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Epstein-Barr Virus EBNA3A and EBNA3C Proteins Both Repress RBP-Jκ–EBNA2-Activated Transcription by Inhibiting the Binding of RBP-Jκ to DNA

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Following infection by Epstein-Barr virus (EBV), the production of viral nuclear proteins EBNA1, EBNA2, EBNA3A, and EBNA3C and the viral membrane protein LMP1 is essential for the permanent proliferation of primary B lymphocytes to occur. Among these, the transcription factor EBNA2 is central to the immortalizing process, since it activates not only the transcription of all the EBNA proteins and LMP1, TP1, and TP2 but also certain cellular genes. EBNA2 is targeted to its DNA-responsive elements through direct interaction with the DNA-binding cellular repressor RBP-Jκ. In a transient-expression assay, the EBNA2-activated transcription was found to be downregulated by EBNA3A, EBNA3B, and EBNA3C. However, since it has been reported that EBNA3C, but not EBNA3A, directly contacts RBP-Jκ in vitro, these proteins appear to repress through different mechanisms. Here, we report for the first time that EBNA3A and EBNA3C both stably interact with RBP-Jκ and most probably repress EBNA2-activated transcription by destabilizing the binding of RBP-Jκ to DNA.

Epstein-Barr virus (EBV) is a human herpesvirus associated with several malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and various lymphomas in immunodepressed individuals (24). In vitro infection of B lymphocytes by EBV, as well as explant culture of lymphocytes from seropositive adults, gives rise to immortalized cell lines. In such immortalized B cells, EBV persists mainly as an episome from which a characteristic and limited set of viral genes is expressed, resulting in type III latency. The products of these genes include six nuclear proteins (EBNA1, -2, -3A, -3B, and -3C and EBNA-LP), three membrane proteins (LMP1, TP1, and TP2), and two small nuclear RNAs (EBER1 and EBER2) (for a review, see reference 20). Of these gene products, the EBV nuclear proteins EBNA2, EBNA3A, and EBNA3C and the EBV integral membrane protein LMP1 are essential for the EBV-induced perpetual proliferation of B cells (7, 12, 18, 27, 36). However, the precise biochemical mechanisms by which these proteins act to induce and maintain the immortalization of B cells are largely unknown.

EBNA2 is necessary for the initiation (32) as well as for the maintenance (19) of immortalization. EBNA2 is a transcription factor that upregulates the expression of cellular CD21, CD23, and c-*fgr* genes (6, 8, 21, 39) and EBV EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C, LMP1, TP1, and TP2 genes (1, 10, 31, 35, 40, 42, 46). However, EBNA2 does not bind directly to DNA but is targeted to its responsive promoter through direct interaction with the cellular binding protein RBP-Jκ (11, 14, 25, 38, 45). The interaction between EBNA2 and RBP-Jκ is essential for the immortalization of primary B lymphocytes (43). By binding to RBP-Jκ, EBNA2 provides an activation domain and interferes with the function of a corepressor that also binds to RBP-Jκ (15, 37).

The EBNA3A, -3B, and -3C proteins are translated from mRNAS transcribed from three tandemly arranged genes in the EBV genome. Although these genes and their protein products have related structures, EBNA3A, -3B, and -3C have unique functions since recombinant EBVs that carry null mutations in either EBNA3A or EBNA3C are nonimmortalizing (36). Of the EBNA3 proteins, EBNA3C is the most studied. Like EBNA2, EBNA3C upregulates the expression of CD21 in non-EBV-infected Burkitt's lymphoma cells (39) and LMP1 in G_1-arrested EBV-infected Raji cells (4). It has also been reported that EBNA3C downregulates EBNA2-mediated activation of the LMP1 promoter in transient-expression assays (28, 30). This latter property, however, is not unique to EBNA3C, since it has been previously shown that EBNA3A, -3B, and -3C are able to individually repress EBNA2-mediated transactivation of the viral terminal protein 1 (TP1) gene promoter (23). Taken together, these results raise the possibility that one of the functions of EBNA3C, and possibly also of EBNA3A and EBNA3B, is to modulate the expression of EBNA2-responsive genes. In the case of EBNA3C, the downregulation of EBNA2-mediated transcriptional activation could be due to a direct interaction between EBNA3C and RBP-Jκ. Indeed, EBNA3C binds to RBP-Jκ and inhibits the specific interaction of RBP-Jκ with DNA in vitro (30). In the case of EBNA3A, as there does not appear to be physical contact with RBP-Jκ in vitro (30), the mechanisms by which EBNA3A downregulates EBNA2-mediated activation remain completely unknown.

In this report, we show that both EBNA3A and EBNA3C efficiently repress the EBNA2-mediated activation of an artificial promoter containing RBP-Jκ binding sites. We then present experimental data demonstrating that although both EBNA3A and EBNA3C make stable physical contact with RBP-Jκ in the absence of EBNA2 in vivo, neither EBNA3A–RBP-Jκ nor EBNA3C–RBP-Jκ complexes bind DNA in vivo. Our results clearly and directly demonstrate that EBNA3A and EBNA3C repress EBNA2-activated transcription by the same mechanism: inhibition of the specific interaction of RBP-Jκ with DNA.

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MATERIALS AND METHODS

Reporter plasmids. The reporter plasmid pTK-CAT-Cp4x has been previously described (37). It contains four copies of an *Xho*I-*Sal*I double-stranded oligonucleotide containing the EBNA2-responsive element of the Cp promoter cloned into the *Xho*I site of plasmid pBLCAT2.

pG4-TK-CAT was generated by inserting six copies of the following double-stranded oligonucleotide containing a Gal4 binding site into the *Sal*I site of pBLCAT2:

5′-TCGAGCGGAGTACTGTCCTCCGC-3′
3′-CGCCTCATGACAGGAGGCGAGCT-5′.

Expression vectors. The pSG5flag-RBP-VP16 expression plasmid, which contains the VP16 activation domain fused to the C terminus of the RBP3 isoform, has been previously described, together with the EBNA2 expression plasmid pSG5-EBNA2 (37). pSG5-EBNA3A was generated by inserting an EBNA3A *Xba*I-*Dra*I fragment from pCMVE4 (23) in the polylinker of the plasmid pSG5flag (37). Subsequently, pSG5-EBNA3A-VP16 was generated in the following way. First, an *Xho*I restriction site was inserted upstream of the initiation codon of EBNA3A in plasmid pSG5-EBNA3A by site-directed mutagenesis (Clontech Transformer kit) using the oligonucleotide 5′-GGTATCGGGCTCG AGACAAAATGG-3′ to generate pSG5-EBNA3A-Xho+. The transcriptional activation domain of VP16 (amino acids 402 to 479) was then PCR amplified from pMCI (2) by using oligonucleotides 5′-CCAATGCATCTCCGGAGGCC CCCCCGACCGATG-3′ and 5′-CTCGGATCCTACCCACCGTACTCG-3′. An *Xho*I-*Bsp*EI fragment from pSG5-EBNA3A-Xho+ containing EBNA3A and the PCR-amplified VP16 *Bsp*EI-*Bam*HI fragment were ligated in frame and subcloned into the pSG5flag polylinker.

pSG5-EBNA3C was generated by inserting the EBNA3C *Hin*dIII-*Bst*EII fragment from pCMVE6 (23) into the pSG5 polylinker. Subsequently, pSG5-EBNA3C-VP16 was generated by inserting a *Hin*dIII-*Nsi*I fragment of pSG5-EBNA3C into pGal4-VP16 (26) which had been digested with *Hin*dIII and *Xho*I. The *Nsi*I extremity of EBNA3C and the *Xho*I extremity of pGal4-VP16 had been blunted with T4 DNA polymerase.

Plasmid pGal4-RBP3 was generated by inserting the full-length human RBP3 cDNA (5) into the *Bam*HI site of plasmid pG4MpolyII (41). pGal4-EBNA3A was generated by inserting a *Bam*HI fragment from pSG5-EBNA3A (containing the EBNA3A coding sequence starting from amino acid 125) into the *Bam*HI site of pG4MpolyII. pGal4-EBNA3C was generated by inserting an *Xba*I-*Sal*I fragment from pSG5-EBNA3C (containing the EBNA3C coding sequence starting from amino acid 10) in pG4MpolyII digested with *Kpn*I and *Sal*I. The *Xba*I extremity of EBNA3C and the *Kpn*I extremity of pG4MpolyII were blunted with Klenow enzyme.

Plasmid pGEX-RBP3, which allows the expression of a glutathione *S*-transferase (GST)-RBP fusion protein in bacteria, was a generous gift of A. Israel (5).

GST-RBP affinity chromatography. GST-RBP and GST proteins were produced in *Escherichia coli* and purified as previously described (26). In vitro-translated ^{35}S-labelled proteins were all synthesized in a reticulocyte lysate coupled transcription-translation system (TNT system; Promega). In vitro-translated ^{35}S-labelled EBNA2, EBNA3A, and EBNA3C were incubated with glutathione-agarose beads coated with equivalent molar amounts of GST or GST-RBP3 proteins for 1 h at 4°C in binding buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5% Nonidet P-40, 400 mM KCl). After incubation, the beads were washed three times in binding buffer. Associated proteins were eluted by being boiled in Laemmli buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

DNA binding analysis. Electrophoretic mobility shift assays (EMSA) were performed by incubating 5×10^4 cpm of 5′-end-labelled double-stranded templates with in vitro-translated proteins for 30 min at room temperature in 10 mM HEPES (pH 7.9)–50 mM KCl–0.5 mM $MgCl_2$–1 mM EDTA–0.5 mM dithiothreitol–8% glycerol–1 μg of poly(dI-dC) in a final volume of 20 μl. Rabbit reticulocyte lysate was added to reaction mixtures as necessary in order to keep the overall amount of rabbit reticulocyte lysate constant. After incubation, the mixture was loaded onto a 4.5% (wt/vol) polyacrylamide gel (29 to 1 cross-linked) with 0.2× Tris-borate-EDTA and run at room temperature at 10 V/cm for 3 h. The protein-DNA complexes were visualized by autoradiography.

Transfections. The plasmids used for transfection were prepared by the alkaline lysis method and purified through two CsCl gradients. The DNAs were in the same topological state, as assayed by agarose gel electrophoresis. HeLa cells were grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% (vol/vol) fetal calf serum and were seeded at 5×10^5 cells per 100-mm-diameter petri dish 8 h prior to transfection. Transfections were performed by the calcium precipitate method. Cells were mixed with the appropriate DNAs: typically, 15 μg of DNA was used, including the expression vectors and plasmids carrying the reporter genes. The amount of simian virus 40 promoter transfected was kept constant by addition of pSG5 to the transfection mixture when necessary. Transfected cells were washed and collected 48 h after transfection. Each series of transfections was repeated at least three times.

CAT-ELISA. Chloramphenicol acetyl transferase (CAT) enzyme-linked immunosorbent assays (CAT-ELISA) were performed with the Boehringer Mannheim CAT-ELISA kit according to the manufacturer's instructions. After transfection, cells were lysed in 1 ml of lysis buffer, and the amount of CAT protein produced was calculated for the totality of the protein extract.

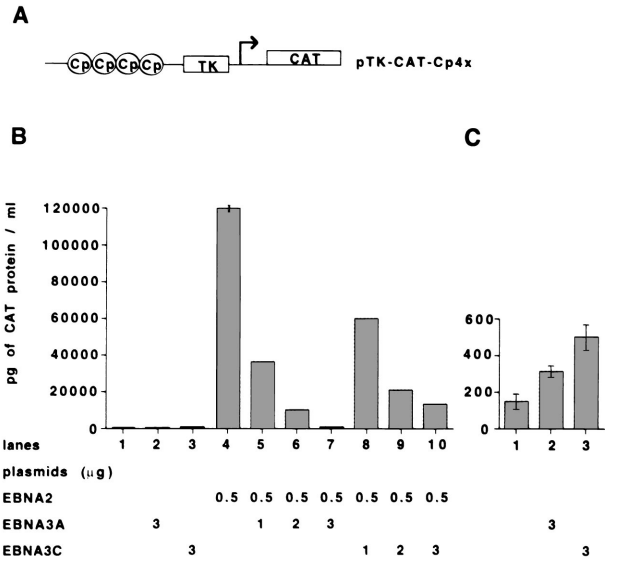

FIG. 1. EBNA3A and EBNA3C inhibit EBNA2-mediated activation. (A) Schematic representation of the reporter plasmid pTK-CAT-Cp4x. pTK-CAT-Cp4x contains four RBP-Jκ binding sites cloned upstream of the herpes simplex virus TK promoter linked to the CAT gene. (B) HeLa cells were transfected with 10 μg of pTK-CAT-Cp4x reporter plasmid and various combinations of expression plasmids for EBNA2, EBNA3A, and EBNA3C proteins as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA. (C) Upscaled representation of the results of transfections 1, 2, and 3.

RESULTS

Both EBNA3A and EBNA3C repress EBNA2-mediated activation from a TK promoter carrying binding sites for RBP-Jκ. Since the EBNA2 transcriptional activation is mediated by RBP-Jκ, it was necessary to determine if EBNA3A and EBNA3C would downregulate EBNA2-mediated activation from a thymidine kinase (TK) promoter carrying binding sites for RBP-Jκ. The reporter plasmid pTK-CAT-Cp4x is shown in Fig. 1A. Upon transfection of pTK-CAT-Cp4x in HeLa cells, transcription of the CAT gene was very inefficient (Fig. 1B, lane 1) but was strongly activated by EBNA2 (lane 4), indicating that endogenous RBP-Jκ binds to the reporter plasmid and recruits EBNA2 to the TK promoter. The EBNA2-mediated activation of CAT transcription was strongly repressed by EBNA3A (Fig. 1B, lanes 5 to 7) and EBNA3C (lanes 8 to 10), the repression being proportional to the amounts of EBNA3A- and EBNA3C-expressing plasmids transfected. These results suggest that EBNA3A and EBNA3C either directly contact RBP-Jκ or prevent the recruitment of EBNA2 by RBP-Jκ or, alternatively, impair the binding of the RBP-Jκ–EBNA2 complex to DNA. Another possibility is that EBNA3A and EBNA3C could titrate a factor required by EBNA2 to activate transcription (squelching), or they could interfere with the activation function of EBNA2.

We have previously shown that in HeLa cells the endogenous RBP-Jκ protein efficiently represses CAT transcription from the TK promoter in the pTK-CAT-Cp4x construct (37). Interestingly, EBNA3A (Fig. 1C, lane 2) or EBNA3C (Fig. 1C, lane 3) detectably increased CAT expression from the TK promoter in plasmid pTK-CAT-Cp4x (Fig. 1C, lane 1), suggesting that both EBNA3A and EBNA3C alleviated the RBP-

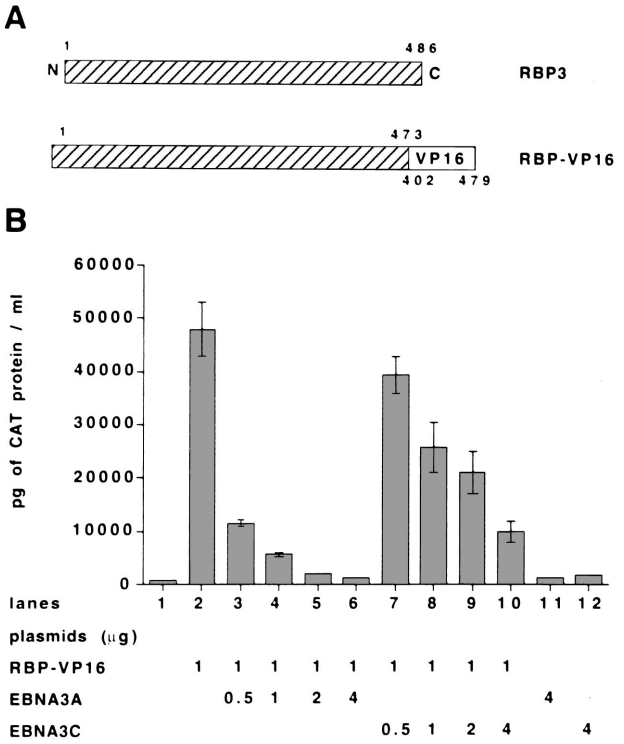

FIG. 2. EBNA3A and EBNA3C inhibit RBP-VP16-mediated activation. (A) Schematic representation of the RBP-Jκ isoform, RBP3, and the RBP-VP16 fusion protein. (B) HeLa cells were transfected with 10 μg of pTK-CAT-Cp4x reporter plasmid (Fig. 1A) and various combinations of expression plasmids for RBP-VP16, EBNA3A, and EBNA3C, as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA.

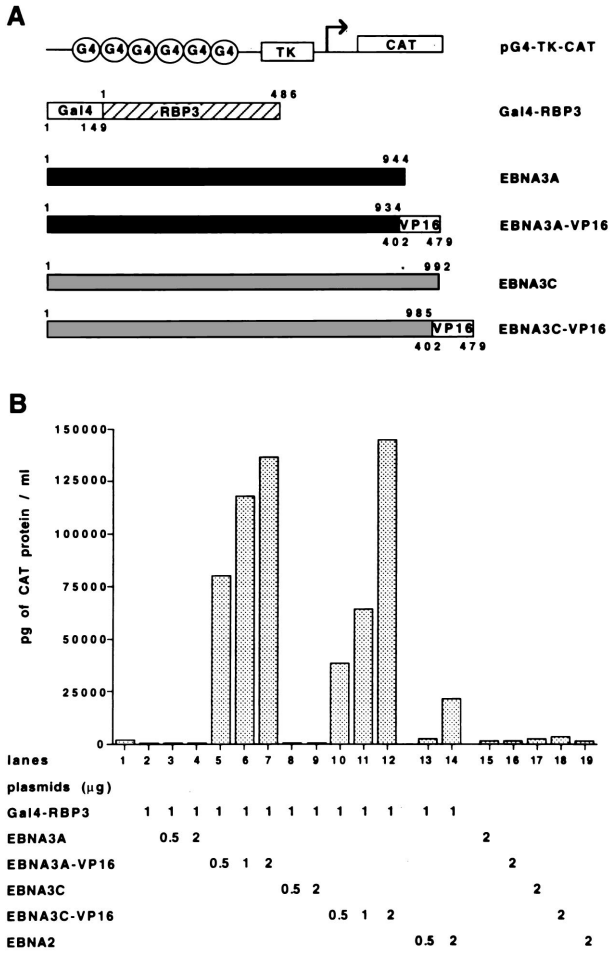

FIG. 3. Both EBNA3A and EBNA3C are recruited by Gal4-RBP3 to a promoter carrying Gal4 binding sites. (A) Schematic representation of the reporter plasmid pG4-TK-CAT and proteins Gal4-RBP3, EBNA3A-VP16, and EBNA3C-VP16. pG4-TK-CAT contains six Gal4 binding sites cloned upstream of the herpes simplex virus TK promoter linked to the CAT gene. (B) HeLa cells were transfected with 10 μg of pG4-TK-CAT reporter plasmid and various combinations of expression plasmids for Gal4-RBP3, EBNA3A, EBNA3A-VP16, EBNA3C, EBNA3C-VP16, and EBNA2, as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA.

Jκ-mediated repression of CAT transcription from the TK promoter. The latter results suggest that both EBNA3A and EBNA3C can contact RBP-Jκ in the absence of EBNA2 in vivo.

Transcriptional activation mediated by RBP-Jκ provided with the VP16 activation domain is strongly repressed by both EBNA3A and EBNA3C. In order to evaluate if RBP-Jκ is indeed a direct target for the repressing effect of EBNA3A and EBNA3C in vivo, we assayed whether the transcriptional activation mediated by RBP-Jκ provided with the VP16 activation domain (RBP-VP16; Fig. 2A) was repressed by EBNA3A or EBNA3C. The RBP-VP16 hybrid protein binds DNA as efficiently as RBP-Jκ and activates transcription (37). As shown in Fig. 2B, when expressed in HeLa cells, RBP-VP16 activated transcription from the TK promoter present in plasmid pTK-CAT-Cp4x (lane 2). Interestingly, both EBNA3A (Fig. 2B, lanes 3 to 6) and EBNA3C (lanes 7 to 10) repressed the RBP-VP16-activated transcription, and the repressing effect was proportional to the amounts of EBNA3A- and EBNA3C-expressing plasmids transfected. The repressing effect observed was unlikely to be due to titration by EBNA3A and EBNA3C of a cellular factor present in limiting amounts in HeLa cells and recruited by the VP16 activation domain. Indeed, neither EBNA3A nor EBNA3C repressed transcription activated by Gal4-VP16 (not shown). Taken together, these results strongly suggest that RBP-Jκ is a direct target for both EBNA3A and EBNA3C. They also indicate that both EBNA3A and EBNA3C can make physical contact with RBP-Jκ.

The hybrid protein Gal4-RBP3 recruits both EBNA3A and EBNA3C to a promoter carrying Gal4 binding sites. In order to evaluate if EBNA3A and EBNA3C make stable contacts with RBP-Jκ in vivo, we examined whether a Gal4-RBP3 hybrid protein (Fig. 3A) would recruit EBNA3A and EBNA3C to the TK promoter carrying Gal4 binding sites (plasmid pG4-TK-CAT; Fig. 3A). In order to visualize this recruitment, the VP16 activation domain was fused to the C termini of the EBNA3A and EBNA3C proteins (EBNA3A-VP16 and EBNA3C-VP16; Fig. 3A). As shown in Fig. 3B, upon transfection of plasmid pG4-TK-CAT in HeLa cells, transcription from the TK promoter was weak but detectable (lane 1). This transcription was repressed by Gal4-RBP3 (Fig. 3B, lane 2), but the Gal4-RBP3-mediated repression was not affected by either EBNA3A (lanes 3 and 4) or EBNA3C (lanes 8 and 9). However, transcription from the TK promoter was strongly enhanced by EBNA3A-VP16 (Fig. 3B, lanes 5 to 7) and EBNA3C-VP16 (lanes 10 to 12) and to a lesser extent by EBNA2 (lanes 13 and 14) when they were coexpressed with

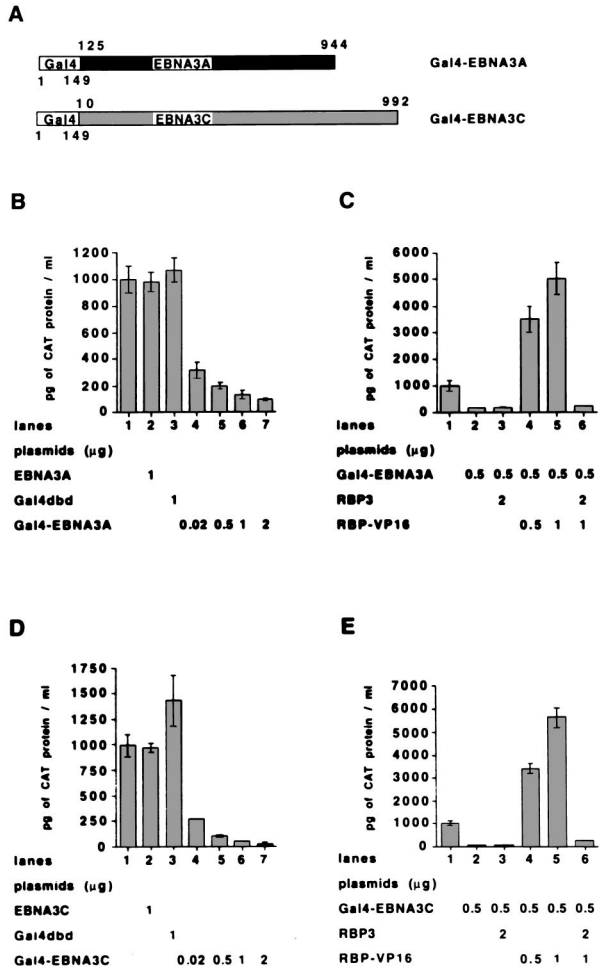

FIG. 4. Endogenous RBP-Jκ (B and D) or cotransfected RBP-VP16 (C and E) is recruited by Gal4-EBNA3A or Gal4-EBNA3C to a promoter carrying Gal4 binding sites. (A) Schematic representation of proteins Gal4-EBNA3A and Gal4-EBNA3C. (B to E) HeLa cells were transfected with 10 μg of pG4-TK-CAT reporter plasmid (Fig. 3) and various combinations of expression plasmids for EBNA3A, EBNA3C, Gal4-EBNA3A, Gal4-EBNA3C, Gal4dbd, RBP3, and RBP-VP16, as indicated below the graphs. Proteins RBP3 and RBP-VP16 are diagrammed in Fig. 2. The control protein Gal4dbd (Gal4 DNA binding domain) is the Gal4 subdomain present in the Gal4-EBNA3A and Gal4-EBNA3C fusions. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA.

Gal4-RBP3. In the absence of Gal4-RBP3, neither EBNA3A (Fig. 3B, lane 15), EBNA3A-VP16 (lane 16), EBNA3C (lane 17), EBNA3C-VP16 (lane 18), nor EBNA2 (lane 19) affected transcription from the TK promoter (lane 1). These results suggest that both EBNA3A and EBNA3C make stable contacts with RBP-Jκ in vivo.

The hybrid proteins Gal4-EBNA3A and Gal4-EBNA3C recruit RBP-Jκ to a promoter carrying Gal4 binding sites. As EBNA3A and EBNA3C are efficiently recruited in vivo by DNA-bound Gal4-RBP3, in the reverse experiment, Gal4-EBNA3A or Gal4-EBNA3C hybrid protein (Fig. 4A) should be able to recruit RBP-Jκ to a reporter promoter containing Gal4 binding sites. As a consequence, Gal4-EBNA3A or Gal4-EBNA3C should repress transcription by recruiting RBP-Jκ, unless the interaction interferes with the repression activity of RBP-Jκ. Thus, in the presence of RBP-VP16, Gal4-EBNA3A or Gal4-EBNA3C should activate transcription by recruiting RBP-VP16.

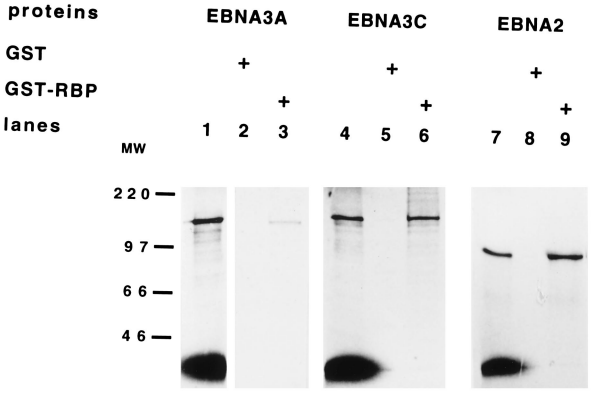

FIG. 5. Direct in vitro protein-protein interaction between RBP3 and EBNA3A or EBNA3C. Proteins EBNA3A, EBNA3C, and EBNA2 were translated in vitro in the presence of [^{35}S]methionine, and their ability to be retained by a GST-RBP3 fusion protein or control GST absorbed to glutathione-agarose beads was analyzed by SDS-PAGE (8% acrylamide gel). Molecular weight markers (in thousands) are indicated on the left. Lanes 1, 4, and 7 show the different in vitro-translated products (half the amount used for each assay) prior to incubation with the beads. The amounts of proteins bound to the GST-RBP3 beads (lanes 3, 6, and 9) are compared with the controls (lanes 2, 5, and 8), which correspond to nonspecific binding of the same samples to GST beads.

As shown in Fig. 4B, transcription from the TK promoter was detectable upon transfection of pG4-TK-CAT in HeLa cells (lane 1). This transcription was not significantly altered by EBNA3A (Fig. 4B, lane 2) or by Gal4dbd (the Gal4 186 N-terminal amino acids) (lane 3). However, the TK promoter basal activity was strongly repressed by Gal4-EBNA3A (Fig. 4B, lanes 4 to 7), suggesting that Gal4-EBNA3A recruited RBP-Jκ to the promoter. If this is so, then Gal4-EBNA3A should activate transcription when coexpressed with RBP-VP16. As shown in Fig. 4C, the basal activity of the TK promoter in plasmid pG4-TK-CAT (lane 1) was repressed by Gal4-EBNA3A (lane 2) and was strongly activated when Gal4-EBNA3A and RBP-VP16 were coexpressed (lanes 4 and 5). Moreover, the Gal4-EBNA3A–RBP-VP16-mediated activation could be repressed by overexpression of RBP3 (Fig. 4C, lane 6). Similar results were obtained with Gal4-EBNA3C (Fig. 4D and E). Taken together, the results presented above demonstrate that both EBNA3A and EBNA3C stably interact with RBP-Jκ in vivo.

Both EBNA3A and EBNA3C make direct contact with RBP-Jκ and impair its binding to DNA in vitro. In order to specifically determine whether EBNA3A and EBNA3C can directly contact RBP-Jκ, we evaluated in vitro the interaction between a GST-RBP3 fusion protein immobilized on glutathione-agarose beads and [^{35}S]methionine-labelled EBNA3A and EBNA3C proteins translated in vitro. As a control of interaction, we used the EBNA2 protein. As shown in Fig. 5, EBNA3A (lane 3), EBNA3C (lane 6), and EBNA2 (lane 9) interacted efficiently with the GST-RBP3 protein, whereas no significant interaction was observed with the GST protein (lanes 2, 5, and 8). However, less EBNA3A bound to the GST-RBP3 protein than EBNA3C and EBNA2.

We then did EMSA using in vitro-translated proteins. RBP-Jκ bound efficiently to a double-stranded DNA fragment containing the EBNA2-responsive sequence found in promoter Cp (Fig. 6, lane 2). Comparable and increasing amounts of EBNA2Δ321-323, EBNA2, EBNA3A, and EBNA3C proteins were then added to RBP-Jκ in the EMSA reaction mixture. EBNA2Δ321-323, which does not interact with RBP-Jκ (37), had no effect on the amount of RBP-Jκ–DNA complexes

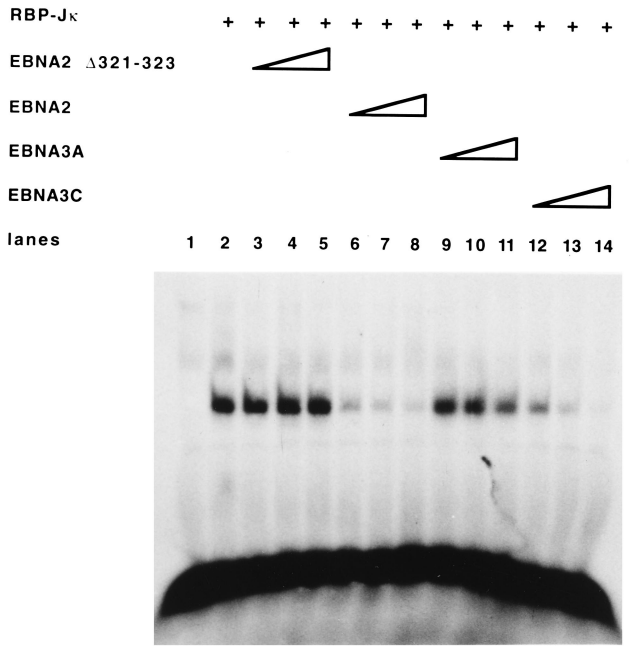

FIG. 6. EBNA3A and EBNA3C inhibit RBP-Jκ DNA binding activity. EMSA was performed with in vitro-translated proteins. The radiolabelled double-stranded probe (TCGAGGTGTAAACACGCC**GTGGGAAA**AAATTTATG TCGA) originates from the Cp promoter and contains one RBP-Jκ binding site (boldface). The probe was incubated with reticulocyte lysate only (lane 1), with RBP3 alone (lane 2), or with RBP3 and increasing amounts of EBNA2Δ321-323 (lanes 3 to 5), EBNA2 (lanes 6 to 8), EBNA3A (lanes 9 to 11), or EBNA3C (lanes 12 to 14). Comparable molar amounts of EBNA2Δ321-323, EBNA2, EBNA3A, and EBNA3C were used in lanes 3, 6, 9, and 12. These amounts were increased by a factor of 2 in lanes 4, 7, 10, and 13 and by a factor of 4 in lanes 5, 8, 11, and 14.

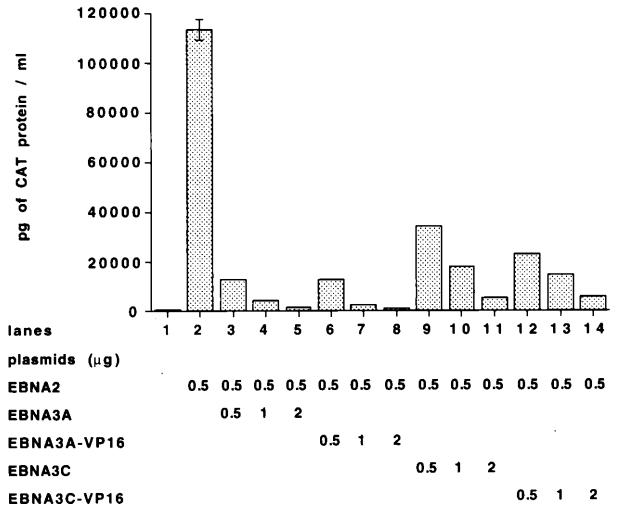

FIG. 7. EBNA3A-VP16 and EBNA3C-VP16, like EBNA3A and EBNA3C, inhibit EBNA2-mediated activation. HeLa cells were transfected with 10 μg of pTK-CAT-Cp4x reporter plasmid (Fig. 1A) and various combinations of expression plasmids for EBNA2, EBNA3A, EBNA3A-VP16, EBNA3C, and EBNA3C-VP16 as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA.

formed (Fig. 6, lanes 3 to 5), whereas EBNA2 strongly impaired RBP-Jκ binding, as previously described (37). Addition of EBNA3A to the EMSA reaction mixture slightly decreased the amount of RBP-Jκ–DNA complexes formed (Fig. 6, lanes 9 to 11). Compared with EBNA3A, addition of EBNA3C to the EMSA reaction mixture strongly impaired the binding of RBP-Jκ to DNA (Fig. 6, lanes 12 to 14). The disappearance of the RBP-Jκ–DNA complexes was not compensated by the appearance of complexes with slower relative electrophoretic mobilities, as might be anticipated for EBNA3A–RBP-Jκ–DNA or EBNA3C–RBP-Jκ–DNA complexes. These results suggest that both EBNA3A and EBNA3C destabilize RBP-Jκ binding to DNA in vitro. If this also occurs in vivo, this destabilization could explain by which mechanism EBNA3A and EBNA3C repress the EBNA2 transcriptional activation mediated by RBP-Jκ. However, addition of EBNA2 to the EMSA reaction mixture also efficiently impaired the binding of RBP-Jκ in vitro, although EBNA2 is stably recruited by DNA-bound RBP-Jκ in vivo (37). The destabilization effects observed in vitro could be due to the in vitro experimental conditions and might not reflect what is occurring in vivo. Thus, the EBNA3A and EBNA3C destabilizing effect has to be demonstrated in vivo.

RBP-Jκ–EBNA3A and RBP-Jκ–EBNA3C complexes do not bind to DNA in vivo. If EBNA3A or EBNA3C impairs the binding of RBP-Jκ to DNA in vivo, then EBNA3A, EBNA3A-VP16, EBNA3C, and EBNA3C-VP16 should equally repress the EBNA2-activated transcription from the TK promoter in plasmid pTK-CAT-Cp4x. Alternatively, if EBNA3A or EBNA3C stably interacts with RBP-Jκ bound to DNA and impairs EBNA2-activated transcription, then EBNA3A-VP16 and EBNA3C-VP16, contrary to EBNA3A and EBNA3C, should overactivate transcription. As shown in Fig. 7, EBNA3A (lanes 3 to 5), EBNA3A-VP16 (lanes 6 to 8), EBNA3C (lanes 9 to 11), and EBNA3C-VP16 (lanes 12 to 14) efficiently repressed the EBNA2-activated transcription from the reporter gene (lane 2) in similar manners. These results strongly suggest that both EBNA3A and EBNA3C repress EBNA2-activated transcription by inhibiting RBP-Jκ's binding to DNA.

DISCUSSION

In this report, we provide experimental data demonstrating that EBNA3A and EBNA3C can both make stable contacts with RBP-Jκ in vivo and that the most likely mechanism for EBNA3A and EBNA3C repression of EBNA2-mediated activation of transcription is the destabilization of RBP-Jκ's binding to DNA. First, we have shown that the effect of EBNA3A and EBNA3C is not exerted on EBNA2 itself. Indeed, a repressing effect of EBNA3A and EBNA3C on RBP-VP16-mediated transcriptional activation was also observed. This repression is unlikely to be due to titration by EBNA3A or EBNA3C of a cellular factor contacted by the VP16 activation domain in RBP-VP16, as neither protein repressed Gal4-VP16-mediated transcriptional activation (not shown). This repression was also not due to masking of the VP16 activation domain by EBNA3A or EBNA3C, because neither protein repressed transcriptional activation mediated by the hybrid protein Gal4–RBP-VP16 (not shown). Taken together, these results suggest that RBP-Jκ could be a direct target for EBNA3A and EBNA3C. We were able to confirm this hypothesis by in vivo and in vitro experiments that strongly suggested that both EBNA3A and EBNA3C interact with RBP-Jκ through direct protein-protein interactions. First, in vivo, we have shown that a Gal4-RBP3 fusion was able to efficiently recruit EBNA3A-VP16 and EBNA3C-VP16 to a promoter bearing Gal4 binding sites. Reciprocally, Gal4-EBNA3A and Gal4-EBNA3C fusion proteins were able to recruit a RBP-

VP16 fusion protein. These in vivo results are in favor of an interaction between RBP-Jκ and EBNA3A and EBNA3C. Furthermore, our in vitro data showing that in vitro-translated EBNA3A and EBNA3C proteins are able to interact specifically with GST-RBP3 purified protein suggest that the interaction is direct.

A previous report by Tomkinson et al. (36) also described in vitro interactions between RBP-Jκ and EBNA3C but not between RBP-Jκ and EBNA3A, and in an EMSA they showed that EBNA3C was able to destabilize RBP-Jκ's binding to DNA, whereas EBNA3A did not detectably affect RBP-Jκ's binding. Those researchers suggested that EBNA3C could inhibit EBNA2-mediated activation by destabilizing RBP-Jκ's binding to DNA, whereas EBNA3A would repress by a different mechanism. Here, we present several lines of evidence showing that in vivo, EBNA3A and EBNA3C both repress EBNA2-mediated transcriptional activation by destabilizing RBP-Jκ's binding to DNA. Indeed, our results clearly demonstrate that although Gal4–RBP-Jκ recruits both EBNA3A-VP16 and EBNA3C-VP16 to a promoter carrying Gal4 DNA binding sites, neither EBNA3A-VP16 nor EBNA3C-VP16 is recruited by RBP-Jκ to a promoter carrying RBP-Jκ DNA binding sites. On the contrary, EBNA3A-VP16 and EBNA3C-VP16 are able to repress EBNA2-mediated activation, to the same extent as EBNA3A and EBNA3C. It remains possible, however, that EBNA3A or EBNA3C has titrated cellular factors essential for EBNA2 to function as a transcriptional activator. This is unlikely, though, since transcription activated by Gal4-EBNA2 was not affected by overexpression of both EBNA3A and EBNA3C (not shown). The more likely explanation for the results is thus that neither the EBNA3A-VP16–RBP-Jκ nor the EBNA3C-VP16–RBP-Jκ complex binds to DNA in vivo. Moreover, the destabilization hypothesis is also compatible with the inhibiting effect of EBNA3A and EBNA3C on the repression of RBP-VP16-mediated activation. This conclusion is also supported by our EMSA data, showing that EBNA3A and EBNA3C both destabilize the binding of RBP-Jκ to DNA.

In summary, there are at least three EBV nuclear proteins, EBNA3A, EBNA3B, and EBNA3C, which counteract EBNA2-mediated activation. EBNA2 is the first latent EBV protein together with EBNA-LP to be expressed following in vitro B-cell infection (3). EBNA2 then transactivates the Cp, LMP1, and TP2 promoters, which leads to expression of all the latency genes, including those for EBNA3A, EBNA3B, and EBNA3C (11, 14, 16, 17, 40, 44, 45). It has been suggested that by inhibiting EBNA2-mediated activation, the EBNA3A, EBNA3B, and EBNA3C proteins could downregulate the expression of EBV latent proteins that bear epitopes provoking the anti-EBV cytotoxic T-cell response, thus allowing EBV to escape host immune surveillance. It could also be important to downregulate LMP1 expression, as LMP1 has been shown to be toxic to the cells when overexpressed (13, 29). However, all the results demonstrating the inhibitory effect of the EBNA3 proteins on EBNA2 activation are based on overexpression assays and may not pertain specifically to effects at physiologic levels of protein. EBNA3A, -3B, and -3C may have other, not yet elucidated, common or specific functions. Indeed, EBNA3C can upregulate the expression of some cellular genes that are also upregulated by EBNA2. In particular, it can upregulate CD21 expression in non-EBV-infected Burkitt's lymphoma cells (39) and LMP1 expression in Raji cells during G_1 arrest (4). As RBP-Jκ appears to be a repressor in mammalian cells (9, 15, 37), EBNA3A and EBNA3C could alleviate a RBP-Jκ-mediated repression and cooperate in the induction of certain cellular genes containing RBP-Jκ binding sites by destabilizing RBP-Jκ's binding to DNA. In agreement with this, we have shown an activating effect, in a transient-transfection assay, of EBNA3A and EBNA3C on a TK promoter containing RBP-Jκ binding sites. On the other hand, it has been reported that EBNA3C contains a Sp1-like glutamine-rich domain which can activate transcription when fused to the Gal4 DNA-binding domain (28). However, our Gal4-EBNA3C fusion protein, which contains most of the EBNA3C coding sequence (amino acids 10 to 992), acts as a repressor, probably by recruiting RBP-Jκ. We therefore suggest that the EBNA3C activation domain is inactive in the full-length protein because of interaction with RBP-Jκ.

In this report, we have focused on the effects of EBNA3A and EBNA3C on the transcriptional activation mediated by EBNA2 targeted to RBP-Jκ binding sites. However, it appears that EBNA2 can possibly be recruited onto promoters by other cellular factors, including proteins from the PU-1 family or proteins containing a POU domain (17, 22, 33, 34). Inhibition of EBNA2-mediated transcriptional activation by the EBNA3 proteins could thus be restricted to promoters activated through RBP-Jκ binding sites. This could be a way to differentially regulate certain viral or cellular genes. Understanding of the common and specific functions of the three EBNA3 proteins will shed further light on the functional interaction between these viral proteins, EBNA2, and cellular factors and their implication in EBV-mediated immortalization of B cells in vitro.

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