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The EBNA-2 Arginine-Glycine Domain Is Critical but Not Essential for B-Lymphocyte Growth Transformation; the Rest of Region 3 Lacks Essential Interactive Domains

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Since deletion of region 3 (amino acids [aa] 333 to 425) of Epstein-Barr virus nuclear protein 2 (EBNA-2) results in EBV recombinants which cannot transform primary B lymphocytes (J. I. Cohen, F. Wang, and E. Kieff, J. Virol. 65:2545–2554, 1991), the role of domains of region 3 was investigated. Deletion of the Arg-Gly repeat domain, R-337GQSRGRGRGRGRGKG354, results in EBV recombinants that transform primary B lymphocytes with modestly decreased activity. The transformed cells grow slowly and are difficult to expand. EBNA-2 deleted for the Arg-Gly domain does not associate with the nuclear chromatin fraction. The Arg-Gly repeat has an intrinsic ability to bind to histone H1, to other proteins, including EBNA-1, and to nucleic acids, especially poly(G). Two independent deletions of each part of the rest of region 3 (aa 359 to 383 and 385 to 430) have little effect on transformation, while deletion of the rest of region 3 (aa 361 to 425) as a single segment substantially reduces transformation efficiency. EBNA-2 deleted for all of region 3 can still transactivate the LMP1 promoter in transient expression assays but is less active than EBNA-2 in transactivating the *Bam*HI-C promoter. EBNA-2 deleted for the Arg-Gly domain is better than EBNA-2 at transactivating the LMP1 promoter and is as active as EBNA-2 in transactivating the *Bam*HI-C promoter. These data are most compatible with a model in which the Arg-Gly domain of region 3 is a modulator of EBNA-2 interactions and activities, while the rest of region 3 is important in positioning the region 2 J kappa binding domain relative to the region 4 acidic transactivating domain. Despite the null phenotype of the region 3 deletion, region 3 is unlikely to mediate essential interactions with other proteins.

The ability of Epstein-Barr virus (EBV) to acutely and efficiently transform primary B lymphocytes into perpetually proliferating lymphoblastoid cell lines (LCLs) is linked to expression from the viral genome of six EBV nuclear proteins (EBNA-1, EBNA-2, -3A, -3B, -3C, and -LP), two latent membrane proteins (LMP1 and -2) and two small RNAs (EBERs) (31). Of these 10 gene products, the EBERs (45), EBNA-3B (46), and LMP2 (33, 34) are not required for latent infection or cell growth transformation in vitro, although LMP2 may be required for the maintenance of latent infection in vivo (37). EBNA-1 is essential for efficient persistence of the EBV genome in latent infection (54), while EBNA-2 (10, 23), EBNA-3A and -3C (47), and LMP1 (27) are essential and EBNA-LP (35) is critical for latent infection or B-lymphocyte growth transformation.

EBNA-2 is one of the first two genes expressed in latent infection. At least one essential function of EBNA-2 is as a transactivator of viral and cell gene expression. EBNA-2 induces expression of the LMP1 (1, 52) and LMP2 (62) genes and of cellular genes, including CD21 (11), CD23 (49–51), and c-*fgr* (29). EBNA-2-responsive elements upstream of the LMP1 (17, 48), LMP2A (61), *Bam*HI-C (Cp) (44), and cellular CD23 (51) promoters have been shown to convey EBNA-2 responsiveness to heterologous promoters.

Molecular genetic and biologic analysis of the role of EBNA-2 in latent growth-transforming infection identified at least four regions of EBNA-2 which are essential for transformation and for transactivation of the LMP1 promoter (9). Region one is defined by a deletion of amino acids (aa) 19 to 110, which consist mostly of a polyproline repeat. Region 2 is defined by the insertion of a linker encoding GRSS between W-320 and P-321. This region mediates EBNA-2 interaction with a 63-kDa cell protein, J kappa, which recognizes a sequence common to EBNA-2 response elements (22, 32). EBNA-2 aa 310 to 336 are sufficient for J kappa interaction, and the short sequence P-317PWWPP-322 conserved between type 1 and type 2 EBNA-2 probably mediates this interaction (22, 55). Region 3 is defined by a deletion of aa 333 to 425, a segment which includes an Arg-Gly repeat at aa 337 to 354. Region 4 is defined by a deletion of aa 426 to 472, a segment which includes a core acidic transactivating domain at aa 449 to 462 (7, 8). The adverse effect of each of these mutations on the transforming and transactivating phenotypes of EBNA-2 is not because the mutant proteins are unstable or fail to translocate to the nucleus (9) and is therefore most consistent with the hypothesis that each region includes an interactive domain(s) involved in viral or cellular gene transactivations or other functions essential for transformation.

This report focuses on region 3 (aa 333 to 425) and the unusual R-337GQSRGRGRGRGRGKG-354 domain in region 3. Although this domain can serve to ensure nuclear translocation when the more usual nuclear localization sequence at the end of EBNA-2 is mutated (9), that is unlikely to be the principal function of this domain. Arg-rich repeat domains in other proteins have been implicated in protein-RNA (13) or protein-protein (59) interactions. We have now investigated the role of the Arg-Gly domain and of the rest of region 3 in EBV-mediated cell growth transformation, trans-

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activation, and interactions with cellular and viral proteins and nucleic acids.

MATERIALS AND METHODS

Cell lines, culture conditions, and labeling of cell proteins. BJAB cells are derived from an EBV-negative (EBV^-) B-cell lymphoma (36). BL30/P3HR-1 is an EBV^- Burkitt lymphoma cell line which has been infected in vitro by EBV strain P3HR-1 (6). The P3HR-1 genome is deleted for an EBV DNA segment which encodes the last two exons of EBNA-LP and the entire EBNA-2 exon. P3HR-1 clone 16 cells were obtained from G. Miller, Yale University (41). IB4 is an EBV-transformed B-lymphoblastoid cell line (28). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells (10^8) were grown to 10^6/ml and labeled overnight in 1 mCi of [^{35}S]methionine (New England Nuclear) per 20 ml in methionine-free RPMI medium (GIBCO BRL) supplemented with 10% dialyzed fetal bovine serum (GIBCO BRL).

Cosmids and plasmids. pSG5-EBNA 2 is an expression vector for EBNA-2 (48). pSG5-E2 plasmids, deleted for various parts of EBNA 2 region 3, were constructed by PCR-facilitated mutagenesis (24). The PCR primer sequences were as follows: 5′-GATTACATTTTTGAGACA-3′ (5′ outside primer) and 5′-ACAAGTTCTGCTTTAATA-3′ (3′ outside primer); for pSG5-E2,d337-354, 5′-CAAGGCCAGAGCAAGTC CAGGGACAAGCAA-3′ (5′ inside primer) and 5′-GCTCT GGCCTTGAGTCTT-3′ (3′ inside primer); for pSG5-E2, d359-383, 5′-AAGTCCAGGGACGTCCTCGGTCTTCATC AG-3′ (5′ inside primer) and 5′-GTCCCTGGACTTGCCC TT-3′ (3′ inside primer); for pSG5-E2,d385-430, 5′-CTAAG TCCAGTCCAGGCTCCCATTCTTTCC-3′ (5′ inside primer) and 5′-GACTGGACTTAGTTCAGG-3′ (3′ inside primer). The *Bst*EII-to-*Bgl*II fragment of pSG5-EBNA 2 was replaced by the deletion fragments from the PCR-amplified DNA. pSG5-E2,d333-425 was made by cloning the *Bsm*I fragment from cosmid T1EBNA 2 d333-425 (9) in place of the wild-type (wt) *Bsm*I fragment of pSG5-EBNA 2. pSG5-E2,d361-425 was made by using a 5′ outside primer (5′-CATCACCACCACG CATGCATC-3′), a 3′ outside primer that was the same as described above, a 5′ inside primer (5′-GCAAGTCTAGAGA CAAGCAATCCCATAATAGCCCAGAGGC-3′), and a 3′ inside primer (5′-GCCTCTGGGCTATTATGGGATTGCTT GTCTCTAGACTTGC-3′). The sites of genetic alterations were sequenced by the dideoxy method (U.S. Biochemical Corp.). Cosmids with EBNA-2 deletions were constructed by cloning the mutated *Nsi*I fragments of the EBNA-2 open reading frame into the *Hin*dIII-*Sst*II fragment from the EBV DNA *Eco*RI A fragment. The mutated *Hin*dIII-*Cpo*I fragments were then used to replace the wt *Hin*dIII-*Cpo*I sequence of cosmid WEAH, a clone of the W91 EBV DNA *Eco*RI A fragment in MAU3 (40). Two cosmid clones of each deletion mutant were independently derived. Glutathione *S*-transferase (GST) fusion protein expression vectors were constructed by PCR amplification of the appropriate part of wt or mutated EBNA-2, using 5′ and 3′ primers which have *Bam*HI and *Eco*RI sites in frame with the GST expression vector pGEX-2TK (26). GST-$(RG)_8$ was made by inserting a synthetic oligonucleotide encoding eight Arg-Gly repeats into the *Bam*HI-to-*Eco*RI site of pGEX-2TK.

Transformation assay and virus passage. Cosmid clones of wt or specifically mutated EBV W91 *Eco*RI-A DNA were digested with *Eco*RI to release the EBV DNA from the vector, and 10 μg of DNA was mixed with 50 μg of pSVNaeIZ DNA. The DNAs were transfected into 15 million P3HR-1 clone 16 cells by electroporation at 200 V and 960 μF in a Bio-Rad Gene Pulser cuvette. The cells were then diluted into 15 ml of RPMI medium with 10% fetal bovine serum and incubated at 37°C for 6 days. On the 6th day, culture supernatant containing the virus was filtered through a 0.45-μm-pore-size filter and used to infect freshly isolated T-cell-depleted human peripheral blood lymphocytes (9, 46, 47). The infected cells were plated on a 96-well plate at 5×10^4 cells in 150 μl of medium per well. The cells were fed once every 2 weeks with 100 μl of medium per well. LCLs were macroscopically visible at 4 to 6 weeks.

Lytic EBV infection was induced by transfecting 15 million cells with 50 μg of pSVNaeIZ DNA and incubating the cells in the presence of 20 ng of phorbol 12-myristate 13-acetate (GIBCO BRL) per ml. Four days after electroporation, culture supernatant containing the virus was used to infect freshly prepared human non-T peripheral blood lymphocytes.

Nuclear fractionation. Cell nuclei were isolated and fractionated as described previously (39). Briefly, cells were lysed in hypotonic buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg of aprotinin per ml), and nuclei were isolated by centrifugation at $1,000 \times g$ for 10 min. The nucleus pellet was extracted twice with nuclear buffer (10 mM HEPES, 120 mM NaCl, 0.5% Nonidet P-40 [NP-40], 1 mM PMSF 2 μg of aprotinin per ml), and the combined supernatants were considered the nucleoplasm fraction. The NP-40-extracted pellet was resuspended in nuclease digestion buffer (10 mM HEPES, 100 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, and 2 μg of aprotinin per ml), digested with DNase I for 30 min, and then extracted with high-salt buffer (10 mM HEPES, 2 M NaCl, 0.5% NP-40, 10 mM EDTA, 1 mM PMSF, 2 μg of aprotinin per ml). The extracts were centrifuged at $15,000 \times g$ for 10 min, and the pellet was washed once in nuclear buffer. The combined supernatants were considered the chromatin fraction. The nuclear matrix fraction was prepared by solubilizing the final pellet in sodium dodecyl sulfate (SDS) protein sample buffer.

GST fusion proteins. GST fusion proteins were expressed in *Escherichia coli* after induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (26). Cells were harvested 3 h after induction. After sonication and centrifugation, the extracts were incubated with glutathione-agarose beads (Pharmacia) for 1 h at 4°C. The beads were precipitated and washed with NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40).

Affinity binding assay. Labeled cells were lysed in buffer (10^8 cells per 2 ml of lysis buffer) containing 150 mM NaCl, 10 mM HEPES (pH 8.0), 1% NP-40, 2 μg of aprotinin per ml, 1 mM PMSF, 1 mM DTT, and 25 mM betaine (Sigma). Insoluble material was removed by centrifugation. The lysates were precleared by incubation with glutathione-Sepharose beads loaded with GST. Aliquots of precleared lysates were incubated with Sepharose beads loaded with various GST fusion proteins as indicated for 1 h at 4°C. The beads were washed in lysis buffer plus 0.1% SDS. Proteins bound to beads were eluted with SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using 8.5% gels.

Far-Western blot analysis. GST fusion proteins were labeled and incubated with nitrocellulose filters containing proteins transferred from gels after SDS-PAGE (26). Briefly, glutathione-Sepharose beads loaded with fusion proteins were resuspended in 1× HMK buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 12 mM $MgCl_2$) containing the catalytic subunit of cyclic

AMP-dependent protein kinase (Sigma), [γ-^{32}P]ATP (New England Nuclear), and 1 mM DTT. The mixture was incubated for 30 min at 4°C. Fusion protein was eluted with 20 mM reduced glutathione (Sigma) in 100 mM Tris (pH 8.0) and 120 mM NaCl. The ^{32}P-labeled fusion proteins were added to hybridization buffer (Hyb75; 20 mM HEPES [pH 7.7], 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40) at 2.5×10^5 cpm/ml. To reduce background, Hyb75 was supplemented with equal volume of lysate from *E. coli* expressing GST.

Nucleic acid binding assay. Single-stranded DNA (ssDNA)-double-stranded DNA (dsDNA)-cellulose, cellulose, and poly(G)-, poly(A)-, poly(U)-, and poly(C)-agarose were purchased from Sigma. GST fusion proteins were ^{32}P-labeled as described above. Nucleic acid coupled to matrix was incubated with labeled GST fusion protein in hybridization buffer (10^5 cpm per sample in NETN) supplemented with 1/10 volume of lysate from bacteria expressing GST (see above). After 30 min of incubation, beads were washed with NETN and Cerenkov counted (Beckman LS 5000TD). For blocking experiments, labeled GST fusion protein was preincubated with blocker for 15 min before addition of the labeled protein to the nucleic acid matrix.

Dimethyl sulfate methylation. Oligonucleotides were methylated by addition of freshly diluted 1% dimethyl sulfate to a final concentration of 0.05% and incubation at 65°C for 15 min (53).

Transfections and CAT assays. BJAB cells (10^7) were suspended in 0.3 ml of RPMI 1640 and placed in a Bio-Rad Gene Pulser cuvette; 5 μg of pUC-βgal, 10 μg of pSG5 or pSG5 with a wt or mutated EBNA-2, and 5 μg of LMP1CAT (−234/+40) reporter plasmid (48) or CpCAT reporter plasmid (55) were added to each cuvette. Cells were electroporated with 220 V at 960 μF. β-Galactosidase and chloramphenicol acetyltransferase (CAT) activities were assayed after 3 days as described previously (48).

RESULTS

The Arg-Gly repeat is important in latent infection and growth transformation of primary B lymphocytes. To evaluate the role of the region 3 (aa 333 to 425) domains in primary B-lymphocyte growth transformation, DNA fragments containing a wt or specifically mutated EBNA-2 gene were compared for the ability to marker rescue transforming virus activity from EBV-infected P3HR-1 cells. P3HR-1 EBV DNA is wt except for a deletion of a DNA segment which includes the last two exons of EBNA-LP and the entire EBNA-2 open reading frame. Transfection of P3HR-1 cells with a wt EBV DNA fragment which spans the deletion and with an expression plasmid for the EBV BZLF1 immediate-early transactivator of lytic infection results in lytic infection and recombination of the transfected DNA with the replicating P3HR-1 genome. Transforming recombinants can then be identified and enumerated by virtue of their ability to infect and to growth transform primary B lymphocytes.

Deletion of all of region 3 (aa 333 to 425) has been previously reported to be a null mutation in such marker rescue experiments (9). The null transforming activity was confirmed with new constructs of the marker-rescuing DNA fragment (E2d333-425). Two different constructs failed to marker rescue transformation in each of three independent experiments in which a similar fractions of the virus from cells transfected with wt DNA gave rise to 19 to 28 transformants. Two different constructs of EBNA-2 deleted for DNA encoding the Arg-Gly domain (E2d337-354) generated 0 to 7 or 0 to 14 transformants in five independent experiments in which wt DNA gave rise to 19 to 84 transformants. The mean value for the E2d337-354 marker rescue efficiency relative to wt controls was 8%. In two independent experiments, two different constructs deleted for the rest of region 3 (E2d361-425) each gave rise to 3 to 8 or 4 to 5 transformants, while the wt DNA generated 61 to 63 transformants. The mean value for the E2d361-425 transforming efficiency relative to the wt value was 8%. Surprisingly, when the rest of region 3 was studied as two separate deletions, E2d359-383 and E2d385-430, each mutated DNA marker rescued transforming activity from P3HR-1 cells with an efficiency fully equal to or greater than that of wt DNA. Since EBV recombinants deleted for EBNA-2 aa 359 to 383 or 385 to 430 had wt transforming activity, these sequences are unlikely to directly mediate important interactions of EBNA-2 with other proteins. Thus, the reduced transforming activity of the combined deletion, E2d361-425, is most likely due to an effect on the folding or presentation of other domains of EBNA-2.

LCLs infected with and transformed by E2d337-354 recombinants also grew differently from wt recombinant-infected LCLs, while LCLs transformed by E2d359-383 or E2d385-430 recombinants were indistinguishable from wt-infected LCLs. Wild-type, E2d359-383, or E2d385-430 recombinant-infected LCLs reached saturation in the original 96-well plates by 4 to 6 weeks. In contrast, E2d337-354 recombinant-infected LCLs reached saturation at 6 to 8 weeks. LCLs infected with E2d337-354 recombinants also continued to grow more slowly than wt recombinant-infected LCLs over the ensuing 4 months. While more than 80% of the wt-infected LCLs could be expanded to $>10^7$ cells, only 50% of the E2d337-354 recombinant-infected LCLs could be expanded.

Putative E2d337-354 mutant recombinant-infected LCLs were confirmed to have the E2d337-354 DNA by PCR with primers corresponding to bp 49228 to 49651 and 49651 to 49670 (3), which amplify a 388-bp fragment across the site of the deletion or a 442-bp fragment from wt EBV DNA (data not shown). E2d359-383-, and E2d385-430-infected LCLs were similarly confirmed to have their respective deletions by PCR using primers corresponding to bp 49443 to 49640 and 49962 to 49979, which amplify 462- and 399-bp fragments from the mutants and 537-bp fragments from wt EBV DNA (data not shown).

To further characterize the E2d337-354 recombinant phenotype, lytic infection was induced in LCLs infected with wt or E2d337-354 EBV recombinants. Dilutions of the resultant virus from wt- and mutant recombinant-infected cells which had been induced to similar levels of permissivity for lytic infection were used to infect primary B lymphocytes. At 4 weeks, E2d337-354 recombinant virus-transformed primary B lymphocytes into macroscopically visible LCLs with 30% of the wt recombinant activity; by 9 weeks, the number of macroscopically visible LCLs generated by E2d337-354 recombinant virus was 80% of the wt level (Fig. 1). Thus, the most evident phenotype was the slower outgrowth of LCLs transformed by E2d337-354 recombinant virus. The near wt ultimate efficiency of the E2d337-354 recombinants in primary B-lymphocyte transformation in these experiments as opposed to experiments with virus derived, de novo, from P3HR-1 cell transfections is probably due to an inhibitory effect of coinfection of the primary B lymphocytes with parental P3HR-1 virus, since a decreased multiplicity of infection of primary B lymphocytes with virus stocks from P3HR-1 cells resulted in an enhanced efficiency of transformation (data not shown).

EBNA, LMP, and BZLF1 expression in LCLs transformed by E2d337-354 or wt EBV recombinants. EBNA and LMP1

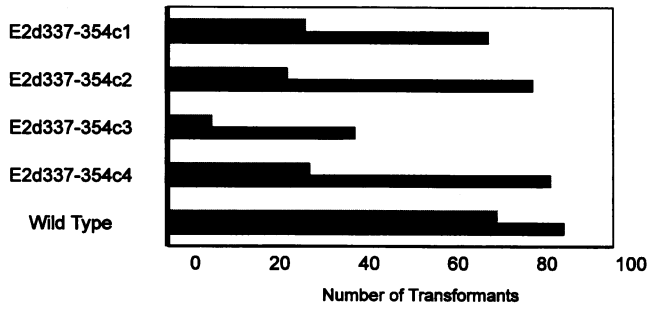

FIG. 1. Summary of outgrowth of LCLs following infection with wt or E2d337-354 recombinant virus. LCLs infected with one wt and four independent E2d337-354 recombinants were rendered similarly permissive for lytic EBV infection. Four days later, culture supernatants containing the virus were used to infect primary human B lymphocytes. The number of macroscopically visible transformants was scored at 4 weeks (gray bars) or at 9 weeks (black bars).

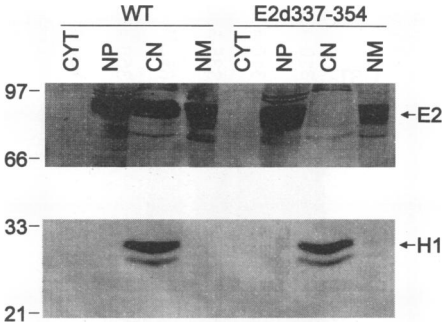

FIG. 3. EBNA-2 and E2d337-354 differentially associate with the nuclear chromatin fraction. LCLs transformed by wt EBV recombinant or E2d337-354 were lysed and fractionated into cytoplasm (CYT), nucleoplasm (NP), chromatin (CN), and nuclear matrix (NM). EBNA-2 was detected by Western blotting with monoclonal antibody PE2; histone H1 was detected by immune rabbit serum. Sizes are indicated in kilodaltons.

expressions were analyzed in LCLs transformed by wt or E2d337-354 EBV recombinants. LCLs infected with E2d337-354 EBV recombinant virus consistently expressed slightly less EBNA-1 relative to EBNA-2 than was found in wt recombinant-infected LCLs. EBNA-2 expression was similar to that in wt recombinant-infected LCLs, and the level of EBNA-1 was reduced less than twofold (Fig. 2A). The EBNA-2 size in the E2d337-354-infected LCLs was also smaller because of the deletion. LMP1 was expressed at similar levels in both mutant and wt LCLs (Fig. 2B).

BZLF1 expression was evaluated as a marker of spontaneous transition of LCLs to lytic infection. As is characteristic of most recently derived LCLs, BZLF1 expression was barely detectable in most of the LCLs. Among five mutant LCL clones tested, one expressed easily detectable BZLF1 (Fig. 2C). Thus, E2d337-354 does not affect the permissivity of LCLs for lytic EBV infection.

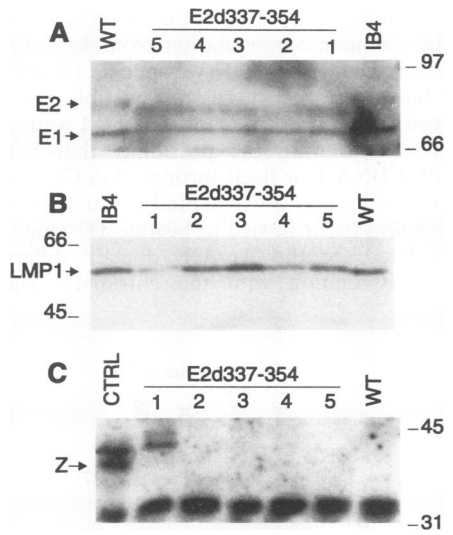

FIG. 2. EBV gene expression in LCLs infected with wt and E2d337-354 EBV recombinants. Lysates from IB4 cells, wt recombinant-infected LCL, or five E2d337-354-infected LCLs were subjected to SDS-PAGE, transferred to nitrocellulose, and probed for EBNA-1 and EBNA-2, using an EBV-immune human serum (A), LMP1, using monoclonal antibody S12 (B), BZLF1, using monoclonal antibody BZ.1 (C). A lysate from cells transfected with BZLF1 was used as a positive control (CTRL). Sizes are indicated in kilodaltons.

By immunofluorescence microscopy, E2d337-354 in mutated recombinant-infected LCLs was slightly more aggregated in nuclear clumps than was wt EBNA-2 in LCLs infected with wt recombinants. E2d337-354-infected LCLs also tended to vary more in immunofluorescence intensity, with some cells expressing higher EBNA-2 levels than any wt EBNA-2 recombinant-infected LCLs (data not shown). However, E2d337-354 was not distinctive enough to be readily identifiable by immunofluorescence microscopy.

CD23 expression was also evaluated by immunofluorescence microscopy, since EBNA 2 transactivates CD23 expression both directly and through LMP1 transactivation (49, 50). Wild-type and mutant recombinant-infected LCLs were equally CD23 positive (data not shown).

Nuclear localization of wt and E2d337-354 from LCLs. EBNA-2 is normally found in the nucleoplasm, chromatin, and nuclear matrix fractions (39). To study the nuclear distribution of E2d337-354, cell lysates from LCLs transformed by wt or E2d337-354 EBV recombinants were fractionated and EBNA-2 was detected by Western blotting. As shown in Fig. 3, wt EBNA 2 was detected in the nucleoplasm, chromatin, and nuclear matrix fractions, while E2d337-354 was absent from the chromatin fraction and was more abundant in the nucleoplasm and nuclear matrix fractions. The absence of E2d337-354 from the chromatin fraction was not due to inefficient extraction, since similar amounts of histone H1 were detected in chromatin fractions from both wt and mutated LCL lysates (Fig. 3). Therefore, the Arg-Gly domain is likely to mediate EBNA-2 association with chromatin.

Interaction of the Arg-Gly domain with cellular and viral proteins. To study the role of the Arg-Gly domain in efficient transformation and chromatin association, the interaction of the Arg-Gly and other region 3 domains with cellular proteins was investigated by using GST fusion proteins. GST fusion proteins with region 2 plus region 3 (GST-E2,310-432), with the rest of region 3 downstream of the Arg-Gly repeat (GST-E2,355-432), with the Arg-Gly domain (GST-E2,335-360), or with a simplified eight Arg-Gly repeats [GST-$(RG)_8$] were incubated with ^{35}S-labeled lysates from IB4 (EBV^+) or BJAB (EBV^-) cells. A similar repertoire of multiple proteins from EBV^- or EBV^+ B lymphocytes bound to GST-E2,310-

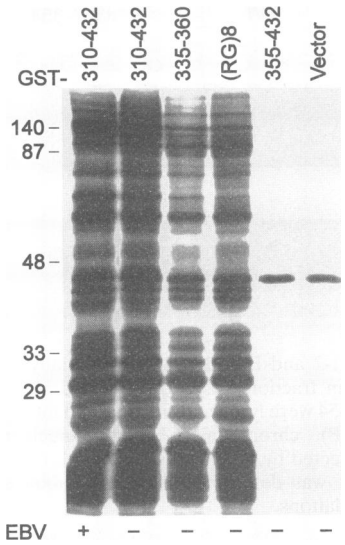

FIG. 4. Cellular proteins bound to EBNA-2 region 3. IB4 cells (EBV^+) or BJAB cells (EBV^-) were labeled with [^{35}S]Met, lysed, and precleared by incubation with glutathione-Sepharose beads loaded with GST. Aliquots of precleared lysate were incubated with beads loaded with GST-E2,310-432, GST-E2,335-360, GST-$(RG)_8$, GST-E2,355-432, or the GST vector control. The cellular proteins bound to the fusion protein beads were subjected to SDS-PAGE and visualized by fluorography. Sizes are indicated in kilodaltons.

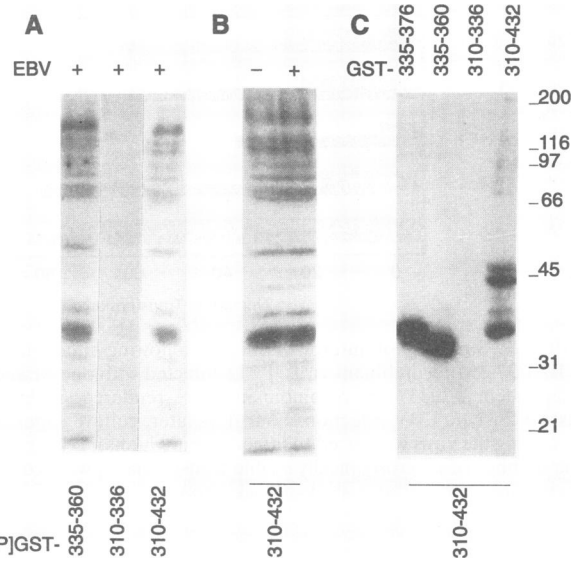

FIG. 5. Far-Western analysis showing direct binding of EBNA 2 region 3 to cell proteins. Total cell proteins were subjected to SDS-PAGE and transferred to nitrocellulose. GST fusion proteins, ^{32}P labeled in vitro by protein kinase A, were used to probe the filter. (A) Proteins from IB4 cells (EBV^+) were probed with labeled fusion protein GST-E2,335-360, GST-E2,310-336, or GST-E2,310-432. (B) Proteins from BJAB (EBV^-) or IB4 (EBV^+) cells were probed with labeled GST-E2,310-432. (C) Purified fusion proteins GST-E2,335-376, GST-E2,335-360, GST-E2,310-336, and GST-E2,310-432 were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with labeled GST-E2,310-432. Sizes are indicated in kilodaltons.

432, GST-E2,335-360, or GST-$(RG)_8$. No protein specifically bound to GST-E2,355-432 compared with GST (Fig. 4). Since so many uninfected cell proteins bound to GST-$(RG)_8$, subtle differences in proteins retrieved from EBV^+ or EBV^- cell extracts could not be detected; also, the differences in proteins bound to larger GST fusion proteins are difficult to discern. Very few proteins were precipitated by the GST-E2,355-432 and GST control under the same condition (Fig. 4). Thus, a large number of cellular proteins bind to the Arg-Gly repeat domain of region 3 or to an even simpler Arg-Gly repeat, while the rest of region 3 does not appear to specifically interact with any B-lymphocyte protein.

To further investigate direct interactions of the Arg-Gly domain with cell proteins, ^{32}P-labeled GST fusion proteins were used to probe an electrophoretic separation of cell proteins immobilized on nitrocellulose filters. The far-Western analysis revealed that the same cell proteins were detected by labeled GST-E2,310-432 and GST-E2,335-360 but not by GST-E2,310-336 or other GST-fusion proteins from region 3 which lack the Arg-Gly repeats (Fig. 5A and data not shown). The same proteins were detected in extracts from EBV-infected or uninfected lymphocytes (Fig. 5B). Further, GST-E2,310-432 bound to itself, to GST-E2,335-360, or to GST-E2,335-376, each of which had the Arg-Gly repeat, but did not bind to GST-E2,310-336 or to GST-E2,355-432 (Fig. 5C and data not shown). Thus, the Arg-Gly repeat can interact with itself on far-Western blots, even though it is highly positively charged.

Since EBNA-2 and EBNA-1 both have Arg-Gly domains, we investigated whether the EBNA-2 Arg-Gly domain could mediate an interaction between EBNA-2 molecules or between EBNA-2 and EBNA-1. EBV-infected cell proteins were incubated with GST-E2,335-360 or with GST control beads, and the proteins which bound to the beads were analyzed with an EBV-immune human serum. About 5% of the EBNA-1 in the lysate bound to GST-E2,335-360 beads and not to GST beads. EBNA-2 was not detected under the same conditions, although a similar level of EBNA-2 bound to GST-E2,335-360 beads would not be detectable with available sera (Fig. 6). Attempts to detect an interaction between EBNA-2 and EBNA-1 by coimmunoprecipitation from EBV-infected cell nuclear extracts with the EBNA-2-specific monoclonal antibody PE2 have not been successful, possibly because of the limited sensitivity of EBNA-2 and EBNA-1 antisera. Nevertheless, these data raise the possibility that EBNA-2 can interact with EBNA-1 or itself through Arg-Gly domains.

Among the cell proteins which bind to labeled GST-E2,310-432 is a protein similar in size to histone H1 (Fig. 5A). Since EBNA-2 deleted for the Arg-Gly domain differs from wt EBNA-2 in association with the chromatin fraction and

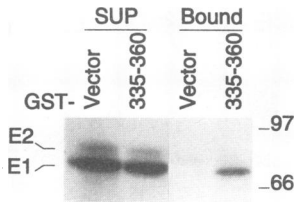

FIG. 6. EBNA-1 is able to associate with the Arg-Gly domain of EBNA-2. Extracts of IB4 cells were incubated with GST-E2,335-360 or with GST (Vector). Proteins bound to GST beads and proteins left in the supernatants (SUP) after incubation were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with an immune human serum. The positions of EBNA-1 and EBNA-2 are indicated. Sizes are indicated in kilodaltons.

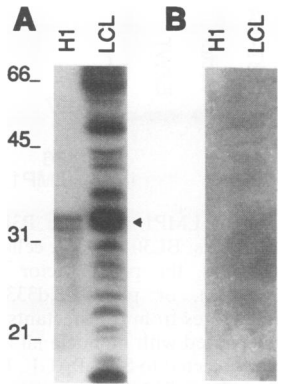

FIG. 7. Histone H1 is detected on far-Western blots by the labeled Arg-Gly repeat. (A) Purified histone H1 (lane H1) or proteins from IB4 cells (lane LCL) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with ^{32}P-labeled GST-E2,335-360. The position of histone H1 is indicated by an arrowhead. In panel B, soluble histone H1 was added to the hybridization buffer. Sizes are indicated in kilodaltons.

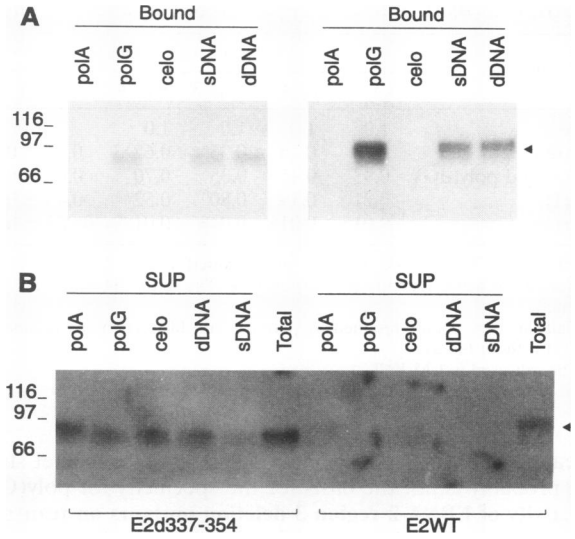

FIG. 8. Binding of wt EBNA 2 or E2d337-354 to nucleic acids. (A) BJAB cells were transfected with pSG5-E2,d337-354. Wild-type EBNA-2 was expressed from IB4 cells (E2WT). Lysates were made from IB4 or from BJAB transfectants, and aliquots were incubated with each type of nucleic acid coupled to matrix: ssDNA- or dsDNA-cellulose (sDNA or dDNA), control cellulose (celo), or poly(A) or poly(G)-agarose (polA or polG). Bound proteins were subjected to SDS-PAGE, and EBNA-2 was detected by Western blotting. In panel B, 10% of the proteins from the starting lysates or from the supernatants (SUP) after incubation with nucleic acid as in panel A were run on a gel, and EBNA-2 was identified by immunoblotting. Positions of EBNA-2 are indicated by arrowheads. Sizes are indicated in kilodaltons.

EBNA-2 has been reported to bind to histone H1 (21), we further investigated whether the Arg-Gly domain might mediate that interaction. In fact, labeled GST-E2,335-360 bound to purified histone H1 or to histone H1 from nuclear extracts (Fig. 7A). Furthermore, soluble histone H1 when added to the probe buffer prevented the binding of labeled GST-E2,335-360 to histone H1 and to other cellular proteins immobilized on filters (Fig. 7B). These experiments indicate that the Arg-Gly domain can bind to histone H1 and that histone H1 can block the binding of the Arg-Gly domain to most other proteins even though histone H1 and the Arg-Gly repeat are both positively charged proteins.

The Arg-Gly repeat can interact with nucleic acids and mediates much of the EBNA-2 interaction with nucleic acids. EBNA-2 also binds to both ssDNA- and dsDNA-cellulose (14), but the DNA binding domain is not known. To investigate whether the Arg-Gly domain could mediate EBNA-2 interaction with nucleic acids, ^{32}P-labeled GST-E2,335-360 was incubated with ssDNA or dsDNA immobilized on cellulose or with poly(A), poly(C), poly(G), or poly(U) immobilized on agarose beads, and the bound fusion protein was quantitated by the amount of radioactivity retained on the matrix. GST-E2,335-360 bound better to ssDNA than to dsDNA and bound best to poly(G) (Table 1). GST-E2,335-360 did not bind significantly to cellulose or to poly(A)-, poly(C)-, or poly(U)-agarose (data not shown).

To evaluate the role of the Arg-Gly domain in EBNA-2 interaction with nucleic acid, extracts from B lymphocytes expressing wt EBNA-2 or E2d337-354 were passed through poly(A)- or poly(G)-agarose or ssDNA- or dsDNA-cellulose. As is characteristic of the Arg-Gly domain alone, EBNA-2 bound to ssDNA- and dsDNA-cellulose and even more strongly to poly(G)-agarose. In contrast, E2d337-354 bound somewhat less to ssDNA- and dsDNA-cellulose and very poorly to poly(G)-agarose (Fig. 8A). Very little wt EBNA-2 passed through ssDNA- or dsDNA-cellulose or poly(G)-agarose, while more E2d337-354 passed through ssDNA- or dsDNA-cellulose and almost all of the E2d337-354 passed through poly(G)-agarose (Fig. 8B). Thus, EBNA-2 binds to ssDNA, dsDNA, or poly(G); moreover, the Arg-Gly repeat is an important component of the ssDNA and dsDNA binding activity and the key component of poly(G) binding activity.

Poly(G), poly(dG), and poly(I) can form very stable quartet structures (43, 53), which could underlie the specificity of the Arg-Gly repeat for poly(G) as opposed to poly(C), poly(U), or poly(A). To investigate whether the ability of poly(G) to form quartet structures underlies the interaction, poly(dG)$_{15}$ or poly(I) was incubated in 0.1 or 1 M salt under conditions which would favor quartet formation and tested as competitors for poly(G) interaction with GST-E2,335-360. While soluble poly(G) in 10-fold excess could effectively block GST-E2,335-360 binding to poly(G)-agarose, poly(dG) and poly(I) were much less effective. Furthermore, although methylation of the N-7 position in G by dimethyl sulfate interrupts hydrogen bonding between neighboring guanine bases and prevents quartet formation (53), methylated poly(dG) had no less blocking activity than poly(dG) (Table 2). Thus, the Arg-Gly

TABLE 1. Binding of the Arg-gly Domain to nucleic acids

Nucleic acid[a]	Degree of binding[b]						
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6	Avg
ssDNA	26.6	126.7	122.6	91	25	32.3	70.7 ± 44.2
dsDNA	9.2	NA	NA	NA	7.2	10.4	89 ± 1.3
Poly(G)	386	534.6	259.9	692	NA	NA	468 ± 161

[a] Nucleic acids coupled to matrix were used for incubation with ^{32}P-labeled GST fusion proteins.

[b] Defined as counts retained by matrix after incubation with GST-E2, 335-360/ counts retained by matrix after incubation with irrelevant protein. NA, not available.

TABLE 2. Block of binding to poly(G) by G-quartet structure[a]

Blocker	Degree of blocking[b]				
	Expt 1	Expt 2	Expt 3	Expt 4	Avg
None	1.0	1.0	1.0	1.0	1.0
Poly(dG)	0.88	0.26	0.35	0.62	0.53 ± 0.24
Methylated poly(dG)	0.93	0.45	0.35	0.70	0.61 ± 0.22
Poly(I)	0.21	0.19	0.80[c]	0.52[c]	0.43 ± 0.25
Poly(G)	0.016	0.015	0.043[c]	0.018[c]	0.023 ± 0.011

[a] ^{32}P-labeled GST-E2,335-360 was preincubated with a 10-fold excess of blocker before being added to poly(G)-agarose. The salt is 100 mM NaCl or as indicated.

[b] Calculated as counts retained by poly(G) with blocker/counts retained by poly(G) without blocker.

[c] The salt used is 1 M KCl.

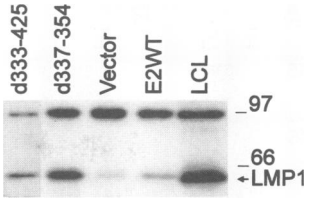

FIG. 9. Transactivation of LMP1 from the P3HR-1 genome by EBNA-2 and region 3 deletions. BL30/P3HR-1 cells were transfected with pSG5-EBNA 2 (E2WT), the pSG5 vector control (Vector), pSG5-E2,d337-354 (d337-354), or pSG5-E2,d333-425 (d333-425). Cells were harvested, and lysates from transfectants or from IB4 cells (LCL) were immunoprecipitated with monoclonal antibody S12. The immunoprecipitates were subjected to SDS-PAGE. LMP1 protein was detected by monoclonal antibody S12. Sizes were indicated in kilodaltons.

repeat specifically binds to poly(G), and the G-quartet structure probably is not the basis for the specificity for poly(G).

Activity of EBNA-2 region 3 deletion mutants on transactivation. Since LMP1 is essential for primary B-lymphocyte growth transformation and EBNA-2 is an important transactivator of the LMP1 promoter, one essential function for EBNA-2 in transformation is through LMP1 promoter transactivation. To determine if E2d337-354 has an adverse effect on LMP1 transactivation which would account for the adverse effects on cell growth transformation, transactivation by E2d337-354, E2d359-383, and E2d385-430 was compared with transactivation by wt EBNA-2 as a positive control (48) or by E2d333-425 as a null mutant control (9). The EBNA-2 open reading frames were cloned into the pSG5 expression vector and transfected into BJAB cells along with a reporter plasmid consisting of the CAT gene under the control of the thymidine kinase promoter and LMP1 upstream sequence (−234/+40LMPCAT) (48). A β-galactosidase expression plasmid was included in each transfection as an internal control. Surprisingly, E2d337-354 induced four- to fivefold-higher CAT activity than wt EBNA-2, while E2d359-383, E2d385-430, and E2d333-425 were nearly as active as wt EBNA-2 (Table 3).

To investigate the relative efficiency of pSG5-E2,d337-354 in transient transactivation of the LMP1 gene from EBV episomes which are in nucleosomal structures (15), pSG5-EBNA 2, pSG5-E2,d337-354, or pSG5-E2,d333-425 was transfected into BL30/P3HR-1 cells. Since the P3HR-1 genome lacks EBNA-2, there is little LMP1 protein in BL30/P3HR-1 cells, but expression can be induced by wt EBNA-2 (9). In BL30/P3HR-1 cells, the effects of wt EBNA-2 or of E2d337-354 on LMP1 expression paralleled the effects on LMP1CAT plasmids. E2d337-354 induced two- to fourfold-higher LMP1 levels than did wt EBNA-2 (Fig. 9 and data not shown). Although EBNA-2 deleted for all of region 3 has been reported to be a null mutant in inducing LMP1 expression in BL30/P3HR-1 cells (9), the previous result was probably due to experimental error, since repetition of the previous experiment showed that E2d333-425 was active in transactivation of LMP1 gene (Fig. 9 and reference 6a). Thus, E2d333-425 has a null transforming phenotype and a near wt LMP1 transactivating phenotype, while E2d337-354 is deficient in transformation and has greater than wt LMP1 transactivating activity.

EBNA-2 has also been shown to transactivate the Cp promoter through an upstream element which is similar to the LMP1 element in having one J kappa binding site (22, 32). EBNA-2, E2d337-354, E2d359-383, E2d385-430, or E2d333-425 was transfected into BJAB cells with a CpCAT reporter plasmid, and CAT activity was assayed (55). In contrast to the results with the LMP1 promoter, E2d337-354 was similar to wt EBNA-2 in Cp transactivation. E2d333-425 and E2d359-383 appeared to be deficient in transactivation, and E2d385-430 was similar to wt EBNA-2. These data suggest that EBNA-2 interacts differently with the LMP1 and Cp promoters, even though they both have a single J kappa binding site.

TABLE 3. Summary of CAT assays

Promoter	CAT activity[a] (mean ± SD)				
	wt	E2d337-354	E2d333-425	E2d359-383	E2d385-430
LMP1	4.9 ± 2.0	27.7 ± 10.6	3.0 ± 1.0	5.2 ± 2.6	5.2 ± 1.4
Cp	2.9 ± 0.6	2.6 ± 0.5	0.8 ± 0.3	1.6 ± 0.3	3.2 ± 1.2

[a] CAT activity from cotransfection with EBNA-2 expression vector divided by CAT activity from cotransfection with the pSG5 vector control. Each value was determined from at least five independent experiments.

DISCUSSION

These experiments confirm that region 3 is critical to primary B-lymphocyte growth transformation and identify the Arg-Gly repeat domain as an important component of region 3. Deletion of the Arg-Gly repeat domain reduces transforming efficiency. The transformed cells grow slowly and are persistently difficult to maintain in culture. In contrast, independent deletions of each part of the rest of region 3 have no adverse effect on primary B-lymphocyte growth transformation or on the growth of the transformed cells. The only region 3 residues not independently investigated in this study are aa 333 to 336, 355 to 358, and 384. These few residues are highly unlikely to constitute essential interactive domains, since deletions of immediately adjacent sequences do not affect transformation. Thus, the data indicate that region 3 does not interact with an essential mediator of cell growth transformation.

What then is the basis for the stringent requirement for region 3 in primary B-lymphocyte growth transformation? From an EBV recombinant genetic perspective, the stringent requirement is probably the sum of several less significant effects. The Arg-Gly domain deletion results in less efficient transformation, and the transformed cells grow poorly. The rest of region 3 when independently deleted as two parts has no significant effect on cell growth transformation, but when deleted as one segment it has a substantial effect on transformation efficiency. The Arg-Gly and other region 3 deletions probably cooperatively result in the null transforming effect characteristic of the whole region 3 deletion.

From a biochemical perspective, region 3 (aa 333 to 425) joins region 2 (aa 310 to 336) to the region 4 (aa 430 to 464) acidic transactivating domain. Region 2 is a proline-rich sequence, L-310HNLPSPPWWPPICDPPPQPSKTQGS-336 through which EBNA-2 binds to the cell protein, J kappa (22, 55). J kappa directs EBNA-2 to a GTGGGAA sequence that is a component of all EBNA-2 response elements. GPPWW PPXXDP is likely to be the core of the interacting domain, since the underlined residues are those conserved between the EBNA-2 genes of EBV types 1 and 2 (12) and mutation of the WW to SS or FF ablates J kappa interaction (22, 55). EBNA-2 with the WW-to-SS mutation is inactive in marker-rescuing transforming activity, providing a genetic link between J kappa interaction and cell growth transformation (55). Residues 333 to 336 are not likely to be important in J kappa interaction, since the type 2 EBV sequence is PPTN instead of QGQS. Therefore, the deletion of these residues in the whole region 3 deletion is not likely to be an important component of the effect of the larger deletion, although this alternative cannot be completely dismissed. Rather, the impressive effects of the region 3 deletion on transformation are more likely to be due to the tight approximation of the acidic and J kappa binding domains which may disrupt the interaction between EBNA-2 and J kappa. The negative effect of deletion E2d361-425 on transformation may have a similar basis. The deletion of the sequences between the acidic domain and the highly charged Arg-Gly domain may affect the activities of the Arg-Gly, the acidic, or the J kappa domain.

An expectation of this simple model is that the region 3 deletion would have a substantial negative effect on transactivation. Such an effect is observed on transient transactivation of the Cp promoter. However, in contrast to a previous report (9), the region 3 deletion has little or no effect on transient transactivation of the LMP1 promoter. The LMP1 and Cp promoters each have a single J kappa binding site (32). Deletion of the site from the Cp promoter ablates transactivation (25), while deletion from the LMP1 promoter does not totally ablate LMP1 transactivation (17). Thus, EBNA-2 must interact with another protein(s) which recognizes the LMP1 promoter.

The region 3 deletion has the expected negative effect on J kappa-mediated transactivation of the Cp promoter and may also have a similar effect on transactivation of the LMP1 promoter. However, the putative negative effect on the LMP1 promoter may be counterbalanced by a positive effect on this promoter from the Arg-Gly deletion.

The Arg-Gly domain is likely to be a negative modulator of EBNA-2 interactions with a second component of the LMP1 promoter. Although deletion of this domain results in decreased transforming efficiency, poor growth of the transformed cells, and decreased EBNA-2 chromatin association, EBNA-2 deleted for the Arg-Gly domain transactivates the Cp promoter and has abnormally high transactivating effects on the LMP1 promoter. The supranormal effect on the LMP1 promoter in transient transactivation assays indicates that an Arg-Gly interaction negatively regulates the LMP1 promoter. The absence of a similar effect on the Cp promoter suggests that the effect is not mediated through J kappa or through interactions between the Arg-Gly and acidic transactivating domains and favors the possibility that the Arg-Gly domain negatively affects the putative second component of the LMP1 promoter.

The Arg-Gly repeat is highly charged and therefore likely to constitute a surface domain of EBNA-2. This domain can interact with many proteins and nucleic acids. EBNA-2 extracted from EBV-transformed cells is part of a large complex (20), and the Arg-Gly domain may mediate some of these interactions. The Arg-Gly domain may also mediate EBNA-2 association with chromatin, possibly through interaction with histone H1.

TABLE 4. Summary of proteins containing the Arg-Gly domain

Protein[a] (reference)	First amino acid of Arg-Gly domain	Sequence
EBNA-2 (3)	337	$RGQS(RG)_6KGKS$
EBNA-1 (3)	356	$RGRERARGGSRERA(RG)_4$
SNF2 transactivator (30)	1505	$(RG)_9RP$
DNA-methyltransferase (4)	990	$(KG)_6KH$
Small nuclear RNP (42)	98	$(RG)_9$
BTF3a (60)	13	RGRGRARG
Papillomavirus E2 protein		
BPV-4 (38)	195	$(RS)_5$
HPV-5 (57)	267	$(RS)_2RH(RS)_5KS$
HPV-8 (18)	302	$RP(RS)_3RGRA$
Splicing factor		
U1-70K snRNP (16)	390	$(RD)_3RR(RD)_3RERD$
$U2AF^{65}$ (58)	27	$RSHS(RS)_2RDRKR(RS)_2$
SF2/ASF (19)	204	$(RS)_8NS(RS)_2$

[a] RNP, ribonucleoprotein; BPV-4, bovine papillomavirus type 4; HPV-5 and HPV-8, human papillomavirus types 5 and 8; snRNP, small nuclear ribonucleoprotein.

The interactions of the Arg-Gly repeats with other proteins and with nucleic acids are not likely to be simply related to positive-negative charge effects. Histone H1 is also a basic protein but binds to the Arg-Gly domain, and the Arg-Gly repeat can bind to itself. Among nucleic acids, only poly(G) has high affinity for the domain. Arginine has a large extended guanidinium side chain which can interact with nucleotides (56). Similar Arg-Gly motifs are also present in other proteins (Table 4) involved in transcription regulation, such as SNF2, a yeast transactivator; BTF3a, a general transcription factor; or papillomavirus E2 protein, a regulator of viral gene expression. Notably, many splicing factors have Arg-Ser-rich regions (Table 4), which mediate interactions among splicing factors (2, 5, 58, 63).

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