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Transcriptional repression by the Epstein–Barr virus EBNA3A protein tethered to DNA does not require RBP-Jκ

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The Epstein–Barr virus (EBV) proteins EBNA1, EBNA2, EBNA3A, EBNA3C, LMP1 and EBNA-LP are essential for the *in vitro* immortalization of primary B lymphocytes by EBV. EBNA2 is a transcriptional activator of viral and cellular genes. Both EBNA3A and EBNA3C have been shown to specifically inhibit EBNA2-activated transcription by direct interaction with RBP-Jκ, a cellular DNA-binding factor known to recruit EBNA2 to EBNA2-responsive genes. This interaction interferes with the binding of RBP-Jκ to DNA *in vitro*, and this is probably the mechanism by which EBNA3A and EBNA3C repress EBNA2-activated transcription *in vivo*. EBNA3A and EBNA3C also directly repress transcription when tethered to a promoter via the DNA-binding domain of the yeast Gal4 protein. As RBP-Jκ has been previously shown to be a repressor in mammalian cells, this repression could be due to the recruitment of RBP-Jκ by Gal4–EBNA3A and 3C. In this study, we have precisely mapped the domain of EBNA3A involved in the interaction with RBP-Jκ and we have shown that interaction with RBP-Jκ is not required for the Gal4–EBNA3A-mediated repression. Furthermore, we have characterized in EBNA3A a domain of 143 amino acids which is necessary and sufficient for EBNA3A-dependent repression.

Introduction

Epstein–Barr Virus (EBV) is a human herpesvirus which is the causal agent for infectious mononucleosis. EBV is also associated with several malignancies including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and B and T lymphomas in immunodepressed individuals. A striking property of the virus is its ability to immortalize B-lymphocytes *in vitro*. In such immortalized cells, the viral genome persists and a limited set of viral genes is expressed, defining a type III latency. The products of these genes include six nuclear proteins (EBNA1, 2, 3A, 3B, 3C and EBNA-LP), three membrane proteins (LMP1, TP1 and TP2) and two small nuclear RNAs (EBER1 and EBER2) (for a review see Kieff, 1996). Of these gene products, the EBV nuclear proteins EBNA2, EBNA-LP, EBNA3A, EBNA3C and the EBV integral membrane protein LMP1 are essential for the EBV-induced proliferation of B cells (Cohen *et al.*, 1989; Hammerschmidt & Sugden, 1989; Kaye *et al.*, 1993; Mannick *et al.*, 1991; Tomkinson *et al.*, 1993).

EBNA2 has been shown to be necessary for both the initiation (Sinclair *et al.*, 1994) and maintenance of immortalization (Kempkes *et al.*, 1995). It is a transcription factor that up-regulates the expression of the cellular genes CD21, CD23 and c-*fgr* (Calender *et al.*, 1987; Cordier *et al.*, 1990; Knuston, 1990; Wang *et al.*, 1990*a*) and EBV genes EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C, LMP1, TP1 and TP2 (Abbot *et al.*, 1990; Fahraeus *et al.*, 1990; Rooney *et al.*, 1992; Sung *et al.*, 1991; Wang *et al.*, 1990*b*; Woisetchlaeger *et al.*, 1990; Zimber-Strobl *et al.*, 1991). EBNA2 does not bind directly to DNA but appears to be targeted to its responsive promoters through direct interaction with the cellular specific DNA-binding protein RBP-Jκ (Grossman *et al.*, 1994; Henkel *et al.*, 1994; Ling *et al.*, 1993; Waltzer *et al.*, 1994; Zimber-Strobl *et al.*, 1994). The interaction between EBNA2 and RBP-Jκ is essential for the immortalization of primary B lymphocytes (Yalamanchili *et al.*, 1994).

More recently, it has been shown that RBP-Jκ is also a direct target for the EBNA3A, 3B and 3C proteins (Krauer *et al.*, 1996; Robertson *et al.*, 1995, 1996; Waltzer *et al.*, 1996; Zhao *et al.*, 1996). EBNA3A, -3B and -3C mRNAs are transcribed from three tandemly arranged genes in the EBV genome. The three EBNA3 proteins have related structures as well as a relatively well-conserved N-terminal domain (up to 28% amino acid identity). However, these proteins probably have unique functions since recombinant EBVs that carry null mutations in either EBNA3A or EBNA3C are non-

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immortalizing (Tomkinson *et al.*, 1993). A number of cellular and viral genes have been reported to be up- or down-regulated by one or the other of the EBNA3s although it is not clear if this is a direct effect: EBNA3C up-regulates the expression of CD21 in non-EBV-infected Burkitt's lymphoma (BL) cells (Wang *et al.*, 1990*a*) and LMP-1 in G1-arrested EBV-infected Raji cells (Allday & Farrell, 1994). EBNA3B up-regulates the cytoskeletal protein vimentin as well as the activation antigen CD40 and down-regulates the Burkitt's lymphoma-associated antigen BLA/CD77 when stably expressed in the EBV-negative BL cell line DG75 (Silins & Sculley, 1994). Stable co-expression of the three EBNA3 proteins in DG75 cells also upregulates pleckstrin (Kienzle *et al.*, 1996).

The most documented and common function of the EBNA3 proteins is their capacity to inhibit EBNA2-mediated activation of the TP1 and LMP1 promoters in transient expression assays (Le Roux *et al.*, 1994; Marshall & Sample, 1995; Robertson *et al.*, 1995). It is now accepted that this inhibition is probably due to a direct interaction between EBNA3A, 3B or 3C and RBP-Jκ, resulting in the inhibition of binding of RBP-Jκ to DNA both *in vitro* and *in vivo* (Robertson *et al.*, 1995; Waltzer *et al.*, 1996). Recently, it has also been shown that both EBNA3A and EBNA3C can act as repressors when tethered to DNA via the DNA-binding domain of the yeast transcription factor Gal4 (Bain *et al.*, 1996; Waltzer *et al.*, 1996). Since RBP-Jκ has been shown to be a repressor in mammalian cells (Dou *et al.*, 1994; Hsieh & Hayward, 1995; Waltzer *et al.*, 1995), it is therefore possible that Gal4–EBNA3A- or 3C-mediated repression is due to the recruitment of RBP-Jκ onto the DNA by these fusion proteins.

In the present study, we have precisely mapped the EBNA3A domain mediating the interaction with RBP-Jκ. Surprisingly, when the interaction between EBNA3A and RBP-Jκ was impaired by specific mutagenesis in EBNA3A, the EBNA3A mutant still acted as a repressor when fused to the Gal4 DNA-binding domain. We then precisely mapped the EBNA3A domain responsible for RBP-Jκ-independent repression. Specific deletion of this domain completely impaired Gal4–EBNA3A repressor activity although RBP-Jκ was still recruited by the mutated fusion protein.

Methods

■ **Plasmids.** Reporter plasmids pG4-TK-CAT and pTK-CAT-Cp4x, and expression vectors for EBNA3A and RBP–VP16 (pSG5Flag-EBNA3A and pSG5flagRBP–VP16) have been described previously (Waltzer *et al.*, 1995). Plasmid pGal4-EBNA3A that contains full-length EBNA3A fused to the Gal4 DNA-binding domain was generated in two steps. Firstly, a *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′ GGTATCGGGCTCGAGACAAAATGG 3′ to generate pSG5-EBNA3A-Xho+. Secondly, a *Xho*I–*Xho*I fragment from pSG5-EBNA3A-Xho+, containing the EBNA3A ORF, was cloned into the *Xho*I site of plasmid pG4MpolyII (Webster *et al.*, 1989).

Expression plasmids for Gal4–EBNA3A deletion mutants were generated in the following way: pGal4-EBNA3A(1–234) was generated by inserting a *Xho*I–*Sac*I fragment from pSG5Flag-EBNA3A between the *Xho*I and *Sac*I sites of pG4MpolyII; pGal4-EBNA3A(234–575) by inserting a *Sac*I (blunted)–*Bss*HII(blunted) fragment from pSG5Flag-EBNA3A into the *Kpn*I site (blunted) of pG4MpolyII; pGal4-EBNA3A(575–944) by inserting a *Bss*HII(blunted)–*Bgl*II fragment from pSG5Flag-EBNA3A between the *Bam*HI(blunted) and *Bgl*II sites of pG4MpolyII; pGal4-EBNA3A(125–944) by inserting a *Bam*HI fragment from pSG5-EBNA3A plasmid into the *Bam*HI site of pG4MpolyII; pGal4-EBNA3A(125–575) by inserting a *Bam*HI–*Bss*HII(blunted) fragment from pGal4-EBNA3A(125–944) between the *Bam*HI and *Sac*I(blunted) sites of pG4MpolyII; pGal4-EBNA3A(1–138) mutant by inserting a *Xho*I–*Nco*I(blunted) fragment from pGal4-EBNA3A between the *Xho*I and *Bam*HI(blunted) sites of pG4MpolyII; pGal4-EBNA3A(1–172) by removing a *Bst*EII–*Sac*I fragment from pGal4-EBNA3A(1–234) and religating the plasmid after blunting of the extremities; pGal4-EBNA3A(172–234) by inserting a *Bst*EII(blunted)–*Sac*I fragment from pGal4-EBNA3A(1–234) between the *Kpn*I (blunted) and *Sac*I sites of pG4MpolyII; pGal4-EBNA3A(172–575) by inserting a *Bst*EII–*Bss*HII blunted fragment from pGal4-EBNA3A into the *Kpn*I(blunted) site of pG4MpolyII; Gal4-EBNA3A(138–234) mutant by inserting a *Nco*I(blunted)–*Sac*I fragment from pGal4-EBNA3A(1–234) between the *Bam*HI(blunted) and *Sac*I sites of pG4MpolyII; pGal4-EBNA3A(125–234) by inserting a *Bam*HI(blunted)–*Sac*I fragment from pGal4-EBNA3A(1–234) between the *Kpn*I(blunted) and *Sac*I sites of pG4MpolyII; pGal4-EBNA3A(234–944) by inserting a *Spe*I–*Xba*I fragment from pGal4-EBNA3A between the *Spe*I and *Xba*I sites of pGal4-EBNA3A(234–575); pGal4-EBNA3AΔ125–138 by inserting a *Nco*I(blunted)–*Spe*I fragment from pGal4-EBNA3A between the *Bam*HI (blunted) and *Spe*I sites of pGal4-EBNA3A; and pGal4-EBNA3A(524–666) by inserting a *Stu*I–*Sca*I fragment from pSG5-Flag-EBNA3A plasmid into the *Kpn*I(blunted) site of pG4MpolyII.

■ **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 at 37 °C in DMEM (Gibco) supplemented with 10% foetal calf serum and were seeded at 5×10^5 cells per 100 mm Petri dish 8 h prior to transfection. Transfections were performed by the calcium phosphate precipitation method. Cells were mixed with the appropriate DNAs: typically 15 μg of DNA was used which included the expression vectors and plasmids carrying the reporter genes. The amount of SV40 promoter transfected was kept constant by the addition of pSG5 to the transfection reaction when necessary. Transfected cells were washed and collected 48 h after transfection.

The EBV-negative human Burkitt's lymphoma-derived cell line DG75 was grown at 37 °C in RPMI 1640 medium, supplemented with 10% foetal calf serum. For transfections, approximately 5×10^6 cells in the exponential phase of growth were pelleted and resuspended in 250 μl of RPMI 1640 medium. The cell suspension was transferred into a chilled cuvette containing the relevant DNA and electroporated using a Bio-Rad gene pulser (220 V, 960 μF). The transfected cells were then resuspended in 10 ml of RPMI 1640 and incubated for 48 h. Each series of transfections was repeated several times.

■ **Immunodetection.** Cells were washed with cold PBS and incubated with 100 μl of RIPA buffer. Equal amounts of protein were loaded and separated on PAGE, and transferred to a nitrocellulose membrane (Schleicher & Schuell) by electroblotting. The membranes were first incubated with a mouse anti-Gal4 monoclonal antibody (RK5C1 from

Santa Cruz Biotechnology) then with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Amersham). Blots were revealed by means of the enhanced chemoluminescence kit (ECL) from Amersham as instructed by the supplier.

■ **CAT-ELISA.** This was done with the Boehringer Mannheim CAT-ELISA kit following 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 total amount of protein extracted.

Results

Mapping of the EBNA3A domain mediating the interaction with RBP-Jκ

A way to evaluate the hypothesis that Gal4–EBNA3A represses transcription by recruiting the cellular repressor RBP-Jκ, would be to examine the effect on Gal4–EBNA3A-mediated repression of a specific mutation in Gal4–EBNA3A that impairs its interaction with RBP-Jκ. In order to achieve this, we first mapped the domain of EBNA3A mediating the interaction with RBP-Jκ by using a two-hybrid type assay in mammalian cells. For this assay we used a fusion protein made between EBNA3A and the yeast protein Gal4 DNA-binding domain (Gal4–EBNA3A, Fig. 1*a*), a fusion protein between RBP-Jκ and the herpes simplex virus VP16 activation domain (RBP–VP16, Fig. 1*a*) and the pG4-TK-CAT reporter plasmid in which transcription of the CAT gene is under the control of the TK promoter and six Gal4 binding sites (Fig. 1*a*). In agreement with our previously published results (Waltzer *et al.*, 1996), the transfection of HeLa cells with pG4-TK-CAT resulted in basal expression of CAT from the reporter plasmid (Fig. 1*b*, lane1). Co-transfection with an expression vector for Gal4–EBNA3A resulted in a decrease in the amount of CAT protein expressed from the reporter plasmid pG4-TK-CAT (Fig. 1*b*, compare lanes 3 and 4 to lane 1). This repression was not induced by expression of the Gal4 DNA-binding domain alone (Fig. 1*b*, lane 2) and correlates with the specific recruitment of Gal4–EBNA3A onto the DNA, as expression of EBNA3A (not fused to the Gal4 DNA-binding domain) did not affect the level of CAT expression (Fig. 1*b*, lane 8). However, when expression vectors for Gal4-EBNA3A and RBP–VP16 were co-transfected together with pG4-TK-CAT, the amount of CAT protein expressed increased (Fig. 1*b*, lanes 5 and 6). As no effect of RBP–VP16 was detected in the absence of Gal4–EBNA3A (Fig. 1*b*, lane 7) this activation of CAT expression can be considered to be the result of the recruitment of RBP–VP16 onto the DNA by Gal4–EBNA3A and is thus a convenient assay for interactions between EBNA3A and RBP-Jκ *in vivo*.

We thus constructed a series of vectors containing overlapping fragments of EBNA3A fused to the Gal4 DNA-binding domain (Fig. 2*a*) to investigate which of the EBNA3A fragments recruit RBP–VP16. Expression plasmids for these fusion proteins were co-transfected into HeLa cells, together with the expression vector for RBP–VP16 and the reporter

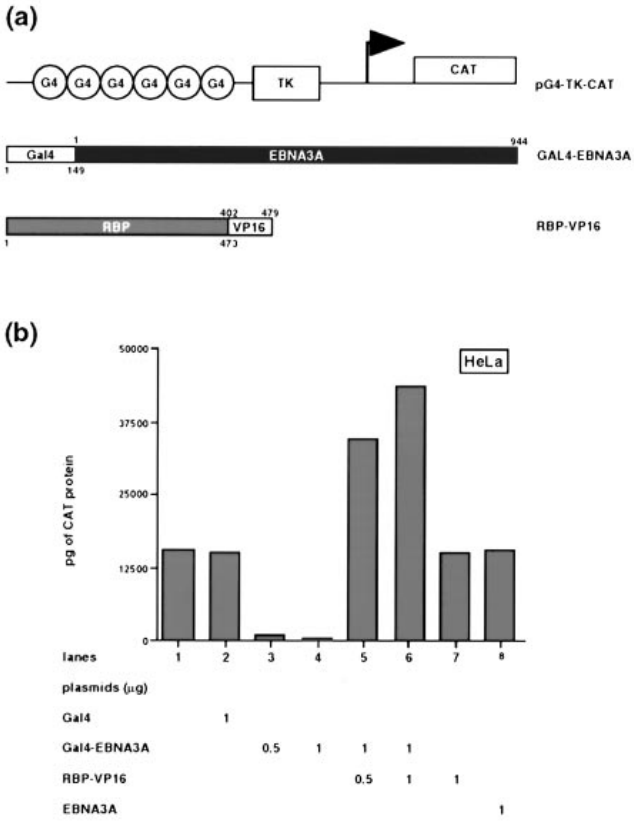

Fig. 1. Specific interaction of EBNA3A with RBP-Jκ in mammalian cells. (*a*) Schematic representation of pG4-TK-CAT, Gal4–EBNA3A, RBP–VP16. The pG4-TK-CAT reporter construct contains four binding sites for the yeast Gal4 protein and the herpes simplex virus thymidine kinase promoter cloned upstream from the CAT gene. Gal4–EBNA3A is a fusion between the yeast Gal4 protein DNA-binding domain [Gal4(dbd)] and EBNA3A. RBP–VP16 is a fusion between RBP-Jκ and the VP16 herpes simplex virus protein activation domain (VP16). (*b*) HeLa cells were co-transfected with 10 μg of pG4-TK-CAT reporter construct and various combinations of expression plasmids for Gal4(dbd), Gal4–EBNA3A, RBP–VP16 and EBNA3A proteins as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA.

construct pG4-TK-CAT. The amounts of the various proteins expressed were evaluated by Western blot analysis using an antibody which recognized the Gal4 moiety of the fusion proteins. All Gal4–EBNA3A mutants were produced in quantities comparable to Gal4–EBNA3A (data not shown). As shown in Fig. 2(*b*), only four Gal4-fusion proteins were able to recruit RBP–VP16: the wild-type Gal4–EBNA3A protein (Fig. 2*b*, lane 3) and the fusion proteins Gal4–EBNA3A(1–234), Gal4–EBNA3A(125–944), Gal4–EBNA3A(125–575) or Gal4–EBNA3A(125–234) (Fig. 2*b*, lanes 4, 7, 8 and 14 respectively). Thus, the smallest EBNA3A fragment interacting with RBP–VP16 lies between amino acids 125 and 234. Interestingly, neither Gal4–EBNA3A(172–234) nor Gal4–EBNA3A(138–234) was able to recruit RBP–VP16 (Fig. 2*b*, lanes 11 and 13 respectively) which suggests that the 13 amino acids between residues 125 and 138 at the N terminus of the

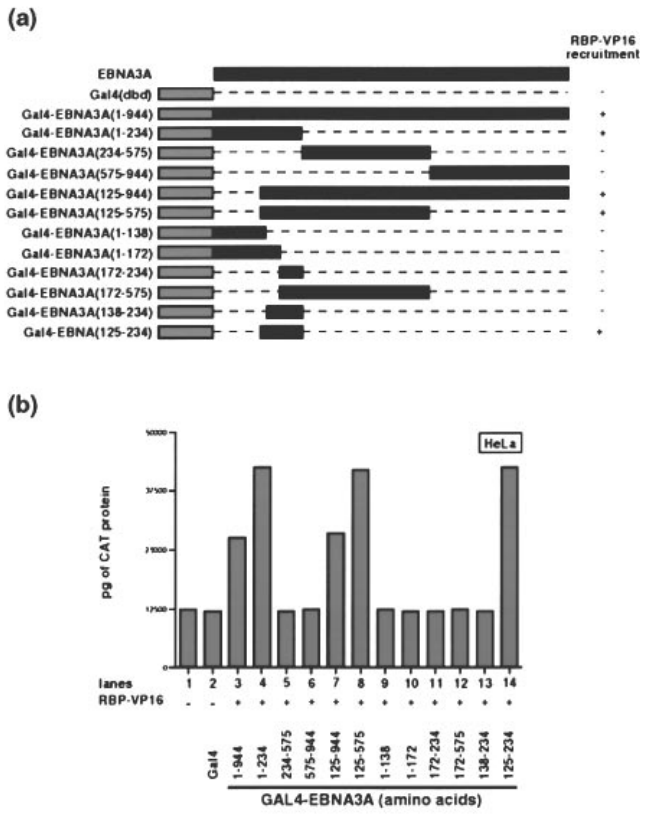

Fig. 2. (*a*) Mapping of the EBNA3A domain responsible for interaction with RBP-Jκ. Schematic representation of the fusion proteins between Gal4(dbd) and EBNA3A deletion mutants used in the experiment. Results of the experiment shown below are summarized on the right. (*b*) HeLa cells were co-transfected with 10 μg of pG4-TK-CAT reporter construct, 1 μg of RBP–VP16 expression plasmid when necessary and 1 μg of expression plasmids for the fusion proteins depicted above, as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA.

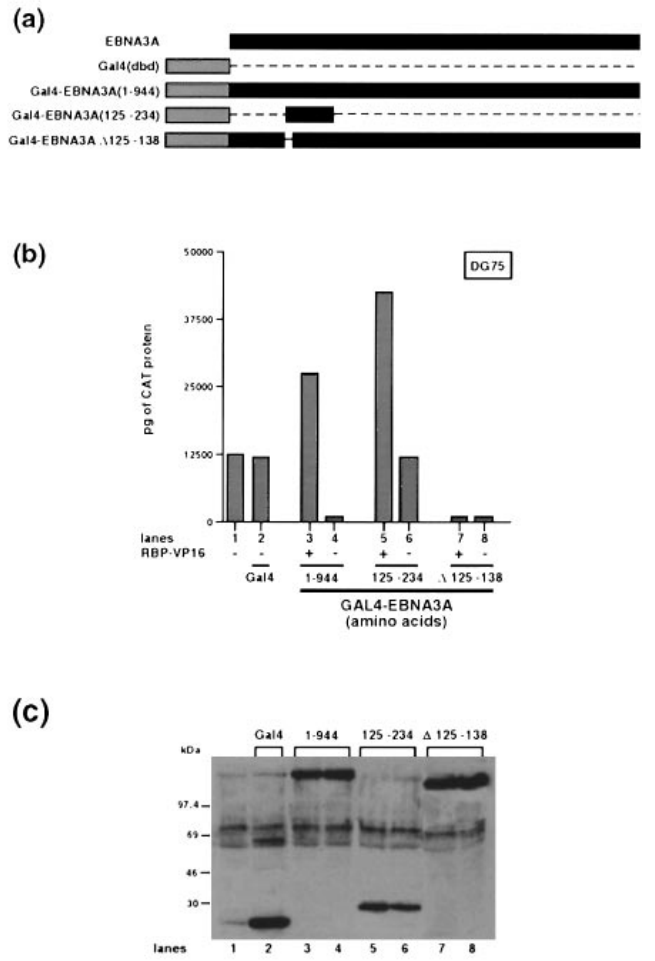

Fig. 3. Repression by the Gal4–EBNA3A mutant proteins does not correlate with recruitment of RBP–VP16. (*a*) Schematic representation of the fusion proteins between Gal4(dbd) and EBNA3A deletion mutants used in the experiment. (*b*) DG75 cells were co-transfected with 10 μg of pG4-TK-CAT reporter construct, 1 μg of expression plasmid for RBP–VP16 when necessary and the fusion proteins depicted above, as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA. (*c*) Immunoblot of the Gal4 fusion proteins expressed in DG75 cells transfected as described in (*b*). Visualization of the proteins was obtained by using mouse anti-Gal4 monoclonal antibody (RK5C1) from Santa Cruz Biotechnology.

domain delimited above are crucial for the interaction with RBP–VP16 and thereby for the interaction with RBP-Jκ.

Gal4–EBNA3A repression of transcription is not mediated by interaction with RBP-Jκ

Having mapped the EBNA3A domain mediating the interaction with RBP-Jκ to amino acids 125–138, we then tested whether a Gal4–EBNA3A protein in which these amino acids were deleted (mutant Gal4–EBNA3AΔ125–138) could still repress transcription and recruit RBP–VP16, as compared to GAL4–EBNA3A(1–944) and Gal4–EBNA3A(125–234) (Fig. 3*a*). Expression plasmids for each protein were cotransfected with the reporter construct pG4-TK-CAT, either alone or together with an expression plasmid for RBP–VP16, in the EBV-negative human lymphoid cell line DG75. All the Gal4 fusion proteins were expressed at comparable levels as shown by immunoblot analysis (Fig. 3*c*). As expected, the full-length EBNA3A fused to the Gal4 DNA-binding domain was able to recruit RBP–VP16 (Fig. 3*b*, lane 3) and act as a repressor in the absence of RBP–VP16 (Fig. 3*b*, lane 4), demonstrating that both recruitment of RBP-Jκ and repression of transcription by Gal4–EBNA3A occurred in B-cells as well as in HeLa cells. Protein Gal4–EBNA3A(125–234), which contains the minimum domain of EBNA3A that is able to recruit RBP–VP16 (Fig. 3*b*, lane 5), was no longer a repressor (Fig. 3*b*, lane 6). Interestingly Gal4–EBNA3AΔ125–138, which did not recruit RBP–VP16 (Fig. 3*b*, lanes 7), was still a repressor (Fig. 3*b*, lanes 8). Thus, although deletion of amino acids 125–138 of EBNA3A completely abolished the interaction with RBP–VP16, this mutant expressed alone was still able to repress expression from the reporter construct with the same efficiency

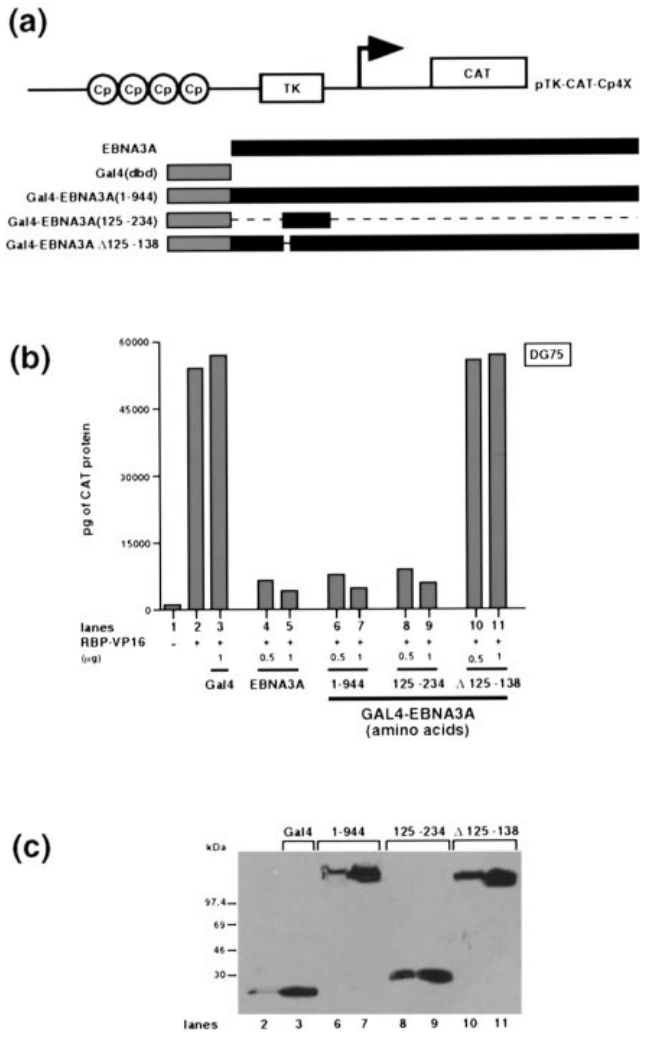

Fig. 4. Effect of Gal4–EBNA3A mutants on RBP–VP16 activation of a reporter construct containing RBP-Jκ binding sites. (*a*) Schematic representation of the pTK-CAT-Cp4x reporter construct, which contains four binding sites for RBP-Jκ and the herpes simplex virus thymidine kinase promoter cloned upstream from the CAT gene, and of the fusion proteins made between Gal4dbd and EBNA3A deletion mutants, used in the experiment. (*b*) DG75 cells were co-transfected with 10 μg of pTK-CAT-Cp4x reporter construct and expression plasmids for RBP–VP16 (1 μg) and the fusion proteins depicted above, as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA. (*c*) Immunoblot of the Gal4 fusion proteins expressed in DG75 cells transfected as described in (*b*). Visualization of the proteins is obtained by using the mouse anti-Gal4 monoclonal antibody (RK5C1).

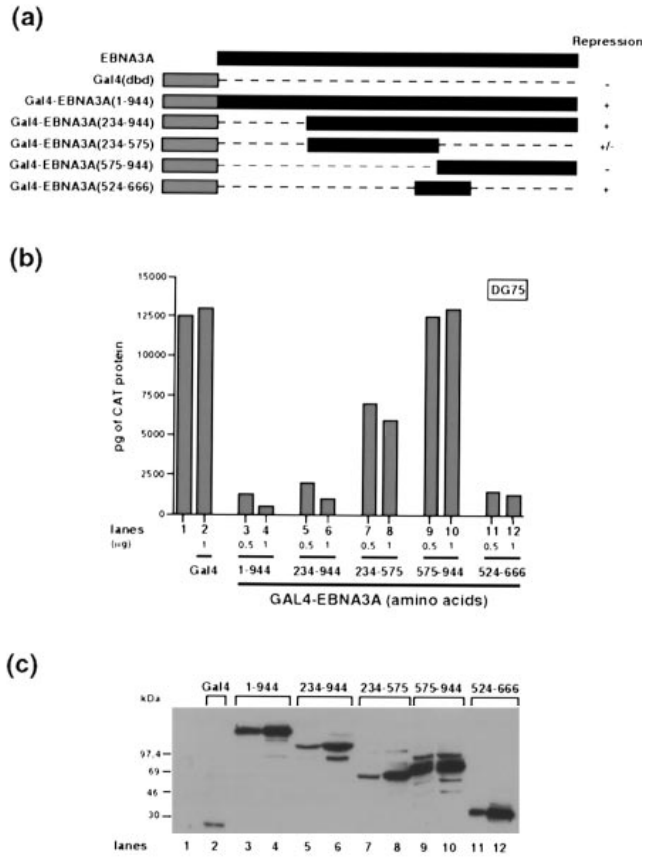

Fig. 5. Mapping of the EBNA3A repression domain. (*a*) Schematic representation of the fusion proteins between Gal4dbd and EBNA3A deletion mutants used in the experiment. The results of the experiment shown below are summarized on the right. (*b*) DG75 cells were cotransfected with 10 μg of pG4-TK-CAT reporter construct and expression plasmids for the fusion proteins depicted above, as indicated below the graph. The promoter activity was assayed by quantifying the amount of CAT protein expressed by CAT-ELISA. (*c*) Immunoblot of the Gal4 fusion proteins expressed in DG75 cells transfected as described in (*b*). Visualization of the proteins was obtained by using the mouse anti-Gal4 monoclonal antibody RK5C1.

as Gal4–EBNA3A. These results strongly suggest that the repression of transcription and the ability of EBNA3A to interact with RBP-Jκ are not directly correlated.

In order to confirm that the Gal4–EBNA3AΔ125–138 protein did not interact with RBP-Jκ, we also performed the following experiment. We used a reporter construct containing four RBP-Jκ binding sites cloned upstream of the TK promoter and the CAT gene (plasmid pTK-CAT-Cp4x; Fig. 4*a*). The RBP–VP16 fusion protein binds and activates transcription from this artificial promoter (Fig. 4*b*, compare lanes 2 and 3 to lane 1) (Waltzer *et al.*, 1995). When EBNA3A was co-expressed in the transfections, activation of transcription by RBP–VP16 was strongly inhibited (Fig. 4*b*, lanes 4 and 5), and this inhibition is likely to be due to destabilization of the binding of RBP–VP16 to DNA (Waltzer *et al.*, 1996). In the same assay, Gal4–EBNA3A(1–944) repressed RBP–VP16 transcriptional activation to the same extent as EBNA3A (Fig. 4*b*, compare lanes 6 and 7 to lanes 4 and 5). As expected, Gal4–EBNA3A(125–234) prevented RBP–VP16 activation (Fig. 4*b*, lanes 8 and 9), whereas Gal4–EBNA3AΔ125–138 did not affect RBP–VP16-activated transcription (Fig. 4*b*, lanes 10 and 11). Taken together, our results strongly suggest that recruitment of RBP-Jκ is not required for Gal4–EBNA3A-mediated repression of transcription.

The domain of EBNA3A mediating repression of transcription by Gal4–EBNA3A is located between amino acids 524 and 666

In order to identify precisely which region of EBNA3A was responsible for the transcriptional repression function of Gal4–EBNA3A, we analysed the repression activity of the Gal4–EBNA3A mutants shown in Fig. 5 (*a*). These constructs were co-transfected in DG75 cells together with the reporter plasmid pG4-TK-CAT. The expression level of each fusion protein was evaluated by Western blot analysis, using a monoclonal antibody directed against the Gal4 DNA-binding domain. As shown in Fig. 5 (*c*), all the proteins were expressed at comparable levels. The activity of the fusion proteins as repressor was then evaluated by measuring the amount of CAT protein expressed from the pG4-TK-CAT reporter. Again, Gal4–EBNA3A strongly repressed expression of the CAT protein (Fig. 5 *b*, compare lanes 3 and 4 to lane 1) and in a manner proportional to the amount (0·5 or 1 μg) of expression vector transfected. Deletion of the first 233 N-terminal amino acids of the protein had no effect on the repression capacity of the fusion protein (Fig. 5 *b*, lanes 5 and 6). Moreover, a fragment encompassing only amino acids 524–666 was still able to repress CAT expression to the same extent as the full-length protein (Fig. 5 *b*, compare lanes 11 and 12 to 3 and 4). Analysis of other overlapping mutants confirmed the localization of the repression domain since fusion proteins Gal4–EBNA3A(234–575) and Gal4–EBNA3A(575–944), which contain only part of the domain delimited above, had respectively strongly reduced or no capacity to repress expression from pG4-TK-CAT (Fig. 5 *b*, lanes 7–8 and 9–10 respectively). Duplicate experiments performed in HeLa cells gave identical results (data not shown). Thus, we could conclude that the repression domain of EBNA3A lies between amino acids 524–666.

Discussion

In this report we provide experimental data demonstrating that EBNA3A tethered to DNA through the Gal4 DNA-binding domain represses transcription from a reporter gene carrying Gal4-DNA binding sites. We have identified a 143 amino acid domain of EBNA3A as being responsible for this repression. We have also precisely mapped the domain of EBNA3A involved in the interaction with the cellular factor RBP-Jκ.

An obvious explanation for the repressing properties of EBNA3A is that it can use endogenous RBP-Jκ as a co-factor for repression, since RBP-Jκ has been reported to be a repressor in mammalian cells. Our results strongly suggest that the EBNA3A repression domain is distinct from the interaction domain with RBP-Jκ. Furthermore, our results also suggest that, although EBNA3A most probably interacts with endogenous RBP-Jκ, the repression domain of RBP-Jκ is inactive when RBP-Jκ is complexed to EBNA3A. Indeed, EBNA3A mutants which did not interact with RBP-Jκ repressed reporter gene expression to the same level as the fusion containing full-length EBNA3A, suggesting that there is no participation of RBP-Jκ in the observed repression. This inactivity of the RBP-Jκ repression domain, when bound to EBNA3A, is not so surprising because the smallest region of RBP-Jκ found to be necessary for RBP-Jκ-induced repression as well as for interaction with EBNA2 (Hsieh & Hayward, 1995) partially overlaps with the RBP-Jκ domain involved in EBNA3A and EBNA3C interaction (Krauer *et al*., 1996; Zhao *et al*., 1996; our report). It is thus probable that the interactions of RBP-Jκ with the different cellular and viral partners described so far are mutually exclusive.

EBNA3s-RBP-Jκ interaction domains had previously been mapped to the N-terminal part of EBNA3A, 3B and 3C by using *in vitro* GST–RBPJκ pull-down assays of C-terminally truncated EBNA3 mutants (Robertson *et al*., 1996). Here, we have delimited a more restricted 110 amino acid domain of EBNA3A (amino acids 125–234) which is necessary for the interaction with RBP-Jκ *in vivo*. This region is well-conserved between EBNA3A, EBNA3B and EBNA3C. In agreement with our results with EBNA3A, the RBP-Jκ interaction domain in EBNA3C has been mapped by Zhao *et al*. (1996) to the same conserved region.

Because of the conserved organization of the EBNA3 genes, it has been postulated that they have been generated by triplication of a common ancestral gene. Interaction with the cellular factor RBP-Jκ appears to be a well-conserved function of the EBNA3 proteins. We have shown here that another function of EBNA3A could be to repress gene expression. Similarly, EBNA3C has also been shown to be a repressor when fused to the Gal4 DNA-binding domain (Bain *et al*., 1996; Waltzer *et al*., 1996). However, in the case of EBNA3A we have mapped a unique 143 amino acid domain which is able to repress as efficiently as full-length EBNA3A when fused to the Gal4 DNA-binding domain. In contrast, for EBNA3C, two separate domains have been mapped which have additive repression activity (Bain *et al*., 1996). Nothing is known about the mechanisms by which Gal4–EBNA3A represses transcription. We have tried to evaluate if a co-factor was necessary for EBNA3A-induced repression. We therefore used the reporter construct pG4-TK-CAT and co-expressed increasing amounts of EBNA3A together with Gal4–EBNA3A in order to titrate a potential co-factor. No difference in the amount of CAT expressed from the pG4-TK-CAT reporter was detected, suggesting that if there is a co-factor it is not present in limited amounts in the cell nucleus (data not shown).

Thus, both EBNA3A and EBNA3C, and possibly EBNA3B, possess characteristics typical of transcriptional regulators. However, it is not yet known if and how they can be targeted to specific promoters. It has been reported that the EBNA3 proteins show some affinity for DNA when crude extracts from EBV-transformed cells are passed over DNA–cellulose columns (Kallin *et al*., 1986), but no direct binding to DNA has

ever been described. The only cellular DNA-binding protein known at present to interact with EBNA3A, 3B and 3C is RBP-Jκ, and in this case the only effect of the interaction observed until now is disruption of the binding of RBP-Jκ to DNA. However, we cannot exclude the possibility that on certain promoters, EBNA3–RBP-Jκ complexes are stabilized by cellular co-factors, possibly binding onto the same promoter. The EBNA3 proteins could also be recruited onto the DNA by uncharacterized cellular DNA-binding factors acting as intermediary.

Moreover, other functions have been proposed for EBNA3C. In effect, EBNA3C can cooperate with activated (Ha-)ras, in co-transfection assays, to immortalize and transform rat embryo fibroblasts (Parker *et al.*, 1996). There is evidence that EBNA3C can act via the pRb-mediated pathway and as EBNA3C is able to directly bind a GST–pRb fusion *in vitro* (Parker *et al.*, 1996), it would be interesting to investigate whether EBNA3A is also able to interact with pRb and cooperate with (Ha-)ras to immortalize rat fibroblasts.

In conclusion, the EBNA3 proteins and especially EBNA3A and EBNA3C play an essential role in the immortalization of B-lymphocytes by EBV, but their precise function is far from being understood. One possibility is that they could exert a retro-control on RBP-Jκ–EBNA2-activated genes. This retro-regulation could be important as overexpression of a protein such as LMP1, for example, appears to be toxic for the cell (Hammerschmidt *et al.*, 1989; Martin *et al.*, 1993). However, the role of the EBNA3 proteins can be expected to be more complex than this. Our work and that of others prompts us to regard the EBNA3 proteins as transcriptional regulators. The identification and characterization of potential cellular partners for these proteins is required for a better understanding of their precise role in EBV immortalization.

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