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Epstein-Barr Virus Nuclear Protein 2A Forms Oligomers In Vitro and In Vivo through a Region Required for B-Cell Transformation

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Epstein-Barr virus nuclear antigen 2 (EBNA-2) has been shown to be indispensable for immortalization of latently infected B lymphocytes, and it has been shown that EBNA-2 exists in a high-molecular-weight complex in these cells. In order to study the components of this protein machinery, we have purified baculovirus-expressed EBNA-2 from insect cells to greater than 95% homogeneity. We have shown by both gel filtration and sucrose gradient analysis that the purified material corresponds to a multimer containing eight EBNA-2 subunits. This multimeric complex is stable in 1.0 M NaCl, suggesting that the self-association is quite strong in vitro. By expressing portions of the EBNA-2 open reading frame to generate fusion proteins in yeast cells, we have used the two-hybrid system to demonstrate that this self-association occurs in vivo and is mediated at least in part by a domain of EBNA-2 encompassing amino acids 122 to 344. Mutational analysis of the self-association function suggests that two subdomains that flank amino acid 232 may each play a role in EBNA-2 protein-protein interaction.

Epstein-Barr virus (EBV) plays a pathogenic role in two human tumors, Burkitt's lymphoma and nasopharyngeal carcinoma (13, 32), and causes the benign lymphoproliferative disease infectious mononucleosis (20). In vitro, EBV can latently infect and immortalize human B lymphocytes, enabling them to proliferate indefinitely in culture as lymphoblastoid cell lines (37). This latent B-cell infection is probably related to the lymphomagenic potential of EBV seen in immunosuppressed patients who demonstrate a high incidence of EBV-associated lymphomas (19, 26, 36).

Of the six nuclear and two membrane proteins expressed in latently infected, growth-transformed B lymphocytes, EBV nuclear antigen 2 (EBNA-2) has been shown to be essential for the establishment of B-cell immortalization (18). The EBV strain, P3HR1, which has suffered a deletion of the EBNA-2 open reading frame, cannot transform B cells (39), while reintroduction of the EBNA-2 open reading frame into P3HR1 virus restores its transforming activity (18). EBNA-2 also acts as a transcriptional activator of both viral and cellular genes, and it has been inferred that the immortalization function of EBNA-2 is related to its potential to act as a transcriptional transactivator. EBNA-2 activates the transcription of the viral genes encoding the terminal protein (53) and the latent membrane protein (1, 49) and also activates expression of cellular genes, including CD21, CD23 (6, 11, 47, 48), and c-*fgr* (27). Peptide domains of EBNA-2 that are essential for lymphocyte transformation coincide with those necessary for transactivation (8), supporting the concept that the immortalization function of EBV is related to its role as a transcriptional activator.

EBNA-2 exists in two allelic forms designated A (found in B95-8 and M-ABA strains of the virus) and B (found in the Jijoye strain of EBV). The type A alleles of EBNA-2 transform B lymphocytes more efficiently (41), and transactivate the expression of CD23 to a higher level (10), than do type B alleles. The A and B forms of EBNA-2 differ in the sequence of a central region of the molecule (12), leading to the conclusion that this region of EBNA-2 plays a critical role in determining the immortalization function of EBV.

Because there is no evidence that EBNA-2 binds to DNA in a sequence-specific fashion but since it does apparently act as a transcription activator, it has been assumed that EBNA-2 acts by forming a functional complex with one or more viral or cellular proteins to activate transcription. Zimber-Strobl et al. (52) have presented evidence that EBNA-2 is included in a complex that binds to a DNA sequence in the terminal protein promoter. Grasser et al. (16) have shown that EBNA-2A sediments in a high-molecular-weight complex from lymphocyte cell extracts. From the Grasser study, it was not possible to determine whether the sedimentation properties of EBNA-2 resulted from oligomerization of EBNA-2, from the formation of a heterooligomeric complex, or from both. Grasser et al. (17) have presented indirect evidence that recombinant EBNA-2 (rEBNA-2) can bind to a 130-kDa cellular protein. More recently, EBNA-2 has been shown to associate with a factor binding to a DNA sequence in the viral *Bam*C promoter which mediates EBNA-2 transactivation (31). Since several transcription factors have been shown to form oligomeric structures (23), it is important to determine the quaternary structure of EBNA-2 in the presumed multiprotein complexes in which it functions.

We have previously expressed EBNA-2 at high levels in insect cells infected with a recombinant baculovirus strain. Here we show the purification of rEBNA-2 and the in vitro formation of a multimeric complex that is stable in both low-salt and high-salt solutions. In order to determine whether this oligomerization may occur in vivo, we have constructed fusion proteins containing regions of EBNA-2 and expressed them in yeast cells. Using the two-hybrid system (15), we have demonstrated that EBNA-2 self-associates through a domain of the molecule that has previously been shown to be important for both transactivation and transformation.

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MATERIALS AND METHODS

Cells and tissue culture. Sf9 cells were grown in Grace's insect medium supplemented with 3.3 gm of both TC Yeastolate and lactalbumin hydrolysate per liter, 10% fetal bovine serum, penicillin, and streptomycin.

Purification of rEBNA-2. The construction of the EBNA-2 baculovirus transfer vector and the purification of recombinant baculoviruses expressing EBNA-2 were described previously (43). Sf9 cells were infected with the recombinant virus stocks at a multiplicity of 10 for 48 h. All maneuvers were carried out at 4°C. The infected cells were harvested and lysed by the addition of 5 pellet volumes of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 20% glycerol, 20 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 μg of aprotinin per ml, 0.5 mM phenylmethylsulfonyl fluoride) and disrupted by 10 strokes in a Dounce homogenizer with a B pestle. The cell lysate was clarified by centrifugation for 10 min at 12,000 × *g*. The lysate was adjusted to a protein concentration of 10 mg/ml, and $(NH_4)_2SO_4$ was added to a final saturation of 20%. The mixture was stirred for 4 h and centrifuged for 30 min at 17,000 × *g*. The supernatant was adjusted to 40% saturation $(NH_4)_2SO_4$ and centrifuged, and the EBNA-2-containing precipitate was suspended overnight in buffer A. The protein suspension was applied to a phenyl-Sepharose column which had been equilibrated with buffer A. The column was washed with buffer A containing 0.2 M $(NH_4)_2SO_4$ and subsequently eluted with 5 column volumes of buffer A lacking NaCl. Peak protein-containing fractions were pooled and dialyzed against buffer B (10 mM sodium phosphate [pH 7.0], 20 mM NaCl, 20% glycerol). The dialysate was applied to a hydroxylapatite column preequilibrated with buffer B. The column was washed with buffer B and eluted with 5 column volumes of buffer B containing 50 mM sodium phosphate, pH 7.0. Peak protein-containing fractions were pooled, adjusted to 50% glycerol, and stored at −20°C. Protein concentrations were determined by the method of Bradford (4). This EBNA-2 preparation was at least 95% homogeneous and was used in the experiments described below.

Gel filtration chromatography. The purified EBNA-2 eluted from the hydroxylapatite column was applied to a Superose 12 (Pharmacia) column preequilibrated with buffer C (50 mM sodium phosphate [pH 7.0], 150 mM NaCl, 20% glycerol) at a flow rate of 0.5 ml/min. Internal molecular weight standards (thyroglobulin, ferritin, catalase, aldolase, and bovine serum albumin) were used to calibrate retention times from the column. The peak fractions were collected and analyzed by discontinuous sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) (29) followed by silver staining.

Sucrose gradient centrifugation of purified EBNA-2. Either purified EBNA-2 or a combination of native molecular weight markers was layered onto an 11.5-ml 10 to 30% sucrose gradient in phosphate-buffered saline with 1.0 M NaCl and 0.5 mM phenylmethylsulfonyl fluoride. The gradient was spun in a Beckman SW41 rotor for 15 h at 35,000 rpm at 4°C. Twenty fractions were collected, and the proteins were precipitated at −20°C with 2 volumes of cold, absolute ethanol. An aliquot of each fraction was analyzed by SDS-PAGE and silver staining.

Construction of EBNA-2 fusion proteins. Plasmids pBTM116 and pGAD424 which contain the complete LexA protein coding sequence (amino acids 1 to 202) and the GAL4 activation domain (amino acids 768 to 881), respectively, were used as yeast vectors in this study (2). These vectors have identical polylinker restriction sites located at the 3' end of the open reading frame of the LexA DNA binding domain and the GAL4 activation domain. The LexA and GAL4 coding sequences of these plasmids are under the control of the yeast ADH1 promoter. Plasmid pBTM116 includes the yeast TRP1 gene and encodes ampicillin resistance. Plasmid pGAD424 encodes ampicillin resistance and the yeast LEU2 gene. The sequence encoding the entire EBNA-2 protein (E2) was removed from the plasmid pE2 by cleavage with *Bgl*II. An *Eco*RI fragment encompassing amino acids 122 to 344 (ΔE2Δ) was removed from plasmid 4 described previously (22). The EBNA-2 sequences lacking the C-terminal acidic activation domain (E2Δ) were retrieved from the plasmid pE2 (42) by amplification using PCR with primers containing *Bgl*II restriction sites at their 5' ends. These EBNA-2 sequences encoded either the entire protein fused to the GAL4 activation domain (GAD-E2) or segments of EBNA-2 fused to the LexA DNA binding domain (BTM-ΔE2Δ and BTM-E2Δ) or the GAL4 activation domain (GAD-ΔE2Δ and GAD-E2Δ). For illustrations of the amino acid coordinates of each of these constructs, see Fig. 2. Plasmid pE2 was used to generate a set of linker insertion mutants in the EBNA-2 open reading frame by the method of Stone et al. (45). Plasmids were linearized by partial digestion with *Hae*III, *Nla*IV, or *Rsa*I, linear molecules were isolated, and an *Xho*I linker was inserted into the resultant blunt-end restriction site. These plasmids were sequenced, and the inserted codons (see Fig. 5) were determined. The mutated sequences were retrieved from the parent plasmids by PCR and inserted into the *Eco*RI site of pBTM116. Fusion proteins containing sequences derived from type B EBNA-2 were retrieved from the Jijoye cell line (American Type Culture Collection) by PCR using primers containing cohesive end restriction sites for cloning into the same vectors used for the EBNA-2A fusion proteins. The PCR primers used to generate some of these clones are as follows: for the 5' ends of the insertion mutants, 5'GGAGAATTCGGACCTCTAGCATCT GCTATGCGA3'; for the 3' ends of the insertion mutants, 5'GGAGAATTCCCCCCTGCTCTGTCCCCGGCTCTG3'; for the 5' end of E2Δ, 5'GGAGATCTCCCATCATGCCTA CATTCTATCTT3'; for the 3' ends of E2Δ and E2B, 5'GG AGATCTCCTCCGGTTCATGTATTGGTGAAA3'; and for the 5' end of E2B, 5'GGAGAATTCCAGCGCAGGGATGC CTGGAC3'.

Yeast transformation. Yeast transformation was performed by the methods of Schiestl and Geitz (42) and Hill et al. (21) as modified by Bartel et al. (2). The *Saccharomyces cerevisiae* CTY10-5d strain (constructed by Rolf Sternglanz, State University of New York at Stony Brook), genotype ***MAT*a** *ade2 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::lexA op-lacZ*, contains four LexA binding sites upstream of the transcription start site of the *GAL1-lacZ* gene from which the upstream activation sequence GAL(UAS_G) has been deleted. It was grown in yeast extract-peptone-dextrose at 30°C with shaking until cells reached mid-log phase. Cells were pelleted, washed once with distilled water, and resuspended in 10 mM Tris (pH 7.5)–1 mM EDTA–0.1 M lithium acetate (TE/LiAc). To 100 μl of yeast suspension, 1 μg of each plasmid DNA and 20 μg of denatured salmon sperm carrier DNA were added prior to the addition of sterile polyethylene glycol (molecular weight of ~3,250) to a final composition of 40% polyethylene glycol in 1× TE/LiAc. The mixture was incubated at 30°C for 30 min, after which dimethyl sulfoxide was added to a final concentration of 10%. Cells were heat shocked at 42°C for 15 min, and pellets were formed by a brief spinning and suspended in 100 μl of Tris-EDTA. The cells were then plated onto selective minimal media lacking tryptophan and leucine (SD-trp-leu) and grown at 30°C for 2 to 3 days until the colonies achieved a size sufficient to assay for β-galactosidase activity.

Assays for β-galactosidase activity. Transformation prod-

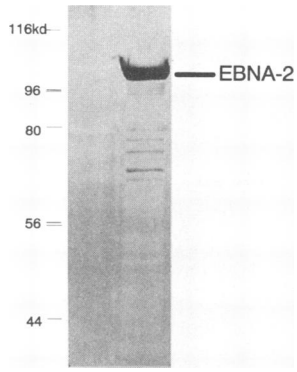


FIG. 1. Silver-stained gel of purified, rEBNA-2 prepared from insect cells. Minor, negatively staining bands are not detectable by Coomassie blue staining. The location of molecular mass standards is indicated at the left.

ucts were assayed for β-galactosidase activity by the replica filter assay of Breeden and Nasmyth (5) utilizing the hydrolysis of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to identify positive, β-galactosidase-producing colonies.

Quantitative assay of β-galactosidase activity was done as follows. Five separate colonies were picked from the transformation plates and grown to mid-log phase in SD-trp-leu medium. The optical density at 600 nm was determined. Following this, 3 ml of culture was centrifuged, washed in Z buffer (100 mM sodium phosphate, 10 mM KCl, 10 mM $MgSO_4$, 40 mM β-mercaptoethanol [pH 7.0]), and suspended in 150 μl of Z buffer. To this was added 50 μl of $CHCl_3$ and 20 μl of 0.1% SDS. The suspension was vortexed for 30 s, and 700 μl of Z buffer containing 1 mg of *o*-nitrophenyl-β-D-galactopyranoside was added prior to incubation at 37°C until a distinct yellow color appeared. The reaction was stopped by the addition of 500 μl of 1.0 M Na_2CO_3. The optical density at 420 nm was determined, and units of β-galactosidase activity were calculated by the formula of Miller (33). The highest and lowest of the five β-galactosidase activity values for each point were discarded, and the means of the three remaining values were used to determine the units of β-galactosidase activity in the quantitative assays.

RESULTS

Purification of rEBNA-2 from insect cells. Insect cell cultures were infected with the recombinant strain of baculovirus directing the expression of EBNA-2 (43) at a multiplicity of 10 for approximately 48 h. The cells were lysed by Dounce homogenization in hypotonic buffer, followed by differential $(NH_4)_2SO_4$ precipitation and sequential chromatography on phenyl-Sepharose CL4B and hydroxylapatite (see Materials and Methods) to yield a product that was greater than 95% pure by silver staining (Fig. 1). Some lower-molecular-weight material represented degradation products of EBNA-2 generated during the purification protocol. This was demonstrated by determining that this material reacted to EBNA-2-specific serum of both human and rabbit origin (data not shown). The apparent retarded migration of EBNA-2 in this and the following experiments is consistent with our prior observation that in this system, rEBNA-2 displays an apparent molecular mass of approximately 98 kDa and comigrates with EBNA-2 expressed from B95-8-infected cell lines (43).

Gel filtration and sucrose gradient analysis of rEBNA-2. The formation of high-molecular-weight complexes of EBNA-2 seen in lymphocytes (16) has been proposed to result from the formation of homo-oligomers, hetero-oligomers involving other viral or cellular proteins, or a combination of these. Because purified, baculovirus-expressed EBNA-2 does not contain any proteins of lymphocyte origin, it should be useful for evaluating the potential for EBNA-2 to form oligomers. To this end, we determined the molecular weight of the purified material by both gel filtration and sucrose gradient analysis. Purified rEBNA-2 was subjected to fast protein liquid chromatography (FPLC) on a Superose 12 gel filtration column and found to elute in a major peak with mobility corresponding to an apparent molecular mass of ~480 kDa (Fig. 2A). Aliquots of the fractions from this column were electrophoresed on SDS-PAGE, silver stained, and found to migrate at the expected position of rEBNA-2. A lower-molecular-weight band centered around fraction 23 represents a breakdown product, since it is immunoreactive with EBNA-2 antisera (data not shown). The calculated molecular mass of EBNA-2 from the M-ABA strain of EBV, from which the baculovirus transfer vector was derived, is 52 kDa. Thus, the bulk of the purified rEBNA-2 is composed of a complex consisting of approximately eight monomeric subunits. To confirm this result and make a qualitative estimate of the strength of the interaction maintaining this oligomeric complex, we performed sucrose gradient centrifugation under conditions of increasing salt concentration, as shown in Fig. 2B. Purified rEBNA-2 was centrifuged through a 10 to 30% sucrose gradient in 1.0 M NaCl. The bulk of the rEBNA-2 sediments in a region of the gradient corresponding to an apparent molecular mass of ~440 kDa, corroborating the results of gel filtration. A series of lower-molecular-weight bands seen with SDS-PAGE probably represent degradation products generated in the sucrose gradient since they were not present in the starting material and these bands are immunoreactive with anti-EBNA-2 antisera (data not shown). Similar results were obtained in 0.15 and 0.5 M NaCl. The stability of the oligomeric complex in high salt concentrations demonstrates that rEBNA-2 has a strong self-affinity under these conditions.

Self-association of EBNA-2 fusion proteins in the yeast two-hybrid system. While purified rEBNA-2 has an intrinsic ability to form oligomers, the results presented in Fig. 2 could have resulted in part from the nonphysiologically high concentration of EBNA-2 present in these experiments or else from some variation in the posttranslational modification of EBNA-2 expressed in insect cells that is distinct from EBNA-2 expressed in lymphocytes. In order to determine whether EBNA-2 has the potential to self-associate in vivo we applied the two-hybrid system (15). This method uses a very sensitive genetic yeast assay that is based on the reconstitution of the function of the yeast transcriptional activator, GAL4. It is based on the modular nature of eukaryotic transcription regulatory factors in which the DNA binding domain and transcription activation domain are separable (25) and has been successfully employed to identify members of transcription complexes (51). Two groups of fusion genes encoding hybrid proteins were constructed: one fuses EBNA-2 sequences to the DNA-binding protein LexA, and the other fuses EBNA-2 to the transcriptional activation domain of GAL4. The plasmids encoding these fusion genes contain distinct selectable markers. Following transformation with expression plasmid pairs, the interaction of these fusion proteins through their EBNA-2 moieties results in reconstitution of a functional transcriptional activator, which can be assayed by the activation of a reporter gene (*Escherichia coli* β-galactosidase) that is placed downstream from four LexA binding sites.

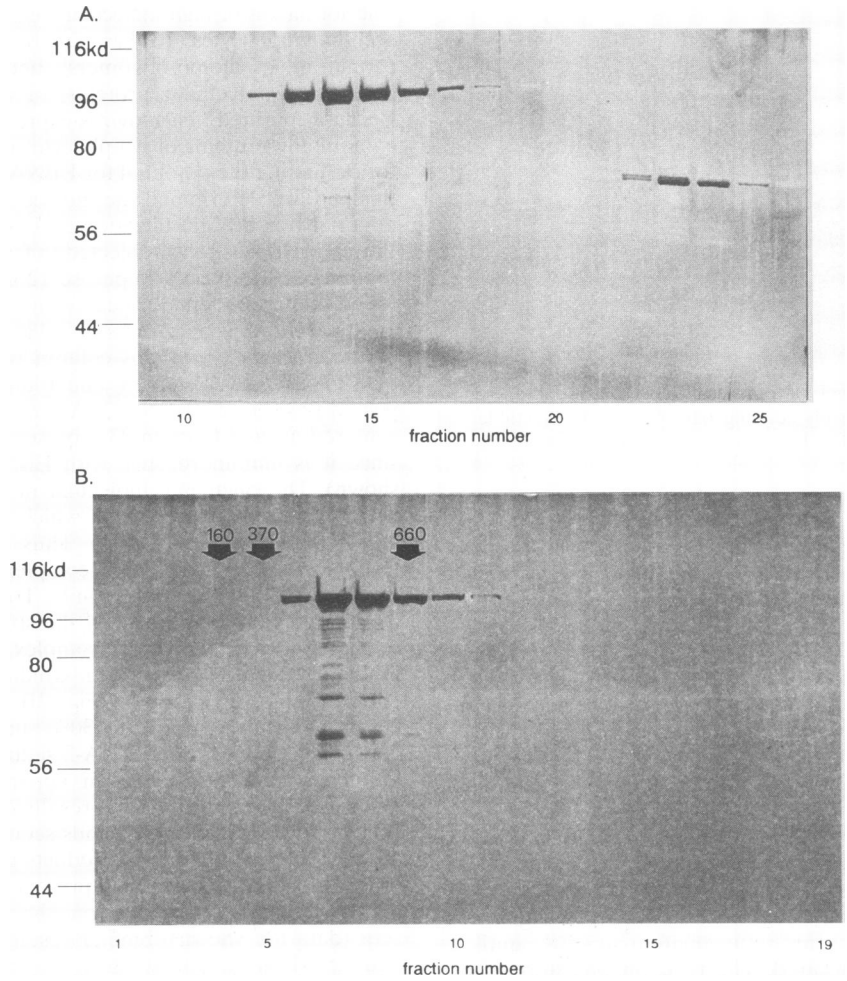


FIG. 2. (A) Superose 12 FPLC gel filtration chromatography of purified rEBNA-2. The material depicted in Fig. 1 was chromatographed as described in Materials and Methods. The column was calibrated by chromatography of molecular mass standards, and purified rEBNA-2 was chromatographed under identical conditions. The protein standards were native thyroglobulin, ferritin, catalase, aldolase, and bovine serum albumin. (B) Sucrose gradient analysis of rEBNA-2. The material depicted in Fig. 1 was applied to a 10 to 30% sucrose gradient in 1.0 M NaCl–10 mM sodium phosphate buffer, pH 7.2. An identical gradient was run with marker proteins. Fractions were collected, and a portion was ethanol precipitated, run on SDS–10% PAGE, and silver stained. The peak positions of sedimentation of the marker proteins are indicated (arrows) at 160 (rabbit muscle aldolase), 370 (phosphorylase *a*), and 660 (thyroglobulin) kDa.

Figure 3 illustrates the constructs generated for these experiments. The plasmid encoding the LexA DNA binding domain, pBTM116, was used to construct fusions containing the regions of EBNA-2 that lack either both the first 122 and final 141 amino acids (BTM-ΔE2Δ) or only the carboxyl-terminal 57 amino acids (BTM-E2Δ). The terminal 57 amino acids of EBNA-2 contain a functional acidic activation domain (9, 22) and were deleted from the LexA fusion proteins in order to eliminate activation of the reporter gene by the DNA-binding EBNA-2 fusion protein alone. The LexA fusion plasmids contain the yeast TRP1 gene as a selectable marker. The plasmid pGAD424, which encodes the activation domain present in amino acids 768 to 881 of GAL4, was used to generate fusion proteins containing those segments of EBNA-2 present in the LexA fusion constructs (GAD-ΔE2Δ and GAD-E2Δ) and an additional fusion protein containing the entire EBNA-2 open reading frame, GAD-E2. This plasmid contains the yeast LEU2 gene as a selectable marker. The plasmid pGAD-ΔE2Δ failed to express an EBNA-2 fusion protein, as determined by immunoblot analysis (data not shown). These plasmids were used to cotransform the yeast strain, CTY10-5d, which contains four LexA binding sites upstream from the *E. coli* β-galactosidase gene and contains mutations in genes encoding both tryptophan and leucine, such that only yeast transformants expressing both the LexA and GAL4 fusion proteins will grow on medium lacking tryptophan and leucine. The degree of transcriptional activation, assayed by expression of β-galactosidase activity, was determined by a filter assay (5). In this assay, the restoration of transcriptional activation of the β-galactosidase reporter gene is qualitatively monitored by hydrolysis of the chromogenic substrate, X-Gal. Figure 4 shows a photograph of this in situ assay for β-galactosidase activity.

Figure 4 shows that transformation by both LexA-EBNA-$2_{122-344}$ plus GAL4-EBNA-2_{1-485} (BTM-ΔE2Δ plus GAD-E2 [Fig. 4e]) and LexA-EBNA-$2_{122-344}$ plus GAL4-EBNA-2_{1-428} (BTM-ΔE2Δ plus GAD-E2Δ [Fig. 4f]) resulted in significant levels of β-galactosidase activity. With negative controls, transformation with the vector plasmids alone (BTM-ADH1 plus GAD [Fig. 4a]) or with the vector plasmid pGAD424 and

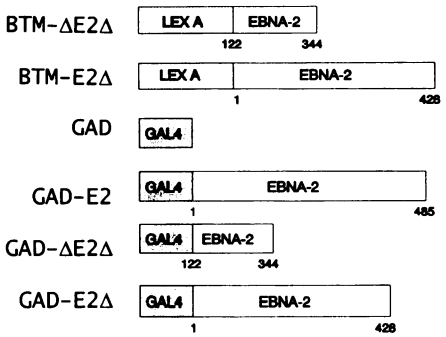


FIG. 3. Fusion proteins generated by constructs used in hybrid association experiments. The constructs BTM-ΔE2Δ and BTM-E2Δ were generated in the vector pBTM116, which encodes the entire LexA protein (the stippled region designated LexA) under control of the ADH1 promoter. The GAD-E2, GAD-ΔE2Δ, and GAD-E2Δ constructs were generated in pGAD424, which expresses amino acids 768 to 881 of the GAL4 protein, containing an activation domain. The amino acid coordinates of the EBNA-2 portion of each hybrid protein are indicated. The GAD construct represents the product of the pGAD424 vector.

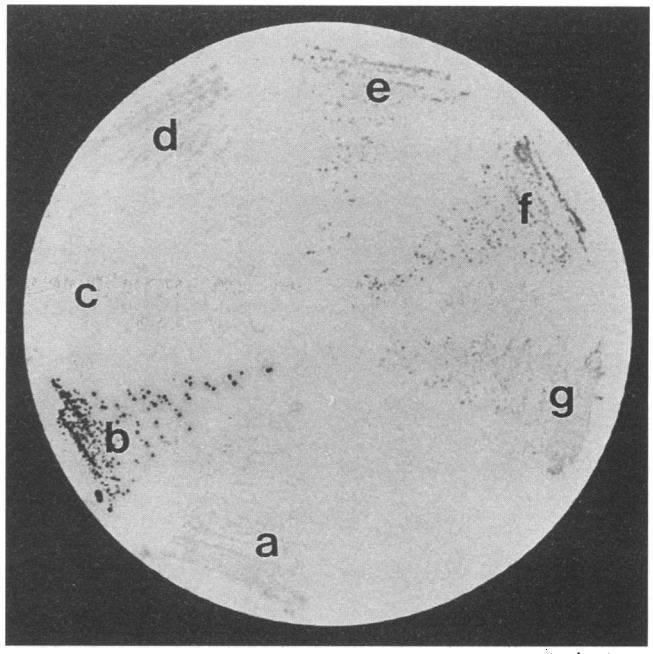


FIG. 4. Interaction of EBNA-2-containing hybrid proteins. A filter was streaked with clones derived from cotransformation of CTY10-5d with plasmids encoding the indicated fusion proteins and overlaid on selective plates for growth before in situ assay for β-galactosidase activity. The dark color resulting from the hydrolysis of X-Gal indicates a protein-protein interaction. (a) BTM116 plus GAD424; (b) BTM-SIR4 plus GAD-SIR4; (c) BTM116 plus GAD-E2; (d) BTM-ΔE2Δ plus GAD424; (e) BTM-ΔE2Δ plus GAD-E2; (f) BTM-ΔE2Δ plus GAD-E2Δ; (g) BTM-E2B plus GAD-E2B.

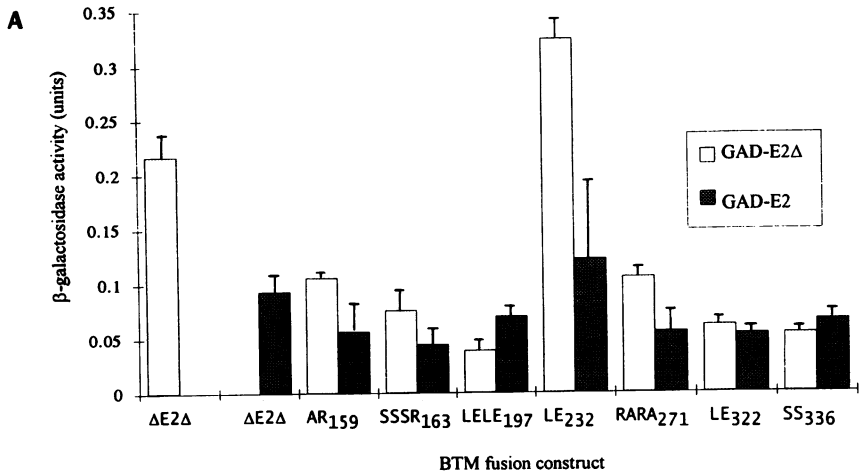
pBTM-ΔE2Δ (BTM-ΔE2Δ plus GAD [Fig. 4d]) and the vector pBTM116 with GAD-E2 (BTM-ADH1 plus GAD-E2 [Fig. 4c]) resulted in no detectable β-galactosidase activity. Cotransformation of the LexA vector BTM-ADH1 with GAD-E2Δ also yielded no detectable activity (data not shown). Thus, in the absence of EBNA-2 sequences in both fusion proteins, no β-galactosidase activity was detected. This result demonstrates that EBNA-2 sequences contained in BTM-ΔE2Δ (amino acids 122 to 344) are sufficient to mediate this interaction in vivo. As a positive control, we used plasmids expressing fusion proteins containing the yeast SIR4 gene (BTM-SIR4 plus GAD-SIR4). This protein is involved in repression of expression of the silent mating-type locus cassette and has been demonstrated to self-associate (7). The same pattern of expression was obtained by using a different host-vector expression system employing the GAL4 DNA binding domain in the EBNA-2 fusion protein constructs (data not shown).

Mutational analysis of the region involved in EBNA-2 self-association. To determine whether a specific segment of EBNA-2 mediates the interaction detected by the two-hybrid system, a set of linker insertion mutants were constructed and were expressed as fusion proteins with the LexA protein in the vector pBTM116. Figure 5B shows the amino acid sequence of the M-ABA strain of EBNA-2, with the underlined portion indicating those amino acids present in the BTM-ΔE2Δ construct. A set of seven mutations were constructed by in-frame linker insertion at the indicated amino acids. It is assumed that the local alteration of secondary peptide structure resulting from the insertion of additional amino acids will alter the affinity of protein pairs. The LexA-mutant fusion proteins were coexpressed with both GAD-E2 and GAD-E2Δ, qualitative filter assays were performed, clones were picked, and quantitative β-galactosidase activity was determined. The results of this analysis are shown in Fig. 5A. Mutations near amino acids 197, 322, and 336 have the largest negative effect on protein-protein interaction, indicating the importance of these regions in EBNA-2 self-association. These mutations flank a mutation at amino acid 232 which does not disrupt the protein-protein interaction. This suggests that two domains, one between amino acids 122 and 232 and another between amino acids 232 and 344, are important for self-association. In this quantitative analysis, the level of β-galactosidase activity resulting from the yeast SIR4 self-interaction (Fig. 4b) was 10-fold higher than that seen with BTM-ΔE2Δ plus GAD-E2Δ.

Since the region of EBNA-2 that we have implicated in mediating oligomer formation is contained within the region that diverges between the EBNA-2A and EBNA-2B alleles (12), we predicted that EBNA-2B, which demonstrates both weaker B-cell immortalization potential (41) and decreased transactivation of the cellular gene CD23 (10), would form weaker oligomers than type A EBNA-2. To test this hypothesis in the two-hybrid system, we generated fusion proteins derived from EBNA-2B, and determined the relative strengths of self-association as determined by the levels of β-galactosidase activity. The fusion partners both contained sequences beginning at the first amino acid following the polyproline stretch and contained the same carboxyl terminal sequence as the E2Δ fusion protein, since this segment is conserved between the EBNA-2A and EBNA-2B alleles. The entire divergent region was thus encompassed in this fusion pair. Figure 4g shows the qualitative level of β-galactosidase activity for BTM-E2B plus GAD-E2B cotransformation. The quantitative level of β-galactosidase activity for this pair was half of that for BTM-ΔE2Δ plus GAD-E2Δ, which is roughly equivalent to that seen for BTM-ΔE2Δ plus GAD-E2.

DISCUSSION

EBNA-2 is essential for EBV-induced B-lymphocyte immortalization. This work demonstrates that highly purified, rEBNA-2 derived from baculovirus forms octomers in vitro. Purified EBNA-2 originating from the baculovirus system lacks



B

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1    MPTFYLALHG GQTYHLIVDT DSLGNPSLSV IPSNPYQEQL STDPLIPLTI

51   FVGENTGVPP PPPPPPPPPP PPPPPPPPPP PPPPPPSPPP PPPPPPPPQR

101  RDAWTQEPSP LDRDPLGYDV GHGPLASAMR MLWMANYIVR QSRGDRGLIL
                AR159 SSSR163                      LELE197
151  PQGPQTAPQA RLVQPHVPPL RPTAPRILSP LSQPRLTPPQ PLMMPSRPTP
                                        LE232
201  PTPLPPATLT VPPRPTRPTT LPPTPLLTVL QRPTELQPTP SPPRMHLPVL
                                RARA271
251  HVPDQSMHPL THQSIPNDPD SPEPRSPTVF YNIPPMMLPP SQLPPPAAPA
                                LE322          SS336
301  QPPPGVINDQ QSHHLPSGPP WWPPICDPPQ PSKTQGQSRG QSRGRGRGRG

351  RGRGKGKSRD KQRKPGGPWR PEPNTSSPSM PELSPVLGLH QGQGAGDSPT

401  PGPSNAAPVC RNSHTATPNV SPIHEPESHN SPEAPILFPD DWYPPSIDPA

451  DLDESWDYIF ETTESPSSDE DYVGGSSKRP RPSIQ
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FIG. 5. Mutational analysis of the interaction domain of EBNA-2. (A) Quantitative levels of β-galactosidase activity were determined as described in Materials and Methods. Absolute β-galactosidase activities for LexA-EBNA-2 fusion proteins resulting from cotransformation with either GAD-E2Δ (light bars) or GAD-E2 (dark bars) are represented. The mutants were generated by linker insertion mutagenesis and encompass the amino acids underlined in B. Error bars represent the standard deviations of the three median values from five separate determinations of β-galactosidase activity. (B) Complete amino acid sequence of the EBNA-2 protein from the M-ABA strain of EBV, as determined in our laboratory. The position and inserted amino acids of each linker insertion mutant are indicated. The indicated amino acids are inserted following the amino acid designated in boldface print.

other proteins of lymphocyte origin that might account for the sedimentation properties of lymphocyte EBNA-2A reported previously by Grasser et al. (16). We demonstrate that highly purified EBNA-2A has an intrinsic ability to oligomerize in vitro. In order to determine whether oligomers form in vivo, we used the yeast two-hybrid system (15). We have shown that fusion proteins containing a region of EBNA-2A encompassing amino acids 122 to 344 are sufficient to promote self-association in yeast cells. Two domains of EBNA-2 appear to be involved. While this analysis defines a domain of EBNA-2A that is sufficient to mediate oligomerization, it is possible that other regions of the molecule could also be involved in oligomer formation. It is also formally possible that the EBNA-2 interaction detected in the two-hybrid system results from association with yeast proteins, resulting in an indirect interaction.

Other transcription factors have been found to form oligomers through a limited set of structural motifs. c-Fos, c-Jun, C/EBP, and heat shock factor oligomerize through an amphipathic helix containing a heptad leucine repeat on the hydrophobic face (leucine zipper) (23, 30, 38). MyoD, E12, and AP4 form oligomers through two amphipathic helices separated by a short loop (helix-loop-helix) (34). Members of the Myc family form homo- and heterodimers through a combination of leucine zipper and helix-loop-helix structures (3, 24, 51). Sp1 oligomerizes through a discrete, glutamine-rich domain (35). The p53 antioncoprotein forms tetrameric and higher-order oligomeric structures through an evolutionarily conserved amphipathic alpha-helical domain that does not contain a leucine zipper (28, 44). Self-interaction of p53 is mediated through hydrophobic residues (46). The same region that mediates oligomer formation is essential for enhancement of transformation by activated *ras* and E1A (40) and is distinct from the transcriptional activation domain of p53 (14). EBNA-2 is

similar to p53 in that it too lacks a leucine zipper and the oligomerization domain is separate from the transcriptional activation domain. Furthermore, deletion of the oligomerization region prevents B-cell immortalization (8).

EBNA-2 lacks any of the structural motifs seen in the transcription factors listed above. The region involved in oligomer formation contains three or four segments that are predicted to form short alpha helices of up to 16 amino acids which are much smaller than the peptide mediating p53 oligomerization. Mutant $LELE_{197}$ disrupts one of these alpha-helical regions and also inhibits self-association in the two-hybrid system. A short amphipathic helix is predicted for the segment including amino acids 152 through 172, and two of our linker insertion mutants (AR_{159} and $SSSR_{163}$) are predicted to disrupt this structure. While these mutations decrease oligomer formation, other mutations elsewhere in the molecule have similar negative effects. The other mutations that disrupt oligomer formation do not directly interrupt specific structural features that are readily predicted from computer modeling. Because no common structural motif apparently dominates the self-interaction, EBNA-2 may present a novel mechanism for self-association distinct from that observed for other proteins involved in transcriptional activation.

Comparison of this work with the results of a previously published mutation analysis of EBNA-2 (8) suggests that oligomer formation may be necessary for B-cell immortalization by EBNA-2. In the earlier study, a virus with most of this segment deleted (amino acids 142 through 342) was unable to confer transformation potential to recombinant viruses. In the same study, a linker insertion mutant similar to LE_{322}, which depresses oligomerization, also was nontransforming and a mutant similar to LE_{232}, which does not affect oligomerization, was fully transforming. Three of the insertion mutations in the present study (AR_{159}, $SSSR_{163}$, and $LELE_{197}$) are not represented in the data in that study; however, a deletion mutation encompassing the AR_{159} and $SSSR_{163}$ mutations showed diminished transforming ability. At variance with this interpretation is the finding that a mutation close to SS_{336}, which reduces oligomerization, was fully transforming. The data in Fig. 5 show that all linker insertion mutants other than LE_{232} reduced oligomerization measured by the β-galactosidase assay. Thus, it is possible that very subtle disruptions of the structure of GAL-4 fusion proteins nonspecifically reduce apparent oligomerization in this system. The weak self-association that results from coexpression of fusion proteins derived from type B EBNA-2 is consistent with the conclusion that oligomer formation may be necessary for the biological function of EBNA-2, since virus strains containing type B alleles display both less potent transcriptional activation activity and B-cell immortalization (10, 41).

Sequence-specific DNA binding by EBNA-2 has not been demonstrated; however, the molecule functions as a direct transcriptional activator and contains a functional acidic transcription activation domain when fused to a heterologous DNA binding domain (9, 22). It has therefore been generally concluded that EBNA-2 acts as an indirect transcription factor, participating in a multiprotein complex in which other members make direct DNA contact with sequences near EBNA-2-responsive genes. This hypothesis has been confirmed by the finding of a cellular factor that targets EBNA-2 to an EBNA-2-responsive element present in the *Bam*HI C latent gene promoter (31). A small domain of EBNA-2 (amino acids 252 to 344) has been implicated in both hetero- and homo-oligomer formation in this study and that of Ling et al. (31); however, the limits of resolution of both analyses prevent a definitive conclusion regarding the discrete domains of EBNA-2 involved in these separate functions.

The in vitro analysis of EBNA-2 demonstrated formation of an oligomeric complex that was quite stable in both low-salt and high-salt solutions. This high-affinity interaction is at variance with the relatively weak interaction of EBNA-2 fusion proteins expressed in yeast cells. Assuming that the level of β-galactosidase activity is an indirect measure of the affinity of the interaction, the self-affinity of EBNA-2 measured in these experiments is approximately 1/10 of that seen in the yeast protein SIR4. The relative weakness of the interaction may be due to aberrant folding of the fusion proteins that partially mask important interacting domains, alteration of the secondary structure of the activation domain of GAL4 by fusion to EBNA-2, differences in posttranslational modification, or the low levels of expression of EBNA-2 fusion proteins seen in yeast cells. Immunoblot analyses demonstrated that the expression of EBNA-2-containing fusion proteins was low and that a fusion between the entire EBNA-2 open reading frame and LexA was not expressed at a detectable level (data not shown).

The function of EBNA-2 oligomers may be to generate a large structure that acts as an effective nucleation site for the assembly of transcription factors. The presence of multiple transcriptional activation domains in this complex may potentiate its effect by the stoichiometric addition of direct transcriptional activation domains which might enhance the induction of altered chromatin structures (50) or the recruitment of transcription factors to the site. The data that we have presented support the conclusion that EBNA-2 shares with other transcription factors the formation of oligomeric structures as a function that is crucial to its mechanism of action.

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