Epstein-Barr Virus mRNA Export Factor EB2 Is Essential for Production of Infectious Virus

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The splicing machinery which positions a protein export complex near the exon-exon junction mediates nuclear export of mRNAs generated from intron-containing genes. Many Epstein-Barr virus (EBV) early and late genes are intronless, and an alternative pathway, independent of splicing, must export the corresponding mRNAs. Since the EBV EB2 protein induces the cytoplasmic accumulation of intronless mRNA, it is tempting to speculate that EB2 is a viral adapter involved in the export of intronless viral mRNA. If this is true, then the EB2 protein is essential for the production of EBV infectious virions. To test this hypothesis, we generated an EBV mutant in which the BMLF1 gene, encoding the EB2 protein, has been deleted (EBV_{BMLF1-KO}). Our studies show that EB2 is necessary for the production of infectious EBV and that its function cannot be transcomplemented by a cellular factor. In the EBV_{BMLF1-KO} 293 cells, *oriLyt*-dependent DNA replication was greatly enhanced by EB2. Accordingly, EB2 induced the cytoplasmic accumulation of a subset of EBV early mRNAs coding for essential proteins implicated in EBV DNA replication during the productive cycle. Two herpesvirus homologs of the EB2 protein, the herpes simplex virus type 1 protein ICP27 and, the human cytomegalovirus protein UL69, only partly rescued the phenotype of the EBV_{BMLF1-KO} mutant, indicating that some EB2 functions in virus production cannot be transcomplemented by ICP27 and UL69.

Epstein-Barr virus (EBV) is a human gammaherpesvirus associated with several malignancies, such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, gastric carcinoma, breast carcinoma, and B- and T-cell lymphomas. EBV also induces the activation and proliferation of quiescent B lymphocytes in vitro. In EBV-associated tumors in vivo as well as in EBV-infected B cells proliferating ex vivo, the transcription of the EBV genome is restricted to a few genes defining a latent state of the virus (for references, see references 23 and 36).

The mechanisms leading to the switch from latency to the productive cycle are now partially deciphered for in vitro-immortalized B cells (15, 47). It is accepted that the expression of a viral transcription factor originally called EB1, but later called ZEBRA, Z or Zta, is essential for the activation of the productive cycle (6). Moreover, expression of EB1 in B cells latently infected with EBV is both necessary and sufficient to activate the transcription of all of the productive-cycle genes and the replication of the viral genome (6, 11, 39).

The functions of the EBV gene products expressed during the productive cycle have been only partially characterized, and very little is known for others, such as the EBV BMLF1 early gene product originally called EB2 (6) but later called Mta or SM. This nuclear protein was first described as a promiscuous transcription factor, as it activates transient expression of the chloramphenicol acetyltransferase gene when

placed under the control of many different promoters (29). In contrast, an accumulation of recent experimental results has given rise to the notion that the EB2 protein is not a transcription factor but rather has properties reminiscent of an RNA export factor. These properties can be summarized as follows. The EB2 protein induces the cytoplasmic accumulation of both intronless and intron-containing RNA (3, 10, 45). The EB2 protein also shuttles between the nucleus and the cytoplasm (2, 10, 45). It seems to carry two adjacent nuclear export signals (NES) (5) and binds to RNA in vivo (40), although no EB2-specific RNA motifs on potential EB2 target RNA have been identified so far.

Most of the EBV early and late mRNAs are transcribed from intronless genes. However, it is now clearly established that nuclear export of mRNA is dramatically increased by splicing (31) which leads to deposition of a multiprotein export complex (EJC), including REF/Aly, Y14, RNPS1, SRm160, and Mago, 20 to 24 nucleotides upstream of the exon-exon junction (22, 24, 26, 27). Cellular mRNAs generated from intronless genes require an alternative mode to be efficiently exported from the nucleus to the cytoplasm. Some contain specific *cis*-acting elements and are exported to the cytoplasm by cellular factors through interaction with mRNA-bound adapters such as REF, SRp20, 9G8, or U2AF, which are targets for cellular nuclear RNA export factors such as TAP (7, 18, 37). Hence, it is tempting to speculate that EB2 is such a viral adapter involved in the export of intronless early and/or late EBV mRNA, similar to its herpes simplex virus type 1 (HSV-1) homolog ICP27 (25). If this is true, the EB2 protein is essential for the production of EBV infectious virions.

To test this hypothesis, we first generated an EBV mutant in

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Escherichia coli in which the BMLF1 gene encoding the EB2 protein had been deleted, introduced the mutated viral genome into 293 cells, and then selected a clonal population called 293-BMLF1-KO. In order to induce the productive cycle of EBV, these cells have to be transfected with an expression plasmid encoding EB1 (8). Our studies show that such cells produced infectious EBV particles only when an expression vector coding for the EB2 protein was cotransfected concomitantly with the expression plasmid coding for EB1. In these cells, *oriLyt*-dependent DNA replication was greatly enhanced by EB2 expression. We also observed that EB2 induced the cytoplasmic accumulation of a subset of EBV early mRNAs coding for essential proteins implicated in EBV DNA replication during the productive cycle. The cytoplasmic accumulation of some late mRNAs is also induced by the EB2 protein. Two herpesvirus homologs of the EB2 protein, the HSV-1 protein ICP27 and the UL69 protein of human cytomegalovirus (HCMV), only partly rescued the phenotype observed with the EBV_{BMLF1-KO} mutant.

MATERIALS AND METHODS

Plasmids. The following expression plasmids have been previously described. When Flag precedes the name of the protein, the corresponding protein has been tagged at its N terminus with the Flag epitope, which can be detected by the M2 monoclonal antibody (MAb) (Sigma catalog no. F3165). pCMV-BZLF1, contains the BZLF1 cDNA encoding the EB1 protein (32). paacFlagEB2 contains the intronless BSLF2-BMLF1 cDNA (4). paacFlagEB2_{ΔRXP} expresses an EB2 protein with the RXP repeat deleted (3), and paacFlagEB2_{ΔNES} expresses an EB2 protein with the two putative NES mutated as described by Chen et al. (5) (the sequence PVSKITFVTLPSPLASLT between amino acids 218 and 236 has been modified to PVSKITF- -AAALAS- -). A. Epstein kindly provided pUL53/54, the HSV-1 ICP27-expressing vector. Briefly, it contains the HSV-1 *Clal/SacI* DNA fragment containing the UL53 and UL54 genes cloned in pBlue-script II SK(+) (Stratagene). The expression plasmid for UL69 was kindly provided by T. Stamminger (30). Plasmids used for transfection were prepared by the alkaline lysis method and purified through two CsCl-ethidium bromide gradients.

Recombinant EBV plasmid. To generate a BMLF1 knockout (KO) mutant in the context of the EBV genome, we used our maxi-EBV technology (8). *E. coli* strain DH10B was transformed with the EBV genome cloned into a mini-F-factor replicon, termed p2089 (8, 20). p2089 is a maxi-EBV plasmid which carries the F factor origin of DNA replication, the chloramphenicol resistance gene, and the gene for the green fluorescent protein (GFP) under the control of the CMV immediate-early promoter/enhancer together with the hygromycin resistance gene as a selectable marker in eukaryotic cells. A second plasmid, termed p2650, was introduced into strain DH10B harboring p2089. p2650 encodes a constitutively expressed *recA* gene to aid homologous recombination and the *redγ* gene to allow transformation of linear DNA fragments. p2650 consists of the plasmid backbone of pST76-amp (34), which carries a temperature-sensitive origin of DNA replication derived from pSC101 and confers resistance against ampicillin. In order to generate p2650, a *SacII/KpnI* fragment derived from pKY102 carrying *recA* (19) was treated with the Klenow fragment of *E. coli* polymerase I in the presence of deoxynucleoside triphosphates and ligated into the *Ecl136I*-cleaved pST76-amp plasmid to yield plasmid p2423. This plasmid was again linearized with *NsiI* and treated with Klenow enzyme to accommodate a Klenow enzyme-treated *HindIII/XhoI* fragment from pBAD-ETγ (51) carrying *redγ* to yield p2650.

In order to delete the coding region of the BMLF1 gene, a subclone of the B95.8 EBV genome was generated in *E. coli*. This plasmid, termed pSM neo/kan (Fig. 1A), contains the *BamHI/EcoRI* fragment (nucleotide coordinates 79535 to 82925) of the B95.8 strain of EBV, the *AbwNI/SspI* fragment from pEGFP-C1 (Clontech) harboring the kanamycin-neomycin resistance cassette, and the *BamHI/BamHI* S-fragment (nucleotide coordinates 84234 to 87654) of B95.8. Insertion of the kanamycin-neomycin resistance cassette at this position removed the BMLF1 coding region from amino acid 1 to 378. A *PmlI/NheI* fragment derived from pSM neo/kan (Fig. 1A) was electroporated into *E. coli* DH10 carrying p2089 and p2650, and the bacteria were coselected for antibiotic resistance against chloramphenicol (30 μg/ml) and kanamycin (50 μg/ml) at 42°C. At

this temperature, p2650 is lost rapidly, and colonies were subsequently analyzed by using various restriction enzymes to confirm correct recombination. One identified clone (p2688.16) was selected, and its plasmid DNA was prepared on CsCl-ethidium bromide gradients. The plasmid DNA was partially sequenced to confirm the anticipated deletion of the BMLF1 gene and the integrity of the flanking regions.

Selection of stable 293 cell clones and induction of the productive phase of the EBV life cycle. The maxi-EBV plasmid p2688.16 was transfected into 293 cells as previously described (8, 20) to establish single-cell clones that carry the BMLF1-KO maxi-EBV plasmid. The 293 cells were selected in the presence of 80 μg of hygromycin per ml for 4 weeks, and single outgrowing clones were expanded. The cells were called 293-BMLF1-KO cells. The single-cell clones were transiently transfected with Lipofectamine in 6-well cluster plates as described previously (8, 20) with 0.5 μg of the expression plasmid p509 (pCMV-BZLF1) (16) encoding EB1 protein to induce the EBV productive phase. In order to *trans-complement* the BMLF1-negative maxi-EBV, 1.0 μg of a second expression plasmid encoding EB2 under the control of the CMV immediate-early enhancer and promoter was cotransfected (pAAC-FlagEB2). Two to 3 days later, the supernatant was collected and filtered through a 0.45-μm-pore-size filter. Infection of Raji cells with the virus stock was carried out as previously described (11, 20). Transient transfections of 293-BMLF1-KO cells were also performed by electroporation as described previously (44), with no apparent variability in the results obtained, in terms both of virus production and protein expression.

Western blot analysis. Cells were lysed in sodium dodecyl sulfate gel sample buffer. The equivalent of 2×10^6 cells was loaded into each lane of sodium dodecyl sulfate–10% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated either with the anti-BZLF1 MAb Z125 or the anti-BRLF1 MAb 8C12 (28) or the anti-BMRF1 MAb NCL-EADE31 (Novocastra) or rabbit polyclonal serum directed against the EB2 protein (3). Anti-mouse or anti-rabbit-horseradish peroxidase conjugate (Amersham) was used as a secondary antibody, and immune complexes were visualized with the ECL reagent (Amersham).

Transient-replication assays. Transient-replication assays were performed with 293-BMLF1-KO cells by using a plasmid carrying the complete wild-type *oriLyt*, termed p968.22, which has been described in detail elsewhere (16). p968.22 was transfected either alone; together with plasmid pCMV-EB1, which expresses the EB1 protein; or together with pCMV-EB1 and paacFlagEB2, an expression plasmid for the EB2 protein. Two days after transfections, DNA was prepared by the Hirt technique, digested with *DpnI* and *BamHI*, subjected to electrophoresis through a 0.7% agarose gel, transferred to a nylon N+ membrane (Amersham), and probed with random-primer ³²P-labeled pUC19. The replication efficiency of the *oriLyt* plasmid p968.22 was quantified by scanning the Southern blot autoradiogram.

RNA extraction and RT-PCR analysis. Transfected cells were washed twice with phosphate-buffered saline, and cytoplasmic RNA was extracted and purified on a cesium chloride gradient according to published protocols (6).

We performed reverse transcription-PCR (RT-PCR) on 0.5 μg of purified RNA by using the Titan one-tube RT-PCR system (Roche Molecular Biochemicals). The positions of the primers used for RT-PCR are the following, according to the B95.8 EBV strain sequence (1): for BALF2, 5'-GGAAGACTGTGTGT TTGATGTGGTG-3' (positions 161726 to 161750) and 5'-TGATAAAGTTGT CCTGGGCTGC-3' (positions 161512 to 161533); for BALF5, 5'-AAGAGGC ACTGGAGGTGTTGG-3' (positions 153763 to 153784) and 5'-AGGGCTA CCCTGTGGGCTTTTGG-3' (positions 154116 to 154137); for BBLF2/3, 5'-C TCAGATTTCACGCCCTATGTCC-3' (positions 18129 to 18151) and 5'-CC ACAAAGCCCAGGATGAATC-3' (positions 117556 to 117577); for BBLF4, 5'-GCATCCGTGACTATGGCATTAGC-3' (positions 112076 to 112098) and 5'-CGTGAGTTCTTTAGGGCATCCAC-3' (positions 111849 to 111871); for BMRF1, 5'-CCAGACATACGGTCAGTCCATCTC-3' (positions 80881 to 80904) and 5'-TGCTTCACTTTCTTGGGGTGC-3' (positions 81071 to 81091); for BSLF1, 5'-AAAACCTTCTGCTACCACATCGC-3' (positions 84786 to 84808) and 5'-CAGCCCTATTATGATTCTGGAGG-3' (positions 84343 to 84366); for BLF1, 5'-TATGGTGGGGTGGTGTAGGTATG-3' (positions 89455 to 89477) and 5'-CAGATTACGGCGGTGATTCAAC-3' (positions 89731 to 89752); for BALF4, 5'-TCGTGATAGCGTCTTCTCGGTAG-3' (positions 156798 to 156820) and 5'-TGAGCAAAACCAGGAGCAAAAG-3' (positions 156896 to 156917); and for BCRF1, 5'-CCACAGGTTCTGCC-3' (positions 10031 to 10045) and 5'-GACATCGCAAGGGG-3' (positions 10236 to 10250). Quantification of the RT-PCR results was done according to a method described previously (3). As a control in our RT-PCR experiments, endogenous expression of the β-actin mRNA was also evaluated by RT-PCR. Primers 5'-G CTGCGTGTGGCTCCCGAGGAG3' and 5'-ATCTTCATTGTGCTGGGTGC

FIG. 1. Mutant EBV lacking its early gene BMLF1. (A) Construction of the BMLF1-negative EBV mutant genome by homologous recombination in *E. coli*. The diagram shows schematically a linearized fragment of the wild-type EBV genome carrying the BMLF1 gene and the same fragment from the EBV_{BMLF1-KO} mutant, in which most of the BMLF1 sequence has been replaced by the kanamycin resistance gene. (B) The 293_{BMLF1-KO} cell clone was used for all of the following experiments. The cells express GFP because the maxi-EBV genome encodes the GFP gene. The cells were visualized by UV light microscopy. (C) Western blot analysis of the EB2 protein and viral early proteins expressed in the 293 cell clone carrying EBV_{BMLF1-KO} mutant maxi-EBV. Total proteins were prepared from Raji cells (EBV-infected cells) or from 293_{BMLF1-KO} cells mock transfected (lane 4), transfected with only an expression vector for the EBV transcription factor EB1 (lanes 1, 2, and 5), or cotransfected with expression vectors for both EB1 and EB2 (lanes 3 and 6). The nitrocellulose filter was probed with a polyclonal serum directed against the EB2 protein (lanes 1 to 3) or a set of MAbs specific for the EBV R transcription factor, the EBV early protein BMRF1 (EA-D), or the EB1 protein.

CAG-3' were used in the PCR to amplify a 690-bp DNA fragment corresponding to the β -actin mRNA.

RESULTS

Construction of a BMLF1-negative EBV. The complete genome of the prototype EBV strain B95.8 was cloned in *E. coli* as described previously (8). In order to facilitate genetic manipulation of the so-called maxi-EBV plasmid in the prokaryotic host, a temperature-sensitive plasmid encoding RecA and Red γ was constructed and termed p2650. This plasmid allows efficient homologous recombination in a *recA*-negative background such as DH10B. In addition, transformation of DH10B with linear DNA fragments is greatly facilitated by the Red γ gene product, which eliminates endogenous exonuclease activity, similar to a recently published strategy (51). To introduce the KO of BMLF1, the plasmid pSM neo/kan was cleaved by two restriction enzymes (*PmlI* and *NheI*) to accommodate a kanamycin resistance gene flanked by two short stretches of homologous EBV sequences of 82 and 76 bp in length (Fig. 1A). The two flanking sequences should target the kanamycin resistance cassette so that most of the coding sequence of BMLF1 is deleted by insertional mutation. The linear DNA fragment of pSM neo/kan was introduced into DH10B harbor-

ing the wild-type maxi-EBV p2089 and the temperature-sensitive, RecA- and Red γ -encoding p2650 plasmids. Colonies were selected on chloramphenicol- and kanamycin-containing plates at 42°C. The maxi-EBV plasmid DNA from single colonies was analyzed with several restriction enzymes, and one clone, termed p2866.16, was selected for further analysis. About 1,000 bp covering the distal parts of the introduced kanamycin resistance cassette and the flanking EBV DNA was sequenced in order to confirm the overall composition of the altered locus and the integrity of the neighboring genes (data not shown). Large-scale plasmid DNA was prepared from *E. coli* and purified on CsCl-ethidium bromide equilibrium density gradients to yield BMLF1-KO maxi-EBV plasmid DNA.

EB2 is essential to produce infectious EBV particles. The production of virus stocks in the maxi-EBV systems requires the establishment of a permissive cell line based on 293 cells that carry the EBV genome. To establish such a cell line, the BMLF1-KO maxi-EBV plasmid DNA prepared from *E. coli* was transfected into 293 cells, and stable hygromycin-resistant cell clones that carry the EBV plasmid extrachromosomally were selected. The correct composition of the maxi-EBV DNA was confirmed by Southern blot hybridization (data not shown). One cell clone, termed 293_{BMLF1-KO}, was used for all

of the following experiments. Since maxi-EBV plasmids encode GFP expressed under the control of the CMV immediate-early promoter and enhancer, the cells were bright green under UV light (Fig. 1B). Upon induction of the productive phase of the EBV life cycle by transient transfection of an expression vector for the EBV productive-cycle transactivator EB1 (8), the 293-BMLF1-KO cells failed to express the EB2 protein (Fig. 1C, lane 2). In contrast, Raji cells transiently transfected with a plasmid encoding the EBV transcription factor EB1 readily expressed the EB2 protein (Fig. 1C, lane 1). The 293-BMLF1-KO cells expressed the EB2 protein only when transfected with an expression plasmid for the EB2 protein (Fig. 1C, lanes 3 and 6).

In order to determine the production of progeny virus, the 293-BMLF1-KO cells were first transiently transfected with an expression plasmid encoding the EBV transcription factor EB1 to induce the productive phase of the EBV life cycle. Three days after transfection, the cellular supernatant was harvested, centrifuged, and filtered to remove cellular debris. This supernatant was then used to incubate Raji cells in order to analyze the presence of infectious EBV, visualized indirectly by GFP expression in Raji cells as described previously (8, 9, 11, 20). No green Raji cells could be found when the cells were transfected with a control plasmid (instead of the EB1 expression vector), indicating that the 293-BMLF1-KO cells do not spontaneously produce infectious viral particles (Fig. 2a). In 293 cells carrying wild-type maxi-EBV, up to 10^6 infectious particles per ml can usually be detected in the cellular supernatant. In sharp contrast to this situation, only very few or no green Raji cells could be found in supernatants from EB1-transfected 293-BMLF1-KO cells, strongly suggesting that the lack of the BMLF1 gene product causes a very dramatic decrease in virus production (Fig. 2b). Next, we cotransfected the 293-BMLF1-KO cells with the expression plasmid for the EB1 protein together with an expression plasmid for the EB2 protein. As expected, an impressive number of green Raji cells could be seen by UV light microscopy, indicating successful transduction of the GFP gene into Raji cells by BMLF1-KO virions (Fig. 2c). The rescue of virus production by cotransfecting an EB2 expression vector and an EB1 expression vector into 293-BMLF1-KO cells was not due to an unusually high expression of the EB1 protein, since its expression level was not altered by concomitant expression of EB2 (Fig. 1C, compare lanes 5 and 6). Similarly, the expression levels of the EBV transcription factor R or the early protein BMRF1 (EA-D) were not significantly modified by the expression of EB2 (Fig. 1C, compare lanes 5 and 6). This result suggests that the EB2 protein is not required for efficient expression of these EBV proteins.

These results clearly demonstrated that the EBV BMLF1 gene is essential for the production of infectious EBV particles and that the disruption of the BMLF1 gene can be functionally *trans*-complemented by transient expression of its product, the EB2 protein.

The EB2 protein strongly enhances *oriLyt*-dependent DNA replication in 293-BMLF1-KO cells. In order to define the molecular level at which the productive cycle of EBV is affected in 293-BMLF1-KO cells, we analyzed the efficiency of the viral DNA synthesis. The 293-BMLF1-KO cells were transfected with the plasmid p968.22, encompassing *oriLyt* together with an expression plasmid for the EB1 protein so as to activate EBV's lytic,

FIG. 2. Infection of Raji cells with different EBV stocks as detected by GFP expression. Raji cells (10^5) were incubated with 1 ml of supernatant from 293-BMLF1-KO cells (a) or with the same cells transfected with an expression plasmid encoding the EB1 protein (b) or transfected with expression plasmids encoding both the EB1 and EB2 proteins (c). GFP fluorescence (corresponding to successful infections) was detected 72 h after incubation with the supernatants as described in the text.

productive cycle. The plasmid p968.22 did not replicate in the absence of the productive cycle (Fig. 3, lane 1). Upon induction of the productive cycle by transfection of the EB1 expression vector, p968.22 replicated in 293-BMLF1-KO cells, although with low efficiency (Fig. 3, lane 2). Coexpression of the EB2 protein in these cells greatly enhanced the signal of newly replicated *oriLyt* DNA (Fig. 3, lane 3), demonstrating that the EB2 protein is not absolutely required for *oriLyt*-dependent replication but enhances its efficiency. These results also showed that 293-BMLF1-KO cells are able to replicate viral DNA in the absence of EB2 but are severely impaired in the production of virus progeny.

EB2 increases the nuclear export of specific EBV early mRNA. Even in the absence of EB2, *oriLyt*-dependent DNA replication was detectable in 293-BMLF1-KO cells induced by EB1. This result suggested that all mRNAs encoding the proteins implicated in DNA replication were transcribed and

FIG. 3. Enhancement of *oriLyt*-dependent DNA replication by the EB2 protein. 293-BMLF1-KO cells were transiently cotransfected with the *oriLyt* plasmid p968.22 together with a control vector (lane 1), an EB1 expression plasmid (lane 2), or the EB1 and EB2 expression plasmids (lane 3). Newly replicated *oriLyt* plasmid DNA that was detected by Southern blot hybridization with a pUC probe after digestion of the Hirt DNA with *Bam*HI and *Dpn*I is indicated by an arrow.

translated. Since the EB2 protein enhanced *oriLyt*-dependent replication considerably, we speculated that EB2 might have an important effect on the level of expression of some of the proteins involved in *oriLyt*-dependent DNA replication, with the exception of the EA-D proteins (BMRF1), which were not affected by the lack of EB2 (Fig. 1C). Since EB2 had an effect on the level of lytic DNA replication, EB2 function could be involved in the expression of other factors, presumably at the level of their mRNA export. To test this hypothesis, we evaluated by semiquantitative RT-PCR the cytoplasmic accumulation of the early mRNAs coding for the proteins necessary for *oriLyt*-dependent replication. The results presented in Fig. 4A provided the following evidence. (i) The amounts of β -actin mRNA detected by RT-PCR in latently infected or induced 293-BMLF1-KO cells were comparable in the absence or presence of EB2 (Fig. 4A, lanes 1 to 3). (ii) As expected, the expression level of the latent gene BYRF1 (EBNA2) was not altered in the latent or productive cycle even when the EB2 protein was expressed (Fig. 4A, lanes 7 to 9). (iii) Expression of the BKRF1 gene (EBNA1) has been shown previously to be increased by the induction of the productive cycle (50). Accordingly, BKRF1 expression was detectable in latently infected cells (Fig. 4A, lane 4), but its steady-state expression level was elevated in the 293-BMLF1-KO cells upon induction of the productive cycle by the EB1 expression plasmid (Fig. 4A, lanes 4 and 5). Cotransfection of the EB2 expression plasmid did not alter this level of the BKRF1 RNA (Fig. 4A, lane 6). These results demonstrated that it is possible to detect variations in the level of expression of EBV genes by RT-PCR and that the RT-PCR conditions used are suitable for quantitation.

During latency the genes coding for the proteins necessary

for DNA replication are not expressed, and their corresponding mRNAs were not detectable by RT-PCR (Fig. 4B, lanes 1, 4, 7, 10, 13, and 16). The faint bands observed lanes 10 and 16 of Fig. 4B are probably the result of some spontaneous reactivation of the productive cycle in the cell culture. When the productive cycle was induced by transfection of the EB1 expression vector into 293-BMLF1-KO cells, all mRNAs from the viral genes involved in DNA replication could be detected by RT-PCR (Fig. 4B, lanes 2, 5, 8, 11, 14, and 17). This result was expected because under the conditions described above, *oriLyt*-dependent replication took place, although at a somewhat reduced level (Fig. 3). Since the EB2 protein enhanced *oriLyt*-dependent DNA replication, we expected that EB2 would increase cytoplasmic RNA accumulation for most of the genes encoding the replication proteins. Unexpectedly, the EB2 protein increased the cytoplasmic accumulation of only a subset of mRNAs corresponding to the BALF2 gene (single-stranded-DNA-binding protein) (Fig. 4B, compare lane 2 with lane 3; see quantification of five independent assays in Fig. 4C) and the BALF5 gene (DNA polymerase) (Fig. 4B, compare lane 5 with lane 6; see quantification of five independent assays in Fig. 4C). Cytoplasmic accumulation of the mRNAs of BBLF2/3, BBLF4, BMRF1, and BSLF1 was not apparently affected by the EB2 protein (Fig. 4B; see quantification of five independent assays in Fig. 4C). Taken together, these results strongly suggest that the EB2 protein specifically exports two out of six mRNAs coding for the viral proteins necessary for *oriLyt*-dependent DNA replication, which implies that EB2 is not a nonspecific viral mRNA export factor.

EB2 also induces the cytoplasmic accumulation of some late mRNAs. It has been shown that the HSV infected protein 27 (ICP27) is required for the expression of certain early viral proteins and many late proteins during productive infection. For example, expression of glycoprotein C is severely restricted at the mRNA level in the absence of ICP27 (21). In order to determine whether the EB2 protein was also required for some late mRNA export, we analyzed the cytoplasmic accumulation of three late mRNAs (BALF4, BLLF1, and BCRF1) by RT-PCR as described above. The quantified results are presented in Fig. 5, and they show that the EB2 protein induced cytoplasmic accumulation of the three mRNAs tested. For these three mRNAs, we observed a twofold increase of the amount of mRNA accumulation in the presence of EB2 protein, as for some early mRNAs such as BALF5. However, since the replication efficiency is affected by the absence of the EB2 protein, we cannot exclude the possibility that the results observed with the late mRNAs were not a consequence of the defect in replication efficiency.

trans-complementation of EBV_{BMLF1-KO} virus by EB2 mutants and functional homologs of other herpesviruses. The EB2 protein contains an Arg-X-Pro tripeptide repeat similar to that described as an RNA-binding motif in the HSV-1 US11 protein (38). The EB2 motif also binds RNA in vitro, but deletion of this particular motif in the EB2 protein does not affect its ability to export nuclear RNA in a transient-expression assay (3). Hence, we wanted to evaluate the effect of this specific deletion in the EB2 protein in the *oriLyt* replication assay in 293-BMLF1-KO cells. The results show that the mutant EB2_{ΔRXP} protein is as efficient as the wild-type EB2 protein in enhancing *oriLyt*-dependent replication (Fig. 6A, compare

FIG. 4. The EB2 protein induces the accumulation of specific mRNAs coding for EBV replication proteins. (A and B) RNAs from 293^{BMLF1-KO} cells transiently transfected with a control vector or expression plasmids coding for proteins, as indicated, were subjected to RT-PCR analysis with specific primer pairs to detect the β -actin cellular mRNA and EBV latent mRNA encoding BKRF1 or BYRF1 (A) or the product of the early genes encoding the EBV replication proteins BALF2, BALF5, BBLF2/3, BBLF4, BMRF1, and BSLF1 (B). The PCR products were loaded onto a 2% agarose gel and visualized by ethidium bromide staining. (C) Quantification of five independent RT-PCR assays. The results are presented as the relative cytoplasmic accumulation of the early mRNA in the cells transfected with expression vectors for EB1 and EB2 compared to their accumulation in the cells transfected only with an EB1 expression vector. Error bars indicate standard deviations.

bars 3 and 4), demonstrating that the Arg-X-Pro motif in EB2 does not support an essential function in this context. This result was also confirmed by the fact that the EB2 _{Δ RXP} protein was as efficient as the wild-type EB2 protein in its capacity to *trans*-complement the EBV_{BMLF1-KO} virus for the production of infectious virions (data not shown).

The EB2 protein shuttles between the nucleus and the cytoplasm and contains a leucine-rich signal sequence, LPSPLA SLTLS, that matches the consensus NES identified in the Rev family of proteins or the HSV-1 ICP27 protein (5). Mutation of this LXL motif alone did not prevent EB2 shuttling (10), but a double mutation of the LXL motif plus an upstream VTL motif was reported to suppress shuttling, suggesting that the EB2 protein has two NES motifs (5). The EB2_{dNES} mutant protein was still capable of enhancing EB1-induced *oriLyt*-dependent replication in our transient DNA replication test, although to a lesser extent than wild-type EB2 (about a 35% reduction in Fig. 6A, lane 5). The EB2_{dNES} protein was also capable of *trans*-complementing EBV_{BMLF1-KO} maxi-EBV for

the production of infectious particles but at a lower level than the wild-type EB2 protein (Fig. 6B, compare panel c to panel b). Results from fluorescence-activated cell sorter analyses showed that the supernatant of 293^{BMLF1-KO} cells obtained after transient transfection of expression vectors coding for EB1 and EB2_{dNES} contained about 1/10 of infectious virions compared to supernatants obtained with wild-type EB1 and EB2 as assessed by GFP fluorescence of infected Raji cells (data not shown). Our observations suggest that the two NES motifs might enhance efficient *oriLyt*-mediated DNA replication and production of progeny virus. It remains to be seen, however, if shuttling of EB2 will be a prerequisite for both functions.

Different functional homologs of the EB2 protein have been found in herpesvirus family members. Among them are the ICP27 protein from HSV-1, which has recently been shown to export intronless viral mRNA in a CRM-1-independent fashion (25), and the UL69 protein from HCMV. Hence, we wanted to assess whether ICP27 or UL69 proteins could *trans*-

complement BMLF1-deficient EBV for *oriLyt*-mediated DNA replication and production of infectious particles. The results from the replication assays showed that ICP27 as well as UL69 proteins enhanced EB1-induced *oriLyt*-dependent replication, although at a lower level than EB2 protein (Fig. 6A, lanes 6 and 7). In sharp contrast, both ICP27 and UL69 proteins only very poorly rescued production of infectious EBV particles in 293-BMLF1-KO cells upon lytic cycle induction with an EB1 expression vector (Fig. 6B, panels d and e). Fluorescence-activated cell sorter analysis of Raji cells incubated with the supernatant of 293-BMLF1-KO cells transiently transfected with the expression vectors for EB1 and ICP27 (or EB1 and EB2 as a control) showed that the supernatants contained only about 1% of infectious particles compared to the wild-type situation. In conclusion, the data show that homologous proteins such as ICP27 or UL69 are capable of partially rescuing *oriLyt*-mediated DNA replication. Unexpectedly, both ICP27 and UL69 were found to be very inferior in reconstituting virus production in 293-BMLF1-KO cells.

ICP27 enhances the cytoplasmic accumulation of BALF2 and BALF5 mRNAs. We have shown that the ICP27 protein, like the EB2 protein, enhances *oriLyt*-dependent replication in 293-BMLF1-KO cells. Our results also show that the EB2 protein increases the nuclear export of two early mRNAs, BALF2 and BALF5. In order to discover whether the ICP27 protein had the same effect on these mRNAs, we repeated the RT-PCR analysis described above with cytoplasmic RNA derived from 293-BMLF1-KO cells transfected with an EB1-expressing vector either alone or in conjunction with an EB2- or ICP27-expressing vector. The results show that the EB1 protein alone causes a weak cytoplasmic accumulation of the products of the BALF2 and BALF5 genes (Fig. 7A, lanes 1 and 4; see quantification of five independent assays in Fig. 7B), which is enhanced by coexpression of the EB2 protein as shown before (Fig. 4 and 7A, lanes 2 and 3; see quantification of five independent assays in Fig. 7B). As expected, the ICP27 protein also enhances cytoplasmic mRNA accumulation of BALF2 and BALF5 transcripts, although the effect is not as profound on the latter (Fig. 7A, compare lanes 2 and 5 to lanes 3 and 6; see quantification of five independent assays in Fig. 7B). Cytoplasmic accumulation of BMLF1 mRNA is not significantly affected by the EB2 or ICP27 protein (Fig. 7, lanes 7 to 9; see quantification of five independent assays in Fig. 7B), confirming our data (Fig. 1C). These observations strongly suggest that the ICP27 protein enhances *oriLyt*-dependent replication in the 293-BMLF1-KO cells by the same mechanism as the EB2 protein.

DISCUSSION

We generated an EBV genome with a deletion of its BMLF1 open reading frame and established a clonal 293 cell line, 293-BMLF1-KO, that stably carries this EBV mutant genome. As expected, no detectable EB2 protein is synthesized from this EBV mutant after induction of the EBV productive cycle with an EB1-expressing vector. Equally, this EBV_{BMLF1-KO} cell line is incapable of producing infectious progeny virus unless the EB2 protein is transiently expressed in the cells. These observations indicate that the EB2 protein is a protein essential in the productive phase of the EBV life cycle and also indicates

FIG. 5. The EB2 protein also induces the cytoplasmic accumulation of late mRNA. RNAs from 293-BMLF1-KO cells transiently transfected with a control vector or expression plasmids coding for EB1 and EB2 proteins, as described Fig. 4, were subjected to RT-PCR analysis with specific primer pairs to detect the early mRNA BALF5 or the late mRNAs BALF4, BLLF1, and BCRF1. Quantification of three independent RT-PCR assays is shown. The results are presented as the relative cytoplasmic accumulation of the early mRNA in the cells transfected with expression vectors for EB1 and EB2 compared to their accumulation in the cells transfected only with an EB1 expression vector.

that a cellular protein cannot substitute EB2's functions during the EBV productive cycle.

Within the family *Herpesviridae*, a set of homologous polypeptides that are implicated in RNA processing and transport has been characterized (10, 14, 43). These proteins were initially described as promiscuous transactivators of viral and cellular gene expression. The most intensively studied member of this group is the posttranscriptional regulator ICP27 of the alphaherpesvirus HSV-1. ICP27 has been reported to shuttle between the nucleus and the cytoplasm and to bind intronless viral mRNA but not spliced viral transcripts (42). Recently, a direct role for ICP27 in viral mRNA export has been demonstrated (25), and it has been shown that ICP27 affects nuclear export of viral RNA via a CRM1-independent pathway (25, 46). ICP27 mutant viruses are defective for virus production and show reduced levels of viral DNA replication (33, 35, 41).

The ICP27 homolog of the betaherpesvirus HCMV is the multifunctional phosphoprotein UL69 (48). UL69 is a nuclear protein that is also able to shuttle between the nucleus and the cytoplasm. Trafficking of UL69 does not involve binding to CRM-1 (30), a property shared by the gammaherpesvirus EB2 protein (10), although other reports suggest the contrary (2). UL69 is also necessary for efficient production of HCMV (17).

In this study, we show that in the absence of the EB2 protein, some of the EBV productive-cycle genes are less efficiently expressed. It has been shown that the EB2 protein is involved in the cytoplasmic accumulation of mRNA. Although the EB2 protein binds to RNA in vivo and shuttles from the nucleus to the cytoplasm (10, 40), the molecular mechanism by which EB2 acts is not known.

The mechanism of cellular mRNA export from the nucleus to the cytoplasm is partially understood. It has been proposed that the splicing machinery positions a protein complex near the exon-exon junction (EJC complex), which interacts with a protein, TAP, that is responsible for targeting the nucleoprotein complex to the nuclear pore complex (26, 27). This mechanism cannot take place for EBV's early and late mRNAs because, in contrast to the majority of the cellular genes, they are transcribed from intronless genes. Since the EB2 protein

FIG. 6. DNA replication analysis and production of infectious particles in 293^{-BMLF1-KO} cells *trans*-complemented with various EB2 mutants and herpesvirus functional homologs. (A) Enhancement of *oriLyt*-dependent replication by the EB2 protein functional homologs from HSV-1 and HCMV. 293^{-BMLF1-KO} cells were transiently cotransfected with the *oriLyt* plasmid p968.22 together with a control vector (bar 1) or expression plasmids coding for proteins as indicated at the bottom of the histogram. The relative replication efficiency of p968.22 in the presence of both EB1 and EB2 was set to 100%. The replication assays were done at least four times, and means and standard deviations are indicated. (B) Infection of Raji cells by various EBV stocks as detected by GFP expression. Raji cells (10^5) were incubated with 1 ml of supernatant from 293^{-BMLF1-KO} cells transfected with expression plasmids encoding either the EB1 protein alone (a), the EB1 and EB2 proteins (b), EB1 and a dNES EB2 mutant protein (c), EB1 and ICP27 (d), or EB1 and UL69 (e). GFP fluorescence (corresponding to successful infections) was detected 72 h after incubation with the corresponding supernatant.

induces cytoplasmic accumulation of intronless mRNA (3), this suggests that the EB2 protein could be a viral mRNA export factor that links intronless viral mRNA to the cellular export machinery. As for ICP27, EB2 might recruit the cellular export factors REF and TAP to viral mRNA (25), providing viral mRNA with access to the cellular mRNA export pathway. This hypothesis seems to hold true for BALF2 and BALF5 transcripts as indicated by our results. Since only a smaller subset of early viral mRNA appeared to be sensitive to the action of the EB2 protein, yet another, and presumably different, mechanism is probably involved in nuclear export of the majority of unspliced EBV mRNA. Cytoplasmic accumulation of spliced viral mRNA such as transcripts encoding the primase-associated factor BBLF2/3 was not affected by the EB2 protein, presumably because this mRNA transcript is exported by a splicing-dependent mechanism similar to cellular mRNA.

It is clear from our results and those reported by Fixman and coauthors (12, 13) that the EB2 protein greatly enhances *oriLyt*-dependent DNA replication. This phenotype is probably the consequence of the effect of the EB2 protein on cytoplasmic accumulation of some early viral mRNAs rather than a

direct effect on viral DNA synthesis. It has been reported previously that the EB2 protein can lead to increased cytoplasmic accumulation of five out of six EBV core replication proteins in a transient-transfection assay (45). With our genetic system we could show that the EB2 protein significantly increased cytoplasmic accumulation of only two EBV core replication proteins, the BALF2 and the BALF5 gene products. Thus, the EB2 protein has a selective effect on unspliced viral mRNA. The differences between our results and those published by Semmes and coauthors (45) are probably due to the fact that the viral genes have been expressed from transfected expression plasmids and thus the corresponding RNAs were not authentically processed (3). By RT-PCR analysis, we observed that the cytoplasmic accumulation of mRNAs from a few late viral genes was also affected (data not shown), but their corresponding mRNAs were also readily detectable in the absence of the EB2 protein. Differences in the level of expression of some specific early and late proteins were also observed in the case of an ICP27 mutant HSV-1 (33).

It has been shown that the HCMV protein UL69, which is a functional homolog of the EB2 protein, is packaged into virus

FIG. 7. The ICP27 protein induces accumulation of the BALF2 and BALF5 mRNAs. (A) RNAs from 293^{-BMLF1-KO} cells transiently transfected with expression plasmids encoding proteins as indicated at the top were subjected to RT-PCR analysis with specific primers to detect the product of the early genes encoding the EBV replication proteins BALF2, BALF5, and BMRF1. The PCR products were loaded onto a 2% agarose gel and visualized by ethidium bromide staining following migration. (B) Quantification of three independent RT-PCR assays. The results are presented as the relative cytoplasmic accumulation of the early mRNA in the cells transfected with expression vectors for EB1 and EB2 (gray bars) or EB1 and ICP27 (black bars) compared to their accumulation in the cells transfected only with an EB1 expression vector. Error bars indicate standard deviations.

particles (17, 49). Although UL69 is a component of the virion, it is dispensable for production of infectious progeny virus (17). Given this report, it appears rather unlikely that cells harboring the BMLF1-KO EBV genome release noninfectious viral particles in the absence of EB2. However, this assumption is supported by our *trans*-complementation system, in which both ICP27 and UL69 proteins, the two EB2 functional homologs in HSV-1 and HCMV, are capable of supporting *ori-Lyt*-dependent DNA replication in the genetic background of an EB2-defective virus but only very poorly support virion production. Efficient DNA replication of *ori-Lyt* is most likely the consequence of efficient mRNA export of viral replication factors, which can be mediated by UL69, ICP27, or BMLF1 similarly. In contrast, UL69 or ICP27 proteins were not able to support all of the functions of the EB2 protein in terms of production of infectious progeny EBV particles. Since virus production and maturation are very complex processes, it appears likely that one or more viral gene products that are limiting in the morphogenesis of EBV particles are also af-

fected by the absence of EB2. As an example, the product of the BALF4 gene is such a candidate (data to be published elsewhere). In summary, our data strongly suggest that EB2 is responsible and indispensable for the export of selected, specific viral mRNAs from the nucleus to the cytoplasm, which could constitute a virus-specific pathway of potential interest for targeted antiviral strategies.

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