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A Novel Nuclear Export Signal and a REF Interaction Domain Both Promote mRNA Export by the Epstein-Barr Virus EB2 Protein*

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A striking characteristic of mRNA export factors is that they shuttle continuously between the cytoplasm and the nucleus. This shuttling is mediated by specific factors interacting with peptide motifs called nuclear export signals (NES) and nuclear localization signals. We have identified a novel CRM-1-independent transferable NES and two nuclear localization signals in the Epstein-Barr virus mRNA export factor EB2 (also called BMLF1, Mta, or SM) localized at the N terminus of the protein between amino acids 61 and 146. We have also found that a previously described double NES (amino acids 213–236) does not mediate the nuclear shuttling of EB2, but is an interaction domain with the cellular export factor REF *in vitro*. This newly characterized REF interaction domain is essential for EB2-mediated mRNA export. Accordingly, *in vivo*, EB2 is found in complexes containing REF as well as the cellular factor TAP. However, these interactions are RNase-sensitive, suggesting that the RNA is an essential component of these complexes.

In cells infected by human herpesviruses, viral mRNAs and proteins are trafficked through the nuclear pore complex. Several cellular factors that mediate the nucleocytoplasmic transport of mRNAs have now been identified (1–6). Interestingly, some human herpesviruses carry, in their genome, genes whose products are also mRNA export factors, such as HSV-1 ICP27 (7) and the EBV BMLF1 early gene product originally called EB2 (8), but later called Mta (9) or SM (10). Such genes are conserved among all human herpesviruses, suggesting a conserved function for their products. At least for HSV-1 and EBV, the inactivation of ICP27 (11) or EB2 (12), respectively, abolishes the production of infectious viral particles, demonstrating that ICP27 and EB2 are essential factors for viral mRNA export and that their function cannot be *trans*-complemented by cellular factors. Moreover, EB2 appears to have an effect on cellular mRNAs because it has transforming proper-

ties when expressed both in established cell lines such as Rat1 and NIH3T3 and in primary rat fibroblasts (13).

Most of the HSV-1 and EBV early and late mRNAs are transcribed from intronless genes. However, it is now clearly established that the nuclear export of mRNAs is dramatically increased when a splicing event occurs (14). In effect, splicing leads to the deposition on the mRNA of a multiprotein export complex (called EJC for exon-exon junction complex), including REF/Aly (Yra1 in yeast), Y14, RNPS1, SRm160, and Magoh, 20–24 nucleotides upstream of the exon-exon junction (2–5). Such a complex is thought to export mRNAs by recruiting TAP/Mex67p (15) to cellular messenger RNPs (16–18). For cellular mRNAs generated from intronless genes, they are likely to be exported to the cytoplasm by cellular factors through nonspecific interactions with mRNA-bound adapters like REF (19) or through sequence-specific interactions with SRp20 or 9G8 (20) or U2AF (21). It is therefore tempting to speculate that EB2, like its HSV-1 functional homolog ICP27 (7), is an adapter of viral origin, involved in the export of intronless early and/or late viral mRNAs.

Interestingly, it has been recently reported that ICP27 recruits the cellular mRNA export factors REF (Aly) and TAP/NXF1 (7) to viral mRNAs generated from viral intronless genes, providing these viral mRNAs with access to the cellular mRNA nuclear export pathway. However, it was clear that in the absence of viral mRNA, ICP27 nucleocytoplasmic shuttling was TAP- and CRM-1-independent. This suggests that ICP27-mediated mRNA export and ICP27 nucleocytoplasmic shuttling follow different pathways and are probably mediated by different ICP27 domains. However, the ICP27 CRM-1-independent nuclear export signal (NES) has not as yet been characterized.

The EBV EB2 protein also shares properties with mRNA export factors: (i) it exports both intronless and intron-containing RNAs (22–24); (ii) it shuttles between the cytoplasm and the nucleus (22, 24, 25); and (iii) it is thought to carry two contiguous CRM-1-dependent NES (26). However, the EB2 nucleocytoplasmic shuttling has also been described to be CRM-1-independent (24). EB2 also appears to homodimerize or multimerize (27) and binds to RNA *in vivo* (28), although no EB2-specific RNA sequences have been identified on EB2 RNA targets.

In this report, we have characterized the EB2 sequences required for nucleocytoplasmic shuttling and mRNA export. We show that the EB2 domain between amino acids 218 and 236, which have been reported to carry a CRM-1-dependent double NES (26), can be deleted without affecting the nucleocytoplasmic shuttling of EB2 and does not constitute a functional NES. However, we demonstrate that this designated double NES region binds REF both *in vitro* and *in vivo* and is

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¹ The abbreviations used are: HSV-1, herpes simplex virus-1; EBV, Epstein-Barr virus; NES, nuclear export signal(s); NLS, nuclear localization signal(s); GST, glutathione S-transferase; hnRNP, heterogeneous nuclear ribonucleoprotein; CAT, chloramphenicol acetyltransferase; LMB, leptomycin B.

essential for EB2-mediated mRNA export. Moreover, we found that the N-terminal region of EB2 carries a CRM-1-independent NES and two nuclear localization signals (NLS) that mediate the nucleocytoplasmic shuttling of EB2.

EXPERIMENTAL PROCEDURES

Plasmids—The following eukaryotic expression plasmids are all SV40 early promoter-based vectors. When F precedes the name of the protein, the corresponding protein was tagged at its N terminus with the FLAG epitope, which can be detected by monoclonal antibody M2 (Sigma). pSG5F.EB2 contains the intronless *BSLF2/BMLF1* cDNA and encodes the wild-type EB2 protein (23). pSG5F.EB2.dNES expresses an EB2 protein deleted of amino acids 225–236 in the two putative NES regions, as described (26). pSG5F.EB2.ΔDN expresses an EB2 protein with the two putative NES (amino acids 218–236) entirely deleted. These two plasmids were obtained by site-directed mutagenesis of pSG5F.EB2 using the QuikChange site-directed mutagenesis kit (Stratagene) and the oligonucleotide primer 5'-CTCCAAGATTACATTGCGGCCGCGAGCCCATCCAAGAC-3' and its complement on the opposite strand for pSG5F.EB2.dNES, 5'-GACATGAGTCTGGTTAAGGAGCCCATCCAAGAC-3', and its complement on the opposite strand for pSG5F.EB2.ΔDN. Mutations in the putative EB2 NLS sequences were introduced by site-directed mutagenesis using the QuikChange kit. For the KR1 mutation (¹²⁷KRRR¹³⁰ → AAAA), the oligonucleotide primer 5'-CACCAGAGGCCACGACGCGGACGCCGAGAGGTCCATGG-3' and its complement on the opposite strand were used. For the KR2 mutation (¹⁴³KRR¹⁴⁵ → AAA), the oligonucleotide primer 5'-GATGAAAGTTATGGCGCGCCGACACCTGCCCCC-3' and its complement on the opposite strand were used. pSG5F.EB2.Cter has been described elsewhere (29). The NLS sequence from the SV40 T antigen (APKKRKV) was introduced between the FLAG and the EB2 C-terminal DNA sequences to generate pSG5F.NLS.EB2.Cter. Seven partially overlapping EB2 DNA fragments (fragments B–H) were cloned into the prokaryotic expression vector pGEX-4T-2 (Amersham Biosciences) to produce GST proteins fused to EB2 peptides B–H (see Fig. 1). EB2 DNA fragments B–H were generated by PCR using the following oligonucleotide primers: 5'-CGGGATCCGATGAAGATCCAAC-3' and 5'-CCGCTCGAGACTTTTCATCGGTGCA-3' for fragment B, 5'-CGGGATCCCTTACACAGAGGC-3' and 5'-CCGCTCGAGGCGTTCTTGCTCGC-3' for fragment C, 5'-CGGGATCCCCCGGTCAGAATC-3' and 5'-CGGCTCGAGCTGCTCAGGCTCCAAT-3' for fragment D, 5'-CGGGATCCGACCGGTTCTACAG-3' and 5'-CCGCTCGAGGTAGGTGATCTCTG-3' for fragment E, 5'-CGGGATCCCTCTGCACCCTGTG-3' and 5'-CCGCTCGAGCTTGTGTTTACGCGGC-3' for fragment F, 5'-CGGGATCCGACTACAACCTTTGTG-3' and 5'-CCGCTCGAGCTGTGCAAAAGGGA-3' for fragment G, and 5'-CGGGATCCCTCTGGGCCACTAC-3' and 5'-CCGCTCGAGTTGATTTAATCCAGG-3' for fragment H. These PCR fragments were subsequently digested with *Bam*HI and *Xho*I for insertion between the *Bam*HI and *Xho*I sites of pGEX-4T-2. The same EB2 DNA fragments (B–H) were also ligated to a DNA sequence encoding the bacteriophage MS2 coat protein in the eukaryotic expression plasmid pCMV-MS2 (a generous gift from Dr. B. R. Cullen) (30) to produce MS2-EB2 peptide fusion proteins. Fragments B–H were obtained by PCR amplification using oligonucleotides similar to those listed above, except for the substitution of the *Bam*HI site present in each upper strand oligonucleotide with an *Eco*RI site. Fragment A was obtained by PCR amplification using the following two oligonucleotides: 5'-CGGAATTCATGTTCTTCTCAG-3' and 5'-CGGAATTCGATGAAGATCCAAC-3'. The PCR fragments were subsequently cut with *Eco*RI and *Xho*I for insertion between the *Eco*RI and *Xho*I sites of pCMV-MS2. The hnRNP-K NLS/NES DNA sequence was generated by PCR using oligonucleotide primers 5'-CGGGATCCATGACAGAAGAGGAGGA-3' and 5'-CCGCTCGAGATAAGCCATCTGCATTC-3', cut with *Bam*HI and *Xho*I for insertion into pGEX-4T-2 to express GST-KNS (where KNS is the *K* nuclear shuttling domain). Plasmid pCMV.NLS.βgal was constructed by inserting the SV40 T antigen NLS into plasmid pCMVβ (Clontech) by site-directed mutagenesis (Stratagene kit) using oligonucleotide 5'-GAGCTGCTCAAGCGCGATGCTAGCGGCGCTAAGAAGAAGCGCAAAGTCAGATCTGTCGTTTACAACGTCGTG-3' and its complement on the opposite strand. pCMV.NLS.B.βgal was then constructed by inserting a PCR-amplified fragment generated from EB2 using the following two oligonucleotides: 5'-CGGGATCCGATGAAGATCCAAC-3' and 5'-CCGGATCCACTTTCATCGGTGCA-3'. PCR DNA fragment B was then cut with *Bam*HI and inserted downstream of the SV40 T antigen NLS into a *Bgl*III site of pCMV.NLS.βgal introduced by site-directed mutagenesis concomitantly with the SV40 T antigen NLS. The REF constructs used in this

study have been described elsewhere (19) and were a generous gift from Dr. E. Izaurralde. The reporter plasmid pDM128/PL has been described by Yang *et al.* (30) and was a generous gift from Dr. B. R. Cullen.

Cell Lines and Transfections—HeLa cells and 293T cells carrying an EBV *BMLF1*-knockout recombinant (293_{BMLF1-KO} cells) were grown at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum. HeLa cells were seeded at 8 × 10⁵ cells/100-mm Petri dish 10 h prior to transfection. Transfections of HeLa cells were performed by the calcium precipitate method as described previously (24). Transient transfections of 293_{BMLF1-KO} cells (12) were performed by electroporation (950 microfarads, 220 mV) using a Bio-Rad electroporator. Plasmids used for transfections were prepared by the alkaline lysis method and purified through two CsCl gradients.

Quantification of CAT Protein—To evaluate CAT protein expression, we used CAT enzyme-linked immunosorbent assay (Roche Molecular Biochemicals). After transfection, cells were collected in phosphate-buffered saline. Half of the cells were used for CAT assays according to the manufacturer's instructions. The other half were used to monitor protein expression by Western blotting using anti-FLAG antibody M2.

Heterokaryon Assays—HeLa cells were transfected in 100-mm Petri dishes by the calcium precipitate method as previously described (31). 24 h post-transfection, the precipitate was washed, and cells were trypsinized. Approximately 2 × 10⁵ HeLa cells were seeded on glass coverslips with an equal number of NIH3T3 cells in 35-mm dishes. The cells were allowed to grow overnight and were then treated for 2 h with 100 μg/ml cycloheximide to inhibit protein synthesis and 25 mM leptomycin B (LMB) (kindly provided by Dr. B. Wolff) when inhibition of CRM-1-dependent protein export was required. Subsequently, cells were washed with phosphate-buffered saline, and heterokaryon formation was carried out by incubating the coverslips for 2 min in 50% polyethylene glycol 3000–3700 (Sigma) in phosphate-buffered saline. Following cell fusion, coverslips were washed extensively with phosphate-buffered saline and returned to fresh medium containing 100 μg/ml cycloheximide and 25 mM LMB when needed. After 2 h at 37 °C, cells were fixed with 4% paraformaldehyde, and indirect immunofluorescence was performed essentially as described previously (24). For the various immunofluorescence experiments, we used anti-FLAG monoclonal antibody M2, anti-hnRNP-C monoclonal antibody 4F4 (kindly provided by Dr. G. Dreyfuss) (32), anti-Rev monoclonal antibody (kindly provided by Dr. B. Wolff) (33), or anti-β-galactosidase antibody (Roche Molecular Biochemicals) as the primary antibody. An Alexa Fluor-conjugated goat anti-mouse IgG (H+L; Interchim) was used as the secondary antibody, and the nuclei of the cells were stained by incubation with a Hoechst 33258 solution (Sigma) at 5 μg/ml.

Microinjections—GST fusion proteins (2 mg/ml) were injected into either the nucleus or the cytoplasm of HeLa cells or HeLa cell polykaryons generated as described above using an Eppendorf Transjector 5246, an Eppendorf micromanipulator (Injectman), and a Nikon Eclipse TE200 microscope. Texas Red-labeled dextran (70 kDa; Molecular Probes, Inc.) was systematically added at a concentration of 2 mg/ml to the microinjection solution so as to mark the injection site. Microinjections were monitored using a Sony digital camera and a Sony TV monitor. 5–10 cells or polykaryons on average per experiment were microinjected, and each experiment was repeated at least twice. After microinjection, cells were incubated for 1 h at 37 °C and then fixed with 4% paraformaldehyde. The GST fusion proteins were visualized by indirect immunofluorescence as described previously (24). Rabbit anti-GST antibody (a generous gift from Dr. J. J. Diaz) was used at a 1:500 dilution. Fluorescein isothiocyanate-coupled goat anti-rabbit antibody (Sigma) was used at a 1:2000 dilution.

In Vitro GST Pull-down Assays—GST and GST fusion proteins were expressed in BL21 pLys bacteria and purified with glutathione-agarose beads following standard procedures. ³⁵S-labeled F.EB2 and F.EB2 mutants were produced in the rabbit reticulocyte lysate *in vitro* transcription/translation TNT system (Promega). Binding reactions were carried out in 500 μl of buffer containing 150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, 100 mM EDTA, and 1% Triton X-100, pH 7.3, for 1 h at 4 °C, and then complexes bound to the glutathione-agarose beads were washed five times with the same buffer. Proteins were eluted in SDS-PAGE loading buffer and analyzed by autoradiography. In some experiments, RNase A (10 μg) and RNase T1 (10 μg) were added to the last wash and incubated for 15 min at room temperature.

Immunoprecipitation of Transfected Cell Extracts—For immunoprecipitation, transfected HeLa or 293_{BMLF1-KO} cells were harvested from 100-mm dishes 48 h post-transfection and lysed in 300 μl of TNE buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, and 1 mM EDTA) containing a protease inhibitor mixture (Roche Molecular Biochemicals). The lysate was then passed five times through a syringe with a 26-gauge needle,

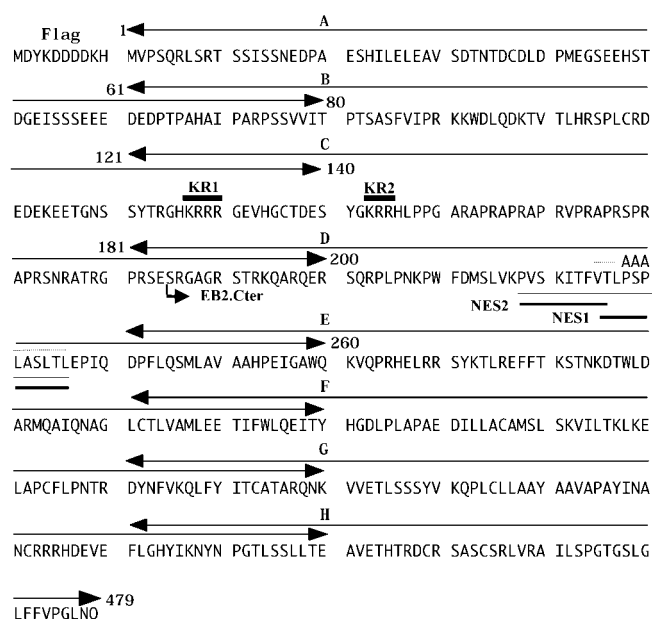


FIG. 1. Location of the various EB2 peptides and mutants used in this study on the EB2 amino acid sequence. The 80-amino acid long EB2 peptides A–H used in this study are represented by double-headed arrows above the EB2 sequence. The two KR-rich motifs (KR1 and KR2) are identified. The N terminus of the truncated EB2.Cter protein is shown at position 185. The locations of the two putative NES (NES1 and NES2) reported previously by Chen *et al.* (26) are indicated, as are the sites of the deleted sequences in F.EB2.ΔNES and F.EB2.ΔNES (dotted line above and thin line below the sequence, respectively).

and cell debris was removed by centrifugation at $13,000 \times g$. Cell extracts were incubated with $50 \mu\text{l}$ of anti-FLAG monoclonal antibody M2-agarose affinity gel (Sigma) for 4 h in TNE buffer at 4°C . The precipitates were washed five times with cold buffer A (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 1% Triton X-100) as described by Koffa *et al.* (7). Proteins were eluted by incubation of the immunoprecipitate with a peptide/FLAG solution at $40 \mu\text{g}/\text{ml}$ in buffer A before loading onto SDS-polyacrylamide gel. Western blot analysis was performed using anti-FLAG monoclonal antibody M2 and rabbit polyclonal antibodies to murine REF (KJ70) (19) and TAP (34) (both a generous gift from Dr. E. Izaurralde). In some experiments, RNase A ($10 \mu\text{g}$) and RNase T1 ($10 \mu\text{g}$) were added to the cell extracts prior to immunoprecipitation for 15 min at room temperature.

RESULTS

Both the NLS and NES of EB2 Are Located within a Domain between Amino Acids 61 and 140—A computer-assisted search for NLS sequences indicated that EB2 contains a putative bipartite NLS (KR1 and KR2) (Fig. 1), which could contribute to the nucleocytoplasmic shuttling of EB2, together with the double NES located in peptide D (NES1/NES2) (Fig. 1) previously characterized by Chen *et al.* (26). To locate more precisely the EB2 NLS and NES, we microinjected a series of fusion proteins between GST and the EB2 peptides represented in Fig. 1 into single nuclei of HeLa cell polykaryons. Texas Red-labeled dextran was co-injected to mark the injection site. As shown in Fig. 2A, GST diffused passively in the cytoplasm, but was not imported in the non-injected nuclei, probably due to the lack of NLS. As expected, GST-peptide C, which contains the putative bipartite NLS, was restricted to the injected nucleus. Surprisingly, GST-peptide B was found in all the nuclei of the injected polykaryons, suggesting that it shuttled between the nucleus and the cytoplasm. Peptide B may thus contain both an NLS and an NES. In contrast, GST-peptide D, which contains the putative double NES mapped by Chen *et al.* (26), was restricted to the injected nucleus. A fusion protein between GST and the KNS domain of the hnRNP-K protein (32), used as

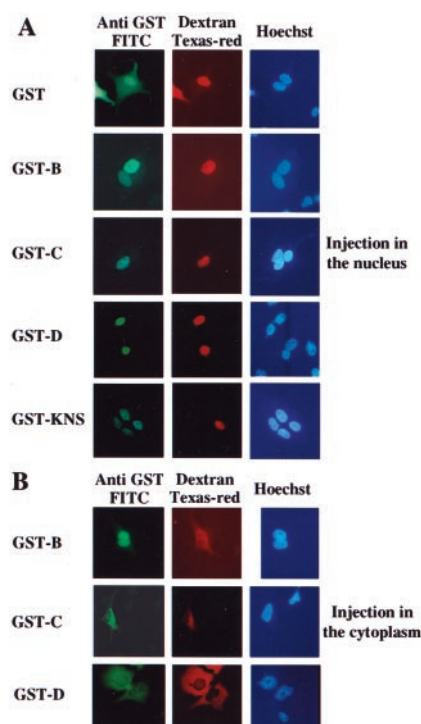


FIG. 2. EB2 peptide B contains both an NLS and an NES. A single nucleus of HeLa cell polykaryons (A) or the cytoplasm of HeLa cells or HeLa cell polykaryons (B) was microinjected with the indicated GST fusion proteins together with Texas Red-labeled dextran (70 kDa) as a marker of the injection site. After a 1-h incubation at 37°C , cells were fixed and analyzed for the localization of the GST fusion proteins using rabbit anti-GST antibody as the primary antibody and fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit antibody. Cell nuclei were labeled by Hoechst staining.

a control shuttling protein, was found in all the nuclei of the injected polykaryons, similar to GST-peptide B.

The GST-peptide B, C, and D fusion proteins were also injected into the cytoplasm of HeLa cell polykaryons (Fig. 2B). Consistent with the previous results, GST-peptide B was exclusively located in the nucleoplasm, whereas both GST-peptide C and GST-peptide D stayed at the injection site, the cytoplasm. It should be noted that in contrast to GST-peptide D, GST-peptide C, which was expected to be localized in the nucleus, did not diffuse into the cytoplasm, suggesting that GST-peptide C might be aggregated at the microinjection site. The GST-peptide E, F, G, and H fusion proteins (Fig. 1) were also injected into one single nucleus or into the cytoplasm of HeLa cell polykaryons and were exclusively localized in the injected cellular compartment, strongly suggesting that GST-peptide B did not diffuse passively (data not shown).

Taken together, these results strongly suggest that peptide B carries an NLS and an NES and that both are transferable. To confirm the localization of the EB2 NLS sequences, peptides A–H were also fused to the bacteriophage MS2 coat protein and expressed in COS-7 cells. Only MS2-peptide B and MS2-peptide C proteins were found to be exclusively nuclear (Fig. 3), confirming that both peptides contain NLS sequences. Because peptide B contains only the KR1 motif of the putative bipartite NLS, this suggests that KR1 functions independently of KR2, rather than being part of a bipartite NLS. Accordingly (Fig. 4), mutation of the KR1 motif (KRRR \rightarrow AAAA in F.EB2.M1) in the full-length F.EB2 protein only slightly impaired the nuclear localization of EB2, whereas mutation of the KR2 motif (KRR \rightarrow AAA in F.EB2.M2) was silent. However, mutation of both KR1 and KR2 (mutant F.EB2.M1.2) almost completely abrogated nuclear translocation. This demonstrates the pres-

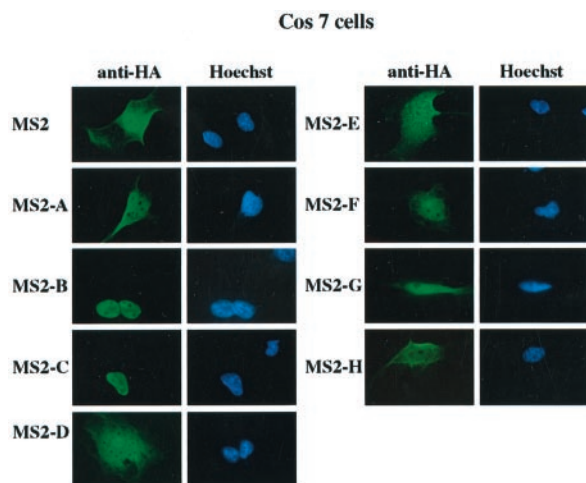


FIG. 3. Both peptides B and C contain functional NLS. COS-7 cells were transfected with plasmids expressing the hemagglutinin (HA)-tagged MS2 fusion proteins as indicated. The cells were immunostained using anti-hemagglutinin monoclonal antibody (Roche Molecular Biochemicals) as the primary antibody and the Alexa Fluor-conjugated goat anti-mouse IgG as the secondary antibody to determine the localization of the proteins and stained with Hoechst dye to visualize the nuclei.

ence of two independent functional NLS regions, suggesting that EB2 contains two independent functional NLS domains.

Peptide B Contains a Novel CRM-1-independent Transferable NES—To ascertain the presence of an NES in peptide B, we wanted to demonstrate that it could confer nucleocytoplasmic shuttling to a heterologous non-shuttling protein whose size was incompatible with passive diffusion between the nucleus and the cytoplasm. Moreover, because we have previously reported that EB2 shuttles via a CRM-1-independent pathway (24), nucleocytoplasmic shuttling of the heterologous protein should be resistant to LMB, a specific inhibitor of CRM-1. We therefore transferred peptide B to the β -galactosidase carrying the SV40 T antigen NLS motif (NLS.B. β gal) (Fig. 5A) and tested this fusion protein in a human-mouse heterokaryon assay in the presence of cycloheximide to suppress *de novo* protein synthesis. In this assay (Fig. 5B), NLS.B. β gal was found both in the transfected HeLa nuclei and in the murine nuclei of heterokaryons (panels e and f), suggesting that this protein shuttles between the nucleus and the cytoplasm. NLS. β gal (used as a control) was exclusively nuclear and, as expected, was found only in the HeLa cell nuclei of heterokaryons (panels c and d). Most interestingly, shuttling of NLS.B. β gal was insensitive to LMB (panels k and l), similar to that of F.EB2 (compare panels g and h with panels a and b). As a control for the functionality of LMB, the HIV Rev protein was expressed in HeLa cells (Fig. 5C). As already reported (33), in transfected HeLa cells, Rev is located in the nucleolus and the cytoplasm; but in the presence of LMB, which inhibits Rev nuclear export, Rev is located mainly in the nucleolus. This is what we also observed (Fig. 5), demonstrating that LMB is active. These results strongly suggest that peptide B contains a CRM-1-independent transferable NES.

Deletion of the Putative NES1/NES2 Region Located between Amino Acids 218 and 236 Does Not Impair the Nucleocytoplasmic Shuttling of EB2—It has been previously reported by Chen *et al.* (26) that EB2 contains a double NES, called NES1/NES2, located in peptide D between amino acids 218 and 236 (Fig. 1). Because we found that peptide B (amino acids 61–140) also harbors a functional NES, we wanted to re-examine the location of the NES in the full-length EB2 protein. Two mutants with deletions in the NES1/NES2 region (amino acids 218–236) of EB2 were generated. One mutant, called

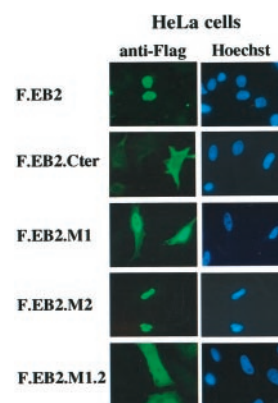


FIG. 4. The EB2 NLS is composed of two functionally independent KR-rich motifs. HeLa cells were transfected with plasmids expressing FLAG-tagged mutant EB2 proteins as indicated. Mutants F.EB2.M1 and F.EB2.M2 have their KR1 or KR2 motif (see Fig. 1) mutated, respectively. Mutant F.EB2.M1.2 has both motifs mutated, and F.EB2.Cter is deleted of the first 184 amino acids. The cells were immunostained using anti-FLAG monoclonal antibody M2 as the primary antibody and Alexa Fluor-conjugated goat anti-mouse IgG as the secondary antibody to determine the localization of the proteins and stained with Hoechst dye to visualize the nuclei.

F.EB2.dNES (Fig. 6A), has been previously reported to be a non-shuttling protein (26). However, in our human-mouse heterokaryon assay, this EB2 mutant shuttled at least as efficiently, if not more efficiently, than the wild-type F.EB2 protein (Fig. 6B). Moreover, a EB2 mutant from which the whole NES1/NES2 region was deleted (F.EB2. Δ DN) (Fig. 6A), also shuttled (Fig. 6B). However, it should be noted that the fluorescence detected in the murine nuclei was always more intense in the case of the mutant EB2 proteins than in the case of wild-type EB2, suggesting a more efficient shuttling of the mutant proteins. It therefore seemed either that the NES1/NES2 region (from now on referred to as the DN region) was not contributing to the EB2 nucleocytoplasmic shuttling or that EB2 contained two independent functional NES, one in peptide B that is CRM-1-independent, as suggested by our results, and a second one in peptide D, as suggested by Chen *et al.* (26). To distinguish between these two hypotheses, we used an EB2 mutant with a deletion of the N-terminal part of the protein, including the peptide B region. Because we have shown that the NLS of EB2 was also located in the N-terminal region of the protein, we generated an N-terminally truncated mutant to which we fused the SV40 T antigen NLS (F.NLS.EB2.Cter) (Fig. 6A). This protein is expected to be nuclear, but if it lacks a functional NES, it should not shuttle in our heterokaryon assay. This is exactly what we observed (Fig. 6B), strongly suggesting that EB2 contains only one functional NES located in the N-terminal part of the protein. The above results were validated by the observation that in our human-mouse heterokaryon assay, the non-shuttling hnRNP-C protein was not transported from the human to the mouse nucleus.

The DN Region Is Essential for EB2-mediated mRNA Export—The various mutant proteins used in our heterokaryon assays were then tested in a functional assay for their capacity to export mRNAs, using the pDM128/PL reporter system (35). In this assay, unspliced CAT-containing mRNA transcribed from pDM128/PL (Fig. 7A) was rarely found in the cytoplasm, whereas the spliced mRNA, which does not contain the CAT sequence, was efficiently exported from the nucleus to the cytoplasm. We have previously demonstrated that EB2 can mediate the nuclear export of unspliced mRNA generated from plasmid pDM128/PL, and this can be quantified at the level of both CAT-expressed protein and unspliced CAT mRNA detected in the cytoplasm (24). Plasmids expressing F.EB2, F.EB2.dNES,

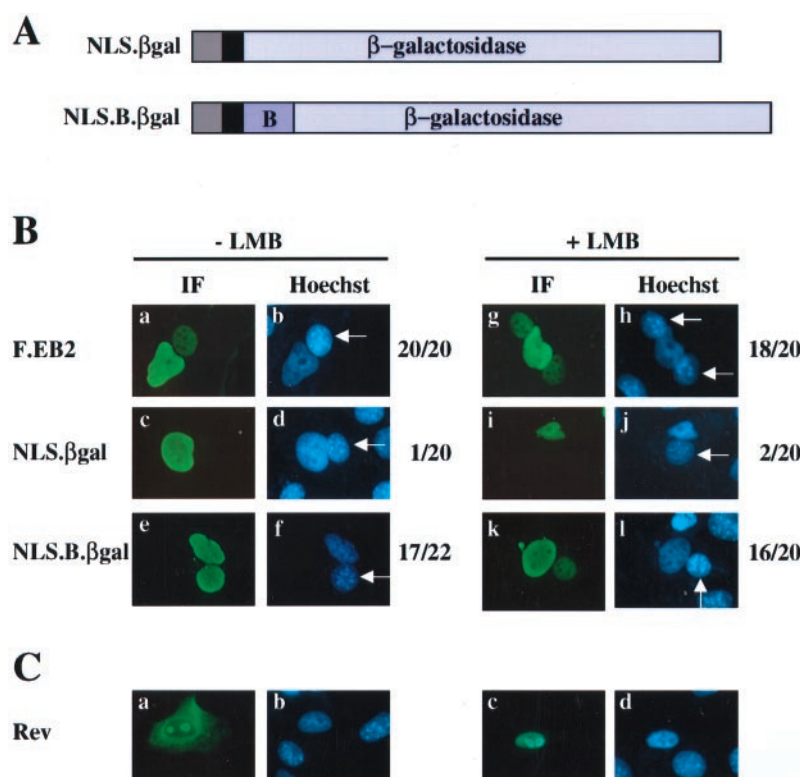


FIG. 5. EB2 peptide B contains a CRM-1-independent transferable NES. A, shown is a schematic representation of the NLS.βgal and NLS.B.βgal proteins. The N-terminal gray boxes correspond to a partial sequence of the *Drosophila melanogaster* alcohol dehydrogenase providing the eukaryotic translation/initiation signals. The black boxes indicate the position of the NLS T antigen SV40 sequence. The peptide B and β-galactosidase sequence localization is indicated. B, expression vectors for F.EB2, NLS.βgal, and NLS.B.βgal were transfected into HeLa cells. After 48 h, HeLa cells were fused with NIH3T3 cells to form heterokaryons and incubated for 2 h with medium containing cycloheximide and leptomycin B where indicated as described under "Experimental Procedures." The cells were immunostained using either anti-FLAG monoclonal antibody M2 or anti-hemagglutinin monoclonal antibody as the primary antibody and Alexa Fluor-conjugated goat anti-mouse IgG as the secondary antibody and then stained with Hoechst dye, which allows differentiation between the HeLa and NIH3T3 nuclei in the heterokaryons. Arrows identify the NIH3T3 cell nuclei. Numbers indicate the number of heterokaryons observed in which the protein is shuttling/the total number of heterokaryons with positive HeLa nuclei scanned. C, an expression vector for the HIV Rev protein was transfected into HeLa cells, and the cells were treated exactly as indicated for B. The cells were immunostained using anti-Rev monoclonal antibody as the primary antibody and Alexa Fluor-conjugated goat anti-mouse IgG as the secondary antibody and then stained with Hoechst dye. IF, immunofluorescence.

F.EB2.ΔDN and F.NLS.EB2.Cter were thus transfected into HeLa cells together with the pDM128/PL reporter construct, and the amount of CAT protein expressed was quantified by CAT enzyme-linked immunosorbent assay. As expected, F.EB2 very efficiently increased the level of CAT protein expressed from pDM128/PL (Fig. 7, B and C, compare bars 1 and 2), whereas the F.NLS.EB2.Cter protein, which did not shuttle, was completely inactive (Fig. 7B, bar 3). Moreover, F.EB2.ΔNES and, more importantly, F.EB2.ΔDN, although not completely inactive, were much less efficient than F.EB2 in this functional assay (bars 3 and 4, respectively), suggesting an important role for the DN region (amino acids 218–236) in mRNA nuclear export.

The C-terminal RGG-rich Domain of REF Binds to the EB2 DN Region in Vitro—As shown above, F.EB2.ΔDN shuttled between the nucleus and the cytoplasm. However, this EB2 mutant did not export mRNA efficiently. We therefore tested whether EB2 could bind known cellular export factors via the DN region. We first tested whether REF/Aly, a promiscuous mRNA export factor, would interact with EB2. To do so, *in vitro* translated F.EB2 (Fig. 8B, lane 1) was incubated with GST-REF1-II or GST-REF2-II (Fig. 8A) immobilized on glutathione-agarose beads. F.EB2 bound both GST-REF1-II and GST-REF2-II (Fig. 8B, lanes 4 and 6), and this interaction was not RNA-dependent (lanes 5 and 7). To map the EB2-binding site in REF, F.EB2 was incubated with various REF1-II subfragments fused to GST and immobilized on glutathione-agarose beads. F.EB2 interacted with both subdomains at amino acids 14–163 and 103–163 of REF1-II (lanes 10–13), but not with the

subregions at amino acids 14–102 and 129–163 (lanes 8, 9, 14, and 15). Again, the interactions between F.EB2 and these subregions of REF were RNase-resistant (lanes 11 and 13). These results suggest that there is a direct interaction between EB2 and REF *in vitro* and that the EB2-binding site in REF is located in the RGG-rich C-terminal variable region of the protein. We then asked whether the REF-binding site in EB2 is located in the DN region. As compared with F.EB2 (Fig. 8C, lanes 1–5), F.EB2.ΔDN did not bind to GST-REF1-II (lanes 6–10). The DN region is thus likely to be an interaction domain with the cellular REF export protein.

EB2 Interacts with REF-containing Complexes in Vivo—The results described above suggesting that the EB2 DN region is a REF interaction domain were obtained by *in vitro* assays. To demonstrate the existence of protein complexes associating with EB2 and REF *in vivo*, F.EB2 or F.EB2.ΔDN was transiently expressed in HeLa cells, and immunoprecipitation from whole cell extracts was carried out with anti-FLAG monoclonal antibody M2. The amount of co-immunoprecipitated REF was then evaluated by Western blot analysis using anti-REF polyclonal antibody. We found that F.EB2 co-immunoprecipitated REF from the transfected HeLa cell extracts much more efficiently than the mutant F.EB2.ΔDN protein (Fig. 9A, first panel, compare lanes 2 and 6). Most importantly, RNase treatment completely abolished the co-immunoprecipitation of REF by F.EB2 (lane 3), whereas endogenous REF was present at comparable levels in every cell extract and was unaffected by RNase treatment (fourth panel). Comparable amounts of

FIG. 6. The N-terminal part of EB2 contains an NES. A, shown is a schematic representation of F.EB2, F.EB2.dNES, F.EB2.ΔDN, and F.NLS.EB2.Cter proteins. The *black box* in the F.EB2.dNES diagram indicates the presence of three alanines in place of the missing EB2 sequence. + or – indicates the capacity of the protein to shuttle between the nucleus and the cytoplasm as determined in B. B, expression vectors for F.EB2, F.EB2.dNES, F.EB2.ΔDN, and F.NLS.EB2.Cter were transfected into HeLa cells. After 48 h, HeLa cells were fused with NIH3T3 cells to form heterokaryons and incubated for 2 h with medium containing cycloheximide as described under “Experimental Procedures.” The cells were immunostained using either anti-FLAG (M2) or anti-hnRNP-C (4F4) monoclonal antibody as the primary antibody and Alexa Fluor-conjugated goat anti-mouse IgG as the secondary antibody and then stained with Hoechst dye, which differentiates between HeLa and NIH3T3 nuclei in heterokaryons. *Arrows* identify the NIH3T3 cell nuclei. *Numbers* indicate the number of heterokaryons observed in which the protein is shuttling/the total number of heterokaryons with positive HeLa nuclei scanned. *IF*, immunofluorescence.

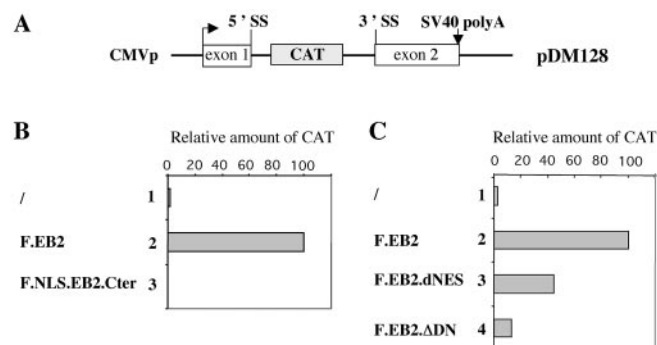
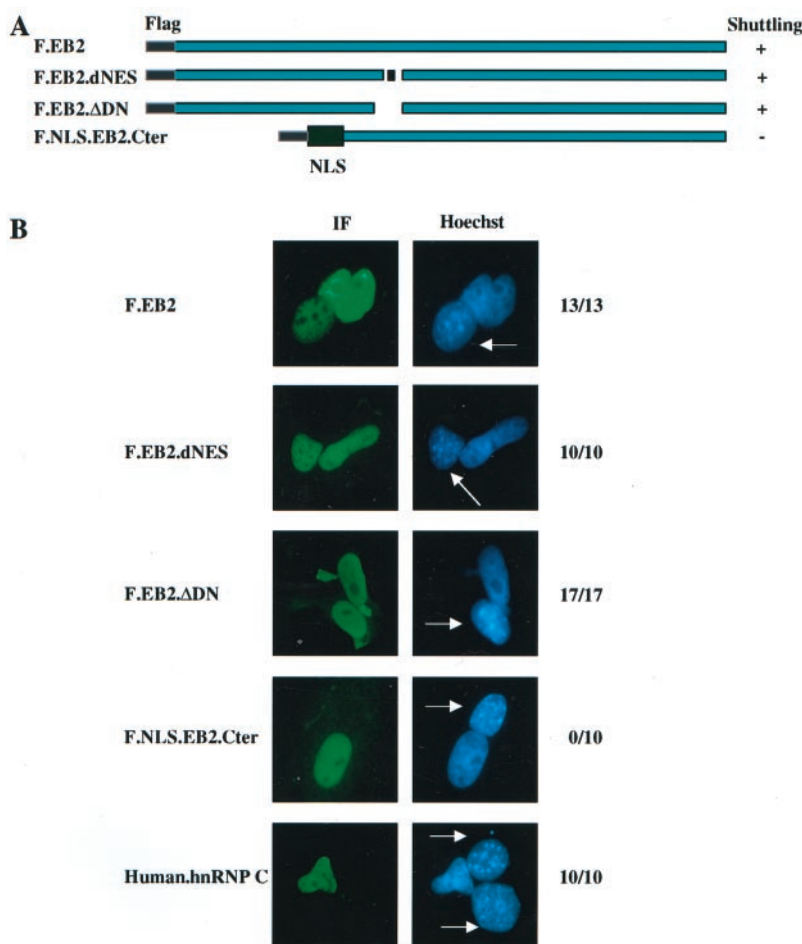


FIG. 7. Both the N-terminal region of EB2 and amino acids 218–236 (DN region) are required for efficient export of mRNA. A, shown is a schematic representation of the pDM128/PL reporter plasmid. The CAT coding sequence is inserted into intronic sequences. The positions of the 5'- and 3'-donor site sequences (SS) are indicated. B and C, expression plasmids for F.EB2, F.NLS.EB2.Cter, F.EB2.dNES, and F.EB2.ΔDN were transfected into HeLa cells together with the pDM128/PL reporter plasmid. CAT protein was quantified by CAT enzyme-linked immunosorbent assay. The results are expressed as relative amount of CAT protein, with a fixed value of 100 given to the amount of CAT protein expressed in the presence of wild-type F.EB2.

F.EB2 and F.EB2.ΔDN were efficiently immunoprecipitated by anti-FLAG antibody M2 (*third panel*).

Because TAP is the REF-interacting factor targeting REF-RNP complexes to the nuclear pore, we also investigated whether EB2 would co-immunoprecipitate TAP. As shown in Fig. 9A (*second panel*), F.EB2 co-immunoprecipitated TAP, and this co-immunoprecipitation was also completely abolished by the RNase treatment. However, contrary to the co-immunopre-

cipitation of REF by F.EB2, the deletion of the DN region in F.EB2.ΔDN did not affect the co-immunoprecipitation of TAP (*lane 6*). Finally, it should be noted that the cytoplasmic EB2 mutant F.EB2.Cter co-immunoprecipitated much less REF and TAP compared with F.EB2 (*lanes 4 and 5*). This latter result is in accordance with the published observations that REF dissociates from messenger RNPs as they travel from the nucleus to the cytoplasm (18) and strongly suggests that the co-immunoprecipitation of REF and TAP by F.EB2 and F.EB2.ΔDN results mainly from interactions occurring in the nuclear compartment.

As shown above, the *in vivo* co-immunoprecipitation of F.EB2 and REF is sensitive to ribonucleases. The HSV-1 EB2 homolog ICP27 has been co-immunoprecipitated with REF from HSV-1-infected cells, and the complexes were found to be resistant to RNase treatment (7). We therefore also evaluated whether FLAG-tagged ICP27 (F.ICP27) transiently expressed in HeLa cells could co-immunoprecipitate REF. We found that REF was co-immunoprecipitated with F.ICP27 in this assay. However, RNase treatment also completely abolished the co-immunoprecipitation of REF with F.ICP27 (Fig. 9A, *first panel*, *lanes 8 and 9*), whereas endogenous REF was unaffected by RNase treatment (*fourth panel*, *lanes 8 and 9*). Comparable amounts of F.ICP27 were efficiently immunoprecipitated with and without RNase treatment (*third panel*, *lanes 8 and 9*).

As the interaction between ICP27 and REF has previously been reported to be RNase-insensitive in the context of HSV-1-infected cells, we then asked whether the interaction between EB2 and REF might also be resistant to RNase in the context of an EBV productive cycle. For this, we used 293_{BMLF1-KO} cells.

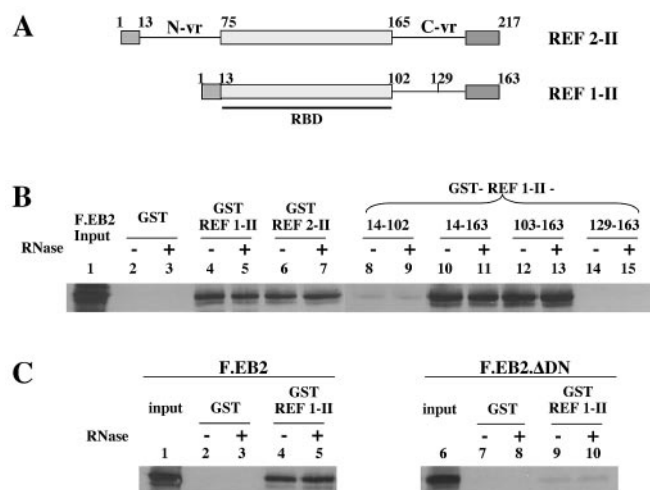


FIG. 8. **EB2 interacts with REF *in vitro*.** *A*, a schematic representation of REF2-II and REF1-II as described by Rodrigues *et al.* (19). *N-vr* and *C-vr*, N- and C-terminal variable regions, respectively; *RBD*, RNA-binding domain. REF conserved N- and C-terminal domains are indicated by gray boxes. Numbers indicate the positions of the different domains in amino acids. *B*, EB2 interacts with a domain in REF1-II located between amino acids 102 and 129. [³⁵S]Methionine-labeled F.EB2 synthesized in rabbit reticulocyte lysate (lane 1) was incubated with beads coated with GST (lanes 2 and 3), GST-REF (lanes 4–7), or GST-REF1-II truncations (lanes 8–15). The input (lane 1) and the bound fractions (lanes 2–15) were analyzed by SDS-PAGE and visualized by autoradiography. RNase was added to the incubation as indicated. *C*, the EB2 DN region is required for *in vitro* interaction with REF1-II. [³⁵S]Methionine-labeled F.EB2 (lane 1) and F.EB2.ΔDN (lane 6) synthesized in rabbit reticulocyte lysate were incubated with GST beads (lanes 2, 3, 7, and 8) or with GST-REF1-II beads (lanes 4, 5, 9, and 10). The inputs (lanes 1 and 6) and the bound fractions (lanes 4, 5, 9, and 10) were analyzed by SDS-PAGE and visualized by autoradiography. RNase was added to the incubation as indicated.

When these cells are transfected with an expression vector for the EB1 transactivator to induce the EBV productive cycle, no infectious viral particles are produced. However, when these cells are cotransfected with expression plasmids for both EB1 and EB2 to *trans*-complement the missing gene product, infectious viral particles are produced (12). These cells were thus transfected with expression plasmids for EB1 alone, for both EB1 and F.EB2, or for EB1 and F.ICP27, and immunoprecipitation from whole cell extracts was carried out using anti-FLAG antibody M2. As shown in Fig. 9B, REF co-immunoprecipitated with both F.EB2 and F.ICP27 (upper panel, lanes 2 and 3, respectively), but this co-immunoprecipitation was sensitive to RNase treatment (lanes 5 and 6, respectively). Thus, even in the context of the viral infection, EB2 interacts with the cellular REF protein in a way that is dependent upon the presence of RNA.

DISCUSSION

We have investigated the signals directing the nucleocytoplasmic shuttling of the EBV mRNA export factor EB2. We have identified the NLS sequences as two KR-rich motifs acting independently and a new CRM-1-independent NES, located 90 amino acids upstream of the putative CRM-1-dependent double NES (NES1/NES2) previously described by Chen *et al.* (26). In our assay, this NES1/NES2 region could be deleted without affecting the nucleocytoplasmic shuttling of EB2. Moreover, we show that this domain is in fact a REF-binding domain, essential for EB2-mediated mRNA export.

We have previously reported that EB2 shuttles between the nucleus and the cytoplasm in an LMB-insensitive manner (24), which suggests that the EB2 NES is CRM-1-independent, whereas others have reported that EB2 carries two contiguous CRM-1-dependent NES (NES1/NES2) (26). Our results clearly

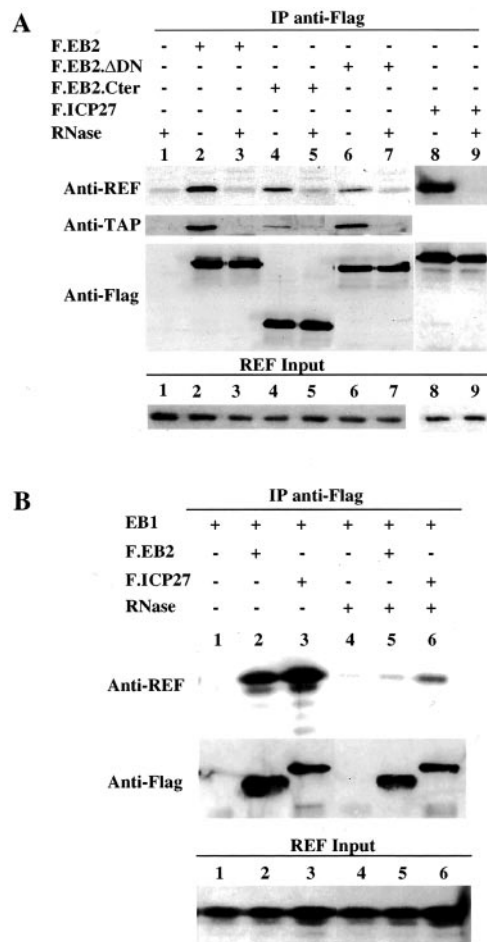


FIG. 9. **F.EB2 interacts with REF and TAP *in vivo*.** *A*, EB2 forms a complex with REF, TAP, and RNA *in vivo*. HeLa cells were transfected with plasmids expressing the FLAG-tagged proteins EB2, EB2.ΔDN, and EB2.Cter, and immunoprecipitation (IP) was performed with agarose beads coated with anti-FLAG antibody M2. Western blot analyses were performed using either anti-FLAG monoclonal antibody M2 or rabbit anti-REF (KJ70) or anti-TAP antibody. RNase was added to the extract prior to immunoprecipitation as indicated. The amount of REF protein in the input was analyzed by Western blotting using anti-REF antibody (REF Input). *B*, EB2 interaction with REF in the context of an EBV productive cycle. 293_{BMLF1-KO} cells were transfected with plasmids expressing EB1 and the FLAG-tagged EB2 and ICP27 proteins as indicated, and immunoprecipitation was performed using agarose beads coated with anti-FLAG antibody M2. Western blot analyses were performed using either anti-FLAG monoclonal antibody M2 or rabbit anti-REF antibody KJ70. RNase was added to the extract prior to immunoprecipitation as indicated. The amount of REF protein in the input was analyzed by Western blotting using anti-REF antibody (REF Input).

show that this double NES, which we now call the DN region, does not direct alone the nuclear export of EB2 because deletion of the DN region did not impair EB2 shuttling. Moreover, a nuclear EB2 protein lacking the N-terminal 185 amino acids, but still carrying the DN domain (F.NLS.EB2.Cter), did not shuttle in a heterokaryon assay. In fact, the shuttling appeared to be even more efficient when the DN region was deleted. This could be explained by a change in the conformation of the protein, which could make the NES located in the N-terminal region of EB2 more accessible. Alternatively, the DN region could overlap with a nuclear retention domain. Taken together, our results suggest that (i) the DN region does not seem to be implicated at all in the shuttling of the EB2 protein; and (ii) the nucleocytoplasmic shuttling function is provided by the N-terminal region of the protein, probably via a motif contained in an EB2 peptide of 80 amino acids called peptide B and located

between amino acids 61 and 140 (Fig. 1). In effect, when peptide B was transferred to a nuclear β -galactosidase, the fusion protein shuttled between the nucleus and the cytoplasm (Fig. 5B). Moreover, we found that this shuttling was CRM-1-independent, which is in agreement with our previous data showing that shuttling of the entire EB2 protein is CRM-1-independent (24). It is possible that the inhibition of EB2 shuttling by LMB observed previously by others (25, 26) was due to nonspecific effects of the drug, *i.e.* because of too long incubation periods or use of too high LMB concentrations. Similarly, the HSV-1 mRNA export factor ICP27 was first proposed to be exported out of the nucleus via a leucine-rich CRM-1-dependent NES (36, 37). However, a recent report demonstrated that neither nucleocytoplasmic shuttling of ICP27 in the absence of viral RNA nor ICP27-dependent viral mRNA export is CRM-1-dependent (7). It should be noted that there are now three functionally conserved human herpes proteins, HSV-1 ICP27, EBV EB2, and human cytomegalovirus UL69 (38), that express proteins containing a CRM-1-independent NES with, however, no obvious sequence homology.

Although not necessary for the nucleocytoplasmic shuttling of the protein, the DN region appears to be important for EB2-mediated mRNA export, through its capacity to bind the cellular REF export factor. In effect, our data demonstrate that *in vitro* synthesized EB2 interacts with bacterially purified GST-REF independently of RNA. They also show that the REF C-terminal RGG repeat region makes contact with the EB2 DN region *in vitro*. In this respect, EB2 differs from HSV-1 ICP27, which interacts with the RNA-binding domain of REF (7). These results therefore suggest a direct interaction between REF and EB2. However, although RNase treatment did not abolish the *in vitro* EB2/REF interaction, it completely abolished the EB2/REF co-immunoprecipitation from both extracts of transfected HeLa cells and extracts of EBV-infected cells in which a productive cycle was induced. The discrepancy between the *in vivo* co-immunoprecipitation and the *in vitro* GST pull-down assays is probably due to the high amount of GST-REF used in the latter, which displaces the equilibrium of the reaction toward an RNA-independent interaction. Moreover, although ICP27 co-immunoprecipitation of REF has previously been shown to be resistant to RNase when performed with cells infected with HSV-1 (7), we found that this interaction was also completely abolished by RNase treatment when we used extracts from transfected HeLa cells. Thus, our results suggest that EB2 recruits REF to mRNAs and that this recruitment is stabilized by RNA.

EB2 also co-immunoprecipitated TAP, a cellular RNA export receptor known to interact with REF (15). However, in transfected HeLa cells, this complex, which was also completely abolished by RNase treatment, was not affected by deletion of the EB2 DN region. These results suggest that TAP is not recruited in the complex by REF, but more likely by RNA-bound EB2 or by other RNA-bound proteins. It is interesting to note that co-immunoprecipitation of TAP by the EJC factor Upf3/3X is also RNase-sensitive (39). Finally, it is also possible that EB2 binds to messenger RNPs, but exports them independently of REF and TAP.

Although we could not demonstrate a direct interaction between EB2 and REF *in vivo*, the REF interaction DN domain is important for EB2-mediated mRNA export. Indeed, we have previously reported that EB2 exports unspliced mRNAs transcribed from pDM128/PL (24), a reporter plasmid widely used and designed to identify factors with mRNA export potential. Using this reporter gene, we have now shown that EB2 deleted

of the REF-binding motif does not export unspliced pDM128/PL mRNAs. Moreover, we have recently produced an EBV virus whose genome is deleted for the EB2 gene. Infectious virus production by cells harboring such a viral genome is only effective when EB2 is provided in *trans*. In this assay, EB2 deleted of the REF-binding site did not *trans*-complement the inactivated wild-type EB2 function (12). This suggests that the REF-binding motif (and probably REF itself) is required both for mRNA export in transient expression assays and for production of infectious virions.

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