

Protein-DNA Binding and CpG Methylation at Nucleotide Resolution of Latency-Associated Promoters Qp, Cp, and LMP1p of Epstein-Barr Virus

DANIEL SALAMON,^{1,2} MARIA TAKACS,³ DORINA UJVARI,¹ JÖRG UHLIG,⁴ HANS WOLF,⁴
JANOS MINAROVITS,^{1*} AND HANS HELMUT NILLER^{4*}

Microbiological Research Group, National Center for Epidemiology, H-1529 Budapest,¹ Division of Virology, National Center for Epidemiology, H-1097 Budapest,³ and Second Department of Pathology, Faculty of Medicine, Semmelweis University, H-1091 Budapest,² Hungary, and Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, D-93053 Regensburg, Germany⁴

Received 20 October 2000/Accepted 12 December 2000

Epstein-Barr viral (EBV) latency-associated promoters Qp, Cp, and LMP1p are crucial for the regulated expression of the EBNA and LMP transcripts in dependence of the latency type. By transient transfection and in vitro binding analyses, many promoter elements and transcription factors have previously been shown to be involved in the activities of these promoters. However, the latency promoters have only partially been examined at the nucleotide level in vivo. Therefore, we undertook a comprehensive analysis of in vivo protein binding and CpG methylation patterns at these promoters in five representative cell lines and correlated the results with the known in vitro binding data and activities of these promoters from previous transfection experiments. Promoter activity inversely correlated with the methylation state of promoters, although Qp was a remarkable exception. Novel protein binding data were obtained for all promoters. For Cp, binding correlated well with promoter activity; for LMP1p and Qp, binding patterns looked similar regardless of promoter activity.

Epstein-Barr virus (EBV) infection is the cause of infectious mononucleosis and is most closely associated with tumor diseases Burkitt's lymphoma (BL) and nasopharyngeal carcinoma. EBV infection of human B lymphocytes in vitro results in B-cell proliferation and transformation into continuously growing lymphoblastoid cell lines (LCL) (for a review, see reference 42). In latently infected cells, viral genomes are maintained as multiple circular episomal copies which are replicated once per cell cycle (2, 103). Several classes of latency have been described depending on the gene expression pattern (41, 77, 78). In strict type I latency, represented by BL cells, viral gene expression is restricted to the two RNA polymerase III-transcribed EBNA RNA genes and the EBNA1 gene (78) that is transcribed from the Q promoter (Qp) (68). The EBNA1 protein is required for the maintenance of the viral plasmid in dividing cells (45, 58). In type III latency, in addition to the EBNA1, EBNA-LP, -2, -3A, -3B, -3C, and -1 are expressed from the C promoter (Cp) (6), whereas LMP-1 and -2B are expressed from the bidirectional LMP1 promoter (46), and a larger splice variant of LMP-2, LMP-2A, is expressed from the TP1 promoter (36). Qp generally is supposed to be silent in type III latency (82, 105), although there is also a different view (93). Among the viral proteins expressed in latency type III, EBNA2 plays a central role in switching EBNA transcription from Wp to Cp (W to C switch) (102, 104)

and in the establishment and maintenance of B-cell transformation (11, 28), as EBNA2 transcriptionally activates the expression of the six nuclear antigens from the C promoter (Cp) and the membrane proteins LMP-1 and -2B from the LMP1 promoter (LMP1p), LMP-2A from the TP1 promoter, and a number of cellular proteins associated with the LCL phenotype (1, 12, 18, 39, 44, 72, 76, 90, 95, 98, 99, 100, 101, 102, 104, 110, 111). A crucial mechanism involved in the silencing of Cp and LMP1p in type I latency has been shown to be methylation of CpG dinucleotides (3, 15, 35, 54, 60, 61, 70, 73, 74, 75, 84, 91, 94). In LCL, the EBV genome is mostly free of CpG methylation, whereas in BL cells, EBV genomes are highly methylated. An essential step in understanding the differences between latency types I and III is to elucidate the patterns of methylation and in vivo protein binding of the latency promoters of EBV at nucleotide resolution. Therefore, we decided to examine Qp, Cp, and LMP1p in cells of both latency types.

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MATERIALS AND METHODS

Cell lines and tissue culture. LCL 721 is a B95-8-transformed LCL with type III phenotype (40, 52, 57). Rael (15, 43, 61) is a group I BL cell line. Mutu BLI-C1216 is a subclone of the BL line Mutu, representative of latency type I (27). Mutu BLIII-C199 is a subclone of the BL line Mutu, representative of latency type III (27). Raji cells express all the type III latency genes but use a thus far unknown promoter, other than Cp, for the EBNA transcripts (29, 96). All cells were maintained in suspension cultures of RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 50 U of penicillin per ml, and 50 µg of streptomycin per ml at 5% CO₂ and 37°C.

Electrophoretic mobility shift assay. Preparation of nuclear extracts from Mutu I cells was essentially based on the standard of Dignam et al. (14). Nuclei were prepared using a combination and modification of two methods (8, 30) as

* Corresponding author. Mailing address for J. Minarovits: Microbiological Research Group, National Center for Epidemiology, Pihenő út 1, 1522 Budapest, Hungary. Phone: 36 (1) 394-5044. Fax: 36 (1) 394-5409. E-mail: mini@microbi.hu. Mailing address for H. H. Niller: Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany. Phone: 49 (941) 944-6451. Fax: 49 (941) 944-6402. E-mail: Hans-Helmut.Niller@klinik.uni-regensburg.de.

TABLE 1. Primers for methylation mapping^a

Primer type	No.	Sequence (nucleotides)
Outer primers for modified Qp ^b	1	GGTTAGTTTGATTAAGGGGTGAGGT (62179–62202)
	2	CCATTACCCCAATACATTCC (62626–62606)
Inner primers for modified Qp ^c	3	Univ-CTCCCAACTACCCAAAATACCA (62504–62483)
	4	Biotin-GTTAGTTTGATTAAGGGGTGAGGTTATAA (62180–62207)
Outer primers for modified Cp ^d	5	GGGTTTAGGTTTTGTAGGGTAGA (10585–10607)
	6	CCCTACRATAAAAACTCTAAAAATCTT (11392–11366)
Inner primers for modified Cp ^e	7	Univ-GTTAGGTTGATAAGGGGATAAG (10610–10631)
	8	Univ-TGAGAGGTTAGTGTTTTAAATATGT (10878–10902)
	9	Univ-GGATTATAGTTAATAAGAGAGTTTAAAGA (11066–11093)
	10	Biotin-ATCCTTATCTCTATACCATAATCTA (11361–11335)

^a Primer positions refer to the nucleotides of the B95-8 sequence (4). Univ indicates the M13 universal primer sequence GTAAAACGACGGCCA. Primers were purchased from Metabion (Martinsried, Germany). Each PCR was cycled 30 times at the temperatures and times indicated. Primers for LMP1p, PCR conditions, and the CpG methylation maps of LMP1p are described in detail by Takacs et al. (91a).

^b Cycled at 95°C for 40 s, 60°C for 40 s, and 72°C for 70 s.

^c Cycled at 95°C for 40 s, 58°C for 40 s, and 72°C for 60 s.

^d Cycled at 95°C for 40 s, 58°C for 40 s, and 72°C for 90 s.

^e Cycled at 95°C for 40 s, 52°C for 40 s, and 72°C for 90 s (7 to 10), 70 s (8 to 10), or 60 s (9 to 10).

already described (64). Complementary double-stranded DNA oligonucleotides (Metabion) containing a consensus binding site for CBF1, 5'-GGATCCGCCG TGGGAAAAGTCGAC-3', and a mutant binding site disabled for CBF1 binding, 5'-GGATCCGCCGTGTTAAAAAGTCGAC-3', (51) were kinase labeled, annealed, and spin column purified for a gel shift probe. Gel retardation assays were performed as described (30, 64): 1 µg of crude nuclear protein was incubated with poly(dI-dC) as indicated, 1 ng of ³²P-labeled probe, and a 50-fold excess of unlabeled competitor fragment in 25 µl of bandshift buffer (10 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 80 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 12.5% glycerol, 0.1% Triton X-100) for 20 min. Protein complexes were resolved by electrophoresis on native 4% polyacrylamide gels (29+1) in 6.7 mM Tris-HCl (pH 7.5)–3.3 mM sodium acetate (pH 7.0)–1 mM EDTA at 20 mA for several hours.

DNA sequences. Oligonucleotides (Metabion, Martinsried, Germany) corresponding to EBV nucleotides (4) 10595 to 10614 and 11364 to 11342 were used for sequencing Cp, and others corresponding to nucleotides 62146 to 62165 and 62548 to 62524 were used for sequencing Qp. Both strands of the two promoters were sequenced from the genomic DNA of all five cell types on an ABI 377 DNA sequencing system using dye-labeled dideoxynucleoside triphosphates (ddNTPs). In the analyzed region of the C promoter (nucleotides 10615 to 11341), a few sequence polymorphisms were noted in Rael (GenBank accession number AJ297541) and in the Mutu subclones, whereas the sequences of LCL 721 and Raji were identical to the standard B95-8 sequence (4). The sequence of Cp between nucleotides 10615 and 11046 in the Mutu subclones is at GenBank numbers AJ000877 and AJ000878 (91), and the 3' part between nucleotides 11047 and 11341 was identical with the standard sequence (4). The sequence of Qp between nucleotides 62166 and 62523 did not show any deviation from the B95-8 standard sequence in all cell types (4). Sequences of LMP1p have been described (91a).

Automated genomic sequencing of sodium bisulfite-treated DNA. We used the method of Frommer et al. (22) and Clark et al. (10) adapted for an automated DNA sequencer (63). A total of 5 µg of genomic DNA in 50 µl of water was denatured by adding 5.5 µl of freshly prepared 3 M NaOH and incubating for 15 min at 37°C. Then 30.5 µl of freshly prepared 10 mM hydroquinone (Sigma), and 530 µl of 3.6 M sodium bisulfite, pH 5 (Sigma), were added to the denatured DNA, mixed gently, divided into five 0.5-ml PCR tubes, overlaid with paraffin oil, and cycled five times at 95°C for 3 min and 55°C for 57 min. After this treatment, the modified DNA was purified using a GeneClean kit (BIO 101) according to the manufacturer's instructions. Then the DNA was desulfonated by adding freshly prepared 3 M NaOH to a final concentration of 0.3 M and incubating the mixture for 15 min at 37°C. After desulfonation, the DNA was ethanol precipitated and dissolved in water. Then 100 ng of freshly modified DNA was used for PCR amplification with the strand-specific outer primer pairs (22) designed for the promoter regions (Table 1). The 50-µl PCR contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl₂, 0.1% Triton X-100, 40 pmol each of the primers, 0.2 mM each of the four dNTPs, and 2 U of *Taq* polymerase (Promega). Then 3 µl of a 1:100 dilution from the first PCR was amplified in a second nested PCR using the primers listed in Table 1. One of the nested primers was biotin labeled, and the other carried 15 bases of the M13 universal primer at its 5' end.

The reaction mixture for the nested PCR was the same as for the first PCR except that the amount of inner primers was 10 pmol each. The product of the second PCR was bound to streptavidin-coated magnetic beads (Dyna), and the purified biotin-labeled strand was sequenced using the AutoRead DNA sequencing kit (Amersham Pharmacia Biotech) and a fluorescein-labeled M13 universal primer as described by Myöhänen et al. (63). The reaction products were separated on acrylamide gels using an automated DNA sequencer (Amersham Pharmacia Biotech). The degree of methylation was estimated as described earlier (63). The bisulfite conversion reaction was complete, since all cytosines outside CpG dinucleotides were converted to uracil and therefore sequenced as thymine instead of cytosine after PCR (see reference 80 and Takacs et al. [submitted] for examples).

DMS in vivo footprinting. Genomic footprinting was performed essentially as described (65). For each footprint reaction, 10⁷ exponentially growing cells were harvested, washed with phosphate-buffered saline (PBS), resuspended in 1 ml of PBS, and incubated at room temperature for 1 min with 5 µl of dimethyl sulfate (DMS). The reaction was stopped by the addition of 5 ml of DMS stop solution, containing 1% bovine serum albumin and 100 µM β-mercaptoethanol in PBS. Cells were washed once more in DMS stop solution and twice more with PBS. Finally, cells were resuspended in 1 ml of PBS, and genomic DNA was prepared. Footprinted DNAs were subjected to piperidine treatment (55). For visualization of footprints by ligation-mediated PCR (LM-PCR), 2 µg of sequenced or footprinted DNA was analyzed as described (26, 62) with modifications (65). The primers for LM-PCR are listed in Table 2. The first-strand primer extension reaction was done in 10 mM KCl–10 mM (NH₄)₂SO₄–20 mM Tris-HCl–2 mM MgSO₄–0.1% Triton X-100 (pH 8.8) at 25°C (Vent buffer; New England Biolabs), containing 0.3 pmol of primer i of each set, 240 µM each dNTP, and 1 U of Vent (exo-) DNA polymerase (New England Biolabs) for 5 min at 94°C, 30 min at 60°C, and 10 min at 72°C. For ligation of the common linker, the sample was transferred to ice, and 5 µl of PCR linker mix as in Mueller and Wold (62), 2 µl of ligation buffer (660 mM Tris-HCl, 50 mM MgCl₂, 10 mM dithioerythritol, 10 mM ATP [pH 7.5] [20°C], Boehringer Mannheim), 1 µl of T4 DNA ligase (5 U/µl; Boehringer Mannheim), and 12 µl of water were added. After overnight incubation at 4°C, the DNA was ethanol precipitated, washed once with 75% ethanol, dried, and then resuspended in water. The PCR amplification was done in 100 µl of Vent buffer containing 10 pmol of each primer ii and the longer linker primer, 240 µM each dNTP, and 1 unit of Vent (exo-) DNA polymerase for 20 cycles using 1 min at 94°C, 1.5 min at 60°C, and 3 min at 72°C. For labeling, the sample was transferred to ice, 5 pmol of T4 kinase [^γ-³²P]ATP-labeled primer iii, 2.5 nmol of each dNTP, and 0.5 U of Vent (exo-) DNA polymerase in a volume of Vent buffer not exceeding 15 µl were added. Then the sample was heated to 94°C for 1.5 min, subjected to eight cycles of 2 min at 94°C, 2 min at 62°C, and 5 min at 72°C, and kept at 72°C for 5 more min. Samples were phenol-chloroform extracted, ethanol precipitated, ethanol washed, and resuspended in loading dye. One fifth of each sample was separated on a 5% sequencing gel, and the gels were dried and autoradiographed at room temperature with Kodak BioMax MR film.

TABLE 2. Primers for LM-PCR^a

Promoter	Primer set	No.	Sequence (nucleotides)
Qp	A	i	GCTATAACGCAGGTCCTGTTCCGGG (62201–62225)
		ii	GCGGTGGATAGAGAGGAGGGGGATC (62229–62253)
		iii	GAGGGGACCACTAGGTCGCCGGAGG (62256–62280)
	B	i	CCCCAACATACACCGTGC AAAAG (62548–62524)
		ii	CCGTGCGAAAAGAAGCACCCCATC (62535–62511)
		iii	CCGCCTCCAGCTGCCAAAATGCC (62508–62484)
Cp	A	i	GTCCCAATTAGAAACCCAAGCGCAG (10845–10869)
		ii	CCCAAGCGCAGAAATTAGTTGAGAGG (10859–10884)
		iii	AACMTGCACCCTAGGCCAGCCAGAG (10896–10920)
	B	i	ACTTTGCGAGCCCTGCGTCTTGAG (11110–11087)
		ii	TATTGGCTATAATCCGTCGCTCCTCCC (11080–11054)
		iii	CCGTCGCTCCTCCAGATAAGGCGT (11067–11043)
	C	i	CTCAAGACGCAGGGCTCGCAAAGT (11087–11110)
		ii	GTATAGTGGCCCCGTGGGACCTTAG (11109–11133)
		iii	TTAGAGGTGGAGCAACGTCTAAAGTGG (11130–11156)
	D	i	GGGCCTACATGGCCGCATGGTAAG (11418–11395)
		ii	GGTAAGAACCCTGCGATGAGGGCTC (11400–11376)
		iii	GATGAGGGCTCTGGGGGTCTTCGGTG (11386–11361)
	E	i	GTGCGTCGAGTGCTATCTTTGGAAC (10981–11005)
		ii	ACCTTGTTGGCGGGAGAAGGMATAAC (11019–11044)
		iii	ACGCCTTATCTGGGAGGAGCGACGG (11043–11067)
LMP1p	A	i	CCCCTCTCAAGGTCGTGTTCCATCC (169452–169476)
		i var	CCCCTCTCAAGTCCAGTCCATGC (169452–169476)
		ii	TCAGGGCAGTGTGTCAGGAGCAAGG (169477–169501)
		ii var	TCAGGGCAGTGTGTCAGGAGCCAGG (169477–169501)
		iii	AGGCAGTTGAGGAAAGAAGGGGGCAG (169489–169524)
	B	i	CTTAGCCCTCTTAGCCGCCTCACC (169966–169943)
		ii	TACGGTTACCCACAGCCTTGCCCTC (169933–169909)
		ii var	TACGGTGAACCCACATCCTTGCCCTC (169933–169909)
		iii	GCCTCACCTGAACCCCTAAAAGCAC (169913–169888)
		iii var	GCCTCACCTGAACCCCTAAAACMC (169913–169888)
	C	i	GCGCCTCTTTGTGCAGATTACACTG (169843–169819)
		ii	CCGCTTCCACAACACTACGCACTC (169818–169794)
		iii	CCTTCTGATTGCCGCACTGCCTTTCC (169791–169716)
	D	i	GTACGGGYRCAGATTTCCCGAAAG (169621–169644)
		ii	GATTTCCCGAAAGCGGGTGTGTG (169632–169656)
		iii	CGGCGGTGTGTGTGTGCATGTAAGCG (169645–169660)
	E	i	AGAGGAGGAGAAGGAGAGCAAGG (169375–169397)
		ii	CCCCTCTCAAGGTCGTGTTCCATCC (169452–169476)
ii var		CCCCTCTCAAGTCCAGTCCATGC (169452–169476)	
iii		TCAGGGCAGTGTGTCAGGAGCAAGG (169477–169501)	
iii var		TCAGGGCAGTGTGTCAGGAGCCAGG (169477–169501)	
F	i	CACACGCTTYCTACTTCCCCTTTYTAC (169696–169670)	
	ii	CGCTTACATGCACACACACCCGCC (169670–169646)	
	iii	CACACACCGCCGCTTTCGGGAAATC (169656–169632)	

^a Primer positions refer to nucleotide numbers of the B95-8 sequence (4). Primers were purchased from Metabion (Martinsried, Germany). Variant primers or primers with wobble bases were used because of minor sequence deviations between EBV strains. With these primer sets, both strands of each promoter were visualized in their entire length, and several promoter parts were seen with more than one primer set.

RESULTS

Methylation patterns at CpG dinucleotides. The methylation data solely reflected the status of tightly latent EBV circular genomes, but not the presence of linear genomes from a possible small amount of lytic replication as was tested by terminal repeat analysis through southern blotting (59, 91a)

and for LCL 721 and Raji cells through Gardella gels, in addition (52). Early antigens or their coding mRNAs associated with productive EBV replication could not be detected either in the above-mentioned cell lines and clones (54, 57; J. Minarorrits, unpublished data). In addition, specific segments of the EBV genome were found to be completely methylated

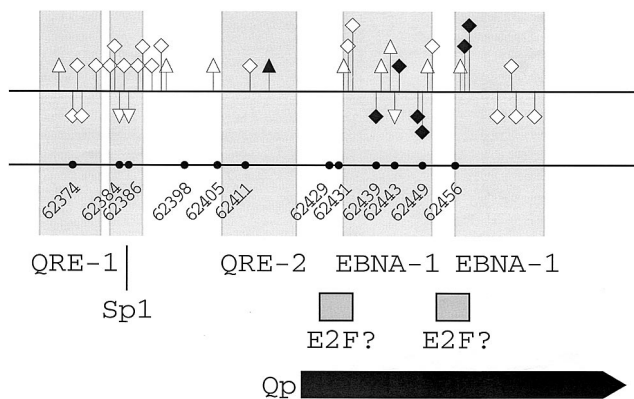


FIG. 1. Summary of genomic footprinting and methylation patterns of Qp. Numbers and circles on the lower line indicate positions of cytosines within CpG dinucleotides and show that all CpG dinucleotides within Qp are totally unmethylated in all cell types. On the upper line, guanines protected from methylation by DMS are indicated by squares and enhanced reactivity to DMS is shown by triangles. Guanines that showed a different reactivity to DMS between cell types are indicated by solid symbols. The upper strand is shown above, and the lower strand is below the line. The positions of important *cis* regulatory elements are indicated by columns and boxes. The transcription initiation site of Qp is shown by a thick arrow.

in all five cell types (data not shown), another indication that there was no lytic cycle viral DNA in the cell lines examined. Cytosines between nucleotides 62264 and 62482 of the Qp region were completely unmethylated in all cell types (Fig. 1). The methylation data on Qp were in agreement with previous observations (84, 93), completed these observations for Mutu I, and extended them to the additional cell types LCL 721 and Mutu III. Previous methylation analyses of Cp (3, 54, 61, 74, 75, 84, 91, 94) could be largely confirmed and extended by our present work. Overall, Cp was nonmethylated in class III cell lines, but highly methylated in class I BL cell lines and Raji (Fig. 2). However, in Mutu I there was a methylation gap of about 100 bp of complete demethylation around the crucial CBF1 and CBF2 binding sites (16, 37, 74, 75), and a further adjoining gap of about 100 bp of partial demethylation (Fig. 2). For Mutu III there was a small contradiction to our earlier work (91), where CpG dinucleotides 10702 and 10799 were found to be highly methylated. Methylation at these two CpGs could not be confirmed anymore. The discrepancy was most likely due to a sequencing artifact in the earlier work. Methylation of LMP1p in the five cell lines has been recently examined by Takacs et al. (submitted). Overall, LMP1p was hypo- or nonmethylated in class III cell lines, as well as Raji, but highly methylated in class I BL cell lines (Fig. 3).

In vivo protein binding. (i) Q promoter. The DMS footprinting was done on both strands of Qp (Fig. 4) according to standard methods (26, 62, 65). The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation is summarized in Fig. 1. The footprint patterns on Qp from the five cell lines were generally identical, with four remarkable features. First, in Mutu I cells the EBNA1 binding sites were more weakly protected than in the other cell lines. Second, there was a hypersensitivity in the type III cell lines on the upper strand at guanine 62416 within QRE2, but not in the

type I cells. Third, there was a strong protein-DNA interaction at a potential Sp1 binding site around nucleotides 62382 to 62394 (67). Fourth, footprints with a typical protection pattern indicative of E2F binding (112), at two previously characterized unconventional E2F sites, interspersed with the EBNA1-sites and the transcriptional start site (13, 89), were not found.

(ii) C promoter. Previous *in vitro* binding and reporter gene experiments have charted a CBF1 site, a CBF2 site, and two CCAAT boxes as transcriptional elements of Cp and identified CBF1/RBP-J κ and AUF1 as the respective binding proteins for the CBF1 and CBF2 sites (23, 25, 37, 47, 50, 71, 75). These binding sites and an additional Sp1 site have been shown to be highly conserved between EBV and two related lymphocryptoviruses of monkeys (24). The promoter area examined here is shown in Fig. 5. The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation is summarized together with the methylation data in Fig. 2. There were two Sp1-like sequences around nucleotides 11176 and 11197 that were protected in all cell lines despite methylation (31). The Sp1 site at 11029 showed slight signs of protein-DNA interaction in all cells. Further, we found a series of sites protected only in 721 and Mutu III cells, but not protected in type I and Raji cells. The two CAAT boxes and several novel protections belonged to this category. The CAAT box at nucleotide 11075 was strongly protected, and the CAAT box at nucleotide 11268 was strongly protected in LCL 721 cells and weakly in Mutu III cells. A novel interaction, called X, was found around nucleotide 11222. Another novel interaction, called Y, carrying no familiar consensus sequence for transcription factor binding, was found around nucleotide 11062. An extended interaction was found between 11120 and 11140, with a methylation interference pattern characteristic of CBF1 binding (56) in the 5' part and a protection, called Z, in the 3' part. Binding at the CBF2 site in LCL 721 and Mutu III cells was weak at best, since there were slight signs of protein-DNA interaction only (Fig. 5). A characteristic protein-DNA interaction was found at the previously described CBF1 site at nucleotide 10959. Since the CBF1 site was unmethylated but the protection pattern was not typical in Mutu I, we performed electrophoretic mobility shift experiments. The gel shifts showed that a CBF1-like binding activity was present in Mutu I and was able to bind to its consensus site in a sequence-specific manner (Fig. 6).

(iii) LMP1 promoter. Many transcriptional elements of LMP1p have been characterized so far by *in vitro* binding and reporter gene experiments. Among these elements were binding sites for CBF1 (38, 51); PU.1, also called Spi-1, and Spi-B (38, 47, 48, 85, 108); AML1, also called LBF1, and several LMP1p binding proteins, named LBF-2 to -7 (38); negative regulatory element NRE (18); the E box carrying a USF binding site (88); a *cis*-inducible element (SIE) (86, 87); and an ATF/CRE that, depending on the distinct proteins binding, was able to activate LMP1p both independently and dependent on EBNA2 (20, 87, 88). The relevant footprinted promoter area is shown in Fig. 7. The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation is summarized together with the methylation data in Fig. 3. Footprints were generally identical for all cell types. Signs for protein-DNA interactions, mostly of low or intermediate strength,

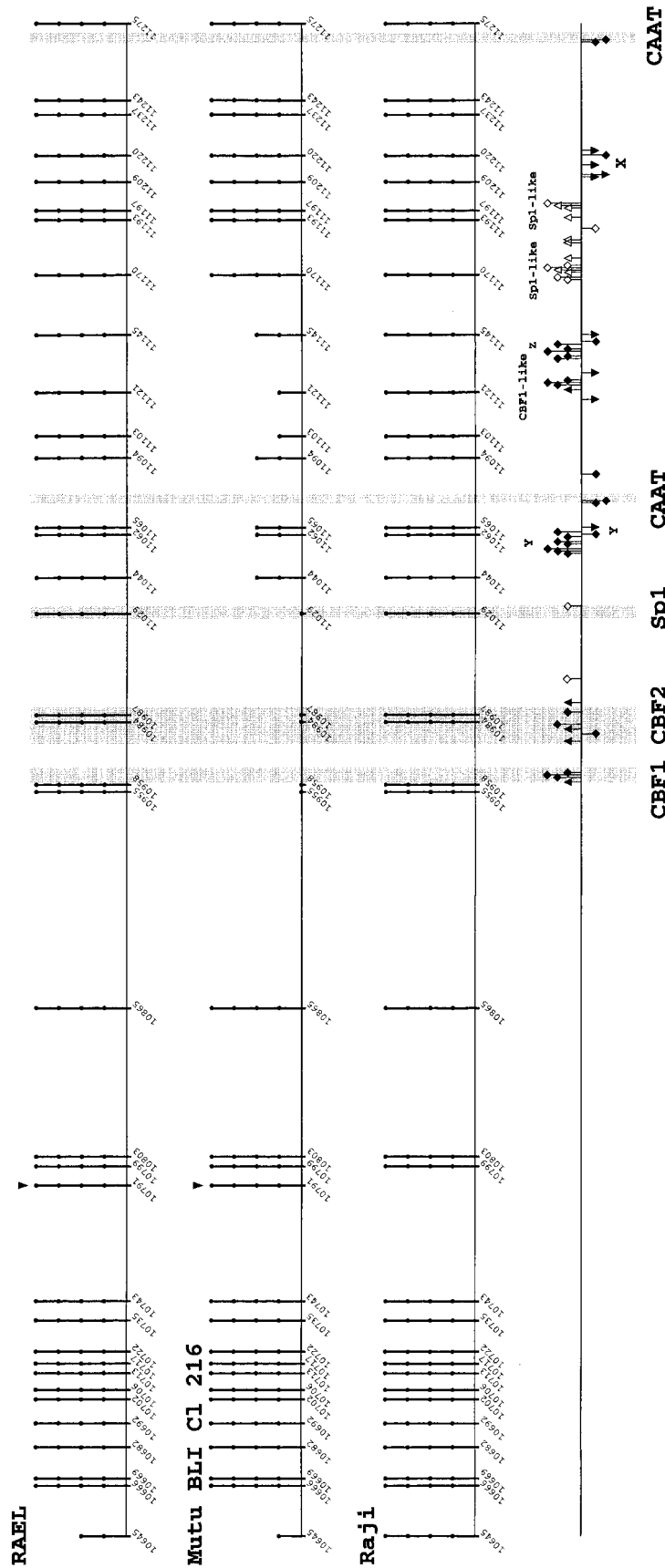


FIG. 2. Summary of genomic footprinting and methylation patterns in the sequenced region of the BCR2 promoter (Cp). Numbers and lollipops indicate positions of cytosines within CpG dinucleotides, based on the prototype B95-8 sequence (4). Triangles above the lollipops mark additional target cytosines for DNA (cytosine-5) methyltransferase (5, 69) present in the cell lines studied. The degree of methylation of cytosines is indicated by the height of the lollipops as follows: spot only, 0%; one lollipop unit, 25 to 50%; two units, 50 to 75%; three units, 75 to 100%. The bottom line shows a summary of genomic footprints for the upper (above the line) and lower (below the line) strand of Cp. Guanines protected from methylation by DMS are indicated by squares, and enhanced reactivity to DMS between cell types are indicated by solid symbols. Novel footprints (X, Y, Z, CBF1-like, and Sp1-like) are indicated above and below the footprint marks. Faint columns represent already published relevant transcription factor binding sites in Cp.

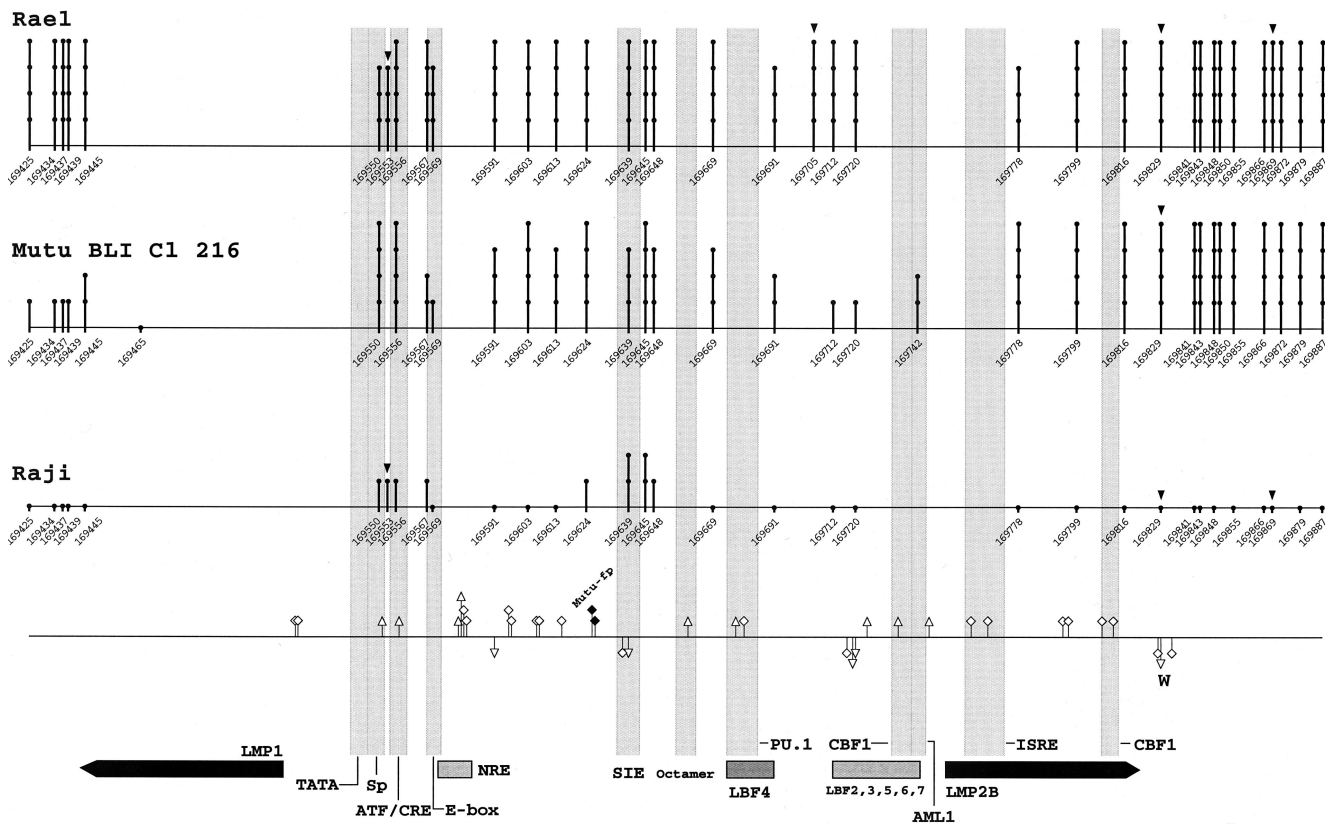


FIG. 3. Summary of genomic footprinting and methylation patterns in the sequenced region of the LMP1 promoter. Numbers and lollipops indicate positions of cytosines within CpG dinucleotides, based on the prototype B95-8 sequence (4). Triangles above the lollipops mark additional target cytosines for DNA (cytosine-5) methyltransferase present in the cell lines studied. The degree of methylation of cytosines is indicated by the height of the as defined in the legend to Fig. 2. The bottom line shows a summary of genomic footprints for the upper (above the line) and lower (below the line) strand of LMP1p. Guanines protected from methylation by DMS are indicated by squares, and enhanced reactivity to DMS is shown by triangles. Some novel footprints (Mutu-fp with solid symbols and W) are indicated above and below the footprint marks. Faint columns represent important transcription factor binding sites in LMP1p. The transcription initiation sites of LMP1 and LMP2B are shown by thick arrows.

were found on either one or both strands of the PU.1, AML1, LBF-3, 5, 6, 7, Oct, ISRE, SIE, NRE, and Sp1-like site, the two CBF1 sites, and the ATF/CRE site. The PU.1/LBF4 binding activity carried the typical methylation interference pattern described for these factors in vitro (38). The CBF1 footprints did not carry the typical methylation interference pattern (56) that was found in type III cells except Raji on Cp. In addition to protein-DNA interactions at already charted elements, we found a footprint at nucleotides 169520 and 169521 around the initiation site of the LMP1 transcript, a hypersensitive site at nucleotide 169591, a footprint at 169596 and 169597, a footprint at 169606 and 169607, a footprint at 169615, and a strong footprint, called W, at nucleotide 169833 for all cell types examined. In addition, we found a footprint, Mutu-fp, specific for both Mutu clones at nucleotides 169626 and 169627. These binding factors await further identification. However, there were no differences in the in vivo binding pattern, with the possible exception of the ATF/CRE-Spl locus. At this locus we found slight differences in the reactivity to DMS between cells.

DISCUSSION

Although under some conditions Mutu I may drift to type III latency, Mutu BLI-C1216 of this study represents a type I cell,

because of its phenotype and EBNA-2 protein could not be found by Western blotting (data not shown). Still, we cannot entirely rule out the possibility that an extremely small proportion of Mutu I cells were drifting towards type III latency. Because of this and because of the limited number of cell types in this study, final conclusions regarding the two latency types may only be drawn after the examination of a larger panel of cell lines and subclones.

Qp behaves like a bacterial promoter. Qp was unmethylated and extensively protein protected in all cell types, regardless of the activity of the promoter (Fig. 1 and 4). The protein binding pattern was generally in congruence with the in vitro (9, 66, 67, 83, 89, 106) and in vivo (33) data described earlier. It is clear now that the Sp1-like sequence just downstream of QRE1 is strongly protein bound. This site has been discussed as a potential unconventional E2F site, but has been shown not to compete for the in vitro binding of E2F-like proteins (79). The overall protection pattern was identical in the five cell types, with minor exceptions: at the QRE2 element there was a hypersensitivity indicative of closer protein binding at QRE2 for the silent promoter state in LCL 721, Mutu III, and Raji cells (Fig. 4). Since Qp is unmethylated and heavily protein protected, the QRE2-bound protein may be key to the silencing of

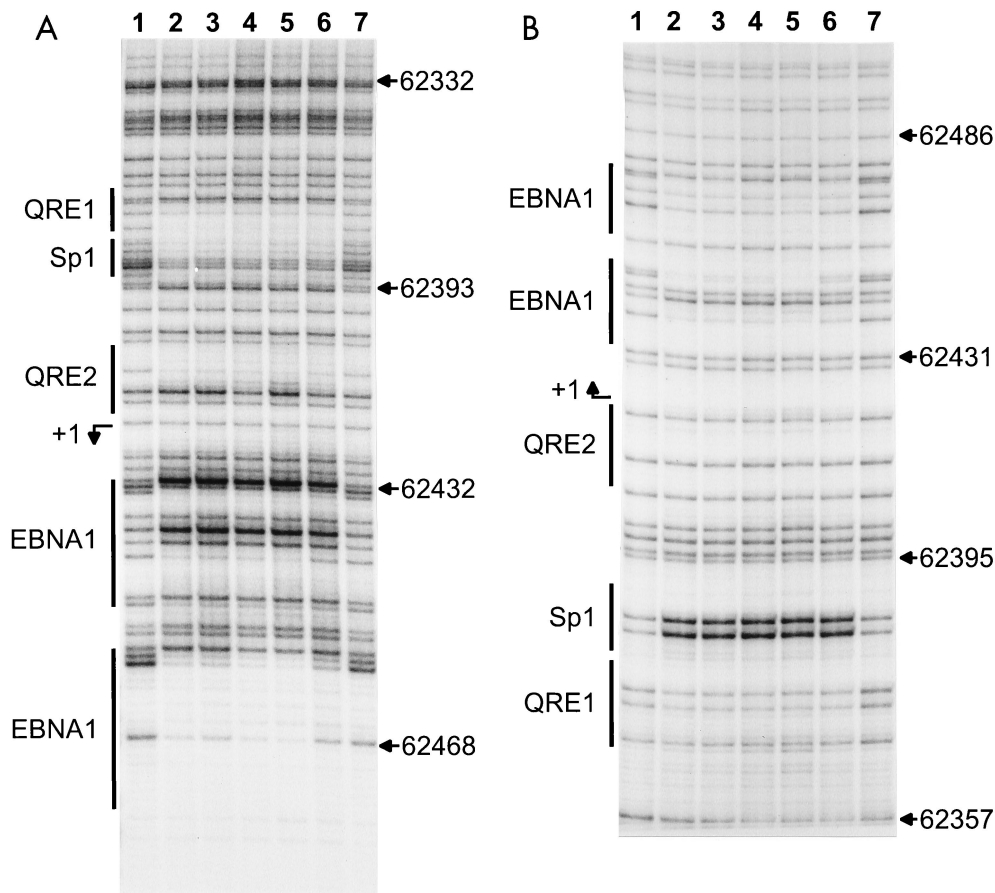


FIG. 4. Genomic footprint analyses of Qp. (A) Upper strand, (B) Lower strand. Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprints. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Mutu I DNA. At the left of each panel, the locations of *in vivo* footprints and previously described *in vitro* binding sites are indicated by vertical bars; at the right of each panel, nucleotide numbers are given according to the EBV sequence of Baer et al. (4).

Qp in type III latency. Candidate factors for this binding activity are IRF-7, a Qp-repressive factor described previously (66, 106), and IRF-2, although there are contrary views on the repressive nature of IRF-2 (83, 107). The EBNA1 binding sites were protected in all cells, in agreement with Hsieh et al. (33), who have already demonstrated the same *in vivo* EBNA1 site protection in Qp for Raji cells. The weaker EBNA1 binding in Mutu 1 may be interpreted in terms of promoter activity in type I cells and a repressive function for EBNA1. However, in Rael, where Qp is active, the EBNA1 protection is as strong as in the type III cells. A clear protection pattern indicating E2F binding (112) could not be found at the sites previously described as *in vitro* E2F binding sites (79, 89). Therefore, the repressive role for EBNA1 (81) and the activating role for E2F in Qp transcription that have been postulated (79, 89) may have to be modified. The constant strong binding of EBNA1 together with Sp1 may cause the constitutive hypomethylation of Qp (7, 32, 49, 53, 80). In summary, Qp activity is likely to be regulated in a comparably simple way, as in bacterial promoters, by the binding or not of a few key transcription factors and a repressor.

Cp is regulated by methylation and protein binding. Cp was unmethylated in the activity promoter state, but methylated in

the inactive state (Fig. 2). The inverse correlation between methylation status and promoter activity was best in the promoter-proximal part, where Cp was completely methylated in the cell types not using Cp (Fig. 2). Therefore, extended alterations in overall CpG methylation seem to be more important than methylation of particular CpG dinucleotides in Cp (75). In agreement with earlier observations (16, 24, 70, 74), additional protein determinants of Cp activity besides CBF1 and CBF2 may play a role (Fig. 2 and 5). In addition to a couple of footprints at Sp1-like sequences that were common to all cells, there were several prominent footprints only found at active Cp that were completely lacking from inactive Cp. Differential footprinting was found at two sites for CBF1 and two CAAT boxes and three sites preliminarily named X, Y, and Z. The identity of these presumably activating transcription factors has yet to be established. CBF1 site protection patterns of Cp were remarkable because they were not identical in all cells, but correlated with promoter activity. Even in Mutu I cells, where the CBF1 binding sites are hypo- or unmethylated, there is the protection pattern of inactive Cp. This pattern is different from the typical CBF1 binding pattern, as demonstrated by methylation interference analysis (56). The difference is not due to the lack of CBF1 binding activity in Mutu I (Fig. 6).

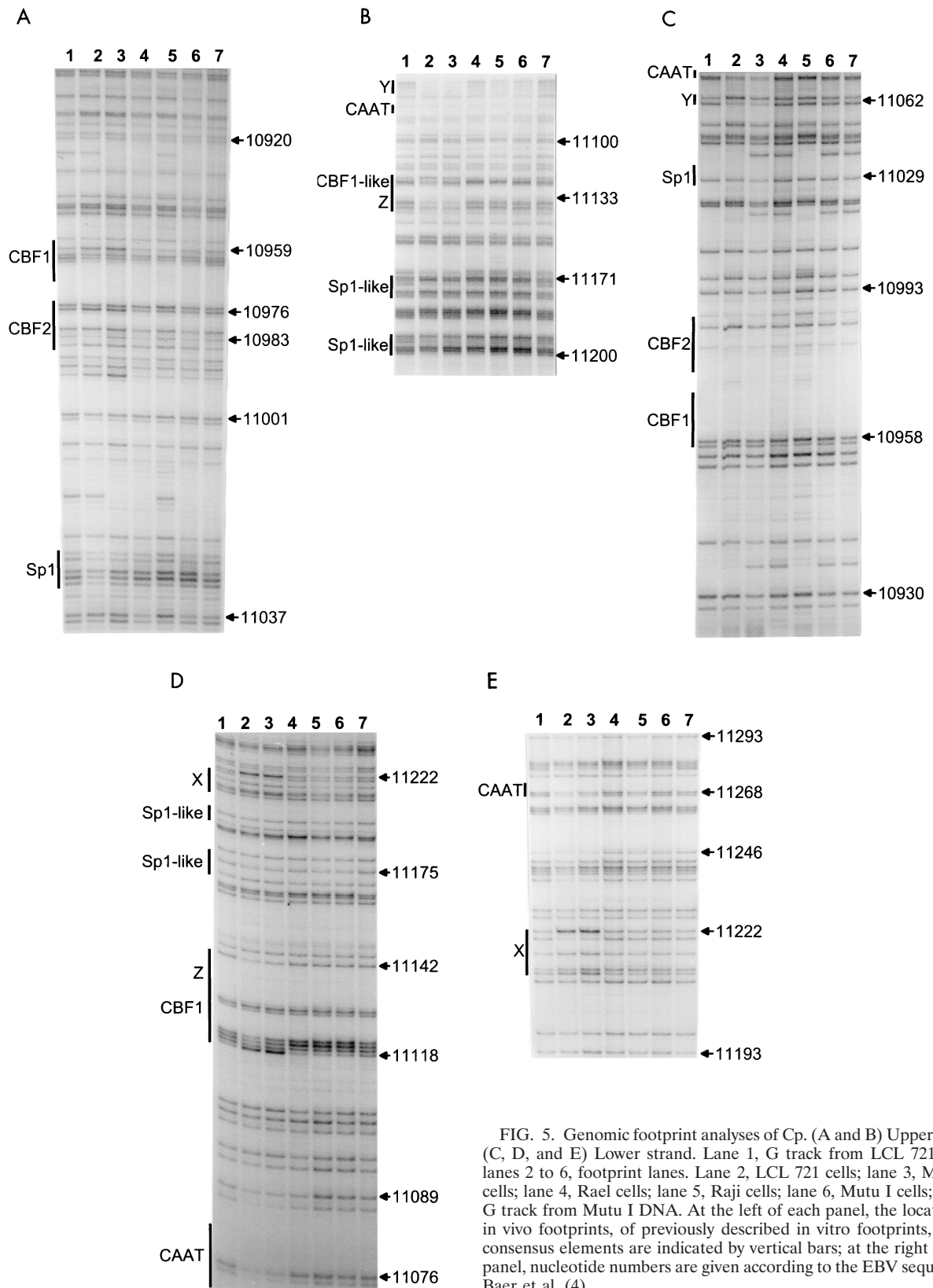


FIG. 5. Genomic footprint analyses of Cp. (A and B) Upper strand. (C, D, and E) Lower strand. Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprint lanes. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Mutu I DNA. At the left of each panel, the locations of *in vivo* footprints, of previously described *in vitro* footprints, and of consensus elements are indicated by vertical bars; at the right of each panel, nucleotide numbers are given according to the EBV sequence of Baer et al. (4).

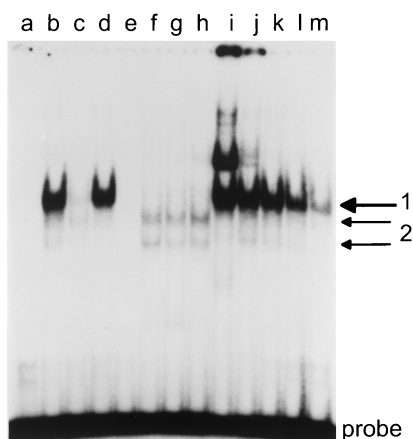


FIG. 6. Electrophoretic mobility shift assay: binding of nuclear proteins from Mutu I cells to the CBF1 site and to a mutant control oligonucleotide. Labeled double-stranded oligonucleotides containing a CBF1 consensus site and a mutant site disabled for CBF1 binding were incubated with 1 μ g of crude nuclear extract from Mutu I cells with the oligonucleotides, amounts of poly(dI-dC), and unlabeled competitor oligonucleotides as indicated. The resulting protein-DNA complexes were separated in a 4% polyacrylamide gel. Lanes a to h and k, 1 μ g of poly(dI-dC) added; lanes a to d and i to m, labeled CBF1 oligonucleotide as a probe; lanes e to h mutant oligonucleotide labeled as a probe. Lane 2, no protein added; lane b, nuclear extract; lane c, shift competed with a 50-fold excess of unlabeled CBF1 oligonucleotide; lane d, competition with a 50-fold excess of unlabeled mutant oligonucleotide; lane e, no protein added; lane f, nuclear extract; lane g, competition with a 50-fold excess of unlabeled CBF1 oligonucleotide; lane h, competition with a 50-fold excess of unlabeled mutant oligonucleotide; lane i, 0.1 μ g of poly(dI-dC) added; lane j, 0.5 μ g of poly(dI-dC); lane k, 2 μ g of poly(dI-dC); lane m, 5 μ g of poly(dI-dC) added.

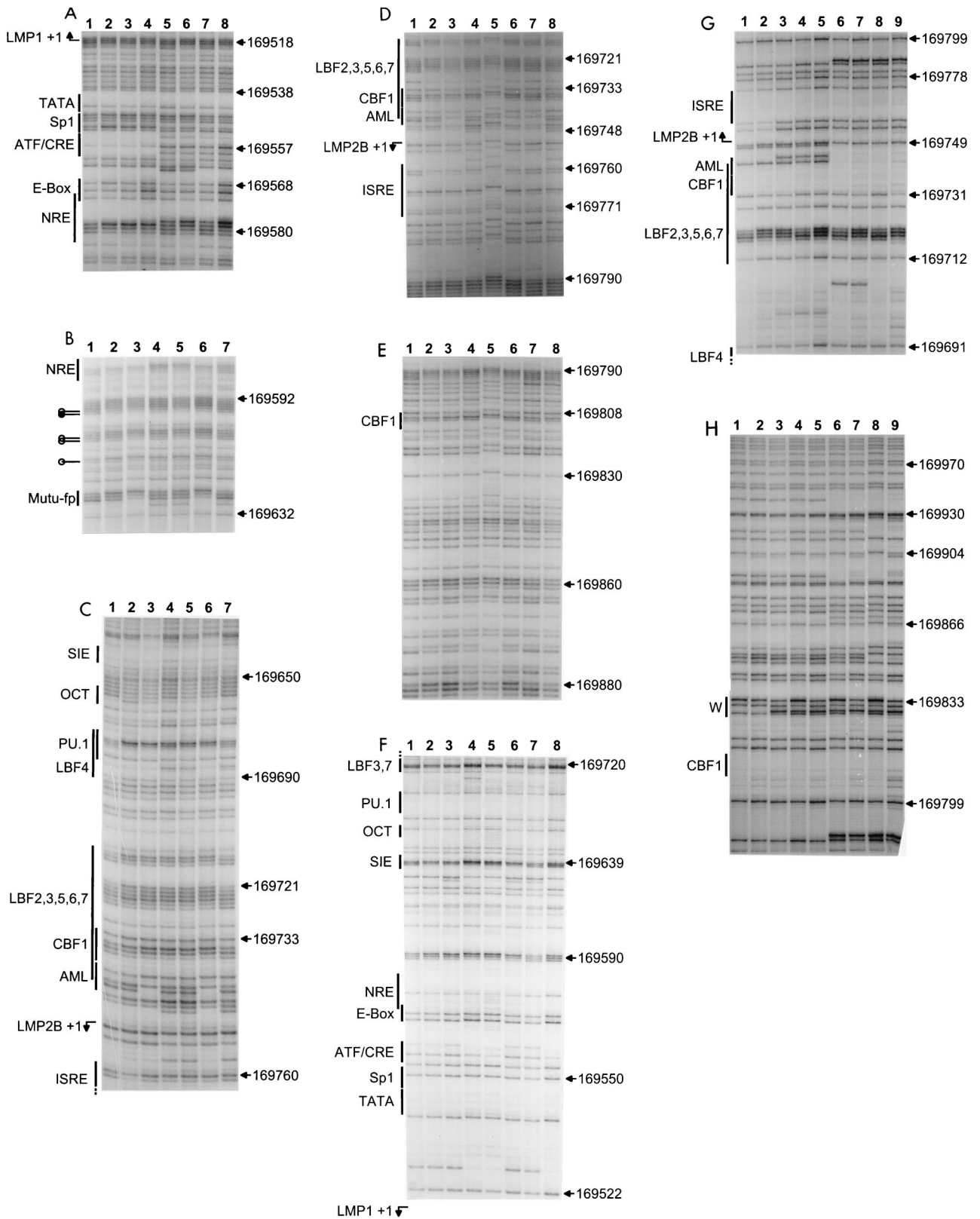
Previously published models (73, 109) supposed that the repressive factor CBF1 is constitutively bound to its binding sites independent of the CpG methylation status of the binding site. In that model, promoter activation occurs when activating transcription factor EBNA2 binds to already promoter-bound repressor CBF1, thereby covering the transcriptionally repressive domain of CBF1 (34). Therefore, the nontypical CBF1 site pattern of inactive Cp may be due to the activity of an additional negative regulator, like KyoT2 (92), or the typical pattern (56) was caused by CBF1 and additional protein. Alternatively, we might assume that CBF1 is not at all bound at the inactive Cp. The major differences in protein binding and

methylation may be a hint for a restructuring between the active and inactive Cp (17).

LMP1p is regulated by methylation. LMP1p has recently been examined in the five cell lines by Takacs et al. (submitted). LMP1p was hypo- or unmethylated in the active promoter state, but methylated in the inactive state. The detailed methylation maps (Fig. 3) were adapted from Takacs et al. (91a). Falk et al. (21) described comparable levels of methylation for both Mutu I and Mutu III cells. Our results for Mutu, however, were quite different. We found zero methylation in Mutu III and a high overall methylation in Mutu I cells. However, methylation in Mutu I was medium or less from nucleotides 169425 to 169465, at 169567 and 169569, and from 169691 to 169742. The discrepancy between the data of Falk et al. (21) and our data may be due to the use of a different clone of Mutu I and a different passage of clone BLIII-C199 for Mutu III. However, our methylation data on LMP1p fit to the promoter activities in the subclones of Mutu. In the previous literature on LMP1p, many binding sites have been characterized (19, 20, 38, 47, 48, 51, 56, 85, 87, 88, 97). Almost all previously characterized *in vitro* binding sites also carry signs of protein binding *in vivo*. However, this *in vivo* protection is visible regardless of LMP1p activity (Fig. 3 and 7). Therefore, since promoter protection patterns are identical in all cell types, the CpG methylation status seems to be the major determinant of promoter activity for LMP1p. Promoter activation is likely to be regulated by CpG demethylation and by alterations at the protein level that are transparent to genomic footprinting. Alternatively, there might be differences in promoter binding at other relevant promoter areas that we did not locate. Another possibility is that binding differences, especially at the ATF/CRE-Sp1 locus, are invisible to *in vivo* footprinting by DMS alone. These differences were very weak at best and were difficult to evaluate because of several sequence polymorphisms in this part of LMP1p. *In vivo* differences might be seen with the use of additional reagents for footprinting. We conclude that binding of all the factors involved in promoter activation at their respective binding sites is not sufficient to activate or repress LMP1p *in vivo*.

In summary, the contributions of CpG methylation and protein binding to promoter activity are in each case different for the three EBV latency-associated promoters Qp, Cp, and LMP1p. It would come as no surprise if the promoters for TP1, W, and the EBER RNAs also presented different pictures.

FIG. 7. Genomic footprint analyses of LMP1p. (A, B, C, D, and E) Upper strand. (F, G, and H) Lower strand. (A) Lane 1, G track from LCL 721 DNA; lane 2, footprint from LCL 721 cells; lane 3, footprint from Mutu III cells; lane 4, footprint from Mutu I cells; lane 5, G track from Rael DNA; lane 6, footprint from Rael cells; lane 7, G track from Raji DNA; lane 8, footprint from Raji cells. (B and C) Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprints. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Raji cells. (D and E) Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprints. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Mutu I cells; lane 8, G track from Rael cells. (F) Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprints. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Mutu I DNA; lane 8, G track from Raji DNA. (G and H) Lane 1, G track from LCL 721 DNA; lane 2, footprint from LCL 721 cells; lane 3, footprint from Mutu III cells; lane 4, G track from Mutu I DNA; lane 5, footprint from Mutu I cells; lane 6, G track from Rael DNA; lane 7, footprint from Rael cells; lane 8, G track from Raji DNA; lane 9, footprint from Raji cells. At the left of each panel, the locations of *in vivo* footprints, of previously described *in vitro* footprints, and of consensus elements are indicated by vertical bars, and at the right of each panel, nucleotide numbers are given according to the EBV sequence of Baer et al. (4).



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