JOURNAL OF VIROLOGY, Mar. 1995, p. 1944–1950 Vol. 69, No. 3
0022-538X/95/$04.00+0

Contribution of Conserved Amino Acids in Mediating the Interaction between EBNA2 and CBF1/RBPJk

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Received 30 August 1994/Accepted 30 November 1994

The Epstein-Barr virus EBNA2 protein is a transcriptional activator that achieves promoter specificity through interaction with the cellular DNA-binding protein CBF1/RBPJk. Within the amino acid 252-to-425 EBNA2 domain that targets CBF1/RBPJk lie three amino acid clusters, conserved regions (CR) 5, 6, and 7, that are retained in the Epstein-Barr virus type A and type B and herpesvirus papio proteins. To further define the important features of the targeting domain, we constructed EBNA2 polypeptides containing deletions in the targeting domain and double or triple point mutations in the conserved motifs. The ability of these polypeptides and the type B and herpesvirus papio domains to interact with CBF1/RBPJk was examined by performing electrophoretic mobility shift assays and correlated with the effect of the mutations on EBNA2 transactivation. Both human type B EBNA2 and herpesvirus papio EBNA2 bound CBF1/RBPJk efficiently. Mutation of hydrophobic residues in CR6 severely impaired CBF1/RBPJk interaction and transactivation, while mutation of CR5 led to a moderate decrease in both activities. Mutation of CR7 had only a minor effect. Synthetic peptides corresponding to each of the conserved motifs were also used as competitors in an electrophoretic mobility shift assay. Only the peptide representing CR6 (amino acids 318 to 327), and not a version of this peptide mutated at the tryptophan residues at positions 323 and 324 (WW323,324), could compete for EBNA2 complex formation with CBF1/RBPJk. Overall, the data indicated that CR5 contributes to an optimal interaction, perhaps through stabilizing contacts, while CR6 forms a crucial interface with CBF1/RBPJk. The peptide competition data are consistent with direct contacts between WW323,324 and CBF1/RBPJk.

Epstein-Barr virus (EBV) is associated with several B-cell malignancies, including Burkitt's lymphoma and a proportion of Hodgkin's lymphoma and lymphomas in immunocompromised individuals (13, 18, 26, 29, 33). EBV immortalizes B lymphocytes with a high degree of efficiency concomitant with establishment of latent infection. EBNA1 is required for maintenance of the EBV genome during latency (43). In addition, genetic studies have shown that EBNA2, EBNA3A, EBNA3C, and LMP1 are required for in vitro immortalization of B cells (2, 7, 16, 20, 25, 27, 35, 36) and that EBNA-LP affects immortalization efficiency (16, 27). EBNA2 is a transcriptional activator that regulates viral latency gene expression and activates expression of cellular genes (1, 4, 6, 9, 24, 30, 31, 40). The viral LMP1 (12, 37), LMP2A (44, 45), and latency C promoters (Cp) (19, 24, 34, 42) are upregulated by EBNA2, as is expression of the cellular CD21, CD23, and c-*fgr* genes (21, 39, 41). Activation of cellular genes by EBNA2 is likely to have an important role in altering B-cell growth control.

The mechanism of EBNA2-mediated transactivation has become an area of intensive investigation. Data from our laboratory (22, 23) and from Zimber-Strobl et al. (44) indicated that EBNA2 did not bind directly to DNA, but rather was tethered to target promoters through interaction with a cellular DNA-binding protein designated CBF1. Peptide sequencing and cloning recently revealed CBF1 to be identical to recombination binding protein J kappa (RBPJk) (15, 17). This latter protein was named on the basis of its ability to bind to the heptamer sequence in the immunoglobulin J kappa gene (28), a property that was subsequently found to be artifactually generated by the addition of a *Bam*HI linker to the heptamer probe (15, 17). CBF1/RBPJk is highly conserved in sequence between species as divergent as humans and members of the genus *Drosophila* (3, 14, 32). The *Drosophila* homolog is encoded by the suppressor of the *hairless* gene and plays a key role in determination of neuronal cell fate (14, 32). An examination of the binding site for CBF1/RBPJk identified an essential core sequence, GTGGGAA, with flanking sequences influencing binding affinity (22). The acceptable flanking sequences have been defined by binding-site selection to yield a consensus, g/cYGTGGGAAa/c (38). A database search with this sequence identifies CBF1/RBPJk-binding sites in a large number of cellular promoters. Thus, EBNA2 has the potential to reprogram B-cell gene expression to a much greater extent than originally recognized.

To obtain comparative information that would aid in identification of important functional domains within EBNA2, we had previously cloned and sequenced the EBNA2 gene of the baboon virus herpesvirus papio (HVP) (24). Comparison of the amino acid sequence with that of the human type A and type B EBNA2 proteins (11) revealed nine conserved regions (CR). CR8 proved to represent the critical hydrophobic segment of the activation domain, and CR9 is a strong karyophilic signal sequence (6, 7, 24). The CBF1/RBPJk interaction domain in EBNA2 was initially located to amino acids (aa) 252 to 425, a sequence which contains three conserved regions, CR5, CR6, and CR7 (23) (Fig. 1). An important role for CR6 had already been indicated by the inability of a polypeptide carrying a double mutation of tryptophans 323 and 324 to interact with CBF1/RBPJk in an electrophoretic mobility shift assay (EMSA) (23). Using the comparative information as a guide, we created a series of deletions and point mutations to further define the CBF1/RBPJk interaction domain.

Contribution of hydrophobic residues in CR5, CR6, and CR7 to the stability of complex formation with CBF1/RBPJk. To further define the critical features within the domain for

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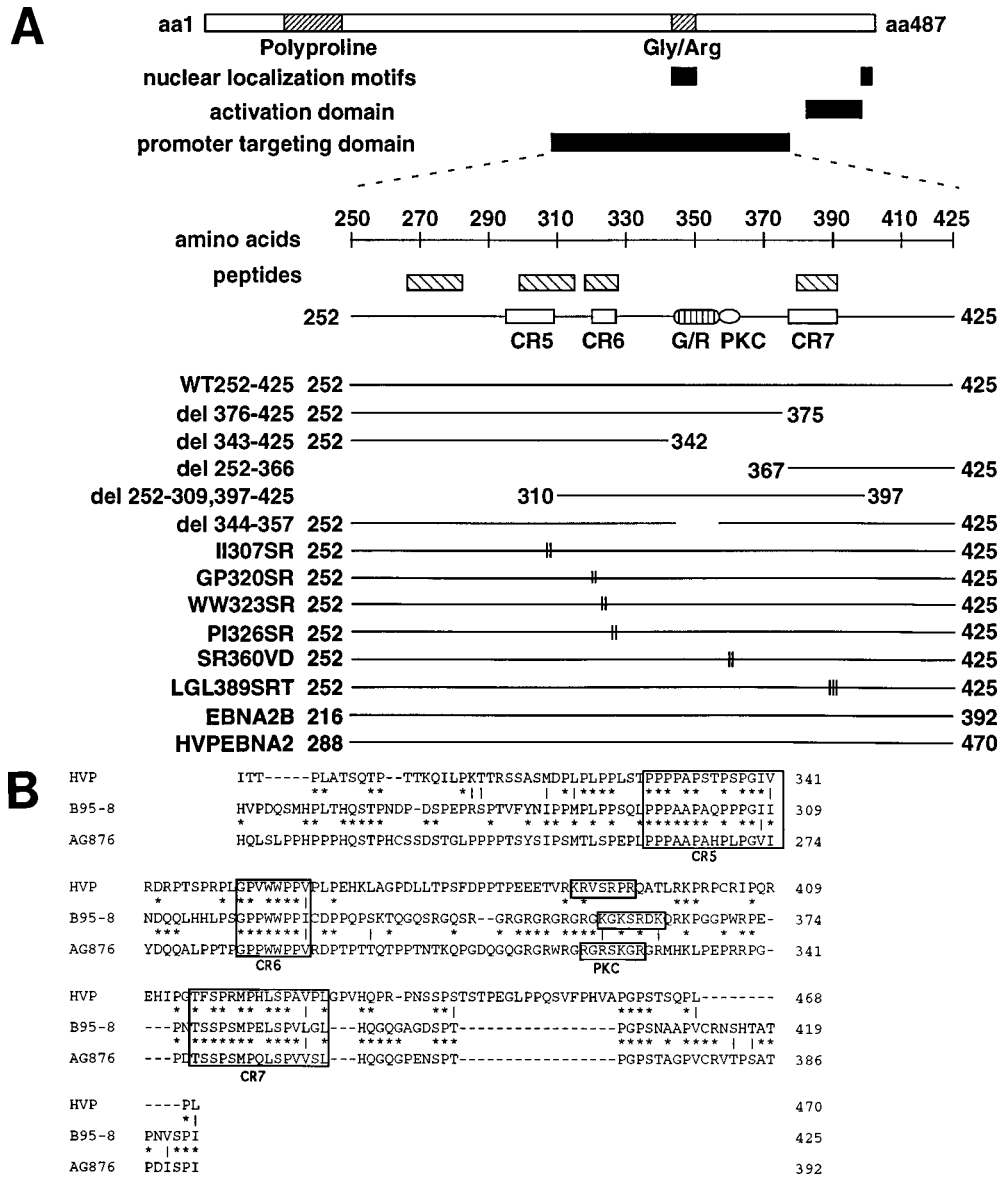


FIG. 1. Features of the EBNA2 domain that interacts with CBF1/RBPJk and summary of mutations created in this domain. (A) Schematic representation of the EBNA2 protein indicating the relative positions of functional domains (black rectangles). The expanded map of the CBF1/RBPJk interaction domain (aa252-425) shows the regions synthesized as synthetic peptides (hatched bars); the locations of conserved motifs (open rectangles); a conserved, putative PKC phosphorylation site (oval); the glycine-arginine repeats (G/R) (hatched oval); the end points of deletions; and the positions of mutations introduced into this domain. For the mutated constructions, wild-type amino acids are shown before the amino acid number and the substituted amino acids are shown after the number. (B) Alignment of the type A (B95-8), type B (AG876), and HVP amino acid sequences from aa 252 to 425. Clusters of amino acids that are conserved between all three EBNA2 proteins are indicated, as is a positionally conserved, putative PKC phosphorylation site (boxes). Asterisks denote amino acid identity, and vertical lines indicate amino acid similarity.

interaction with CBF1, the truncated and mutated polypeptides diagrammed in Fig. 1A were synthesized by in vitro transcription-translation and tested for their ability to form complexes with CBF1 that could be detected in an EMSA. The polypeptides were labeled with [^{35}S]methionine and checked for integrity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To ensure that equal amounts of the EBNA2 polypeptides were added to each binding reaction mixture, the SDS-PAGE bands were quantitated with a Molecular Dynamics PhosphorImager and the volume of the samples was adjusted on the basis of polypeptide size and the number of methionine residues. As illustrated in Fig. 2, a polypeptide lacking CR7 (aa 376 to 425 deleted) (del376-425) could inhibit the CBF1 complex by competition as effectively as could EBNA2(252-425) (EBNA2 containing aa 252 to 425), but the E2/CBF1 complex itself was reduced in amount and apparently was less stable than that formed with the larger aa 252-to-425 polypeptide (aa252-425). Further truncation at the carboxy terminus resulted in a polypeptide, del343-425, that could partially compete for the CBF1 complex but was unable to generate a stable E2/CBF1 complex. Polypeptides lacking CR5 and CR6 (del252-366) or lacking CR5 (del252-309,398-425) neither competed for the CBF1 complex nor formed a supershifted E2/CBF1 complex.

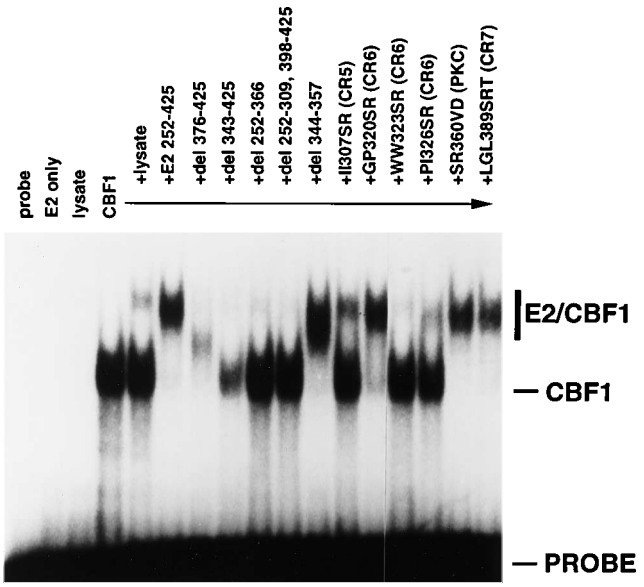


FIG. 2. Effect of mutations on the ability of EBNA2(252-425) to interact with CBF1/RBPJk. Results of an EMSA using CBF1 purified by heparin-agarose chromatography and a 30-bp oligonucleotide probe containing the Cp CBF1 binding site (23) are shown. EBNA2(252-425) and mutant EBNA2 polypeptides were in vitro transcribed and translated, and equimolar amounts were added to the binding reaction mixtures. Addition of unprogrammed reticulocyte lysate to the CBF1 extract (+lysate) produced a minor band shift that migrated in the range of the E2/CBF1 complex. The EBNA2/CBF1 complexes vary in mobility depending on the size of the added EBNA2 polypeptide.

A concern with the use of truncated polypeptides is that the smaller polypeptide size might affect the ability to form stable complexes with CBF1 and influence the results obtained. We therefore generated aa252-425 polypeptides carrying double and triple point mutations within CR5, CR6, and CR7 and within a conserved, putative protein kinase C (PKC) phosphorylation site (Fig. 1). Mutation of two hydrophobic residues in CR7 (LGL at position 389 changed to SRT) (LGL389SRT) produced a 50% reduction in the ability of aa252-425 to form an EBNA2/CBF1 complex (Fig. 2). In contrast to this polypeptide, whose mutation resulted in a relatively small effect, polypeptides carrying mutations in hydrophobic residues in CR5 (II307SR) and CR6 (WW323SR and PI326SR) did not compete for the CBF1 complex and produced little or no EBNA2/CBF1 supershifted complex. Mutation of two nonhydrophobic residues in CR6 (GP320SR) had no effect on the behavior of the polypeptide in the EMSA, indicating that the hydrophobic residues in CR6 are functionally dominant. The combined results suggested that CR7 makes a small contribution to the stability of the interaction with CBF1 but is not essential, while CR5 and CR6 are more important contributors.

We also examined the contribution of the glycine-arginine repeats (aa346-356) and a putative PKC phosphorylation site (aa357-363). Removal of the glycine-arginine repeats (del344-357) did not negatively affect EBNA2/CBF1 complex formation (Fig. 2), and mutation of the PKC site (SR360VD) had only a small effect, reducing EBNA2/CBF1 complex formation by 40% relative to the level for the wild-type polypeptide.

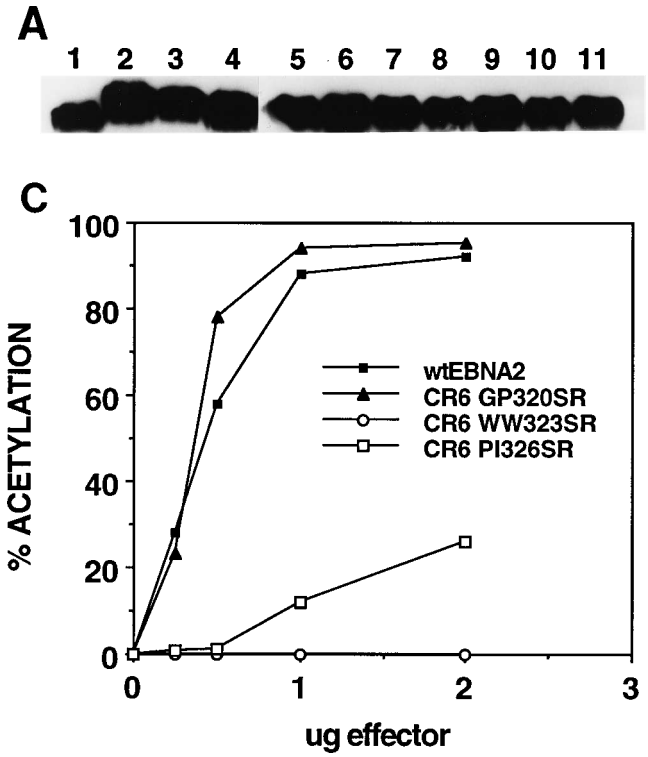
Mutations in CR5 and CR6 also affect EBNA2 transactivation function. In order to correlate the effect of the mutations on CBF1 interaction measured in the in vitro binding assays with the effects on CBF1 interaction in vivo in cultured cells, the point mutations and the glycine-arginine deletion were incorporated into full-length EBNA2 proteins which were used to study EBNA2 targeting in transactivation assays. The mutant proteins were synthesized in equal abundance in transfected cells, as assessed by Western immunoblot analysis (Fig. 3A). Figures 3B and C compare the abilities of wild-type and mutant EBNA2 proteins to transactivate expression from the EBV latency C promoter in a dose-response assay.

The CR7 and the PKC mutations, which had a small (approximately twofold) effect in reducing the amounts of EBNA2/CBF1 complex formed, did not affect transactivation, and the dose-response curves obtained with these mutants were indistinguishable from the wild-type EBNA2 response (Fig. 3B). It is possible that these mutations have some effect on protein conformation in the smaller aa252-425 polypeptides used in the EMSA but that this perturbation is compensated for in the context of the intact protein. The glycine-arginine deletion did not negatively affect complex formation in the EMSA, and the deleted EBNA2 protein transactivated the Cp-CAT target plasmid at 150% of wild-type levels in the linear range (0.5 μg of effector DNA).

The correlation between binding affinity as measured by EMSA and transactivation effectiveness was also observed with the CR5 and CR6 mutations. Mutation of the hydrophobic residues in CR5 impaired the ability of the aa252-425 polypeptide to form an EBNA2/CBF1 complex, and transactivation in the linear range was only 10% that of the wild-type EBNA2 (Fig. 3B). Interestingly, the deficit could be overcome by increasing the amount of effector DNA and at the highest dose of EBNA2 (2 μg), transactivation by the CR5 mutant approached wild-type levels. We interpret these results to indicate that CR5 contributes to a local polypeptide conformation that stabilizes the EBNA2/CBF1 interaction or mediates stabilizing contacts with CBF1.

Mutation of nonhydrophobic residues in CR6 had no effect on complex formation in the EMSA, and as expected, transactivation by this mutant (CR6 GP320SR) mirrored that of wild-type EBNA2 (Fig. 3C). The CR6 mutation WW323SR had previously been shown to abolish both complex formation with CBF1 and transactivation of the Cp (23) (Fig. 2), and the data in Fig. 3C confirm the inability of this mutant to transactivate the Cp even at high doses of effector. Mutation of the only other hydrophobic residue in CR6 (PI326SR) severely impaired the ability to form a complex with CBF1 in an EMSA, and transactivation at 0.5 μg of effector was only 2% that of wild-type EBNA2 (Fig. 3C). In contrast to what was observed with the CR6 WW323SR mutant, transactivation was observed at the highest levels of effector DNA, but even then the response was only 20% that of the wild type. Table 1 gives a quantitative summary of results of EMSAs and transient transfection experiments. The data are consistent with a model in which the hydrophobic residues in CR6 play a critical role in forming the contact interface between EBNA2 and CBF1, and tryptophans 323 and 324 are absolutely essential for this interaction.

The homologous domain of HVP binds CBF1. The rationale for focusing attention on CR5, CR6, and CR7 lay in the conservation of these regions between the human type A and type B and the HVP EBNA2 proteins and the low level of amino acid sequence conservation (23% identity) between HVP EBNA2 and the human proteins outside these three conserved regions (Fig. 1B). Outside CR5, CR6, and CR7, there are only five positionally conserved hydrophobic residues between aa 252 and aa 425. Two of these lie downstream of CR7 in a region that is unlikely to contribute to CBF1 interaction on the basis of the behavior of del376-425, and the other three occur immediately upstream of CR5. To validate the thesis that CR5



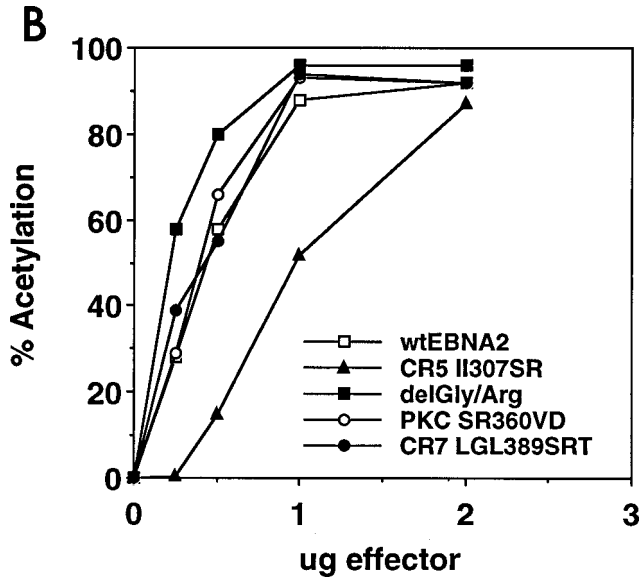


FIG. 3. Effect of mutations in CBF1 interaction domain on EBNA2 transactivation. (A) Immunoblot analysis demonstrating equal expression of the different EBNA2 effector constructions in transfected cells. Lane 1, type B EBNA2; lanes 2 and 11, wild-type (type A) EBNA2; lane 3, CR7 LGL389SRT; lane 4, delGly/Arg; lane 5, PKC mutant SR360VD; lane 6, CR6 PI326SR; lane 7, CR6 WW323SR; lanes 8 and 9, CR6 GP320SR; lane 10, CR5 II307SR. (B and C) Transactivation, in DG75 cells, of a Cp-CAT construction (24) cotransfected at 2 μg with increasing amounts (0, 0.25, 0.5, 1.0, and 2.0 μg) of the indicated EBNA2 effector DNAs. wtEBNA2, wild-type EBNA2.

and CR6 contain the most important sequences for EBNA2/CBF1 complex formation, it was important to show that the same domain of HVP EBNA2 did indeed mediate interaction with CBF1. The homologous segment of HVP EBNA2 (288-470) was in vitro translated, and the ability of this polypeptide to form an EBNA2/CBF1 complex was examined by EMSA (Fig. 4A). The HVP polypeptide bound CBF1 as efficiently as did the type A EBNA2, converting all the DNA-bound CBF1 complex to the supershifted EBNA2/CBF1 complex. In view of the very low level of amino acid homology between HVP and the human isolates in the aa252-425 targeting domain, the emphasis on CR5 and CR6 appears to be justified.

Type B EBNA2 binds CBF1 and transactivates in a manner comparable to that of type A EBNA2. Type A EBV immortalizes B cells more efficiently than does type B virus, and this phenotype maps to EBNA2 (8). It has been reported that type A EBNA2 transactivates the cellular CD23 promoter more efficiently than does type B (41), raising the possibility that transactivation ability might contribute to the immortalization phenotype. Since the activation domains of the two proteins have been shown to have equivalent activity (5), we tested whether there might be a difference in the abilities of the type A and type B proteins to interact with CBF1. In an EMSA (Fig. 4A), type B EBNA2 (aa216-392) and type A EBNA2 bound CBF1 comparably, although twofold more of the type B polypeptide was required to achieve conversion of all the CBF1 to the supershifted complex. To evaluate whether this small difference in in vitro binding was biologically significant, we reexamined the transactivation efficiencies of the two proteins in dose-response assays using the Cp and the CD23 promoters as targets (Fig. 4). The type A and type B proteins are synthesized equally in transfected cells (Fig. 3A). Type B EBNA2 transactivated expression from a Cp reporter plasmid slightly better than did the type A protein at the lower doses of EBNA2 and equally at higher doses (Fig. 4B). The type B protein has also been reported to be more efficient at transactivating the LMP1 promoter (41). Expression from our CD23 promoter construction was transactivated equally by the type A and type B proteins over a range of input effector DNA concentrations (Fig. 4C). On the basis of the observation that the type A and type B proteins interacted comparably with CBF1 and that the type B protein was not at a disadvantage in transactivating either a viral or a cellular promoter in cotransfection assays, it seems likely that other activities mediated by

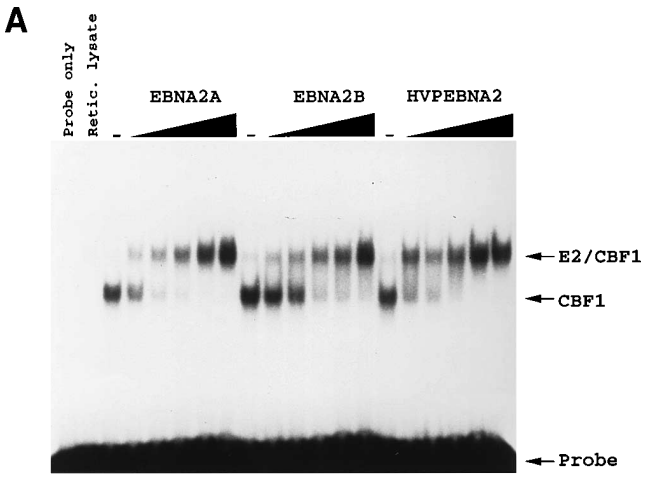
TABLE 1. Quantitative summary of results of EMSAs and transient transfection experiments

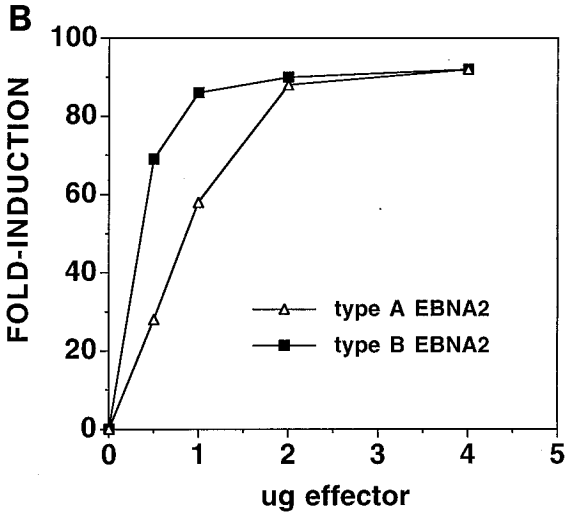
Protein	% Complex formation[a]	% Transactivation[b]
aa252-425 (wild type)	100	100
del376-425	10	NT
del343-425	<1	NT
del252-366	<1	NT
del252-309,398-425	<1	NT
del344-357	100	150
II307SR (CR5)	20	20[c]
GP320SR (CR6)	90	120
WW323SR (CR6)	<1	0
PI326SR (CR6)	5	2[c]
SR360VD (PKC)	60	110
LGL389SRT (CR7)	50	100

[a] Complex formation relative to that obtained with wild-type EBNA2(252-425). The amount of EBNA2/CBF1 supershifted complex was quantitated by using a phosphoimager. The amount of nonspecific complex formation by the reticulocyte lysate alone was subtracted.

[b] Relative EBNA2 transactivation of the Cp determined from Fig. 3. Values were calculated at DNA concentrations that gave 50% acetylation for wild-type EBNA2. NT, not tested.

[c] Mutant showed increased transactivation at higher concentrations of effector DNA.





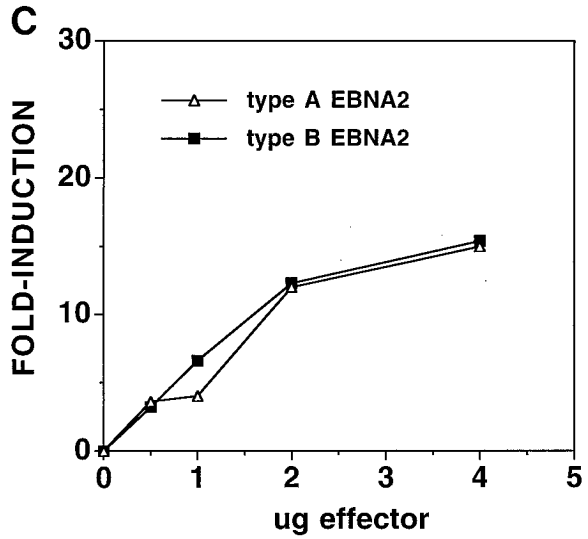


FIG. 4. Comparison of the CBF1/RBPJk-binding properties of HVP EBNA2 and human type A and type B EBNA2 and transactivation by the human proteins. (A) EMSA comparing the abilities of type A EBNA2(252-425) (EBNA2A) and the homologous domains of type B EBNA2 (aa216-392) (EBNA2B) and HVP EBNA2 (aa288-470) (HVPEBNA2) to form complexes with CBF1/RBPJk. The EBNA2 polypeptides and CBF1/RBPJk were prepared by in vitro transcription and translation. EBNA2 polypeptides were added with twofold increases in amount (black triangles) to a binding mixture containing a constant amount of CBF1/RBPJk and the 30-bp Cp CBF1 binding site probe. Retic., reticulocyte. (B and C) Cotransfection assays comparing the abilities of type A EBNA2 and type B EBNA2 to transactivate expression from Cp-CAT (B) and CD23p-CAT (C) (22) constructions in DG75 cells. The target plasmids were transfected at a constant amount (2 μg), and the effector DNAs were transfected at increasing amounts of 0, 0.5, 1.0, 2.0, and 4.0 μg.

the amino-terminal half of EBNA2 may be responsible for the inefficient immortalizing phenotype.

A synthetic peptide, CR6 aa318-327, can compete for EBNA2/CBF1 interaction, whereas a peptide mutated at WW323,324 cannot. Overall, the EMSA and transactivation data implicated CR5 as contributing to the stability of the EBNA2/CBF1 interaction and suggested that CR6, and in particular the tryptophan residues at positions 323 and 324, formed a contact interface with CBF1. CR6 could perform this function by generating a specific secondary or tertiary structure that facilitated interaction (a lock-and-key model of interaction). Alternatively, CR6 could mediate direct protein-protein contacts, perhaps through the formation of a tryptophan bridge (10). To further examine the proposed contributions of CR5, CR6, and CR7 and to probe the role of CR6, we synthesized five 10- to 15-mer peptides and tested them for the ability to compete with EBNA2 for complex formation with CBF1. The relative locations of the peptides are illustrated in Fig. 1A, and the exact positions of the sequences can be determined from Fig. 1B. The peptides are as follows: control, aa265-279 (STPNDPDSPEPPSPT); CR5, aa299-314 (APA QPPPGIINDQQL); CR6, aa318-327 (PSGPPWWPPI); CR6 WW323SR, aa318-327 (PSGPPSRPPI); and CR7, aa380-391 (PSMPELSPVLGL).

Increasing amounts of the peptides were added to the binding reaction mixtures, which were then analyzed by using an EMSA (Fig. 5). Neither the control peptide nor the peptides spanning CR5 or CR7 had any effect on the formation of the EBNA2/CBF1 complex. In contrast, addition of the 10-mer CR6 peptide gave an interesting result. The CR6 peptide eliminated by competition 40% of the EBNA2/CBF1 complex while at the same time regenerating the DNA-bound CBF1 complex to the levels seen in the absence of added EBNA2. It is known that EBNA2 can interact with CBF1 in solution (15, 17), and a typical binding reaction mixture presumably contains both DNA-bound and non-DNA-bound EBNA2/CBF1 complexes. Glutathione *S*-transferase (GST) affinity assays using GST–EBNA2(252-425) and ^{35}S-labeled, in vitro-translated CBF1 confirmed that the CR6 peptide could also compete for EBNA2 interaction with CBF1 in solution (data not shown). The amount of CBF1/EBNA2 complex detected by EMSA is therefore likely to be influenced by the equilibrium between DNA-bound and free CBF1/EBNA2 complexes and by any difference in the affinity for DNA that CBF1 may exhibit when binding alone versus binding as a CBF1/EBNA2 complex. Mutation of the tryptophan residues at positions 323 and 324 within an otherwise identical CR6 peptide abolished the ability of the peptide to compete with EBNA2 for interaction with CBF1 as assayed both by EMSA (Fig. 5) and in a GST affinity assay (data not shown).

In summary, we conclude that CR5 plays a role in generating an optimal local conformation for interaction with CBF1 and may make stabilizing protein-protein contacts. Critical protein-protein contacts are made by CR6. A 10-amino-acid peptide is too small to establish a stable secondary structure. The fact that the 10-mer CR6 peptide was able to compete for EBNA2 binding to CBF1 and the inability of the peptide carrying the

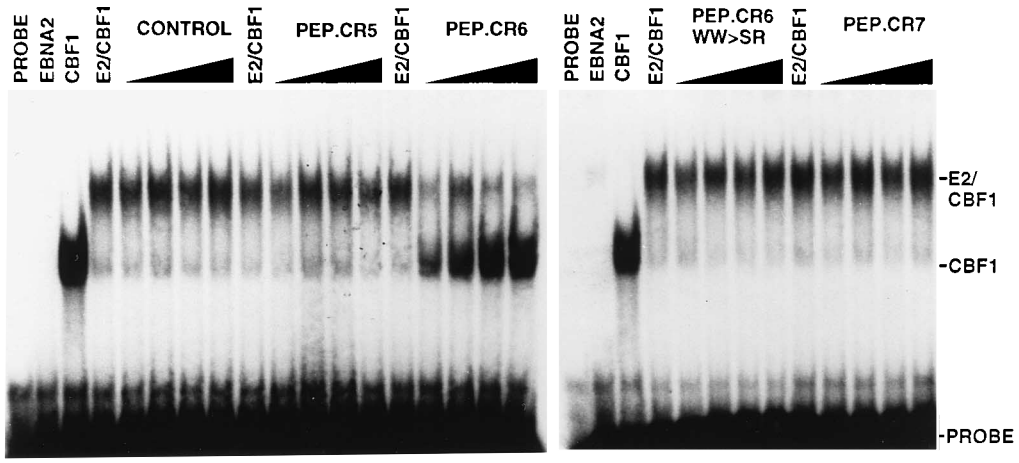


FIG. 5. Synthetic 10-amino-acid peptide representing CR6 competes with EBNA2 for complex formation with CBF1/RBPJk. Results of an EMSA examining the abilities of synthetic peptides representing CR5 (PEP.CR5), CR6 (PEP.CR6), mutant CR6 (CR6 WW323SR) (PEP.CR6 WW>SR), CR7 (PEP.CR7), and a control, nonconserved region (aa265-279) (CONTROL) to compete for EBNA2/CBF1 complex formation are shown. Increasing amounts of peptide (1.25, 2.5, 5.0, and 10 μg [black triangles]) were added to the binding reaction mixtures containing the Cp CBF1 binding site probe, in vitro-translated wild-type EBNA2(252-425), and heparin-agarose-purified CBF1.

WW mutation to perform this function strongly suggest that the two tryptophan residues in CR6 are responsible for directly contacting CBF1. This interpretation is compatible with the transactivation data which showed that only the WW mutation in CR6 could completely abolish transactivation function. The ability of such a small peptide to compete for EBNA2 binding to CBF1 also suggests that targeting the disruption of this interaction may be a viable antiviral strategy.

We thank Jie Tan for technical assistance and Feng Chang for manuscript preparation. The type B EBNA2 gene was kindly provided by Elliott Kieff.

This work was supported by grant RO1 CA42245 from the National Institutes of Health. P.D.L. was partially supported by a Leukemia Society of America special fellowship. S.D.H. is the recipient of an American Cancer Society award (FRA 429).

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