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Crucial Sequences within the Epstein-Barr Virus TP1 Promoter for EBNA2-Mediated Transactivation and Interaction of EBNA2 with Its Responsive Element

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***EBNA2* is one of the few genes of Epstein-Barr virus which are necessary for immortalization of human primary B lymphocytes. The EBNA2 protein acts as a transcriptional activator of several viral and cellular genes. For the *TP1* promoter, we have shown previously that an EBNA2-responsive element (EBNA2RE) between −258 and −177 relative to the *TP1* RNA start site is necessary and sufficient for EBNA2-mediated transactivation and that it binds EBNA2 through a cellular factor. To define the critical *cis* elements within this region, we cloned EBNA2RE mutants in front of the *TP1* minimal promoter fused to the reporter gene for luciferase. Transactivation by EBNA2 was tested by transfection of these mutants in the absence and presence of an EBNA2 expression vector into the established B-cell line BL41-P3HR-1. The analysis revealed that two identical 11-bp motifs and the region 3′ of the second 11-bp motif are essential for transactivation by EBNA2. Methylation interference experiments indicated that the same cellular factor in the absence of EBNA2 binds either one (complex I) or both (complex III) 11-bp motifs with different affinities, giving rise to two different specific protein-DNA complexes within the left-hand 54 bp of EBNA2RE. A third specific complex was shown previously to be present only in EBNA2-expressing cells and to contain EBNA2. Analysis of this EBNA2-containing complex revealed the same protection pattern as for complex III, indicating that EBNA2 interacts with DNA through binding of the cellular protein to the 11-bp motifs. Mobility shift assays with the different mutants demonstrated that one 11-bp motif is sufficient for binding the cellular factor, whereas for binding of EBNA2 as well as for efficient transactivation by EBNA2, both 11-bp motifs are required.**

Epstein-Barr virus (EBV), a widespread human herpesvirus, is the causative agent of infectious mononucleosis, a self-limiting lymphoproliferative disorder. This virus is also associated with several malignancies, including Burkitt's lymphoma (20), nasopharyngeal carcinoma (18), Hodgkin's disease (4, 39), and lymphomas in immune-deficient individuals (22, 24). EBV has a dual tropism; the virus is produced in epithelial cells of the oropharynx, whereas B lymphocytes are nonproductively infected. Infection of B lymphocytes in vitro leads to the growth of permanently proliferating cell lines. In these lymphoblastoid cell lines, the virus is episomally maintained in a high copy number (32), although only a limited number of EBV genes are expressed, including six nuclear antigens, EBNA1, -2, -3a, -3b, -3c, and LP, and three membrane proteins, LMP (LMP1), TP1 (LMP2A), and TP2 (LMP2B) (28).

EBNA2, a phosphorylated polypeptide of 487 amino acids, is absolutely necessary for the establishment of B-cell immortalization. EBV strain P3HR-1, containing a 6.6-kb deletion encompassing the *EBNA2* gene and part of the *EBNA-LP* gene, has lost the ability to transform B cells (7, 26). After reintroduction of the *EBNA2* gene by homologous recombination into the P3HR-1 genome, a transformation-competent virus could be reconstituted (13, 25). EBNA2 is necessary not only for B-cell immortalization in vitro but also for B-cell tumor growth in vivo in SCID (severe combined immunodeficiency) mice (12). In natural EBV isolates, two alleles of the *EBNA2* gene code for the two proteins EBNA2A and EBNA2B, which have 57% homology (2, 17, 46). Viruses carrying the *EBNA2A* gene have higher transforming capacity than EBNA2B-expressing strains (13, 36).

It is likely that EBNA2 contributes to B-cell immortalization by its ability to transactivate cellular and viral genes. It increases transcription of the B-cell activation markers CD21 and CD23 and of the tyrosine kinase c-fgr (8, 14, 29, 42). In addition, EBNA2 can transactivate the promoters of the viral genes *LMP*, *TP1*, and *TP2* and the *Bam*HI-C promoter, which is necessary for transcription of the nuclear antigens (1, 21, 23, 27, 40, 44, 45, 48). Furthermore, EBNA2 has been shown to transactivate the human immunodeficiency virus type 1 long terminal repeat (38). After in vitro infection of B cells, *EBNA2* and *EBNA-LP* are the first viral genes to be expressed (3, 37). EBNA2 subsequently induces expression of the EBNA2-responsive viral and cellular genes. Therefore, elucidation of the mechanism by which EBNA2 is able to transactivate other genes will contribute to the understanding of the immortalization process.

Until now, the mechanism of EBNA2-mediated transactivation has been poorly understood. An acidic transactivation domain with similarities to that of the herpes simplex virus transactivator VP16 could be mapped at the C terminus (10, 11). Recently, we could demonstrate EBNA2 interaction with the *TP1* promoter via a cellular protein (47). Ling et al. (33) have demonstrated an indirect interaction of a glutathione-*S*-transferase–EBNA2 fusion protein with the *Bam*HI-C-promoter.

To further elucidate the function of EBNA2, we chose the

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TP1 promoter, which contains an 81-bp element between −258 and −177 relative to the *TP1* RNA start site, responsible for EBNA2-mediated transactivation (EBNA2RE) (47). The element contains a duplicated 11-bp motif with homologies to the EBNA2-responsive regions of the *LMP*, *Bam*HI-C, and *CD23* promoters. The *TP1* promoter is particularly well suited for studying the mechanism of EBNA2 action for several reasons. This promoter is tightly regulated and is, in contrast to the *LMP1* promoter (15), strictly dependent on EBNA2 in B lymphocytes. Compared with the *Bam*HI-C and *LMP* promoters, EBNA2 appears to interact more stably with the EBNA2RE of the *TP1* promoter, thus allowing the visualization of the EBNA2-containing complex in gel shift assays with EBNA2-positive nuclear cell extracts. In contrast, it was not possible to demonstrate the presence of EBNA2 in protein-DNA complexes formed at the EBNA2REs of the *Bam*HI-C or *LMP* promoter (27, 31, 33). Addition of the glutathione-*S*-transferase–EBNA2 fusion protein is necessary to detect binding of EBNA2 to its responsive element at the *Bam*HI-C promoter (33).

A detailed knowledge of the crucial DNA sequences conferring EBNA2 responsiveness is a prerequisite for understanding the mechanism of EBNA2-mediated transactivation. We therefore determined the sequences within the EBNA2RE of the *TP1* promoter which are protected by DNA-binding proteins. This study revealed that the two 11-bp elements are contacted by a cellular protein with different affinities. In addition, we present a detailed analysis of the sequences vital for EBNA2-mediated transactivation and binding to its EBNA2RE through a cellular protein.

MATERIALS AND METHODS

Cell lines and culture conditions. The cell line M-ABA is a marmoset lymphoblastoid cell line transformed by a virus originally derived from a nasopharyngeal carcinoma (16). BL41-P3HR-1 and BL41-B95-8 were obtained after infection of the EBV-negative Burkitt's lymphoma cell line BL41 with virus strains P3HR-1 and B95-8, respectively (8).

All cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cultures were incubated at 37°C in 5% CO_2. Cells were diluted 1:3 with fresh medium twice a week.

Plasmids. For cloning of mutated or deleted forms of the 81-bp EBNA2RE in front of the *TP1* minimal promoter, we have inserted a polylinker containing an *Sna*BI and *Nco*I site in the *Stu*I site of pTP1-LUC/-45 (pGa59/19) (47). pGa59/19 was digested with *Sna*BI and *Nco*I and ligated with oligonucleotides containing an *Sna*BI and *Nco*I site at their ends, respectively. The accuracy of the cloned oligonucleotides was controlled by sequencing. Sequencing reactions were carried out with a sequencing kit (US Biochemicals). The EBNA2 expression vector pU294-6 has been described previously (47).

Oligonucleotides. The positions of the oligonucleotides used in relation to the EBV genomic sequence according to Baer et al. (5) are as follows: O40, 166281 to 166320; O54, 166236 to 166289; O60, 166241 to 166300; O80, 166241 to 166320; O5′-del, 166253 to 166320; O1, 166236 to 166268; and O2, 166259 to 166289.

Transfection of cells. Electroporation of cells was carried out in a Bio-Rad gene pulser by the method of Cann et al. (9). Briefly, 10^7 cells with a viability of more than 90% were washed once and resuspended in 0.25 ml of RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were placed on ice in a 4-mm Gene Pulser cuvette, and 20 μg of the corresponding DNA was added. BL41-P3HR-1 cells were electroporated at 250 V and 960 μF; BL41-B95-8 were done at 260 V and 960 μF. Ten minutes after electroporation, the cells were resuspended in 10 ml of warm RPMI with 20% fetal calf serum.

Luciferase assay. Cells were harvested 48 h after transfection, washed once in ice-cold phosphate-buffered saline (PBS), and resuspended in 100 μl of 91 mM K_2HPO_4–9 mM KH_2PO_4–1 mM dithiothreitol (DTT)–1% Triton X-100 (pH 7.8). After 15 min on ice, debris was removed by centrifugation at 14,000 × *g* for 10 min. Then, 10 μl of the supernatant was mixed with 350 μl of 25 mM glycylglycine (pH 7.8)–5 mM ATP–15 mM $MgSO_4$–100 μl of 11 mM luciferin–0.5 M Tris-HCl (pH 7.8). The bioluminescence in relative light units (RLU) was measured with a Lumat LB9501 (Berthold, Wildbach, Germany).

Nuclear extract preparation. Nuclear extracts were prepared by a modification of the method of Dignam et al. (19). The pellet (3×10^7 to 5×10^7 cells) was washed once in ice-cold PBS, resuspended with 3 to 4 volumes of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9)–10 mM KCl–1.5 mM $MgCl_2$–5 mM DTT–0.5 mM phenylmethylsulfonyl fluoride (PMSF) (buffer A) and incubated on ice for 1 h. Lysis of cells was achieved by 10 to 20 strokes with a Dounce homogenizer and was microscopically controlled by trypan blue staining. Nuclei were pelleted for 10 s at maximal speed in an Eppendorf minifuge, washed once in buffer A, resuspended in 3 volumes of buffer B (20 mM HEPES [pH 7.9], 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM DTT, 0.5 mM PMSF) and incubated on ice for 30 min. Nuclei were removed by centrifugation at 14,000 × *g* for 20 min. The supernatant was stored in liquid nitrogen.

Radioactively labeled probes. Luciferase constructs containing wild-type, mutated, or deleted forms of the 81-bp EBNA2RE in front of the *TP1* minimal promoter were digested with *Sna*BI and *Nco*I. The 81-bp or mutated fragments were isolated, labeled with Klenow polymerase in the presence of [^{32}P]dCTP (3,000 Ci/mmol), and purified on a nondenaturing polyacrylamide gel.

Gel shift analysis. Binding reactions were performed in a volume of 20 μl containing 5 μl of binding buffer (41 mM HEPES [pH 7.9], 200 mM KCl, 4 mM EDTA, 1.6% Ficoll, 4 mM DTT, 0.5 mM PMSF), 2 μl of poly(dI-dC) (1 mg/ml), 2 μl of bovine serum albumin (10 mg/ml), 5 μg of protein extract, and 0.1 to 0.2 ng of radioactively labeled DNA. After incubation at room temperature for 30 min, the reaction products were separated on a 4% polyacrylamide gel. In competition and supershift experiments, either unlabeled oligonucleotide or 1 μl of tissue culture supernatant containing monoclonal antibodies was added to the reaction mixture. Anti-EBNA2 monoclonal antibody R11 (rat immunoglobulin G2a [IgG2a]), which recognizes EBNA2A (29a), was used. Anti-mouse CD45 (rat IgG2a) was used as an isotype control antibody. Gel shift analysis in the presence of sodium deoxycholate was performed as described by Baeuerle and Baltimore (6).

Methylation interference. The methylation interference assay is based on a G reaction of the Maxam-Gilbert sequencing reaction (34). About 100 ng of a prepared DNA fragment, labeled at one end only with Klenow polymerase in the presence of [^{32}P]dCTP (3,000 Ci/mmol), was methylated together with 1 μg of unspecific carrier DNA by adding 1 μl of dimethyl sulfate in 200 μl of 50 mM sodium cacodylate (pH 7.0)–1 mM EDTA and incubating for 3 min at 20°C. The reaction was timed to yield approximately one modification per DNA molecule and was stopped by adding 50 μl of 1.5 M

sodium acetate (pH 7.0)–1 M 2-mercaptoethanol. Methylated DNA samples were precipitated with ethanol, washed, dried under vacuum, and resuspended in water. A gel shift assay as described above was performed with the modified DNA. The free oligonucleotide and DNA-protein complexes were excised from the polyacrylamide gel and isolated by electroelution. The recovered DNA was cleaved at the methylated positions by heating at 90°C in 100 μl of 1:10 freshly diluted piperidine for 30 min. Positions of cleavages were determined by separating samples on an 8% denaturating polyacrylamide gel.

RESULTS

Both 11-bp motifs of EBNA2RE are important for EBNA2-mediated transactivation. For the *TP1* promoter, it was shown that an 81-bp element (EBNA2RE) located between positions −258 to −178, corresponding to the RNA start site, is sufficient for transactivation by EBNA2 (47). To identify the sequences within EBNA2RE that are responsible for transactivation, we made deletion mutants of the 81-bp element. Plasmids containing these deletions, which are linked to the luciferase (LUC) reporter gene under the control of the *TP1* minimal promoter, were constructed (Fig. 1A) and transiently transfected into EBNA2-negative BL41-P3HR-1 cells with or without the EBNA2A expression vector pU294-6. A cytomegalovirus enhancer-human T-cell lymphotropic virus long terminal repeat-luciferase construct was used as the positive control. One of four representative experiments is shown in Fig. 1A. Cotransfection of the EBNA2 expression vector with a plasmid containing the entire EBNA2RE induced 27.7-fold luciferase expression, whereas a construct containing only 54 bp of the 5′ part of EBNA2RE (−262 to −209) was not able to mediate transactivation by EBNA2. Analysis of an elongated construct (−257 to −198) resulted in an approximately 10-fold activation by EBNA2. An oligonucleotide containing the 3′ part of the EBNA2RE sequence (−217 to −178) lacking the two 11-bp motifs was incapable of mediating transactivation. A deletion at the 5′ end of EBNA2RE, which removes nearly all of the first 11-bp motif (−245 to −178), resulted in a dramatic decrease in induction by EBNA2. This indicates that the crucial sequences lie somewhere between −257 and −198. However, full EBNA2 transactivation was only obtainable with the complete sequence.

Analyzing EBNA2RE in greater detail, we created various *TP1* promoter-luciferase constructs carrying consecutive substitutions of four to six nucleotides in the EBNA2RE. These constructs were tested in the luciferase assay after cotransfection in BL41-P3HR-1 cells with or without an EBNA2 expression plasmid (Fig. 1B). A representative experiment presenting the original luciferase activities of the constructs in the absence and presence of EBNA2 is given in Fig. 1C. EBNA2 inducibility was dramatically decreased in constructs mutH, mutI, and mutK, which have mutations within the second 11-bp motif. Constructs containing mutations within the first 11-bp motif also showed a decrease in EBNA2 inducibility (mutB, mutC, and mutD), although to a lesser extent. This indicates that the necessary sequences for EBNA2-mediated transactivation lie within the 11-bp motifs. The core sequence appears to be 1-CGTGGGAAA-9, since substitution of the last two bases (10-AT-11) of the 11-bp motif had no effect on EBNA2 inducibility (mutL and mutE). Substitutions outside these 11-bp motifs did not significantly impair induction by EBNA2. Only three plasmids (mutN, mutP, and mutQ) containing nucleotide substitutions within the 3′ part of the EBNA2RE were slightly less inducible by EBNA2.

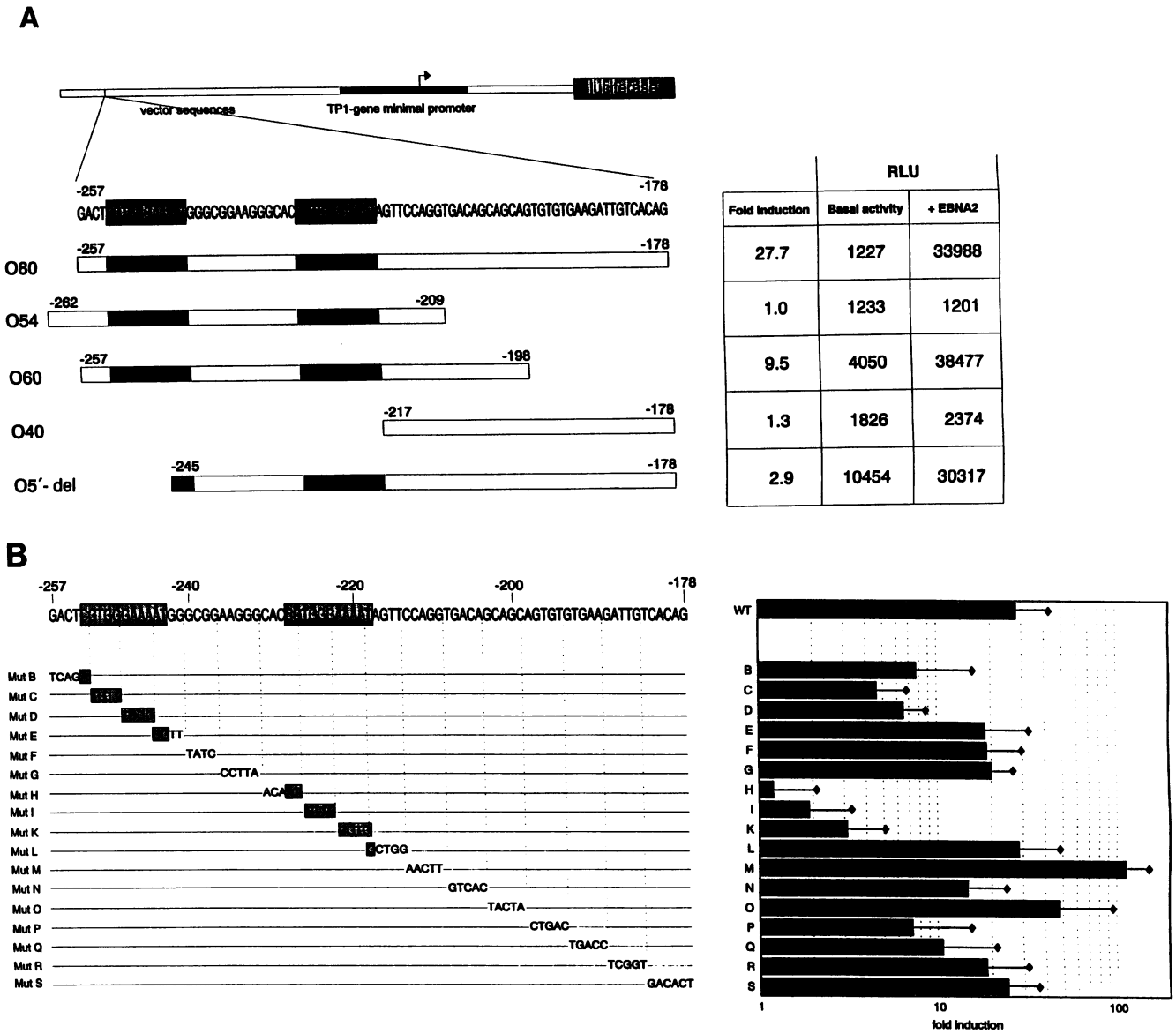
To confirm these data, we transiently transfected the constructs into EBNA2-positive BL41-B95-8 cells and compared the luciferase activities with those obtained after transfection into EBNA2-negative BL41-P3HR-1 cells with exogeneously added EBNA2. The results were virtually the same for both (data not shown). To corroborate that the induction rates result from an EBNA2 effect and not from other gene products upregulated by EBNA2, we transiently transfected the wild-type construct together with the EBNA2A expression vector into EBV-negative DG75 cells, leading to an induction rate comparable to that in the experiment carried out with the EBV-positive cell line BL41-P3HR-1 (data not shown).

The experiments revealed that EBNA2-mediated transactivation of the *TP1* promoter requires both 11-bp motifs and at least 20 bp downstream of the second 11-bp motif. The 14-bp spacer between the two 11-bp motifs appears not to be relevant for transactivation by EBNA2.

Specific protein-DNA complexes within the EBNA2RE are confined to the left-hand 54 bp. To analyze specific DNA-protein interactions within the EBNA2RE, we performed gel retardation assays. The results of an electrophoretic mobility shift assay (EMSA) with the −257 to −178 wild-type sequence as a radioactive probe and the nuclear extracts from EBNA2A-positive M-ABA cells are shown in Fig. 2. The EMSA revealed four major complexes (I, II, III, and IV) and two weaker complexes (* and **). The wild-type 80-bp oligonucleotide successfully competed in protein-DNA binding for all complexes except complex II. The 54-bp −262 to −209 oligonucleotide, lacking the 3′ part of the EBNA2RE, could also compete for binding. All specific complexes are therefore due to protein-DNA interactions within the left-hand 54-bp sequence containing both 11-bp motifs. In accordance with earlier results (47), EBNA2 must be a component of complex IV, since addition of the monoclonal anti-EBNA2A antibody R11 (29a) but not the anti-CD45 isotype control caused a supershift of complex IV.

Cellular EBNA2-binding protein binds to the two 11-bp motifs with different affinities. To analyze which nucleotides of the EBNA2RE are involved in the formation of protein-DNA complexes I, III, and IV, methylation interference experiments were performed. The 54-bp −262 to −209 wild-type fragment was used as a probe with M-ABA nuclear extracts. Complexes I, III, and IV and the free oligonucleotide were recovered after gel retardation. In Fig. 3A, the methylation interference pattern is shown. Analysis of complex I revealed a pronounced underrepresentation of the bands corresponding to three consecutive guanines within the second 11-bp motif and an intensification of the band corresponding to the first guanine residue. This indicates that in complex I, the second 11-bp motif is predominantly occupied by a cellular protein. However, overrepresentation of the band representing the first guanine within both 11-bp motifs indicates, to a lesser extent, the protection of the first 11-bp motif as well.

To determine whether each 11-bp motif is in itself sufficient to bind the cellular protein, we performed methylation interference experiments with mutants of the 80-bp wild-type oligonucleotide in which the first or second 11-bp motif was displaced (Fig. 3B and C). In both mutants tested (11mut/2 and 11mut/1), the unmutated 11-bp motif was protected, whereas the mutated motif was not. We therefore conclude that complex I formed with the wild-type oligonucleotide is actually a mixture of complexes, in which either the first or the second 11-bp motif is protected. The cellular protein seems to have a higher affinity for the second 11-bp motif than for the first, thus predominantly protecting the second 11-bp motif.



Fold induction	RLU Basal activity	RLU + EBNA2
27.7	1227	33988
1.0	1233	1201
9.5	4050	38477
1.3	1826	2374
2.9	10454	30317

FIG. 1. Crucial sequences within the EBNA2RE of the *TP1* promoter mediating EBNA2 responsiveness. (A) Deletion analysis of the EBNA2-dependent enhancer. Different portions of the EBNA2RE were cloned, surrounded by vector sequences, in front of the *TP1* minimal promoter fused to the reporter gene luciferase. The regions of EBNA2RE contained in different luciferase constructs are schematically indicated. Positions correspond to the *TP1* RNA start site. The characteristic 11-bp motifs are marked by stippled boxes. The constructs were transfected into EBNA2-negative BL41-P3HR-1 cells in the absence and presence of an EBNA2 expression vector. The luciferase activities of one of four representative experiments are presented. (B) Mutational analysis of the EBNA2RE. Luciferase activities of constructs containing the 80-bp EBNA2RE (WT) and various scanning mutants (mutB to mutS) were determined after transfection into BL41-P3HR-1 cells with and without an EBNA2 expression vector. The average induction rates by EBNA2, including standard deviations, from at least 13 independent luciferase assays are shown at the right. (C) Basal luciferase activities and those after induction by EBNA2 of one representative experiment are given for the wild type (WT) and mutants.

Analyzing the oligonucleotide bound in complex III (Fig. 3A), the same protection pattern was observed for both 11-bp motifs; however, protection of the first motif was not as complete as that of the second. The identical contact pattern within both 11-bp motifs suggests that the same protein binds to both elements. Unlike that seen in complex I, an additional guanine 3′ of the second 11-bp motif (at the bottom of Fig. 3A) was protected in complex III. Analysis of the oligonucleotide bound in the EBNA2-containing complex IV revealed the same protection pattern as in complex III, with two protected 11-bp motifs and no additional protection within the EBNA2RE (Fig. 3A). This provides additional evidence that EBNA2 interacts indirectly with DNA via a cellular protein.

To demonstrate the different affinities of each 11-bp motif for the EBNA2-binding protein, we performed gel retardation assays with the 54-bp oligonucleotide as a radioactively labeled probe with nuclear extracts of EBNA2A-positive M-ABA cells. The affinities of the two 11-bp motifs for the protein were

C

	RLU	
	Basal activity	+ EBNA2
WT	3602	92952
B	20418	174135
C	7081	40782
D	18614	147392
E	5274	68846
F	11486	302440
G	3854	99516
H	18145	19306
I	16909	11601
K	2703	2777
L	2524	95766
M	2153	316446
N	6198	56144
O	1415	32410
P	7486	61541
Q	1813	22245
R	3022	41136
S	2874	47952

FIG. 1—*Continued.*

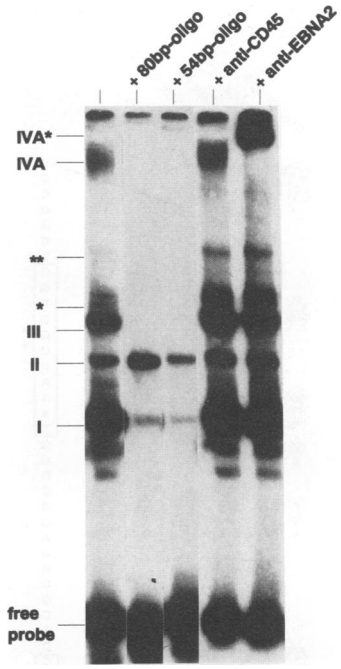


FIG. 2. Analysis of DNA-protein interactions within the *TP1* promoter. EMSA with nuclear extracts of EBNA2A-positive M-ABA cells, which were incubated with the ^{32}P-labeled 80-bp wild-type EBNA2RE from positions −178 to −257 relative to the *TP1* transcription start site. For competition, a 100-fold molar excess of the unlabeled oligonucleotides from −178 to −257 (80bp-oligo) or from −209 to −262 (54bp-oligo) was added to the gel shift reactions. To show that EBNA2 is a component of complex IV, the monoclonal anti-EBNA2A antibody R11 was added. The monoclonal anti-mouse CD45 antibody of the same isotype as the anti-EBNA2A R11 antibody was used as a negative control. Complexes were separated on a 4% polyacrylamide gel. The positions of complexes I, II, III, IVA, IV*, and ** are shown.

analyzed by addition of a 5- to 125-fold molar excess of unlabeled oligonucleotides O1 or O2, which contain the first and second 11-bp motif and adjacent sequences, respectively (Fig. 4). Addition of unlabeled oligonucleotides in 5- to 125-fold molar excess revealed that O2 competed more efficiently than did O1. These results correspond to our data from methylation interference analysis and demonstrate a greater affinity of the cellular protein for the second 11-bp motif than for the first.

Interaction of EBNA2 with the cellular protein is disrupted by sodium deoxycholate treatment. The fact that EBNA2 binds indirectly to DNA via a cellular factor led us to question whether this interaction could be destroyed by deoxycholate, a detergent which usually inhibits protein-protein interactions more readily than protein-DNA interactions. Therefore, the 54-bp fragment from −209 to −262 was end labeled and incubated with EBNA2A-positive M-ABA cell extracts in the presence or absence of 0.4% deoxycholate (Fig. 5). Without deoxycholate, all three specific complexes (I, III, and IV) were formed in which binding of the labeled probe could be inhibited by the addition of a specific, unlabeled oligonucleotide. Addition of a monoclonal anti-EBNA2A antibody resulted in a supershift of complex IV. However, in the presence of 0.4% deoxycholate, only complexes I and III, not complex IV, were formed. Therefore, the interaction of EBNA2 and the cellular protein by formation of complex IV is sensitive to deoxycholate treatment.

Binding of the EBNA2-interacting protein to both 11-bp motifs is a prerequisite for detection of EBNA2 in the complex. To determine which structures within the EBNA2RE are necessary to allow detection of a specific EBNA2-containing complex, EMSAs were performed with different scanning mutants of the 80-bp fragment (see Fig. 1B) as radioactively labeled probes and extracts of EBNA2A-positive M-ABA cells. To demonstrate the binding specifity and to verify the presence of EBNA2 in the complexes, EMSAs were performed for each mutant in the presence and absence of excess competitor DNA and in the presence and absence of monoclonal anti-EBNA2 and control antibodies (data not shown). The results with oligonucleotides mutB to mutM are shown in Fig. 6. Except for mutL, all oligonucleotides carrying mutations within one of the two 11-bp motifs have fully or partially lost the ability to form EBNA2-containing complex IV. In the EMSAs with oligonucleotides mutC, mutH, and mutI, neither complex III nor complex IV could be detected, whereas in EMSAs with oligonucleotides mutB, mutD, mutE, and mutK, complex III was reduced. Oligonucleotides mutD, mutE, and mutK have not totally lost their ability to form complex IV.

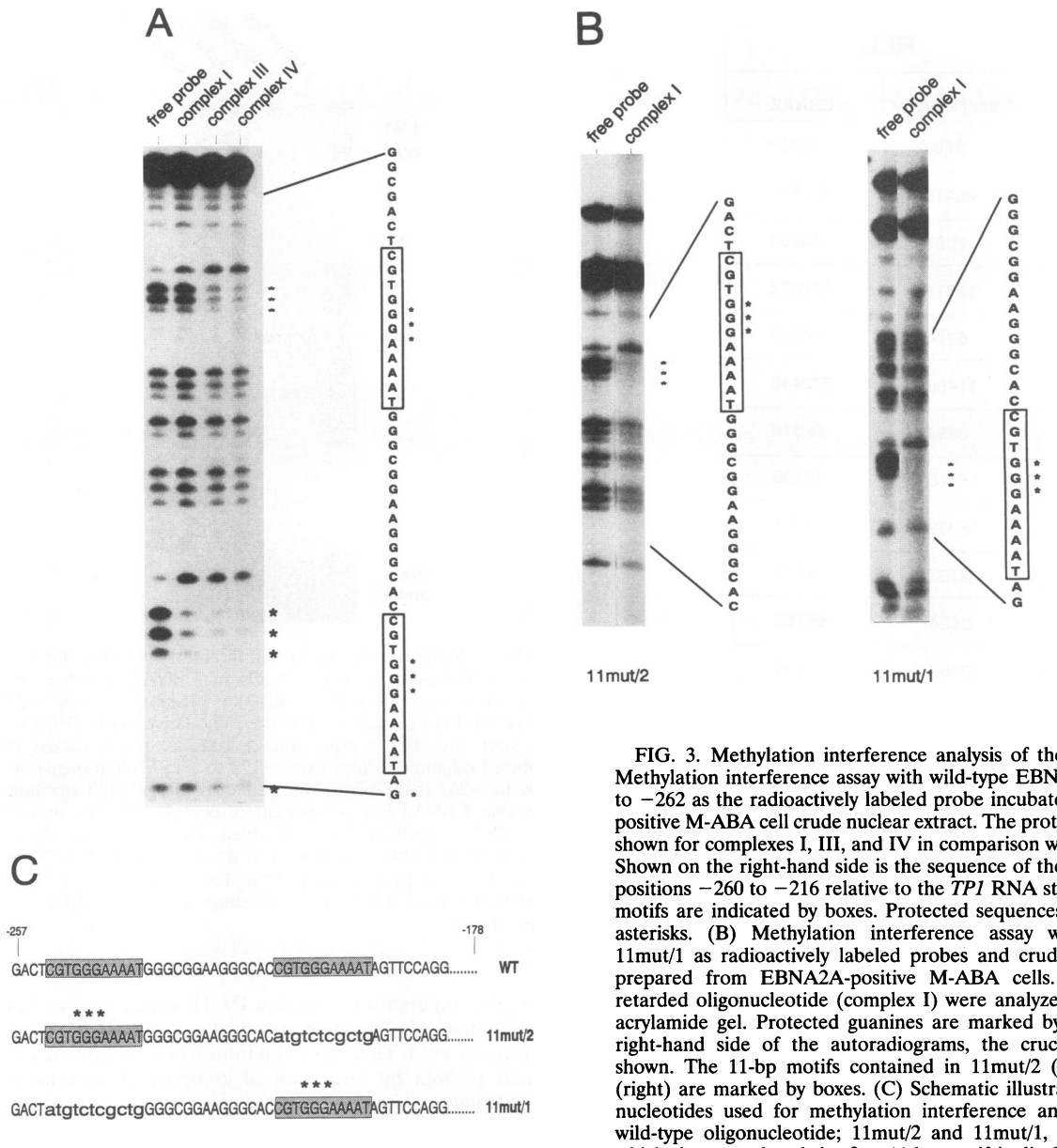


FIG. 3. Methylation interference analysis of the EBNA2RE. (A) Methylation interference assay with wild-type EBNA2RE from −209 to −262 as the radioactively labeled probe incubated with EBNA2A-positive M-ABA cell crude nuclear extract. The protected guanines are shown for complexes I, III, and IV in comparison with the free probe. Shown on the right-hand side is the sequence of the EBNA2RE from positions −260 to −216 relative to the *TP1* RNA start site. The 11-bp motifs are indicated by boxes. Protected sequences are marked with asterisks. (B) Methylation interference assay with 11mut/2 and 11mut/1 as radioactively labeled probes and crude nuclear extracts prepared from EBNA2A-positive M-ABA cells. Free probe and retarded oligonucleotide (complex I) were analyzed on an 8% polyacrylamide gel. Protected guanines are marked by asterisks. At the right-hand side of the autoradiograms, the crucial sequences are shown. The 11-bp motifs contained in 11mut/2 (left) and 11mut/1 (right) are marked by boxes. (C) Schematic illustration of the oligonucleotides used for methylation interference analysis. WT, 80-bp wild-type oligonucleotide; 11mut/2 and 11mut/1, mutated forms in which the second and the first 11-bp motif is displaced, respectively. The mutated sequences of 11mut/2 and 11mut/1 are indicated by lowercase letters; the 11-bp motifs are marked by stippled boxes. Positions correspond to the *TP1* RNA start site.

This is indicated by a faint band representing complex IV* upon stabilization of the complex with the monoclonal anti-EBNA2A antibody. Oligonucleotides mutated within the 14-bp spacer between the two 11-bp motifs or 3′ of the second 11-bp motif showed the normal EMSA pattern. Therefore, the data for mutN to mutS are not included in Fig. 6. The two weaker complexes (* and **) were detected with all mutants tested.

We conclude that the essential sequences for DNA-protein interactions lie within the 11-bp motifs. Binding of the cellular protein to both 11-bp motifs is necessary to visualize complex III and the EBNA2-containing complex IV, whereas for complex I formation, binding of one 11-bp motif is sufficient.

DISCUSSION

EBNA2 plays a central role in B-cell immortalization. It most likely participates in this process by its ability to transactivate several cellular and viral genes. Further elucidation of the molecular mechanism of EBNA2-mediated transactivation is therefore required and should include analysis of the EBNA2-responsive regions of different EBNA2-inducible viral and cellular promoters.

We present here a detailed analysis of the EBNA2RE of the *TP1* promoter. This element had previously been localized to the region from −258 to −178 relative to the RNA start site. We first generated a series of deletion mutants of the

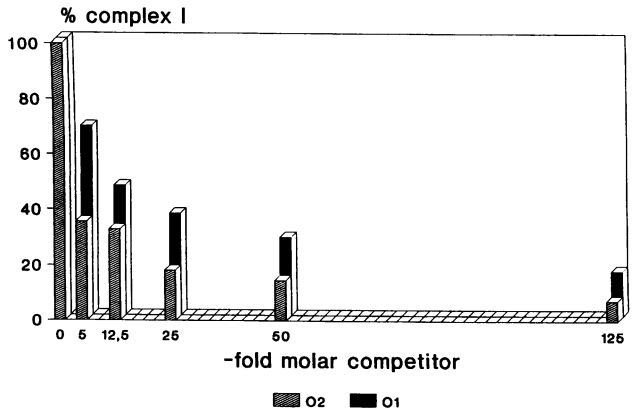


FIG. 4. Competition assays demonstrating the higher affinity of the second 11-bp motif for the cellular protein interacting with EBNA2. An EMSA was performed with nuclear extracts from EBNA2A-positive M-ABA cells, which were incubated with the ^{32}P-labeled 54-bp oligonucleotide. Unlabeled oligonucleotides O1 and O2, representing the first and the second 11-bp motif and adjacent sequences, respectively, were added to the reaction mixture in stepwise increasing amounts from 5- to 125-fold molar excess. The amounts of specifically shifted oligonucleotide (complex I) were quantified with the Fuji-BAS system and compared with the uncompeted lanes (100%) to determine relative binding of the wild-type 54-bp oligonucleotide.

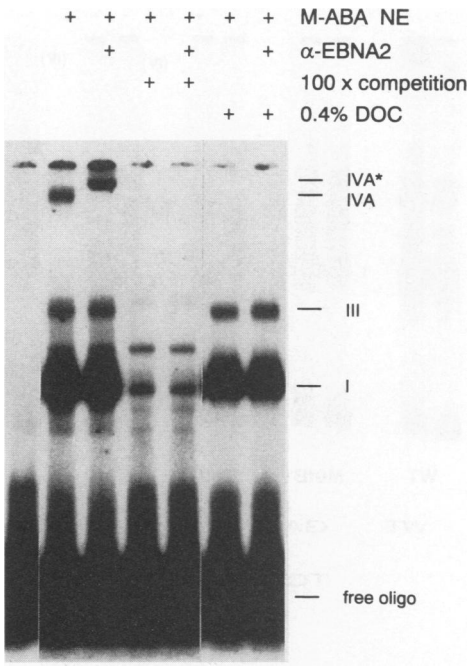


FIG. 5. Disruption of the interaction between EBNA2 and the cellular factor by sodium deoxycholate (DOC). Nuclear extracts (NE) of M-ABA cells (EBNA2A positive) were incubated with the labeled 54-bp fragment from −209 to −262 with and without 0.4% deoxycholate. Competition was performed by adding a 100-fold molar excess of the unlabeled 54-bp oligonucleotide. That EBNA2 is a component of complex IV was shown by addition of the monoclonal anti-EBNA2A antibody R11. Protein-DNA complexes were separated on a 4% polyacrylamide gel.

EBNA2RE fused to the luciferase reporter gene and transfected them into BL41-P3HR-1 cells in the presence and absence of EBNA2. Deletion analysis revealed that the region between −262 and −209, which is sufficient to target EBNA2 to its response element (47), cannot mediate transactivation by EBNA2. By transfecting an elongated *TP1* promoter-luciferase construct, additional sequences required for transactivation were localized between positions −209 and −198. Transfection of *TP1* promoter-luciferase constructs carrying 4- or 5-bp substitutions allowed us to narrow down more precisely which sequences within the EBNA2RE are crucial for EBNA2-mediated transactivation.

The most important sequence motifs for EBNA2-mediated transactivation within the 80-bp EBNA2RE are the duplicated 11-bp motifs, since the integrity of both motifs is necessary for full EBNA2 responsiveness. Mutations in either 11-bp motif not only impaired transactivation by EBNA2, but also seemed to increase the basal activity of the constructs in the absence of EBNA2. More work is needed to establish that this is indeed a specific effect of the 11-bp motif on basal transcription and does not reflect experimental variation. Scanning mutations of the 3′ portion of EBNA2RE had little or no effect on the EBNA2 inducibility of reporter constructs. This is in contrast to the effect of 3′ deletions, which completely abolished EBNA2 inducibility. There are several possibilities to explain this discrepancy. First, if the sequence requirements for the 3′ region are less stringent, mutation of only a few bases may be more easily tolerated. Second, if we assume that the five CAG triplets observed in the EBNA2RE between −212 and −178 are important for EBNA2 responsiveness, mutation of only one of them might still allow transactivation by EBNA2. The same argument holds true if we assume that several elements unrelated to the CAG repeat cooperate synergistically for EBNA2 transactivation. Sequences between −198 and −178 are not absolutely required for EBNA2 responsiveness but do intensify the EBNA2 effect; a luciferase construct containing the EBNA2RE from positions −257 to −178 resulted in an approximately 28-fold induction by EBNA2, whereas the shorter construct, containing EBNA2RE from positions −257 to −198, conferred only an approximately 10-fold induction. Sequences intensifying the EBNA2 effect could be located between positions −198 and −189, since scanning mutants mutP and mutQ showed a lower inducibility by EBNA2. Since full EBNA2 responsiveness could only be detected after transfection with a luciferase construct containing the whole 80-bp element, it is most likely that the EBNA2RE element reflects an enhancer composed of several different regulatory elements. This is in accord with the other EBNA2-responsive regions described so far. Both in the *Bam*HI-C and in the *LMP* promoters, it could be demonstrated that different regions are necessary for EBNA2 responsiveness (21, 23, 27, 41).

To analyze whether the same sequences necessary for transactivation are also involved in protein-DNA interactions, we studied specific protein-DNA interactions within the EBNA2RE. With a radioactively labeled probe from positions −257 to −178, five specific complexes (I, III, IV, *, and **) could be detected. All specific complexes could be inhibited by an unlabeled probe spanning the EBNA2RE from positions −262 to −209, implying that all observed protein-DNA interactions are taking place at the 5′ part of the EBNA2RE. Although sequences between −189 and −209 participate in transactivation by EBNA2, we were not able to detect protein-DNA complexes binding to this region. It is possible that additional protein-DNA complexes exist but, because of instability, cannot be detected in the standard EMSA with the whole 80-bp element. We are now using DNase I and exonuclease footprinting in the search for additional protein-DNA

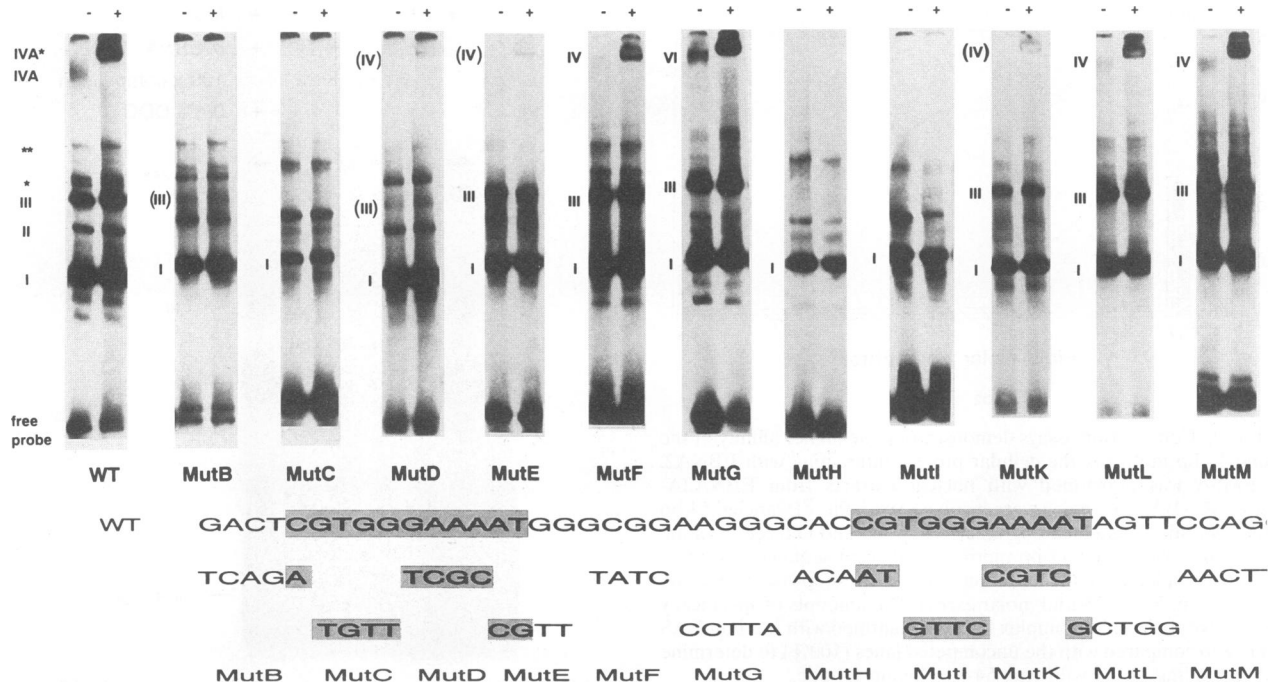


FIG. 6. Analysis of protein-DNA interactions in mutants of the EBNA2RE. EMSAs with the different scanning mutants of the EBNA2RE from −178 to −257 as radioactively labeled probes and nuclear extracts from EBNA2A-positive M-ABA cells are shown with (+) and without (−) addition of the monoclonal anti-EBNA2A antibody R11. A 4% polyacrylamide gel was used for separating protein-DNA complexes and the free oligonucleotide. On each gel, an EMSA reaction with wild-type oligonucleotide was run to class the complexes. At the bottom of the figure, base exchanges of the different mutants are indicated compared with the wild-type (WT) sequence. The 11-bp motifs are marked by shaded boxes.

complexes formed in the 3′ part of the EBNA2RE. Yet another possibility is that, in fact, no additional protein-DNA complexes exist and that structural constraints, for example, DNA bending, influence the activity of the EBNA2-dependent enhancer. DNA bending may be particularly important in facilitating or modulating looping between regulatory elements (35).

Analysis of the different DNA-protein complexes in methylation interference assays revealed that in complex I, the three consecutive guanines of the second 11-bp motif are predominantly protected by a cellular protein. Full protection of the first 11-bp motif was only visible if the second motif was mutated by base-pair substitutions. This indicates that the 11-bp motifs are necessary not only for transactivation but also for protein-DNA interaction. The cellular protein binding to this motif has a higher affinity for the second than for the first 11-bp motif, pointing to the contribution of adjacent sequences to the affinity of binding. The functional transactivation data corroborate the finding that the second 11-bp motif is more important, since mutations within the second motif resulted in a more dramatic decrease in transactivation by EBNA2 than mutations within the first. The higher binding affinity of the second 11-bp motif was confirmed by competition assays with the 54-bp oligonucleotide (−262 to −209) as the radioactively labeled probe and an excess of unlabeled oligonucleotides representing the first or the second 11-bp motif and adjacent sequences. The oligonucleotide carrying the second 11-bp motif was the significantly better competitor. In complexes III and IV, both 11-bp motifs were protected, although complete protection could only be detected at the second motif. The EBNA2-containing complex IV revealed a protection pattern identical to that seen with complex III, which is devoid of EBNA2. We have, therefore, as yet no evidence that EBNA2 creates contact sites to the DNA itself adjacent to the sequence recognized by the cellular protein. This is different from the interaction of herpes simplex virus immediate-early protein VP16 (Vmw65) with Oct-1, since VP16 seems to interact directly, albeit very weakly, with DNA (30). However, a more sophisticated footprint analysis must be carried out to provide a definite answer to this question.

Analysis of different scanning mutants in EMSAs emphasized the importance of the 11-bp motifs. Nearly all oligonucleotides containing mutations within one of the two 11-bp motifs were able to form complex I but failed partly or completely to create complexes III and IV (except mutL). Only the oligonucleotides mutD, mutE, and mutK, carrying mutations within the second half of the 11-bp motif, were able to form complex III and complex IV. Formation of these complexes became visible only after addition of the monoclonal anti-EBNA2 antibody, indicating a very weak protein-DNA interaction, which is stabilized in the presence of the anti-EBNA2 antibody. All mutants carrying base-pair substitutions outside the 11-bp motifs showed no difference in shift pattern compared with the wild type, demonstrating that only the 11-bp motifs are necessary for targeting EBNA2 to its response element. Examination of these mutants showed that for detecting complex III and, in particular, complex IV binding of the cellular protein to both 11-bp motifs is absolutely required, whereas occupation of one 11-bp motif is sufficient for formation of complex I.

Sequence homologies of the 11-bp motif are found in several EBNA2-responsive promoters, such as *LMP* (41), *Bam*HI-C (40), and *CD23* (43). Sequence comparison of the different promoters suggests that the core sequence GTGGGAA is

essential for EBNA2 responsiveness. This may indicate that a common cellular protein binds to all EBNA2REs of different promoters and serves as an adaptor molecule between DNA and EBNA2. Purification of this protein by affinity chromatography with the 11-bp motif as the probe is currently in progress. Identification of this protein will provide a clearer insight into the mechanism of EBNA2-mediated transactivation. In contrast to the *TP1* promoter, all other EBNA2-responsive promoters known so far contain only one copy of the common motif. For the *TP1* promoter, we could clearly demonstrate the importance of both 11-bp motifs for EBNA2-mediated transactivation as well as for detection of EBNA2 in the protein-DNA complexes formed at the EBNA2RE. Our data provide an explanation of why EBNA2 has not yet been detected in protein-DNA complexes formed at the EBNA2RE of the *Bam*HI-C and *LMP* promoters. They do not explain, however, why the *Bam*HI-C and *LMP* promoters can be transactivated by EBNA2. Given the structural organization of the *Bam*HI-C and *LMP* promoters, there is no doubt that one motif must be sufficient in some instances to mediate the contact to EBNA2 and EBNA2 inducibility. If so, the interaction may be too labile to be detected.

There are several possibilities to explain the appearance of two 11-bp motifs within the *TP1* promoter versus a single motif within all the other EBNA2-responsive promoters so far characterized. First, the *TP1* promoter has developed a system, perhaps by DNA duplication, resulting in two identical 11-bp motifs, which leads to more efficient transactivation by EBNA2. To test this hypothesis, comparative studies of the EBNA2 inducibility of the *TP1* promoter and other promoters should be performed. Functional transactivation studies of the EBNA2RE within the *TP1* promoter revealed three regions important for optimal EBNA2-mediated transactivation: both 11-bp motifs and the 3′ portion. If we assume that two elements generally suffice for EBNA2-mediated transactivation and that the presence of a third element has a synergistic effect, the presence of only one 11-bp motif in other EBNA2-inducible promoters may be compensated for by another sequence element which could possibly bind yet another cellular protein. This more complex composition of promoters and the involvement of different factors in its regulation may explain the strict EBNA2 dependence of the *TP1* promoter in B cells as opposed to that seen with the *LMP* promoter, which, depending on the cellular background, can be activated without EBNA2 (15), perhaps by overriding the EBNA2-dependent regulation by another factor which interacts with the promoter.

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