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Epstein-Barr Virus Nuclear Antigen 3C Is a Transcriptional Regulator

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Epstein-Barr virus (EBV) nuclear antigen 3C (EBNA-3C) is one of five viral nuclear proteins that are essential for EBV-induced immortalization of primary human B lymphocytes in vitro. Previous studies have implied that EBNA-3C acts as a transcription factor. Using transient transfection assays, we demonstrate that EBNA-3C has two effects on reporter genes that are linked to the latent membrane protein 1 promoter, (i) low-level activation by EBNA-3C alone, as well as potentiation of EBNA-2-mediated transactivation, and (ii) inhibition of the normally strong activation mediated by EBNA-2. These two disparate effects seem to be mediated at different stages following cell feeding. The inhibitory effect of EBNA-3C was localized to a known EBNA-2 response element that had previously been shown to be recognized by the DNA-binding protein RBP-Jκ. In addition, direct interaction between RBP-Jκ and EBNA-3C was observed by coimmunoprecipitation. Activation by EBNA-3C, however, seems to be achieved via sequences that are distinct from RBP-Jκ sites, since activation remained even after these sites had been mutated. Consistent with its ability to activate transcription, a region of EBNA-3C which has homology to the glutamine-rich activation domain of Sp1 can function as a transcription activation domain when it is fused to the heterologous DNA-binding domain of Gal4 and can partially restore the activity of a mutant EBNA-2 protein with a deletion in the transactivation domain. Collectively, these data strongly support the role of EBNA-3C as a transcriptional regulator.

Epstein-Barr virus (EBV) transforms human B lymphocytes into immortalized cells. Like other herpesviruses, EBV establishes a latent infection during which only a small number of viral genes, six nuclear antigens (EBNAs), two latent membrane proteins (LMPs), and two small nuclear RNAs (for reviews, see references 20 and 33), are expressed. Deletion of individual genes by genetic recombination has demonstrated that five EBNAs and one LMP are required for immortalization of human B cells in vitro (6, 13, 19, 28, 30, 44–46).

One of the essential nuclear proteins, EBNA-2, activates the expression of genes which are involved in B-lymphocyte growth and transformation, including viral protein LMP-1, B-lymphocyte activation antigens CD21 and CD23, and proto-oncogene c-*fgr* (23, 47–49). These EBNA-2-responsive promoters share a 7-bp region of homology which binds a cellular protein, CBF-1 (11, 15, 26, 27); this cellular protein, previously identified as protein RBP-Jκ, was initially thought to play a role in gene rearrangement by binding to the recombination recognition sequence (12). It is now believed to be a transcriptional regulatory protein with some properties of a transcriptional repressor (7, 9). EBNA-2 binds to DNA via RBP-Jκ and provides an activation domain within the carboxyl terminus to contact the transcriptional machinery and activate expression from its target promoters (4).

EBNA-2 also activates expression of the other EBNAs, including the EBNA-3 proteins (18, 38, 43, 50). The EBNA-3 gene family consists of EBNA-3A, -3B, and -3C, which have related gene structures and are proposed to have arisen from gene duplication (16, 35, 36). However, these proteins have clearly evolved unique functions since both EBNA-3A and -3C are essential for B-lymphocyte growth and transformation (46). Like EBNA-2, EBNA-3C increases expression of CD21 and LMP-1 (1, 2, 47). We have previously demonstrated that whereas EBNA-3C exhibits DNA-binding activity in crude extracts from EBV-infected cells, the recombinant baculovirus-expressed protein cannot bind to double-stranded DNA columns (40). This suggests that EBNA-3C may interact with DNA in an indirect manner via a cellular protein, although binding of EBNA-3C to specific DNA sequences has not been demonstrated. EBNA-3C sequence analysis reveals a region that is homologous to the basic leucine zipper motif that is found in many mammalian transcription factors which functions as a DNA-binding and protein dimerization domain (25, 34). Collectively, these data suggest that EBNA-3C functions as a transcriptional regulator.

Since EBNA-3C increases expression of LMP-1, we examined the ability of EBNA-3C to activate expression from the LMP-1 promoter in transient transfection assays. EBNA-3C increased expression from the LMP-1 promoter both alone and in conjunction with EBNA-2. In other cell lines, EBNA-3C inhibited activation of the LMP-1 promoter by EBNA-2. We have localized the sequences that are required for inhibition by EBNA-3C to the known EBNA-2-responsive sequence which binds RBP-Jκ. When both EBNA-2 and -3C are cotransfected with a reporter gene construct under the control of a promoter that consists only of repeats of this EBNA-2 response element, EBNA-3C completely represses the strong activation that is normally elicited by EBNA-2. However, the sequences involved in activation seem to be distinct since EBNA-3C can activate a mutant LMP-1 promoter in which both RBP-Jκ sites have been mutated. In accordance with its role as a transcriptional activator, we identified a glutamine-rich region with bulky hydrophobic residues which is characteristic of the transactivation domain of the mammalian transcription factor Sp1 (10). By creating fusion proteins with heterologous DNA-binding domains and testing the ability of these fusion proteins to activate transcription, we established that the glutamine-rich domain of EBNA-3C functions as an activation domain in both Gal4 and EBNA-2 chimeric proteins. These data clearly pro-

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vide evidence to support the proposed role of EBNA-3C as a transcription factor.

MATERIALS AND METHODS

Cell lines. Louckes and BL2 are EBV-negative Burkitt lymphoma (BL) cell lines. IB4 is an EBV-positive lymphoblastoid cell line obtained by infection of cord blood B cells in vitro (22). HSB-2 is a T-lymphoblastoid cell line. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The epithelial cell line HEp-2 was maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Plasmid DNA. The pSG5-3C and pSG5-Jκ plasmids were generated by cloning EBNA-3C cDNA (provided by Jeff Sample) and Jκ cDNA, respectively, into expression vector pSG5 (Stratagene). p-2350LMP-CAT was derived by cloning the *Xho*II-*Mst*II LMP-1 promoter fragment (extending from −2350 to +40) into pCAT3M. p-512LMP-CAT was generated by cloning the *Hin*fI-*Mst*II LMP-1 promoter fragment (extending from −512 to +40) into pCAT3M. These LMP-1 promoter fragments were also cloned into pΦGH to generate human growth hormone (hGH) reporter plasmids. The following deletions of the −2350 LMP promoter-hGH construct were also generated by restriction endonuclease digestion: p-1277LMP-hGH (*Eco*RI), p-924LMP-hGH (*Xma*III), p-634LMP-hGH (*Sac*II), p-413LMP-hGH (*Bst*EII), p-335LMP-hGH (*Bss*HII), p-225LMP-hGH (*Bsm*I), and p-54LMP-hGH (*Mlu*I). In the RBP-Jκ-deleted LMP-CAT construct generated by recombinant PCR (17), both RBP-Jκ sites, located at −298 and −223, have been mutated from GTGGGAA to GGATCC (39a). pGal4-3CGln was generated by ligating the 306-bp *Bam*HI e_1 fragment of the B95-8 EBV genome into the *Bam*HI site of pM3, an expression vector for generation of fusion proteins with the DNA-binding domain of the yeast transcription factor Gal4 (39). pSG5-EBNA-2Δ/3CGln was derived by ligating the 306-bp *Bam*HI e_1 fragment of the B95-8 EBV genome into the *Bgl*II site of pSG5-EBNA-2Δ, deleted of amino acids 456 to 485 (4). All plasmids were purified by anion-exchange chromatography (Qiagen, Inc.) and subsequent purification on a cesium chloride density gradient.

Transfections. Lymphoblastoid cell lines were transfected at a concentration of 8×10^6 cells in 250 μl of RPMI 1640. Ten micrograms of reporter plasmid and 10 μg of expression vector were introduced into cells by electroporation at 250 V and 960 μF; total DNA was 30 μg in all cases. HEp-2 cells were transfected by standard calcium phosphate precipitation. Cells were harvested at 40 to 48 h posttransfection. The hGH reporter gene under control of the cytomegalovirus immediate-early promoter, pCMV-hGH, was used as a transfection control for chloramphenicol acetyltransferase (CAT) reporter plasmids, and the CAT reporter gene under control of the cytomegalovirus promoter, pCMV-CAT, was used as a transfection control for hGH reporter plasmids.

Reporter assays. CAT activities were determined by the standard two-phase partition method. CAT activities were calculated as ratios of acetylated [^{14}C]chloramphenicol to total acetylated and unacetylated [^{14}C]chloramphenicol. Then these values were presented relative to that obtained with the expression vector that contained no insert (which was set to 1). The levels of hGH in culture supernatants were quantitated with a radioimmunoassay system according to manufacturer's recommendations (Nichols Institute). Immunofluorescence was used to confirm the expression of EBNA-2 and EBNA-3C in transfected cells. Following fixation in methanol-acetone (1:1) for 4 min at −20°C, EBNA-3C expression was detected with human EBV-positive serum and subsequently with biotinylated goat anti-human secondary antibody and fluorescein isothiocyanate-conjugated streptavidin. PE2, a mouse monoclonal antibody to EBNA-2, as well as biotinylated rabbit anti-mouse antibody and fluorescein isothiocyanate-conjugated streptavidin, was used to detect EBNA-2.

In vitro transcription, translation, and immunoprecipitation. pSG5-EBNA-3C and pSG5-Jκ were used for in vitro transcription with T7 polymerase (mCAP kit; Stratagene) according to the manufacturer's recommendations. RNAs were in vitro translated with rabbit reticulocyte lysate (Promega) and [^{35}S]methionine (Dupont). Proteins were incubated in 20 mM Tris-Cl (pH 8.0)–150 mM NaCl–0.5 mM EDTA–0.5% Nonidet P-40 at 4°C for 30 min and immunoprecipitated with human EBV-positive serum. Immunoprecipitates were collected with protein A-Sepharose (Pharmacia) blocked with 5% bovine serum albumin (Promega). Proteins were eluted from protein A-Sepharose by boiling in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer that contained 10% β-mercaptoethanol and were electrophoresed on 7.5% SDS-polyacrylamide gels. Following electrophoresis, gels were stained with 0.5% Coomassie blue G-250 in 40% methanol–7% acetic acid and destained in 45% methanol–1% acetic acid. Gels were dried and exposed to Kodak XAR-5 film at −70°C.

RESULTS

EBNA-3C transactivates the LMP-1 promoter. Previous reports had suggested that EBNA-3C increases expression of LMP-1 (2). Therefore, we used transient transfection assays with the LMP-1 promoter linked to reporter genes to determine whether this effect was at the level of transcription. Our initial reporter constructs contained a region of the LMP-1 promoter extending 2,350 bp upstream of the site of transcriptional initiation (+1) linked to a CAT reporter gene and were transfected into the EBV-negative BL cell line BL2. As expected from previous reports, EBNA-2 was able to efficiently transactivate this reporter gene construct (Fig. 1A). EBNA-3C was also able to transactivate this construct, although to a lesser extent. EBNA-3C-mediated activation ranged from 2- to 10-fold in different experiments and was approximately 10-fold weaker than that obtained with EBNA-2, a strong transcriptional activator of LMP-1. In addition, EBNA-3C was able to potentiate EBNA-2-stimulated transcription two- to fivefold, clearly demonstrating the ability of EBNA-3C to activate expression from the LMP-1 promoter.

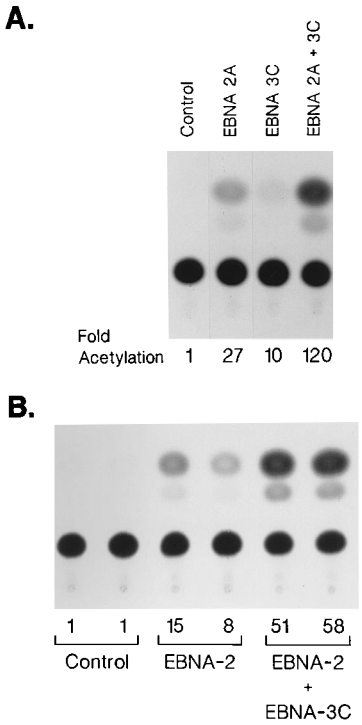

FIG. 1. EBNA-3C can activate expression from the LMP-1 promoter. (A) The EBV-negative cell line BL2 was transfected with a CAT reporter gene linked to a 2,350-bp upstream sequence of the LMP-1 promoter plus either a control expression vector (pSG5), EBNA-2 expression vector, EBNA-3C expression vector, or both EBNA-2 and -3C expression vectors. Activities are expressed as fold increases in acetylation over the value obtained by cotransfection of the CAT reporter plasmid with the control vector. (B) Activation of the RBP-Jκ-deleted LMP-1 promoter under the same conditions used in panel A.

To localize the region of EBNA-3C responsiveness within the LMP-1 promoter, we constructed a series of deletions from the 5′ end of the −2350 promoter linked to the hGH reporter gene and tested the ability of EBNA-3C to activate these reporter constructs both in the presence and in the absence of EBNA-2 (Table 1). EBNA-3C alone transcriptionally activated all of these LMP-1 promoter constructs, except −54LMP-hGH. This activation was quite low, and its significance is unclear. In the presence of EBNA-2, however, EBNA-3C significantly augmented expression from the longer constructs (−2350 to −512), relative to that with EBNA-2 alone. Although constructs which contained less than 335 bp of upstream sequence remained responsive to EBNA-3C alone, no additional increase was seen in the presence of both EBNA-2 and -3C. Neither EBNA-2 nor EBNA-3C was able to stimulate expression from the shortest construct, −54LMP-hGH.

TABLE 1. Activation of LMP-1 promoter constructs by EBNA-3C in BL2 cells

LMP-1 promoter[a]	Fold activation[b]		
	EBNA-2	EBNA-3C	EBNA-2 and -3C
−2350	11	1.5	35
−1277	18	1.5	30
−924	19	2	46
−634	39	3.5	42
−512	13	3.3	23
−335	20	2.5	23
−225	23	3.5	18
−54	1	1	1

[a] 5′ coordinate relative to the transcription start site (+1). All promoter constructs contained LMP-1 sequences to +40.

[b] Data are fold activation over values obtained with the expression vector alone and with the LMP-hGH construct (which were set to 1) and are the means of a representative experiment. All transfections were done a total of four times.

Since there were RBP-Jκ binding sites at −298 and −223, we tested the ability of EBNA-3C to activate a mutant LMP-1 promoter in which both RBP-Jκ sequences had been specifically mutated to *Bam*HI sites by recombinant PCR; this construct was weakly activated by EBNA-2 (39a). Deletion of the RBP-Jκ sites did not affect the ability of EBNA-3C to activate expression of the LMP-1 promoter (Fig. 1B). This suggested that the sequences that are required for EBNA-3C-mediated activation are independent of the RBP-Jκ binding sites.

EBNA-3C can inhibit EBNA-2 activation via the RBP-Jκ element. We tested the ability of EBNA-3C to increase transcription from the LMP-1 promoter in other EBV-negative BL cell lines. In Louckes BL cells, EBNA-3C alone did not activate the LMP-1 promoter but surprisingly downregulated the effect of EBNA-2 when both EBNA-2 and -3C were expressed (Fig. 2A). In the presence of both EBNA-2 and EBNA-3C, activity was approximately 50% of that obtained with EBNA-2 alone; in different experiments, this downregulation ranged from 15 to 50%. This is in contrast to transcriptional activation of the LMP-1 promoter by EBNA-3C in BL2 cells.

EBNA-3C was able to downregulate expression from the shortest promoter deletion, −225LMP-CAT (data not shown). One RBP-Jκ binding site through which EBNA-2 activates this same promoter is located at −223. Since this EBNA-2-responsive element has been well characterized, we examined the ability of EBNA-3C to modify expression via this element alone with the reporter plasmid E2RSP-CAT, which contains 10 copies of the EBNA-2-responsive RBP-Jκ binding site. EBNA-2 was able to efficiently transactivate this construct (Fig. 2B). EBNA-3C was not able to transactivate via these sequences alone (data not shown). Surprisingly, when both EBNA-2 and EBNA-3C were cotransfected with E2RSP-CAT, EBNA-3C completely abolished the ability of EBNA-2 to stimulate expression. Since these data suggested that inhibition of expression was mediated via the RBP-Jκ sites, we tested the effects of RBP-Jκ deletions in the LMP-1 promoter. Mutation of the RBP-Jκ sites prevented inhibition by EBNA-3C in Louckes cells (Fig. 2C). These data suggest that interaction between EBNA-3C and RBP-Jκ or its target sequences results in a loss of EBNA-2-mediated stimulation.

EBNA-3C effects on the LMP-1 promoter vary with feeding conditions. To clarify the different responses seen in these two cell lines, we tested whether the inhibition seen in Louckes cells could be reproduced in BL2 cells under different growth conditions. Normally, cells are diluted 1:10 with fresh media 2 days prior to transfection; at the time of transfection, cells are

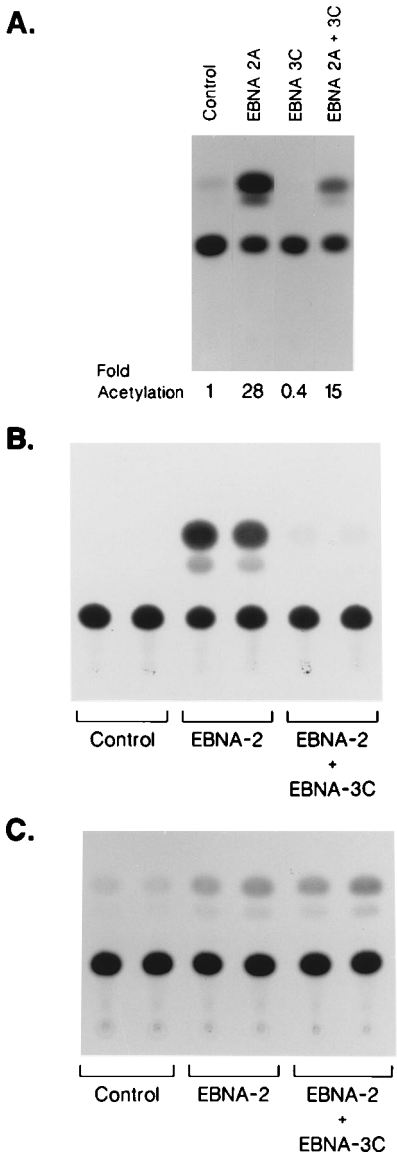

FIG. 2. EBNA-3C can downregulate the EBNA-2 response. The EBV-negative BL cell line Louckes was transfected with various reporter constructs together with either a control expression vector, EBNA-2 expression vector, EBNA-3C expression vector, or both EBNA-2 and -3C expression vectors. Activities are expressed as fold increases in acetylation over that of the control vector as described in the legend to Fig. 1. (A) The reporter construct was −2350LMP-CAT. (B) A plasmid that contained 10 copies of the EBNA-2 response element, GTGGGAA, linked to a CAT reporter gene was used as the reporter construct. (C) RBP-Jκ-deleted LMP-CAT was the reporter construct.

in logarithmic growth. For this transfection, cells were fed on the day prior to transfection and transfected with either a wild-type LMP-1 promoter construct (Fig. 3A) or one in which both RBP-Jκ sites had been mutated (Fig. 3B). EBNA-3C downregulated EBNA-2-induced activation of the wild-type LMP-1 promoter in BL2 cells that had been transfected under these conditions. This downregulation was approximately threefold and was similar to the results obtained with Louckes cells (Fig. 2A). This clarifies the differences seen in various cell lines and suggests that these differences may occur as a result of cell cycle modifications. EBNA-2 was still able to transactivate the RBP-Jκ-deleted LMP-1 promoter (Fig. 3B); this ac-

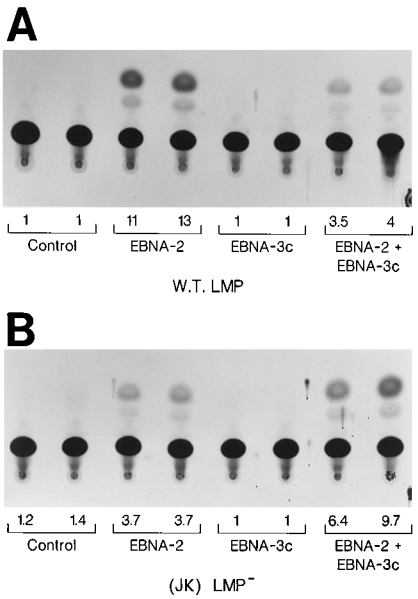

FIG. 3. EBNA-3C effects vary with feeding conditions. A wild-type (W.T.) LMP-1 promoter (A) or one in which both RBP-Jκ sites had been mutated [(JK) LMP⁻] (B) was transfected into the EBV-negative cell line BL2, which had been fed on the day prior to transfection, in the presence of an empty expression vector (control) or one that expressed either EBNA-2, EBNA-3C, or both EBNA-2 and -3C. Activities are expressed as fold increases in acetylation over that of the control vector.

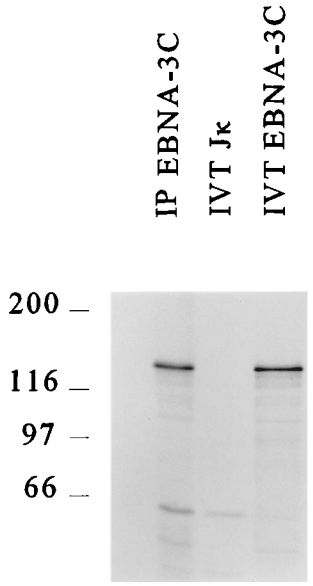

FIG. 4. EBNA-3C interacts with RBP-Jκ. In vitro translated (IVT) EBNA-3C (lane 3) was combined with IVT Jκ (lane 2) and immunoprecipitated (IP) with human EBV-positive serum (lane 1). Molecular size standards (in kilodaltons) are given on the left.

tivation was equal to that obtained with the wild-type promoter in the presence of both EBNA-2 and EBNA-3C. The presence of both EBNA-2 and EBNA-3C led to increased expression, relative to that obtained with EBNA-2 alone. This suggests that net promoter activity is a balance between the negative effect that is mediated through the RBP-Jκ elements and the positive effect which is not mediated via these sequences and that the predominant effect varies as a result of changes that are induced by feeding and/or the cell cycle.

EBNA-3C binds directly to RBP-Jκ. Since our data suggested that EBNA-3C-mediated inhibition was achieved through RBP-Jκ binding sites, it is possible that EBNA-3C interacts directly with RBP-Jκ protein. To examine this possibility, we combined in vitro translated EBNA-3C and RBP-Jκ and immunoprecipitated EBNA-3C with an EBV-positive human serum which had been extensively absorbed against an EBV-negative B-cell lysate. This serum had previously been demonstrated to have major reactivity against EBNAs by both immunoprecipitation and immunoblotting (40). Immunoprecipitates of EBNA-3C with human serum also contained RBP-Jκ protein (Fig. 4). In addition, we have demonstrated direct binding of EBNA-3C to RBP-Jκ by using the yeast two-hybrid system (51). These data indicate that EBNA-3C may interact directly with RBP-Jκ protein, which may prevent it from either binding to DNA or interacting with the basal transcriptional machinery.

EBNA-3C contains a transcriptional activation domain. The ability of EBNA-3C to stimulate transcription of the LMP-1 promoter supports the likely role of EBNA-3C as a transcription factor. If EBNA-3C is indeed a transcription factor, it should have a transactivation domain. EBNA-3C has an amino acid domain which is similar to a transactivation domain in the mammalian transcription factor Sp1. This sequence was initially noticed for being rich in glutamine residues, although it also contains many proline residues, and is located at the opposite end of the molecule from the basic leucine zipper and putative DNA-binding domain (Fig. 5B). These glutamine-rich sequences were cloned into plasmid pM3, adjacent to the DNA-binding domain of the yeast Gal4 protein, to create a

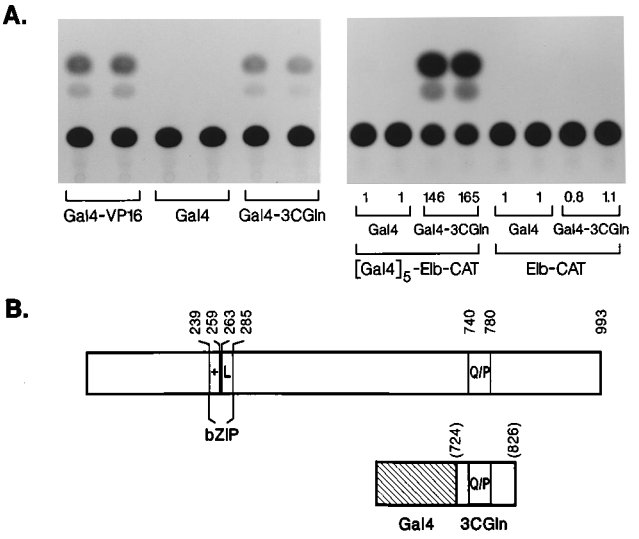

FIG. 5. EBNA-3C contains an activation domain. (A) (Left panel) EBV-negative B cells were transfected with plasmid pG5EC, which contained the CAT reporter gene under the control of Gal4 binding sites, and either pGal4VP-16 (positive control), pGal4 (negative control), or the Gal4-3C chimera pGal4-3CGln. (Right panel) Data are fold acetylation, relative to the background obtained with pGal4 (which was set to 1). The EBV-positive cell line IB4 was transfected with Gal4 or Gal4-3CGln together with a reporter plasmid that contained Gal4 binding sites or a control plasmid that contained only the E1b promoter linked to the CAT reporter gene. (B) Schematic diagram of the EBNA-3C protein sequence. The putative basic leucine zipper (bZIP), as well as the glutamine-rich region that is homologous to the Sp1 transcription factor activation domain (Q/P), is shown. Numbers indicate amino acid residues of EBNA-3C. The region of EBNA-3C fused to Gal 4 is also indicated.

TABLE 2. Putative EBNA-3C transactivation domain

Cell line	Cell type	Fold activation by Gal4-3CGln[a]
Loukes	EBV$^-$ BL	27.5
BL2	EBV$^-$ BL	7
BJAB	EBV$^-$ BL	7.6
IB4	EBV$^+$ lymphoblastoid	6
HSB-2	T cell	19
HEp-2	Epithelial	11

[a] Data are presented as fold percent acetylation, relative to that of the negative control, Gal4 (which was set to 1). Data are from a total of four transfections.

3C-Gal4 fusion protein in plasmid pGal4-3CGln. This 3C-Gal4 chimeric construct was transfected into cells together with a CAT reporter gene under the control of Gal4 binding sites. Representative results are presented in Fig. 5A (left panel). Gal4-3CGln was able to activate expression of the reporter gene with approximately 25% activity, relative to that of the very strong transactivator Gal4-VP16. The Gal4 binding domain alone had no effect on the activity of the reporter gene. As a control, we demonstrated that Gal4-3CGln had no effect on the reporter plasmid without Gal4 binding sites (Fig. 5A, right panel). The effects of this EBNA-3C activation domain were tested in a variety of cell types, and it was active in all of the cells tested (Table 2). The activity of the positive control, Gal4-VP16, was 2- to 20-fold greater than that of Gal4-3CGln, depending on cell type (data not shown). This demonstrates that this region of EBNA-3C can indeed act as a transcription activation domain and does not function in a cell type-specific manner.

In addition, we tested the ability of the putative EBNA-3C transactivation domain to restore the transactivation domain of an EBNA-2 deletion mutant. Previous characterization of EBNA-2 has demonstrated that there is a transactivation domain in the carboxyl terminus of EBNA-2 (4). Deletions in this region of EBNA-2 prevent EBV-mediated transformation (5) and transactivation of EBNA-2-responsive promoters (39a). We cloned the glutamine-rich domain of EBNA-3C described above into this EBNA-2 deletion mutant to create pSG5-EBNA-2Δ/3CGln. As seen in Fig. 6, the resulting EBNA-2Δ/3C chimera transactivated E2RSP-CAT sevenfold, relative to that of pSG5-EBNA-2Δ. Although the chimeric molecule was approximately threefold weaker than wild-type EBNA-2 in transcriptional activity, these data indicate that the glutamine-rich region of EBNA-3C is able to partially restore the function of deleted EBNA-2 as a transcriptional activator and, therefore, can function as a transactivation domain in a known EBV transcription factor.

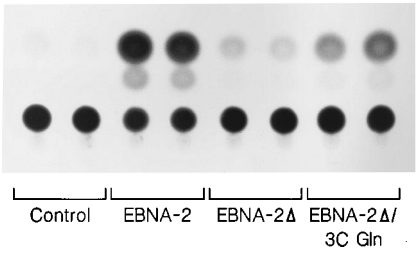

FIG. 6. The EBNA-3C transactivation domain can partially substitute for the deleted transactivation domain of EBNA-2. The EBV-negative cell line Loukes was transfected with the EBNA-2-responsive promoter E2RSP-CAT together with either a control expression vector, EBNA-2 expression vector, expression vector for EBNA-2 with a deletion in its transactivation domain (EBNA-2Δ), or EBNA-2Δ with its deleted residues replaced by the EBNA-3C activation domain (EBNA-2Δ/3CGln).

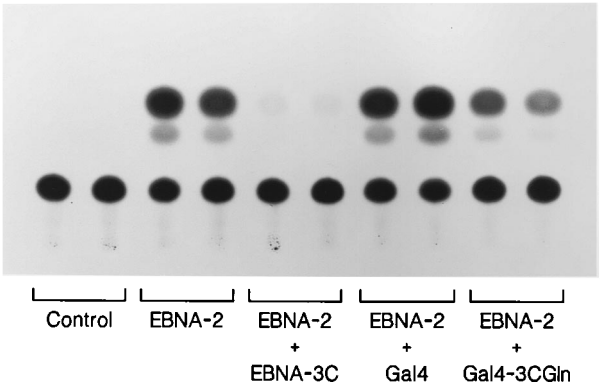

FIG. 7. Downregulation of EBNA-2-responsive sequences is not due to the EBNA-3C transcription activation domain. The EBV-negative B-cell line BL2 was transfected with E2RSP-CAT and either the control expression vector or an expression vector for EBNA-2 alone or in the presence of EBNA-3C, Gal4, or the Gal4 expression vector that contained the putative EBNA-3C activation domain, Gal4-3CGln.

The EBNA-3C transactivation domain is not responsible for EBNA-3C-mediated inhibition of EBNA-2 responsiveness. One possible explanation for the EBNA-3C-mediated inhibitory effect on EBNA-2 is that these proteins compete for the basal transcriptional machinery, a phenomenon known as squelching (3). To address this possibility, we examined the ability of Gal4-3CGln to interfere with activation of E2RSP-CAT by EBNA-2. This fusion protein should not be able to bind to the promoter, but it does contain an activation domain. Therefore, if interference is due to the sequestering of basal transcription factors, inhibition of EBNA-2-induced activity should be observed with this construct. In contrast to the total abrogation of activity in the presence of EBNA-3C, the Gal4-3CGln fusion protein had only a slight effect on EBNA-2-stimulated transcription from the EBNA-2 response element (Fig. 7). Gal4 alone had no effect. In addition, the effects of EBNA-3C on EBNA-1-mediated activation were tested by using a reporter construct that was preceded by EBNA-1-responsive sequences. EBNA-3C had no effect on EBNA-1-mediated activation. These data suggest that residues other than those in the transactivation domain of EBNA-3C are critical for downregulation and that this effect is specific for EBNA-2-mediated transactivation.

DISCUSSION

EBNA-3C has been proposed to act as a transcriptional regulator on the basis of its ability to activate CD21 and LMP-1 expression, its nuclear location, and the presence of a domain that is homologous to a transcription factor DNA-binding motif. We have used transient transfection assays to demonstrate that the effect of EBNA-3C occurs at the level of transcription. Here, we have reported two effects of EBNA-3C on the LMP-1 promoter, inhibition of the activation normally elicited by EBNA-2 and activation in the presence of EBNA-2 which is greater than that seen with EBNA-2 alone. Although these effects seem paradoxical, our data suggest that these activities are mediated by distinct DNA sequences and that each of these activities plays a role in the regulation of LMP-1 expression. The fact that both inhibition and activation can be demonstrated in the same cell line suggests that the different effects preside at different stages in the cell cycle and reflect changes

in the abundance or activity of various transcription factors. Since activation was not seen in Louckes cells under any conditions or with the RBP-Jκ-deleted LMP-1 promoter, it is possible that activation is mediated by a factor(s) that is not present in this cell line.

The ability of EBNA-3C to inhibit the LMP-1 promoter is clearly linked to the RBP-Jκ transcription factor since RBP-Jκ binding sites are both required and sufficient for EBNA-3C-mediated inhibition. The ability of EBNA-3C to inhibit EBNA-2-mediated activation of −225LMP-CAT demonstrates that a single RBP-Jκ site, located at −223, is sufficient for this effect. Our data suggest that this is a result of direct physical association between EBNA-3C and RBP-Jκ.

However, the DNA sequences through which EBNA-3C exerts activation of the LMP-1 promoter are likely to be distinct from the RBP-Jκ binding sites. The fact that EBNA-3C represses EBNA-2-stimulated transcription from the isolated EBNA-2 response element, which is composed of RBP-Jκ binding sites, clearly demonstrates that EBNA-3C does not act in a manner analogous to that of EBNA-2 by binding to the promoter via RBP-Jκ and providing an activation domain, even though the experiments described here demonstrate that EBNA-3C contains sequences which can act as a transcriptional activation domain. Since RBP-Jκ protein has been characterized as an inhibitor, one possible explanation for EBNA-3C-mediated activation is the relief of inhibition by RBP-Jκ, leading to increased transcription as a result of the binding of other transcription factors to the promoter. Although this may explain activation by EBNA-3C alone, it does not explain augmented activity by EBNA-3C in the presence of EBNA-2, since mutation of the RBP-Jκ sites leads to inhibition rather than increased activation of the promoter by EBNA-2, relative to that of the wild-type promoter. The most convincing demonstration that EBNA-3C mediates its activation via distinct sequences is its ability to activate the RBP-Jκ-deleted LMP-1 promoter. The ability of EBNA-3C to activate expression from the LMP-1 promoter is consistent with the findings of Allday and Farrell (2), who demonstrated that stable expression of EBNA-3C in Raji cells, which normally express only a truncated EBNA-3C protein, prevents a decrease in LMP-1 which otherwise occurs in growth-saturated arrest.

The fact that EBNA-3C contains sequences that are homologous to the activation domain of the cellular transcription factor Sp1 is consistent with our demonstration that EBNA-3C can activate expression from the LMP-1 promoter. Indeed, this domain can function as an activation domain in two heterologous proteins (Fig. 6 and 7), Gal4 (which binds directly to DNA) and EBNA-2 (which interacts indirectly with DNA). The glutamine-rich activation domain of the transcription factor Sp1 also contains bulky hydrophobic residues but few charged residues (10). In all of these respects, the putative activation domain of EBNA-3C is similar to that of Sp1. This region of EBNA-3C is also rich in prolines; proline-rich transactivation domains are present in a number of transcription factors, such as CTF-1 (32). In addition, there are several examples of proteins which have both glutamine- and proline-rich activation domains, such as yeast glucose-regulated transcription factor CCR4 and rat hepatocyte nuclear factor 1 (8, 37). Activation domains generally interact with components of the basic transcriptional machinery; different classes of activation domains have different targets, such as the TATA box-binding protein, TFIIB, and various TATA box-binding protein-associated factors (10, 21, 42). Conclusive demonstration that the putative activation domain of EBNA-3C indeed functions as such in intact EBNA-3C protein will require a demonstration that this domain interacts with one of these basal transcription factors. Similar residues are also present in EBNA-3A and EBNA-3B, which may indicate that these EBNAs are also involved in transcriptional regulation. Indeed, EBNA-3B has recently been shown to be associated with increased expression of certain cellular proteins, though it is not clear that this is a direct effect (41).

The ability of EBNA-3C to both activate and inhibit LMP-1 gene expression underscores the complex nature of LMP-1 gene regulation. It is possible that EBNA-3C interacts with both RBP-Jκ and an activating factor but that only one protein can be bound at a time or only one complex can interact with DNA because of adjacent binding sites in the promoter. Interestingly, greater activation by EBNA-3C can often be obtained when the RBP-Jκ site is deleted (Fig. 4 and data not shown), suggesting that net promoter activity is the result of balancing inhibition and activation. These activities may be regulated through posttranslational modifications, such as protein phosphorylation, which can alter the ability of a transcription factor to bind to DNA as well as to interact with other proteins (24, 29). Although EBNA-3C is a phosphoprotein (40), nothing is known about the kinase(s) that is responsible for phosphorylation, whether phosphorylation varies during the cell cycle, or whether such modification has a resultant regulatory effect. In addition, the LMP-1 promoter contains potential binding sites for numerous cellular transcription factors, some of which may regulate LMP-1 expression in a cell cycle-dependent manner. LMP-1 is clearly an important protein in EBV-mediated immortalization of primary B cells. This protein is known to activate expression of the cellular proto-oncogene bcl-2 (14), as well as the B-cell activation antigen CD23 (47). Overexpression of LMP-1, however, is toxic to cells in culture (31). It is very likely, therefore, that expression of LMP-1 is tightly regulated. However, the regulation of LMP-1 expression is not likely to be the only function of EBNA-3C; of greater interest is the likelihood that EBNA-3C also activates expression of cellular genes that are critical to immortalization and proliferation of EBV-infected B cells. Further delineation of the EBNA-3C-responsive sequences will allow us to identify such cellular genes.

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