic transport pathways (13, 24, 45). Therefore, we decided to test whether Nup214 could be involved directly or not with EB2 nuclear export. Δcan, a Nup214 C-terminal fragment including the phenylalanine-glycine repeat (FG repeat)-rich region, has been previously described to have a transdominant negative phenotype and to
inhibit Rev function in the CAT.RRE system (2, 23). However, when the RRE is replaced by the TAP binding sequence (CTE), TAP also induces the cytoplasmic accumulation of CAT-CTE intron-containing RNAs, but overexpression of Δcan has no effect in this assay (2, 23). A similar Δcan mutant was overexpressed in our CAT.RRE reporter system to determine whether it could affect EB2-mediated RNA export. As expected, Δcan efficiently repressed Rev activity to about 10 to 20% of control level (Fig. 6A, lanes 3 and 4). Similarly, Δcan expression resulted in a dramatic inhibition of EB2 transactivation, indicating that Nup214 could be involved in the EB2 export pathway (Fig. 6A, lanes 6 and 7).
Inhibition of CAT.RRE RNA nuclear export by \( \Delta \text{can} \) was not due to a general effect on mRNA export since (i) \( \Delta \text{can} \) overexpression has only a slight effect on CAT expressed from pAAC-CAT, a basic CAT reporter plasmid (Fig. 6A, lanes 9 and 10), and (ii) endogenous \( \beta \)-actin mRNA expression appeared to be insensitive to \( \Delta \text{can} \) overexpression as revealed by RT-PCR analysis using different amplification conditions (Fig. 6B). These observations therefore indicate that Nup214 participates in the nuclear export of the EBV protein EB2.

**DISCUSSION**

The experiments reported here clearly indicate that HIV-1 Rev and EBV EB2 proteins efficiently induce the nuclear export of an intron-containing RNA. It was reported previously that the HIV-1 tat/rev intron, present in the CAT.RRE RNA, was inefficiently removed due to suboptimal signals in the 3′ splice site (43). Therefore, and as documented recently (4), our data confirm that EB2 has the ability to induce cytoplasmic accumulation of intron-containing RNAs when they are poor substrates for spliceosome assembly. Recently, EB2 was found to shuttle between the nucleus and the cytoplasm (reference 39 and this work), and a leucine-rich region with high homology to the Rev NES was identified in the protein primary sequence. Therefore, it was tempting to propose that EB2 nuclear export of RNA was, similar to Rev, dependent on the Crm-1 pathway (39, 40). To address this issue, we focused on the EB2 LRR and investigated the requirement for the Crm-1 protein in nuclear export of EB2 and its target RNA. As the Rev protein was included as a positive control, the CAT RRE reporter gene derived from pDM128 was found to be a valuable model to study EB2-dependent nuclear export. Our data indicate that (i) the EB2 putative leucine-rich NES is not an NES per se, as it could be mutated without affecting the function of EB2 and could not substitute for the Rev NES in the context of the Rev protein; (ii) EB2 transactivation is not affected by LMB (a Crm-1-specific inhibitor) under conditions where Rev activation is dramatically reduced; and (iii) EB2 nucleocytoplasmic shuttling visualized by an interspecies heterokaryon assay is also not LMB sensitive. Our results appear to be rather different from those which have recently been published by Boyle and coworkers (3). They have reported, by performing transient expression assays with lymphoblastoid B cells (BJAB), that (i) EB2 activation of an intronless CAT reporter gene is inhibited by LMB and potentiated by overexpression of Crm-1; (ii) complete deletion of the EB2 LRR resulted in an 80% reduction in transactivation, whereas point mutations of leucines in the EB2 LRR reduced activation by only 40%; and (iii) EB2 can be pulled down with Crm-1 in coimmunoprecipitation experiments. According to previous work on leucine-rich NESs, we believed that if the EB2 LRR (LPSPLASLTLPSP) was the EB2 NES, it should be completely inactivated by mutating leucines 234 and 236 to alamines as exemplified for p53 (44), PKI (46), FMRP (15), IxBe (22), and Rev (27). However, we show that mutation of these leucines does not significantly affect EB2 transactivation, indicating that these residues are not essential for function. Furthermore, although Boyle et al. reported that complete deletion of the LRR (mutant LRR-\( \Delta \)) reduced EB2 activation to about 20% of wild-type EB2 levels, they also showed that mutant LRR-\( \Delta \) is insoluble in 1% Triton, suggesting a tight association with nuclear structures. We have previously described a similar EB2 deletion mutant called \( \Delta \text{7} \) lacking the LRR. We found that mutant \( \Delta \text{7} \) is not functional in different transactivation assays (4) and localizes, similarly to LRR-\( \Delta \), to large nuclear dots (data not shown). We believe...
that the Δ7 mutant as well as most of our C-terminal deletion mutants is a nonfunctional activator (4), probably because it does not fold properly and aggregate in the nucleus to give rise to these large nuclear substructures, which are also observed by visible light microscopy (data not shown). Therefore, it is not surprising that the LRR-Δ mutant is not fully active. The significant discrepancy between our results and those obtained by Boyle and coworkers could be also partially explained if EB2 contains more than one NES, one dependent on Crm-1 and active in both B lymphocytes and HeLa cells, and the other being Crm-1 independent and active only in HeLa cells. We also cannot rule out at this point the possibility that the LRR participates in the nuclear export of EB2 through a Crm-1-independent mechanism.

In agreement with Boyle and coworkers (3), we show that a negative transdominant mutant of Nup214, called Δcan, is an efficient inhibitor of EB2 trans activation. This result suggests that Nup214 is an essential component of the EB2 export pathway. This last observation is not in contradiction to our observation that Crm-1 is not involved in EB2 nuclear export, since Nup214 was proposed to be involved in different export and import pathways (13, 24, 45). For example, the cellular TAP protein has been identified as the export factor for the CTE-containing RNA of type D retrovirus (18). Although TAP function is insufficient to LMB, it binds to Nup214 both in vitro and in yeast cells (23, 24). In conclusion, our results favor a mechanism for the nuclear export of EB2 distinct from the Rev pathway but also involving Nup214. The EB2 NES as well as the EB2 export pathway has now to be carefully identified and characterized.

The EBV EB2 protein is not unique in its capacity to affect mRNA nuclear export. Other herpesviruses express EB2-like factors, i.e., HSV-1 ICP27, human herpesvirus 8 ORF57, herpesvirus saimiri ORF57, bovine herpesvirus 4 HORF1/2, etc., that act posttranscriptionally to facilitate early and lytic viral gene expression. Although these factors have been shown to activate CAT reporter genes, their mechanism of action as well as their role in viral pathogenesis is still not clear. For HSV-1, it was shown that HSV-1 ICP27 null mutants did not replicate their DNA and were unable to grow in Vero cells (41). By use of temperature-sensitive mutants, ICP27 was found to simultaneously activate viral intronless genes and repress intron-containing ones. Furthermore, it is now well documented that ICP27 is a shuttling protein which activates nuclear export of intronless mRNA (32, 33, 38, 41). Export of ICP27 is mediated by an LRR located at the N terminus of the protein, but the ICP27 export receptor has not been identified yet (38). The EB2 protein is somewhat different, since in our hands it does not inhibit expression of intron-containing genes but activates nuclear export of intron-containing polyadenylated RNA possessing suboptimal splice sites (reference 4 and this work). However, the mechanisms of action of these two proteins may not be so different. Indeed, it is believed that splicing is a prerequisite for nuclear export of most mRNAs. Accordingly, intron-containing RNAs are not normally found in the cytoplasm and very few RNAs, including histones, c-jun, alpha interferon, and hepatitis B virus RNA, do not contain introns. These particular RNAs are nevertheless exported to the cytoplasm, and for the histone H2a and the HSV-1 thymidine kinase, RNA sequences that induce efficient cytoplasmic accumulation of these intronless transcripts have been identified (21, 31). Therefore, poor expression of viral intronless RNA may be explained by the presence of cryptic splice sites that would allow nonproductive assembly of splicing factors. In the absence of RNA transport elements, such as those found in the histone H2a and the HSV-1 thymidine kinase RNA (21, 31), intronless RNA would be retained in the nucleus and eventually degraded. Interestingly, such a cryptic 5′ splice site is present in the bacterial CAT gene used to study EB2 function (4, 39). Similarly, intron-containing transcripts with suboptimal splice sites are retained in the nucleus until fully spliced. EB2 and ICP27 proteins would efficiently interact with these nucleaus-entraped RNAs and promote their export to the cytoplasm, therefore competing with spliceosome assembly. In this respect, the data presented here are relevant to EBV biology since, as for HSV-1, most EBV early and late mRNAs are synthesized from intronless genes, whereas genes expressed during latency harbor introns. Furthermore, several intron-containing RNAs appear to accumulate in the cytoplasm of infected cells during the productive cycle (5, 28). We believe that, similarly to Rev and TAP, EB2 is a nuclear export factor that facilitates cytoplasmic accumulation of both intronless RNA and intron-containing RNA with suboptimal splice sites.

The question of the interaction of EB2 with its target RNA is also important to address. Although the extent of the EB2 effect is dependent on the RNA template, no specific EB2-interacting sequences have been identified so far on the RNA targets, and this is also true for ICP27. Different groups including ours have reported the binding of recombinant EB2 to various RNA probes in vitro (4, 37, 39). However, by North-western analysis we noted that RNA does not interact with full-length EB2 but with C-terminally truncated EB2 protein species (data not shown). We found this interaction to be mediated by the RXP region in vitro, but surprisingly, we also demonstrated that this domain was dispensable in vivo since it could be deleted without affecting EB2-mediated nuclear RNA export (4). Therefore, it is tempting to speculate that EB2 does not bind RNA directly in vivo but, instead, interacts with target RNA via an adapter protein that could be either hnRNPs, SR proteins, or even components of the basal splicing machinery. This would explain why many different mRNAs are sensitive to EB2, including the CAT transcripts, but also cellular RNA, as the EB2 protein was reported to induce transformation of rodent fibroblasts by a mechanism which involves overexpression of the myc proto-oncogene (9).

Further work is now needed to precisely map functional domains in the EB2 protein and to identify cellular proteins directly involved in EB2 shuttling and EB2-mediated RNA export.

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