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Epstein-Barr Virus Nuclear Protein 2 Mutations Define Essential Domains for Transformation and Transactivation

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Epstein-Barr virus (EBV) nuclear protein 2 (EBNA-2) is essential for B-lymphocyte growth transformation. EBNA-2 transactivates expression of the EBV latent membrane protein (LMP-1) and also transactivates expression of the B-lymphocyte proteins CD21 and CD23. In order to analyze the functional domains of EBNA-2, we constructed 11 linker-insertion and 15 deletion mutations. Each of the mutant EBNA-2 proteins localized to the nucleus, and each was expressed at levels similar to wild-type EBNA-2. Deletion of both EBNA-2 basic domains was required to prevent nuclear localization, indicating that either is sufficient for nuclear translocation. The mutant EBNA-2 genes were assayed for lymphocyte transformation after recombination with the EBNA-2-deleted P3HR-1 EBV genome and for LMP-1 transactivation following transfection into P3HR-1-infected B-lymphoma cells. Cell lines transformed by recombinant EBV carrying EBNA-2 mutations were assayed for growth properties and LMP-1, CD21, and CD23 expression. The mutational analysis indicates that at least four separate EBNA-2 domains are essential for lymphocyte transformation. Two other domains are necessary for the full transforming phenotype. Two deletion and eight linker-insertion mutations did not reduce transforming activity. Mutations which diminish or abolish lymphocyte transformation also diminish or abolish LMP-1 transactivation, respectively. Cells transformed by recombinant EBV carrying EBNA-2 genes with diminished or normal transforming activity all expressed high levels of LMP-1, CD23, and CD21. These findings suggest that transactivation of these viral and cellular genes by EBNA-2 plays a critical role in lymphocyte transformation by EBV. Furthermore, these results indicate that the transformation and transactivation functions of EBNA-2 may not be separable.

Epstein-Barr virus (EBV) causes an acute lymphoproliferative disease in immunosuppressed patients (45), cotton-top tamarins (22), and mice with severe combined immunodeficiency (24) and is associated with Burkitt's lymphoma and nasopharyngeal carcinoma (20). Primary B-lymphocyte infection in vitro results in expression of EBV genes and perpetual cell proliferation. The EBV genes expressed in latently infected lymphocytes encode nuclear proteins EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP, latent membrane proteins LMP-1, LMP-2A, and LMP-2B, and two small RNAs (15). These genes are likely to maintain latency or lymphocyte proliferation.

EBNA-2 is particularly interesting because it is essential for lymphocyte transformation. EBNA-2 reduces the serum requirements of rodent fibroblasts (8) and transactivates CD23 (36), CD21 (37), c-*fgr* (17), and LMP-1 (1, 39). Recombinant viruses with EBNA-2 deletion (6) or truncation (12) mutations fail to transform B cells. In addition, the two naturally occurring EBV types, EBV-1 and EBV-2, differ widely in their ability to growth-transform B lymphocytes (29), and EBNA-2 is a major determinant of this type-specific difference (6).

Other EBV genes are also likely to be essential for lymphocyte growth transformation. LMP-1 is a key mediator of EBV's effects in inducing B-lymphocyte activation and adhesion molecules (35) and is a transforming oncogene in rodent fibroblasts (3, 33, 34). LMP-2 associates with LMP-1 and may contribute to the latter's effect (18). EBNA-3C upregulates CD21 (37), while EBNA-1 is necessary for EBV episome maintenance (44).

The amino acid sequences of EBV type 1 and 2 EBNA-2 are remarkably divergent. This divergence is potentially useful for predicting essential domains of the protein. The EBV type 1 and 2 EBNA-2 proteins (7) (Fig. 1) consist of a slightly negatively charged, well-conserved amino terminus (amino acids 1 to 58), a polyproline domain which differs in length (amino acids 59 to 95), a short conserved charged domain (amino acids 96 to 134), a long divergent domain (amino acids 135 to 281), a moderately well conserved proline-rich domain (amino acids 282 to 330), a moderately well conserved basic domain, including an arginine-glycine repeat motif (amino acids 331 to 369), a moderately well conserved acidic domain (amino acids 370 to 475), and a short, basic, conserved carboxy terminus (amino acids 476 to 483). A portion of the acidic domain near the carboxy terminus (amino acids 457 to 469 [29a]) is homologous to the retinoblastoma protein-interactive motif of adenovirus E1A (41), human papillomavirus E7 (11), and simian virus 40 (SV40) large T antigen (9). EBNA-2 undergoes extensive posttranslational modification, including phosphorylation, and is associated with the nuclear matrix, nucleoplasm, and chromatin (26, 38).

EBNA-2 may be essential for transformation because of its transactivating function, or transformation may be partially or completely separable from transactivation. A molecular genetic analysis of EBNA-2 transforming and transactivating functions should indicate whether the same or different EBNA-2 domains are required for these two functions. Therefore, linker-insertion and deletion mutations were constructed throughout the EBNA-2 gene so that the essential and dispensable transforming and transactivating

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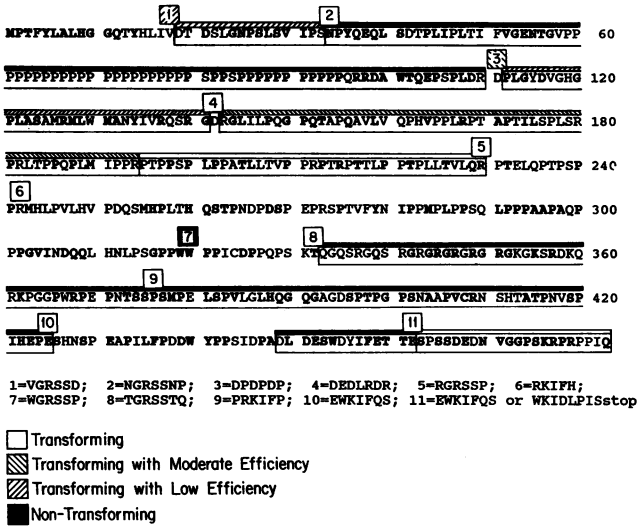

FIG. 1. Predicted amino acid sequence of EBNA-2 from the W91 EBV-1 strain. Numbered boxes above the sequence indicate the sites of linker-insertion mutations. Open boxes (without numbers) indicate deletion mutations with wild-type transformation phenotype, and hatched boxes (without numbers) indicate deletion mutations with reduced transformation efficiency. Solid boxes (without numbers) indicate deletion mutations that are nontransforming. Transformation with moderate efficiency is defined as ≤50% of the number of wells positive for transformation compared with wild-type EBNA-2; transformation with low efficiency is defined as ≤30% of the number of wells positive for transformation compared with wild-type EBNA-2. Amino acids indicated by boldface letters are conserved between W91 (EBV-1) and AG876 (EBV-2); amino acids not shown in boldface are not conserved between W91 and AG876. The amino acid sequence resulting from each linker-insertion mutation is shown below the complete sequence, beginning with the amino acid underneath the number and ending with the next amino acid in the original sequence. For example, the linker-insertion indicated by a 1 in the figure results in insertion of the sequence GRSS between amino acids 18 (V) and 19 (D) of EBNA-2. Each of the linker-insertion mutations except two results in insertion of new amino acids without loss of amino acids from the wild-type sequence. Linker-insertion 6 results in loss of an amino acid (M at position 243) and insertion of the sequence KIF; linker-insertion 9 results in loss of two amino acids (SM at positions 377 and 378) and insertion of the sequence RKIF.

domains could be identified. Transformation-competent EBNA-2 mutations were assayed for LMP-1, CD21, and CD23 transactivation in lymphoblastoid cell lines infected with mutant recombinant virus. Both transforming and nontransforming mutants were assayed for LMP-1 transactivation by transient transfection of the mutant EBNA-2 genes into B-lymphoma cells containing an EBV genome deleted for EBNA-2. These experiments identify EBNA-2 domains necessary for B-cell transformation and for LMP-1 transactivation.

MATERIALS AND METHODS

Cell lines. P3HR-1 clone 16 (kindly provided by G. Miller) is a Burkitt's lymphoma cell line that contains a transformation-incompetent EBV that lacks the EBNA-2 gene (28). BL30/P3HR-1 (kindly provided by G. Lenoir) is an EBV-negative Burkitt's lymphoma cell line that is infected with the P3HR-1 virus (4). IB4 is an EBV-infected lymphoblastoid cell line. W91 is an EBV-1 isolate from an African case of Burkitt's lymphoma (21).

Cosmids and plasmids. Cosmid T1EBNA-2 contains the *Eco*RI A fragment from W91 DNA inserted into plasmid MUA3 (27). Plasmid HK-EBNA-2 (6) contains the EBNA-2 gene of W91 EBV with adjacent sequences in pBluescript KS(+) (Stratagene). The nucleotide sequence of the EBNA-2 gene from W91 EBV was determined from both strands of plasmid DNA (46). Plasmid SVNaeIBamZ was constructed by inserting the 1.2-kb *Nae*I-*Bam*HI fragment from the *Bam*HI Z fragment of B95-8 EBV DNA into the *Bam*HI site of plasmid pSG5 (Stratagene).

Plasmids containing linker-insertion mutations were constructed by cutting plasmid HK-EBNA-2 with the appropriate restriction enzyme, producing blunt ends with the Klenow fragment of DNA polymerase (protruding 5′ ends) or T4 DNA polymerase (protruding 3′ ends) when necessary, and ligating a double-stranded oligonucleotide linker. Linkers containing *Bgl*II restriction endonuclease sites were chosen since there is no *Bgl*II site in the EBNA-2 or vector sequence. The linker was 8, 10, or 12 nucleotides in length as necessary to preserve the open reading frame. The nucleotide sequences of the linkers and adjacent sequences were determined. An *Hin*dIII-*Rsr*II fragment from the mutant plasmid was inserted in place of the wild-type fragment in cosmid T1EBNA-2. Restriction enzymes *Hin*cII, *Bam*HI, *Bst*EII, and *Bal*I were used to construct the linker-insertion mutations in cosmids T1EBNA-2li18, T1EBNA-2li111, T1EBNA-2li142, and T1EBNA-2li320, respectively. Partial restriction digests with *Hin*fI were used to generate cosmids T1EBNA-2li34, T1EBNA-2li332, T1EBNA-2li425, and T1EBNA-2li462; with *Sph*I to generate T1EBNA-2li242 and T1EBNA-2li376; and with *Stu*I to generate T1EBNA-2li230. Numbers refer to the amino acid site of the linker-insertion mutation in the EBNA-2 protein based on the sequence of W91 EBNA-2.

Plasmids containing deletion mutations were constructed by cutting plasmid HK-EBNA-2 with one or two restriction enzymes, producing blunt ends (as described above), and ligating the DNA ends together. The mutant EBNA-2 gene was then inserted in place of the wild-type gene in cosmid T1EBNA-2 (see above). Restriction enzyme pairs *Hin*cII-*Bam*HI, *Bam*HI-*Bst*EII, *Bst*EII-*Stu*I, and *Stu*I-*Bal*I were used to construct the deletion mutations in cosmids T1EBNA-2d19-110, T1EBNA-2d112-141, T1EBNA-2d143-230, and T1EBNA-2d195-320, respectively. The construction of cosmids T1EBNA-2d195-230, T1EBNA-2d244-378, and T1EBNA-2d143-320 was described previously (6). (The numbering of the deletions has been changed to reflect the sequence of W91 EBNA-2.) Cosmids T1EBNA-2d19-33, T1EBNA-2d321-378, T1EBNA-2d333-425, and T1EBNA-2d426-462 were constructed by using linker-insertion mutations from *Hin*cII and *Hin*fI (first site), *Bal*I and *Sph*I (second site), *Hin*fI (second and fourth sites), and *Hin*fI (fourth and fifth sites), respectively. Cosmid T1EBNA-2d398-473 was constructed by inserting the *Pfl*MI-*Ppu*MI EBNA-2 deletion mutation from B95-8 virus (25) into plasmid HK-EBNA-2 and subsequently replacing the EBNA-2 gene of T1EBNA-2 with the mutant EBNA-2. Cosmid T1EBNA-2d463-483 was constructed by cutting a plasmid containing the linker-insertion mutation from T1EBNA-2li462 with *Bgl*II, producing blunt ends with DNA polymerase I (Klenow fragment), and ligating the ends together. The resulting EBNA-2 gene in cosmid T1EBNA-2d463-483 encodes eight new amino acids (Trp-Lys-Ile-Asp-Leu-Pro-Ile-Ser) after amino acid 462, followed by a stop codon.

Cosmids T1EBNA-2d447-472 and T1EBNA-2d449-479 were constructed by cutting a plasmid containing the linker-insertion mutation from T1EBNA-2li462 with *Bgl*II, digesting with *Bal* 31, producing blunt ends with T4 polymerase, inserting a *Bgl*II linker, and ligating the ends together. Cosmid T1EBNA-2d244-378,d463-483 was constructed by cutting a plasmid containing the T1EBNA-2d463-483 with *Sph*I and ligating the ends of the large fragment together.

The junctions spanning each of the deletions in the EBNA-2 mutants were sequenced from plasmid DNA. Two separate cosmid clones were prepared for cosmids T1EBNA-2li18, T1EBNA-2li34, T1EBNA-2li111, T1EBNA-2li142, T1EBNA-2li320, and T1EBNA-2li462 and for each of the deletion mutants except T1EBNA-2d447-472 and T1EBNA-2d449-479.

The EBNA-2 gene for wild-type W91 EBNA-2, the EBNA-2 linker-insertion mutation at amino acid 320, and the EBNA-2 deletion mutants deleted for amino acids 19 to 33, 19 to 110, 143 to 230, 143 to 320, 195 to 230, 195 to 320, 321 to 378, 333 to 425, 449 to 479, and 463 to 483 were each isolated from their respective plasmids by using restriction enzymes *Bst*UI and *Dra*I and cloned into plasmid pZip-Neo-SV(X)1 (5) at the *Bam*HI site.

Transfections and infections. For production of recombinant EBV, cosmids (20 μg) were precipitated with plasmid pSVNaeIBamZ (40 μg) and transfected into P3HR-1 cells by use of an electroporator (Gene Pulser; Bio-Rad) with a pulse of 0.2 V at 960 μF. Four days after transfection, the virus was harvested and used to infect human umbilical cord mononuclear cells as described previously (6). At least two separate virus preparations were obtained for each cosmid that yielded recombinant virus with transforming activity equivalent to wild-type virus; multiple virus preparations (from at least two independent bacterial clones) were obtained for each cosmid that yielded nontransforming or weakly transforming recombinant virus.

For immunoprecipitations, cosmids or plasmids (25 μg) were transfected into BL30/P3HR-1 cells by electroporation. After 6 days, cells were counted and equal numbers of cells were used for immunoprecipitations.

Immunoprecipitations. LMP-1 was immunoprecipitated from an aliquot of the transfected cells as described previously (39). EBNA-2 was immunoprecipitated from another aliquot of cells (containing the same number of cells as for LMP-1). For EBNA-2 immunoprecipitations, cells were pelleted, resuspended in 0.5 ml of buffer A (phosphate-buffered saline with 0.2% Nonidet P-40), 0.5 ml of buffer B (10 mM Tris [pH 8.0], 100 mM NaCl, 0.5% Nonidet P-40, 1% deoxycholate) was added, and the supernatant was sonicated. The supernatant was incubated with PE2 anti-EBNA-2 monoclonal antibody (45) for 3 h at 4°C, protein G-Sepharose 4 (Pharmacia) was added, and the incubation was continued for 30 min at 4°C. Immunoprecipitates were washed three times with buffer B, resuspended in protein gel loading buffer, boiled, and run on a polyacrylamide gel.

Immunofluorescence, immunoblotting, and flow cytometry. EBNA-2 expression was analyzed by indirect immunofluorescence microscopy with an anti-EBNA-2 monoclonal antibody (45) or by immunoblot with human serum (36). LMP-1 was detected with the S12 anti-LMP-1 monoclonal antibody (19). Flow cytometry for cell surface expression of CD23 was performed with monoclonal antibody EBVCS 1 (16) and for surface expression of CD21 with monoclonal antibody HB5 (31).

TABLE 1. Comparison of EBNA-2 sequence of EBV W91, EBV B95-8, EBV AG876, and EBV Jijoye

Nucleotide position	Nucleotide[a]			Amino acid[a]			
	W91	B95-8	AG876	W91	B95-8	AG876	Jijoye
185	C	T	d	Pro	Leu	d	d
198–212	d	N_{15}[b]	d	d	Pro_5[b]	d	d
219	T	C	d	Pro	Pro	d	d
228	T	C	d	Pro	Pro	d	d
237	T	C	d	Pro	Pro	d	d
246	T	C	d	Pro	Pro	d	d
256	T	C	G	Ser	Pro	Ala	Ala
487[c]	G	A	A	Val	Arg	Thr	Thr
488[c]	T	G	C				
495	A	C	A	Val	Val	Gln	Gln
554	G	A	C	Arg	Gln	Ala	Ala
588	T	G	C	Ile	Met	Ala	Ala
610	T	A	A	Ser	Thr	Thr	Thr
633–634	CTC	d	d	Leu	d	d	d
667	T	C	C	Leu	Leu	Gln	Gln
946	A	C	C	Asn	His	Pro	Pro
1110	A	T	A	Pro	Pro	Pro	Pro
1251	A	G	A	Thr	Thr	Ser	Ser
1410	T	A	T	Ser	Ser	Ser	Ser
1420	C	T	A	Asn	Tyr	Asn	Asn
1427	G	A	G	Gly	Glu	Gly	Gly
1453	C	T	T	Pro	Ser	Ser	Ser

[a] d, Nucleotide or amino acid deleted.
[b] CCCACCACCACCCCC, encoding five Pro residues in the context of adjacent sequence.
[c] Nucleotides 487 and 488 are part of the same codon.

RESULTS

Parent EBNA-2 gene nucleotide sequence. EBNA-2 mutants were constructed from an EBV-1 (W91 strain [21]) cosmid DNA clone (27). Since the degree of divergence in EBV-1 EBNA-2 gene DNA sequences is unknown, the sequence of W91 EBNA-2 was determined and compared with the previous EBV-1 (B95-8 strain) EBNA-2 sequence and the two previous EBV-2 (AG876 and Jijoye strain) EBNA-2 sequences (7, 10). The predicted W91 EBNA-2 amino acid sequence is shown in Fig. 1. Comparison of the W91 and B95-8 EBV-1 EBNA-2 sequences shows 38 (2.6%) nucleotide and 16 (3.3%) amino acid differences (Table 1). W91 has a five-amino-acid deletion and a one-amino-acid insertion relative to B95-8. In contrast, the two predicted type 2 EBNA-2 amino acid sequences are identical. With the exception of amino acids 204, 485, and the amino acid insertion (between amino acids 211 and 212 of B95-8), W91 differences from B95-8 coincide with the differences between B95-8 and type 2 EBNA-2.

Lymphocyte transformation by EBV containing EBNA-2 mutations. P3HR-1 cells were used to generate recombinant EBV containing EBNA-2 mutations. These cells are infected with an EBV strain, P3HR-1, which is deleted for EBNA-2 and therefore cannot transform B lymphocytes. Transfection of these cells with EBV DNA fragments followed by induction of P3HR-1 virus replication results in homologous recombination of the viral genome with the transfected DNA (6, 12). Recombinant P3HR-1 EBV with a wild-type EBNA-2 gene can transform primary B lymphocytes. The transfection, induction of viral replication, recombination, and primary B-lymphocyte transformation processes are sufficiently reproducible so that transformation by the resultant recombinant EBV can be quantitated. Therefore, transformation by recombinant EBV containing EBNA-2 muta-

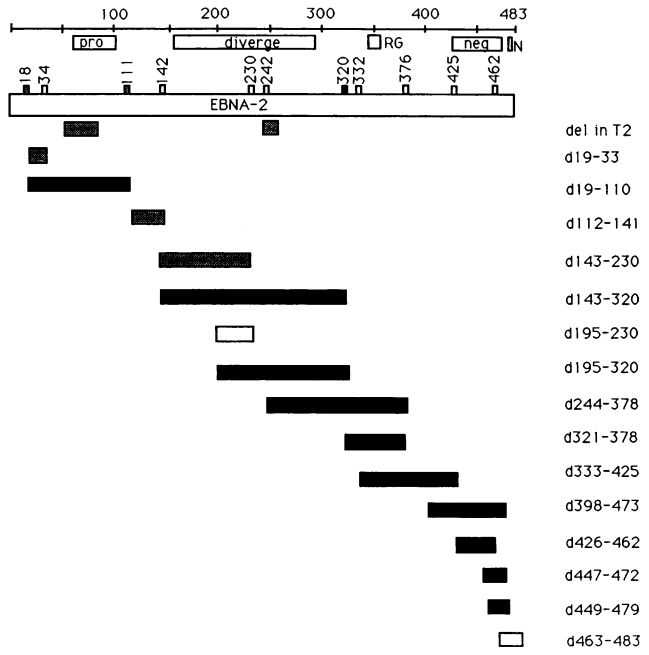

FIG. 2. Map of EBNA-2 linker-insertion and deletion mutants. The scale at the top refers to the EBNA-2 amino acid sequence shown in Fig. 1. Below the scale, pro indicates the polyproline domain, diverge indicates the least-conserved sequence between types 1 and 2, RG indicates the arginine-glycine repeat motif, neg indicates the negatively charged domain, and N indicates the putative nuclear localization signal at the carboxy terminus. The vertical bars above the boxed EBNA-2 indicate the sites of the linker-insertion mutations. Boxes below indicate deletion (del) mutations which were constructed or occur naturally in type 2 EBNA-2 (T2). Numbers after d refer to the amino acids deleted from the predicted W91 EBNA-2 sequence shown in Fig. 1. Open bars indicate wild-type or nearly wild-type transformation phenotype, stippled bars indicate reduced transformation efficiency, and solid bars indicate nontransforming mutations.

TABLE 2. Transformants obtained and LMP transactivation for recombinant EBV containing EBNA-2 mutations

Cosmid	Mean no. of wells containing transformants (no./plate)	Time to colony formation (wk)	LMP transactivation[a]	Staining with PE2 monoclonal antibody
Linker-insertion mutants				
T1EBNA-2li18	6 (1, 1, 17)	4–8	1+	+
T1EBNA-2li34	39 (32, 38, 48)	3–4	2+	+
T1EBNA-2li111	22 (5, 12, 24, 45)	3–4	1+	+
T1EBNA-2li142	43 (38, 44, 46)	3	2+	+
T1EBNA-2li230	36 (19, 53)	3	3+	+
T1EBNA-2li242	52 (46, 46, 64)	3	3+	+
T1EBNA-2li320	0 (0, 0, 0, 0)		0	+
T1EBNA-2li332	59 (54, 64)	3	2+	+
T1EBNA-2li376	54 (53, 54)	3	3+	+
T1EBNA-2li425	59 (54, 64)	3	3+	+
T1EBNA-2li462	64 (64, 64)	3	4+	+
Deletion mutants				
T1EBNA-2d19-33	7 (1, 2, 2, 12, 20)	4–6	+/−	+
T1EBNA-2d19-110	0 (0, 0, 0, 0)		0	+
T1EBNA-2d112-141	3 (1, 1, 2, 6)	4–8	2+	+
T1EBNA-2d143-230	19 (11, 20, 20, 24)	3–5	2+	+
T1EBNA-2d143-320	0 (0, 0, 0, 0, 0)		0	+
T1EBNA-2d195-230	40 (18, 46, 55)	3	4+	+
T1EBNA-2d195-320	0 (0, 0, 0, 0)		0	+
T1EBNA-2d244-378	0 (0, 0, 0)		0	+
T1EBNA-2d321-378	0 (0, 0)		0	+
T1EBNA-2d333-425	0 (0, 0)		0	+
T1EBNA-2d398-473	0 (0, 0, 0, 0, 0, 0)		0	−
T1EBNA-2d426-462	0 (0, 0, 0, 0)		0	−
T1EBNA-2d447-472	0 (0, 0)		0	+
T1EBNA-2d449-479	0 (0, 0)		0	+
T1EBNA-2d463-483	55 (54, 55, 55)	3	5+	+
Wild-type T1EBNA-2	55 (50, 53, 55, 61)	3	4+	+

[a] LMP transactivation was scored from 0 to 5+ relative to transactivation by the vector control (0) or wild-type EBNA-2 (4+).

tions can be compared with recombinant EBV with wild-type EBNA-2 (6).

The EBNA-2 linker-insertion and deletion mutations which were constructed for these experiments are shown in Fig. 2. Each mutation was inserted into an EBV cosmid, T1EBNA-2, which substantially overlaps the EBV P3HR-1 deletion. The recombinant cosmid was transfected into P3HR-1 cells, and virus replication was induced by cotransfection with plasmid pSVNaeIBamZ. The resultant viruses, consisting of nontransforming parent EBV P3HR-1 and EBV which has undergone recombination with the transfected cosmid DNA, were used to infect primary B lymphocytes. A cosmid containing wild-type EBNA-2 was transfected in parallel with the cosmids containing mutant EBNA-2 to control for the transfection, recombination, and transformation efficiency in each experiment. The number of transforming EBV recombinants was assessed by infecting primary B lymphocytes with the recombinant virus preparation and counting the number of microwells that were positive for transformation. B lymphocytes were infected with an undiluted preparation of recombinant virus so that mutants with diminished transforming activity could be detected. At this concentration of virus, the number of microwells that were positive for transformation with recombinant EBV containing wild-type EBNA-2 was just beyond the linear range. Almost all of the wells were positive with an undiluted preparation of wild-type EBNA-2 recombinant virus; about one-half of the wells were positive with a fourfold dilution of the wild-type EBNA-2 recombinant virus preparation. The undiluted preparation of recombinant EBV containing wild-type EBNA-2 was estimated to contain about 100 to 200 transforming viruses, based on the number of transformed wells obtained with serial dilutions of this virus preparation (data not shown). The number of transformed wells obtained when primary B lymphocytes were infected with each recombinant virus preparation is shown in Table 2.

Ten of 11 linker-insertion and 5 of 15 EBNA-2 deletion mutation recombinant viruses transformed lymphocytes. One linker-insertion mutant (amino acid 18) and two deletion mutants (amino acids 19 to 33 and 112 to 141) consistently had a lower transforming efficiency than wild-type EBNA-2 ($\leq$30% of the number of wells transformed with wild-type recombinant virus [Table 2]). This is a minimal estimate of the reduction in transforming activity, since the wild-type EBNA-2 recombinant virus preparation, which resulted in nearly all wells being positive for transformation, corresponded to 100 to 200 transforming viruses in endpoint dilution assays. Transformants with recombinant EBV containing these mutations also took longer to appear as macroscopically visible colonies (Table 2) and grew at a slower rate than recombinants containing wild-type EBNA-2 (data not shown). One linker-insertion mutant (amino acid 111)

and one deletion mutant (amino acids 143 to 230) showed a moderately reduced transforming efficiency compared with wild-type EBNA-2 (≤50% of the number of wells transformed with wild-type recombinant virus [Table 2]). Many of the transformants with these mutations took slightly longer to appear as macroscopically visible colonies, and cell lines from these transformants grew at a slower rate than recombinant EBV with wild-type EBNA-2 (data not shown). These two mutants were classified as transforming with moderate efficiency, since the data suggest that they were not fully wild type and not as clearly impaired as those with a low transforming efficiency. Eight of the linker-insertion mutants and two of the deletion mutants resulted in approximately the same number of positive wells as recombinants with wild-type EBNA-2; however, a twofold difference would not be detected in this assay.

One linker-insertion mutant (amino acid 320) and 10 deletion mutants (amino acids 19 to 110, 143 to 320, 195 to 320, 244 to 378, 321 to 378, 333 to 425, 398 to 473, 426 to 462, 447 to 472, and 449 to 479) were unable to transform lymphocytes (Table 2). To verify that these results were not due to experimental variability or to unexpected mutations occurring during recombinant DNA cloning, all experiments which yielded few or no transformants were repeated several times with at least two independently derived recombinant DNA clones. Independently derived clones were not obtained, however, for two EBNA-2 deletion mutants (amino acids 447 to 472 and 449 to 479). These deletions are nearly identical and are in that respect controls for each other.

Detection and localization of EBNA-2 mutants. Transfection of each cosmid containing an EBNA-2 mutant into Burkitt's lymphoma cell lines induced EBNA-2 antigen in similar numbers of cells (0.5 to 2%) 48 h after transfection as determined by immunofluorescence microscopy with an anti-EBNA-2 monoclonal antibody. The EBNA-2-specific monoclonal antibody PE2 detected each mutant except for two of the deletion mutants (amino acids 398 to 473 and 426 to 462 [Table 2]). Affinity-purified EBV-immune human serum specific for EBNA-2 (26) detected these EBNA-2 mutants. Since the mutant with deletion of amino acids 447 to 472 was detected with PE2 and the mutant with deletion of amino acids 426 to 462 was not, PE2 likely recognizes an epitope between amino acids 426 and 446. Since PE2 reacts with both type 1 and type 2 EBNA-2, and amino acids 429 to 443 are largely conserved between type 1 and type 2 EBNA-2, the PE2 epitope is likely to lie between amino acids 429 and 443.

Each mutant, including those with deletions of potential nuclear localization signals (amino acids 244 to 378 or amino acids 463 to 483), localized to the nucleus (see above and Table 2). To demonstrate that either putative nuclear localization signal was sufficient for nuclear translocation, a mutant deleted for both sequences was constructed. A double mutant with deletion of amino acids 244 to 378 and 463 to 483 showed diffuse cytoplasmic staining, with occasional cells showing a perinuclear pattern (Fig. 3).

Transient EBNA-2 expression from the cosmids after transfection into BL30/P3HR-1 cells was sufficient to allow quantitation of EBNA-2 expression on immunoblots after immunoprecipitation. A representative immunoblot is shown in Fig. 4. Cosmids with EBNA-2 mutants expressed EBNA-2 at levels similar to the parent wild-type cosmid. There was comparable EBNA-2 expression for each of the mutants when the transfected cells were stained for EBNA-2 expression with the anti-EBNA-2 monoclonal antibody (data not shown). The linker-insertion mutant cosmids expressed slightly larger EBNA-2 (data not shown), while the deletion mutant cosmids expressed smaller EBNA-2, compatible with the size of the deletion (Fig. 4). Deletions involving the polyproline or the negatively charged carboxy-terminal region of EBNA-2 resulted in proteins with disproportionately increased electrophoretic mobility. These results are compatible with the hypothesis that the polyproline and acidic domains contribute to the slower than expected electrophoretic mobility of wild-type EBNA-2.

Induction of LMP-1 by EBNA-2 mutants. Two series of experiments were done to assay the ability of the EBNA-2 mutants to transactivate LMP-1. In the first set of experiments, cosmids containing wild-type EBNA-2, each of the EBNA-2 linker-insertion mutants, and each of the EBNA-2 deletion mutants were used. In the second set of experiments, wild-type EBNA-2 and most of the deletion mutants (especially those that failed to transform B lymphocytes) were cloned into a smaller plasmid, pZip-Neo-SV(X)1. Plasmids or cosmids containing wild-type or mutant EBNA-2 were transfected into BL30/P3HR-1 cells, and transactivation of the P3HR-1 LMP-1 gene was assayed by immunoprecipitation and immunoblot. Experiments with plasmids and cosmids were each done in triplicate; thus, a total of six independent experiments were performed for each of the mutants shown in Fig. 5. The relative amounts of LMP-1 expressed with each of the mutants was consistent between experiments; however, the absolute amount of LMP-1 expressed varied between experiments. Therefore, LMP-1 was not quantitated directly but was graded from 0 to 5+ relative to transactivation with the vector control (0+) or with wild-type EBNA-2 (4+). Levels of LMP-1 transactivation (relative to wild-type EBNA-2) obtained for EBNA-2 mutants were similar for cosmid and plasmid vectors; however, the transfection efficiency with the plasmid vector (2 to 5%) was higher than with the cosmid vector (0.5 to 2%). An immunoblot of immunoprecipitations from transfections with each of the plasmids is shown in Fig. 5.

LMP-1 transactivation generally correlated with transforming activity for each of the mutants (Table 2). The one linker-insertion mutant and 10 deletion mutants that failed to transactivate LMP-1 also failed to transform B lymphocytes. Two deletion and five linker-insertion mutants that had wild-type (4+) or nearly wild-type (3+) LMP-1 transactivating activity had a wild-type transforming phenotype. Three linker-insertion and two deletion mutants had moderately reduced (2+) LMP-1-transactivating activity. The three linker-insertion mutants had a wild-type transformation phenotype; however, the two deletion mutants had moderate (amino acids 143 to 230) or reduced (amino acids 112 to 141) transforming activity. Two linker-insertion mutants had low (1+) LMP-1-transactivating activity; one had moderate (amino acid 111) and one had low (amino acid 18) transforming activity. A single deletion mutant (amino acids 19 to 33) had barely detectable (+/−) LMP-1 transactivating activity and low transforming activity. Thus, all EBNA-2 domains that were essential for LMP-1 transactivation were essential for B-lymphocyte transformation. Furthermore, all EBNA-2 domains that were essential for transformation were also essential for LMP-1 transactivation. However, some mutations which showed reduced LMP-1 transactivation had a wild-type transformation phenotype.

Recombinant viruses containing EBNA-2 mutants which retained the transforming phenotype could be assayed for LMP-1 expression in the resultant transformed lymphoblastoid cell line. At least two independent clones of each mutant were analyzed and two separate cell extracts were prepared

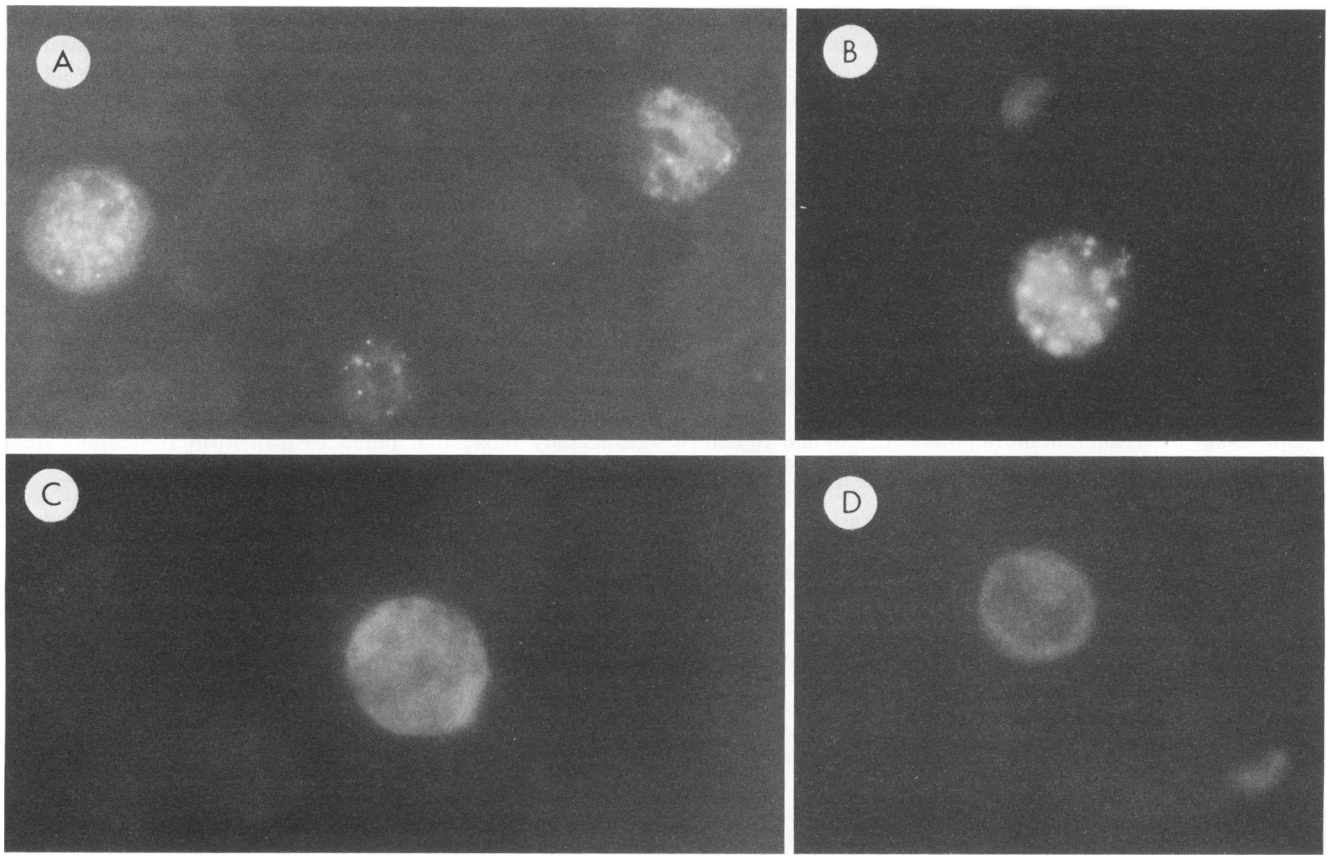

FIG. 3. Immunofluorescence staining of BL30/P3HR-1 cells transfected with EBNA-2 mutants deleted for amino acids 244 to 378 (A), amino acids 463 to 483 (B), or amino acids 244 to 378 and 463 to 483 (C and D). Note the punctate nuclear staining with nucleolar exclusion (A and B) compared with the diffuse cytoplasmic staining (C) and perinuclear staining (D) for cells expressing EBNA-2 deleted for amino acids 244 to 378 and 463 to 483.

from each clone. EBNA-2 mutants which had normal or nearly normal LMP-1-transactivating activity in transient assays yielded lymphoblastoid cell lines that expressed LMP-1 at levels similar to those in cell lines obtained from recombinant virus containing wild-type EBNA-2. However, EBNA-2 mutants with markedly reduced LMP-1-transactivating activity in transient assays also yielded lymphoblastoid cell lines that expressed LMP-1 at levels similar to those in cell lines obtained from recombinant virus with wild-type EBNA-2. The same samples were used for the EBNA and LMP-1 immunoblots (Fig. 6 and 7, respectively). EBNA-1 (Fig. 6) is a useful internal control for the amount of protein loaded in these immunoblots. While the IB4, P3HR-1, deletion mutant 19-33, and mutant 143-230 samples are slightly overloaded relative to most of the others, the level of LMP-1 expression in the cell lines containing the EBNA-2 mutants was at least as high as the level in the wild-type EBNA-2 recombinant. Thus, the LMP-1 transactivation activity of the EBNA-2 mutants in transient assays did not always correlate with the level of LMP-1 expression in lymphoblastoid cell lines derived from the mutants.

The level of EBNA-2 in all of the cell lines was similar except for T1EBNA-2d463-483. The apparent low level of expression of this mutation in both transient transfection (Fig. 4) and stable cell lines (Fig. 6) may have been due to poorer transfer of the protein from the gel to the nitrocellulose. The difference in EBNA-2 expression between

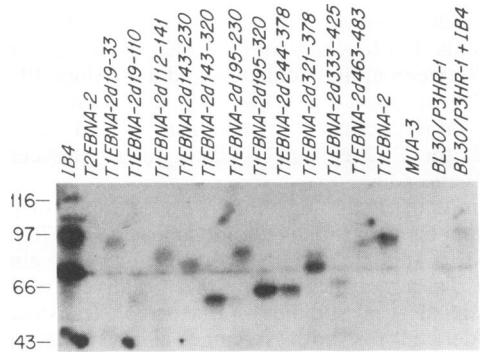

FIG. 4. Immunoblots of EBNA-2 expressed in BL30/P3HR-1 cells following transient transfection with cosmids containing EBNA-2 or EBNA-2 mutants. EBNA-2 was immunoprecipitated with the PE2 monoclonal antibody and detected by immunoblot with an EBV-1 human immune serum. A small amount of EBNA-1 carries over in the immunoprecipitate and is evident at about 73 kDa in each lane. EBNA-2 is the band at about 92 kDa in the IB4 lane, and EBNA-2 is the band above or below EBNA-1 in the lanes from cells transfected with cosmids containing EBNA-2. The IB4 lane is a protein extract from the IB4 lymphoblastoid cell line that has not been immunoprecipitated. Other lanes are immunoprecipitates from cells transfected with cosmids T1EBNA-2 or T2EBNA-2, cosmid vector MUA3, untransfected BL30/P3HR-1 cells, or BL30/P3HR-1 cells mixed with IB4 cells (last lane).

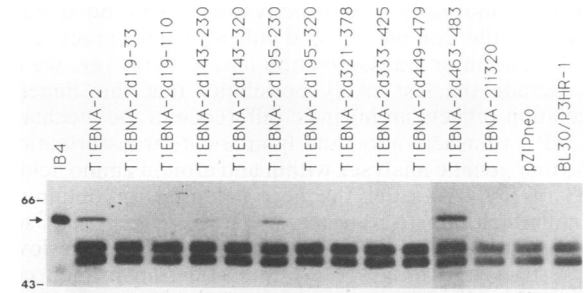

FIG. 5. LMP-1 transactivation in BL30/P3HR-1 cells transfected with wild-type or mutant EBNA-2 in pZip-Neo-SV(X)1. LMP-1 was immunoprecipitated and detected with the S12 monoclonal antibody. The arrow indicates LMP-1. The IB4 lane is a protein extract from the IB4 lymphoblastoid cell line that has not been immunoprecipitated. All other lanes are immunoprecipitates from cells transfected with EBNA-2 mutants (T1EBNA-2) cloned in pZip-Neo-SV(X)1, the vector control (pZIPneo), or untransfected BL30/P3HR-1 cells. Since BL30/P3HR-1 expresses a low level of LMP-1, a very faint band can be seen in both the BL30/P3HR-1 and vector control lanes. The two bands below the arrow are derived from the heavy chain of the S12 antibody which is recognized by the secondary antibody used to probe the blot. Sizes are indicated in kilodaltons.

T1EBNA-2d463-483 and the other mutants was less evident when EBNA-2 was detected by immunofluorescence with the monoclonal antibody to EBNA-2.

Induction of CD23 and CD21 by EBNA-2 mutants. Lymphoblastoid cell lines transformed by recombinant EBV containing EBNA-2 mutations were also analyzed for CD23 and CD21 expression because these B-cell activation molecules are induced by EBNA-2 (36, 37). All of the transformed lymphoblastoid cell lines showed high-level CD23 and CD21 surface expression. There was substantial quantitative variation in the high CD21 and CD23 expression levels both among different mutants and in different lymphoblastoid cell lines derived from the same mutant (data not shown). The mean CD23 surface expression for lymphoblastoid cell lines containing EBNA-2 deletion mutants was 55 to 88% of that seen in lines containing wild-type EBNA-2; the mean CD21 surface expression for cell lines with EBNA-2 deletion mutants was 78 to 96% of that seen with lines containing wild-type EBNA-2.

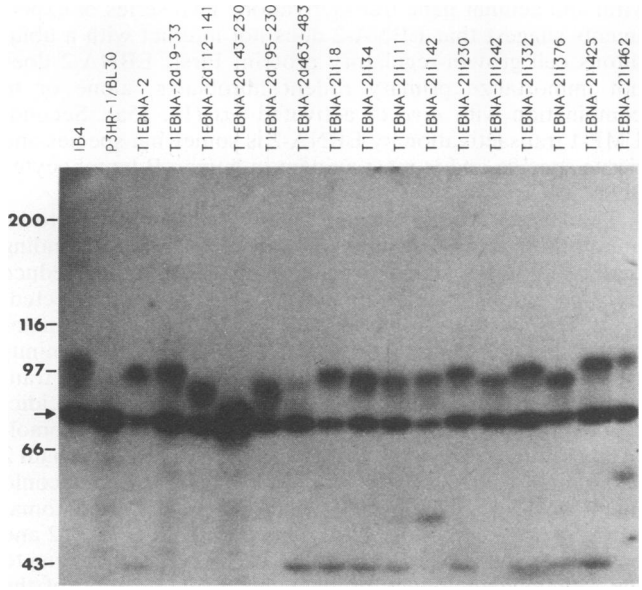

FIG. 6. Immunoblots of EBNA-1 and EBNA-2 in protein extracts from lymphoblastoid cell lines transformed by wild-type or mutant EBNA-2 recombinant viruses. Controls include EBNA-2-positive IB4 cells (lane 1) and EBNA-2-negative BL30/P3HR-1 cells (lane 2). The arrow indicates EBNA-1. EBNA-2 is the band above the arrow and varies in size as in Fig. 4; EBNA-LP is the band below the arrow. Sizes are shown in kilodaltons.

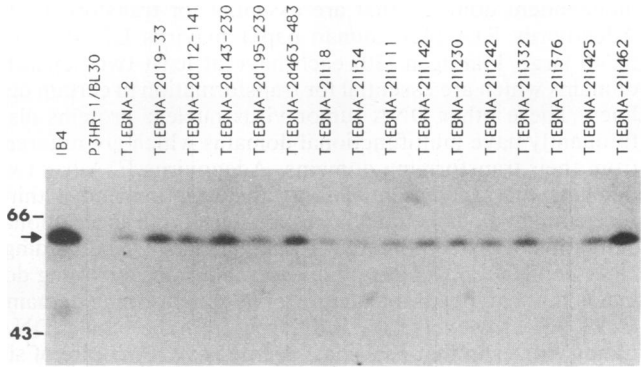

FIG. 7. Immunoblots of LMP-1 in protein extracts from lymphoblastoid cell lines transformed by wild-type or mutant EBNA-2 recombinant viruses. Controls include LMP-1-positive IB4 cells (lane 1) and BL30/P3HR-1 cells, which express low levels of LMP-1 (lane 2). The arrow indicates LMP-1. Sizes are shown in kilodaltons.

DISCUSSION

These mutational analyses indicate that at least four separate EBNA-2 domains are essential for transformation (Fig. 1). Since appropriate levels of EBNA-2 were expressed by each of the mutants, these mutations likely define transforming domains rather than domains which affect protein stability or nuclear localization. The essential domains are most precisely defined by deletions (amino acids 34 to 110, 333 to 425, and 449 to 462) and a linker-insertion mutation (amino acid 320). All four domains involve type-common motifs. The first domain (amino acids 34 to 110) consists of a 25-amino-acid sequence, followed by a polyproline sequence, and a 15-amino-acid sequence. The polyproline region is unlikely to be essential, since type 2 EBV has a much shorter polyproline region and the length of this region varies among type 1 isolates. Since a linker-insertion mutation at the beginning of the first domain had no effect on transformation, the type-common sequences before or after (Val-Gly-Glu-Asn-Thr-Gly [before] or Arg-Arg-Asp-Ala-Trp-Thr-Gln-Glu-Pro [after]) the polyproline region are likely candidates for essential motifs. The other three essential domains are in the carboxy third of EBNA-2, which is largely conserved between EBV types 1 and 2. Two of these domains include the arginine-glycine repeat and the acidic carboxy domain, which are hydrophilic sequences that are likely to be accessible for interactions with other molecules and functionally significant. The third domain is defined by a linker-insertion mutation (amino acid 320) which results in insertion of a hydrophilic sequence (Gly-Arg-Ser-Ser) into a relatively hydrophobic sequence and thus could affect protein folding and impair transformation.

Nuclear proteins of other DNA tumor viruses also have

independent domains that are essential for transformation. Adenovirus E1A (42), human papillomavirus E7 (40), and SV40 large T antigen (30) each have at least two separate domains which are essential for transformation in certain cell lines. These other DNA tumor virus nuclear proteins also frequently have multifunctional domains which are different from their transforming domains. Adenovirus E1A has two domains that are essential for transformation and a third independent domain that is responsible for transcriptional activation (23). SV40 large T antigen has DNA binding, DNA replication, ATPase, helicase, and transactivating domains that can be dissociated from its transforming domains (2, 13, 32). However, EBV is different from the smaller DNA tumor viruses in that EBV has an extensive repertoire of six different nuclear and two different membrane proteins expressed in latently infected, growth-transformed B lymphocytes. Furthermore, EBV has numerous immediate-early and early viral replication cycle genes, so that EBNA-2 (and probably other proteins expressed in latent infection) is not essential for virus replication. The P3HR-1 strain of EBV is deleted for EBNA-2 and is able to replicate in B lymphocytes. Therefore, each virus protein expressed in B lymphocytes transformed by EBV might be expected to have fewer functions than the transforming proteins of the smaller DNA tumor viruses.

At least one-third of the EBNA-2 molecule is dispensable for B-lymphocyte transformation if one includes deletions which reduce but do not abolish transformation. Two deletions (amino acids 195 to 230 and 463 to 483) did not reduce transformation, while a larger deletion (amino acids 143 to 230) which overlaps the former deletion removed primarily type-specific sequences and had only a slight effect on transformation. Deletions in two other independent domains (amino acids 19 to 33 and 112 to 141) reduced but did not eliminate transformation. These latter two deletions involve sequences common to type 1 and type 2 EBV. Assuming that most of the polyproline region is dispensable for transformation (as described above), our deletion analysis indicates that at least 65% of the amino half of the EBNA-2 protein is probably dispensable for transformation. Thus, a large part of the EBNA-2 molecule, including sequences which are highly conserved between type 1 and type 2 EBV (amino acids 19 to 33 and 463 to 483), is dispensable for transformation and for transactivation.

By defining the role of EBNA-2 domains in transformation and LMP-1 transactivation, our data are consistent with the hypothesis that transformation and transactivation are closely linked. Each important transforming domain was also important in LMP-1 transactivation. All 11 deletion and linker-insertion mutations which abolished transformation also abolished LMP-1 transactivation in transient-transfection assays. Similarly, the five mutants with a low or moderate transforming efficiency also showed reduced LMP-1 transactivation compared with wild-type EBNA-2.

While high-level LMP-1 transactivation (or nontransactivation) in transient assays correlated with a wild-type transforming (or nontransforming) phenotype, intermediate LMP-1-transactivating activity did not always correlate with the results of the transformation assays (Table 2). Three of the mutants with a wild-type transforming phenotype showed reduced LMP-1 transactivation. In addition, one of the deletion mutants (amino acids 19 to 33) resulted in few transformants and barely detectable LMP-1 transactivation, while another deletion mutant (amino acids 112 to 141) resulted in fewer transformants but greater LMP-1-transactivating activity. The imperfect correlation between transformation and LMP-1 transactivation could be a consequence of the complexity and inherent inaccuracy of the transformation or transactivation assay. However, we cannot exclude the less likely possibility that this imperfect correlation reflects an intrinsic difference in the mechanism of LMP-1 transactivation and lymphocyte transformation.

Further genetic analyses within and around amino acids 19 to 33 of EBNA-2 offer the best potential for obtaining a mutant which fails to transactivate LMP-1 but retains some transforming activity. This deletion mutant had the lowest LMP-1-transactivating activity and still transformed cells, albeit with reduced transforming activity. However, despite the reduced LMP-1-transactivating activity of this mutant and other EBNA-2 mutants in transient-transfection assays, lymphoblastoid cell lines obtained from recombinant EBV containing this mutation and other EBNA-2 mutations had wild-type levels of LMP-1 expression. The high-level expression of LMP-1 in lymphoblastoid cell lines containing the EBNA-2 mutants may be due to selection for cells with high LMP-1 levels during transformation or during subsequent passage. These findings are consistent with the hypotheses that LMP-1 is an essential transforming gene and that other viral or cellular genes can complement those EBNA-2 mutations with diminished LMP-1 transactivation activity.

While we cannot exclude the possibility that further mutational analysis could partially separate transformation from transactivation domains, our experiments strongly favor the hypothesis that transformation and transactivation are largely overlapping functions. Thus, transactivation of viral and cellular genes may be the primary mechanism by which EBNA-2 mediates transformation. Alternatively, EBNA-2 may induce transformation by another mechanism and the upregulation of LMP-1 and specific cell proteins may be a consequence of transformation. For example, EBNA-2 interaction with a cellular growth-regulatory protein could be an indirect mechanism by which EBNA-2 could mediate viral and cellular gene transactivation. Two series of experiments suggest that EBNA-2 does not interact with a ubiquitous cell growth-regulatory protein. First, EBNA-2 does not immortalize primary rodent fibroblasts, alone or in combination with *myc* or activated *ras* (14, 35a). Second, LMP-1 transactivation by EBNA-2 is somewhat species and tissue specific and is most evident in human B lymphocytes (39).

The EBNA-2 type-common acidic domain is likely to be important in transactivation and transformation. The finding that deletion of amino acids 463 to 483 did not reduce transformation or transactivation was therefore unexpected, since this mutation deletes part of the conserved major acidic and the entire conserved basic carboxy terminus which could interact with cellular proteins involved in transcription or growth control. The well-conserved, acidic, carboxy EBNA-2 domain (amino acids 457 to 469) is homologous to the acidic portion of the adenovirus E1A domain 2 and human papillomavirus E7 region 2 (29a) and thus could interact with cellular proteins such as the retinoblastoma, p107, and p300 proteins. The adenovirus E1A domain 2 and human papillomavirus E7 region 2 are involved in binding to the retinoblastoma gene product; however, deletion of the carboxy part of E1A domain 2 does not affect retinoblastoma, p107, or p300 protein interactions (43). Since deletion of the carboxy portion of adenovirus E1A domain 2 does not affect binding to these cell proteins, the absence of an effect of the homologous EBNA-2 deletion (amino acids 463 to 483) might be expected and does not exclude the possibility that

EBNA-2 might interact with these cell proteins. Deletion of the EBNA-2 sequence immediately amino terminal to amino acid 463 abolishes transformation, as is observed with deletion of the amino-terminal portion of adenovirus E1A domain 2. However, these latter sequences of EBNA-2 and adenovirus E1A are only weakly homologous.

Analysis of amino acid sequences and phenotypic characteristics which are common or specific to EBV-1 and EBV-2 EBNA-2 genes are also useful for relating primary sequence to function. Fifty-six percent of the predicted amino acid sequence is identical in type 1 and type 2 EBV EBNA-2 (as is indicated in Fig. 1). Type-common sequences are likely to be important in the phenotypic features shared by type 1 and type 2 EBV, including B-lymphocyte transformation and LMP-1, CD23, and CD21 transactivation. Similarly, type-specific sequences are likely to be important in the transforming and transactivation phenotypes specific for type 1 or type 2 EBV. Compared with type 1 EBV, type 2 EBV has a lower transforming efficiency, poorer initial outgrowth, and higher cell density dependence for cell viability. Compared with type 1 EBNA-2, type 2 EBNA-2 also has less effect in transactivating CD21 or CD23 expression in stably transfected cell lines (37), and EBV type 2 transformants are more variable in CD23 expression (6). However, type 2 EBNA-2 does not differ from type 1 EBNA-2 in LMP-1 transactivation in acute transfection experiments (39) or in transformed cell lines (6). These type-common and type-specific effects are compatible with the working hypothesis that transformation and transactivation are closely linked and that transformation or CD21 and CD23 transactivation may be partly affected by type-specific EBNA-2 domains that are not involved in LMP-1 transactivation.

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REFERENCES

1. **Abbot, S. D., M. Rowe, K. Cadwallader, A. Ricksten, J. Gordon, F. Wang, L. Rymo, and A. B. Rickinson.** 1990. Epstein-Barr virus nuclear antigen 2 induces expression of the virus-encoded latent membrane protein. J. Virol. **64**:2126–2134.
2. **Auborn, K., M. Guo, and C. Prives.** 1989. Helicase, DNA-binding, and immunological properties of replication-defective simian virus 40 mutant T antigens. J. Virol. **63**:912–918.
3. **Baichwal, V. R., and B. Sugden.** 1988. Transformation of Balb 3T3 cells by the BNLF-1 gene of Epstein-Barr virus. Oncogene **2**:461–467.
4. **Calendar, A., M. Billaud, J. Aubry, J. Banchereau, M. Vuillaume, and G. Lenoir.** 1987. EBV induces expression of B cell activation markers on in vitro infection of EBV negative B lymphoma cells. Proc. Natl. Acad. Sci. USA **84**:8060–8064.
5. **Cepko, C., B. Roberts, and R. Mulligan.** 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell **37**:1053–1062.
6. **Cohen, J. I., F. Wang, J. Mannick, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86**:9558–9562.
7. **Dambaugh, T., K. Hennessy, L. Chamnankit, and E. Kieff.** 1984. U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2. Proc. Natl. Acad. Sci. USA **81**:7632–7636.
8. **Dambaugh, T., F. Wang, K. Hennessy, E. Woodland, A. Rickinson, and E. Kieff.** 1986. Expression of the Epstein-Barr virus nuclear protein 2 in rodent cells. J. Virol. **59**:453–462.
9. **deCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston.** 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell **54**:275–283.
10. **Dillner, J., and B. Kallin.** 1988. The Epstein-Barr virus proteins. Adv. Cancer Res. **50**:95–158.
11. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–936.
12. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalising functions of Epstein-Barr virus in human B lymphocytes. Nature (London) **340**:393–397.
13. **Kalderon, D., and A. E. Smith.** 1984. *In vitro* mutagenesis of a putative DNA binding domain of SV40 large-T antigen. Virology **139**:109–137.
14. **Kieff, E., and D. Liebowitz.** 1989. Oncogenesis by herpesviruses, p. 259–280. *In* R. Weinberg (ed.), Oncogenes and the molecular origins of cancer. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr virus and its replication, p. 1889–1920. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed. Raven Press, New York.
16. **Kinter, C., and B. Sugden.** 1981. Identification of antigenic determinants unique to the surfaces of cells transformed by Epstein-Barr virus. Nature (London) **294**:458–460.
17. **Knutson, J. C.** 1990. The level of c-*fgr* RNA is increased by EBNA-2, an Epstein-Barr virus gene required for B-cell immortalization. J. Virol. **64**:2530–2536.
18. **Longnecker, R., and E. Kieff.** 1990. A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and colocalizes with LMP1. J. Virol. **64**:2319–2326.
19. **Mann, K. P., D. Staunton, and D. Thorley-Lawson.** 1985. Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. J. Virol. **55**:710–720.
20. **Miller, G.** 1990. Epstein-Barr virus, p. 1921–1958. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed. Raven Press, New York.
21. **Miller, G., D. Coope, J. Neiderman, and J. Pagano.** 1976. Biological properties and viral surface antigens of Burkitt lymphoma and mononucleosis-derived strains of Epstein-Barr virus released from transformed marmoset cells. J. Virol. **18**:1071–1080.
22. **Miller, G., T. Shope, D. Coope, L. Waters, J. Pagano, G. Bornkamm, and W. Henle.** 1977. Lymphoma in cotton-top marmosets after inoculation with Epstein-Barr virus: tumor incidence, histologic spectrum, antibody responses, demonstration of viral DNA, and characterization of viruses. J. Exp. Med. **145**:948–967.
23. **Moran, E., and M. B. Matthews.** 1987. Multiple functional domains in the adenovirus E1A gene. Cell **48**:177–178.
24. **Mosier, D. E., R. J. Gulizia, S. M. Baird, and D. B. Wilson.** 1988. Transfer of a functional human immune system to mice with severe combined immunodeficiency. Nature (London) **335**:256–259.
25. **Murray, R. J., M. G. Kurilla, H. M. Griffin, J. M. Brooks, M. Mackett, J. R. Arrand, M. Rowe, S. R. Burrows, D. J. Moss, E. Kieff, and A. B. Rickinson.** 1990. Human cytotoxic T cell responses against Epstein-Barr virus nuclear antigens demonstrated by using recombinant vaccinia viruses. Proc. Natl. Acad. Sci. USA **87**:2906–2910.
26. **Petti, L., C. Sample, and E. Kieff.** 1990. Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins. Virology **176**:563–574.
27. **Raab-Traub, N., T. Dambaugh, and E. Kieff.** 1980. DNA of Epstein-Barr virus VIII:B95-8, the previous prototype, is an unusual deletion derivative. Cell **22**:257–267.
28. **Rabson, M., L. Gradoville, L. Heston, and G. Miller.** 1982. Nonimmortalizing P3J-HR-1 Epstein-Barr virus: a deletion mu-

tant of its transforming parent, Jijoye. J. Virol. **44**:834–844.
29. **Rickinson, A. B., L. S. Young, and M. Rowe.** 1987. Influence of the Epstein-Barr virus nuclear antigen EBNA2 on the growth phenotype of virus-transformed B cells. J. Virol. **61**:1310–1317.
29a. **Sample, C.** Unpublished data.
30. **Srinivasan, A., K. W. C. Peden, and J. M. Pipas.** 1989. Large tumor antigen of simian virus 40 encodes at least two distinct transforming functions. J. Virol. **63**:5459–5463.
31. **Tedder, T. F., L. T. Clement, and M. D. Cooper.** 1984. Expression of C3d receptors during human B cell differentiation: immunofluorescence analysis with the HB5 monoclonal antibody. J. Immunol. **133**:668–673.
32. **Thompson, D. L., D. Kalderon, A. E. Smith, and M. J. Tevethia.** 1990. Dissociation of Rb-binding and anchorage-independent growth from immortalization and tumorigenicity using SV40 mutants producing N-terminally truncated large T antigens. Virology **178**:15–34.
33. **Wang, D., D. Liebowitz, and E. Kieff.** 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell **43**:831–840.
34. **Wang, D., D. Liebowitz, and E. Kieff.** 1988. The truncated form of the Epstein-Barr virus latent-infection membrane protein expressed in virus replication does not transform rodent fibroblasts. J. Virol. **62**:2337–2346.
35. **Wang, D., D. Liebowitz, F. Wang, C. Gregory, A. Rickinson, R. Larson, T. Springer, and E. Kieff.** 1988. Epstein-Barr virus latent membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. J. Virol. **62**:4173–4184.
35a. **Wang, F., E. Bushman, and E. Kieff.** Unpublished data.
36. **Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, H. Kikutani, T. Kishimoto, and E. Kieff.** 1987. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. Proc. Natl. Acad. Sci. USA **84**:3452–3456.
37. **Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP-1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP-1 cooperatively induce CD23. J. Virol. **64**:2309–2318.
38. **Wang, F., L. Petti, D. Braun, S. Seung, and E. Kieff.** 1987. A bicistronic Epstein-Barr virus mRNA encodes two nuclear proteins in latently infected, growth-transformed lymphocytes. J. Virol. **61**:945–954.
39. **Wang, F., S.-F. Tsang, M. G. Kurilla, J. I. Cohen, and E. Kieff.** 1990. Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein (LMP1). J. Virol. **64**:3407–3416.
40. **Watanabe, S., T. Kanda, H. Sato, A. Furuno, and K. Yoshike.** 1990. Mutational analysis of human papillomavirus type 16 E7 functions. J. Virol. **64**:207–214.
41. **Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow.** 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature (London) **334**:124–129.
42. **Whyte, P., H. E. Ruley, and E. Harlow.** 1988. Two regions of the adenovirus early region 1A proteins are required for transformation. J. Virol. **62**:257–265.
43. **Whyte, P., N. M. Williamson, and E. Harlow.** 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell **56**:67–75.
44. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis*-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81**:3806–3810.
45. **Young, L., C. Alfieri, K. Hennessy, H. Evans, C. O'Hara, K. Anderson, J. Ritz, R. S. Shapiro, A. Rickinson, E. Kieff, and J. I. Cohen.** 1989. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. N. Engl. J. Med. **321**:1080–1085.
46. **Zagursky, R. J., K. Baumeister, N. Lomax, and M. L. Berman.** 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gene Anal. Tech. **2**:89–94.