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Transcriptional Activation of the Epstein-Barr Virus Latency C Promoter after 5-Azacytidine Treatment: Evidence that Demethylation at a Single CpG Site Is Crucial

KEITH D. ROBERTSON,[1] S. DIANE HAYWARD,[1] PAUL D. LING,[1] DVORIT SAMID,[2] AND RICHARD F. AMBINDER[1]*

Departments of Oncology and Pharmacology, Johns Hopkins School of Medicine, Baltimore,[1] and Pharmacology Laboratory, National Institutes of Health, Bethesda,[2] Maryland

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The Epstein-Barr Virus (EBV) latency C promoter (C_P) is the origin of transcripts for six viral proteins. The promoter is active in lymphoblastoid B-cell lines but silent in many EBV-associated tumors and tumor cell lines. In these latter cell lines, the viral episome is hypermethylated in the vicinity of this promoter. We show that in such a cell line (Rael, a Burkitt's lymphoma line), 5-azacytidine inhibits DNA methyltransferase, brings about demethylation of EBV genomes, activates C_P transcription, and induces the expression of EBNA-2. Investigation of the phenomenon demonstrates the importance of the methylation status of a particular CpG site for the regulation of the C_P: (i) genomic sequencing shows that this site is methylated when the C_P is inactive and is not methylated when the promoter is active; (ii) methylation or transition mutation at this site abolishes complex formation with a cellular binding activity (CBF2) as determined by electrophoretic mobility shift analyses, competition binding analyses, and DNase I footprinting; and (iii) a single C→T transition mutation at this site is associated with a marked reduction (>50-fold) of transcriptional activity in a reporter plasmid. Thus, the CBF2 binding activity is shown to be methylation sensitive and crucial to EBNA-2-mediated activation of the C_P.

Seventy percent of all CpG's are methylated at the 5 position of cytosine in mammalian cells. In vivo, levels of methylation are generally inversely related to levels of transcriptional activity. In vitro, methylation of plasmid DNA prior to introduction into tissue culture cells generally inhibits reporter gene expression. These observations suggest that methylation plays an important role in the regulation of gene expression (27).

The regulation of latency promoters on the Epstein-Barr virus (EBV) episome in B lymphocytes is an attractive system to use for studying the mechanisms by which methylation might inhibit transcription. In contrast to cellular genes, for which potential *cis*-acting sequences are incompletely characterized, the sequence of the entire EBV genome is known (1). Furthermore, by virtue of its episomal nature, gene regulation is expected to be independent of the larger chromosomal context. In EBV-associated Burkitt's lymphoma, a B-cell malignancy, the viral genome which encodes more than 80 proteins is largely silent and only a single viral protein is expressed. In contrast, in EBV-immortalized B lymphoblastoid cell lines, nine viral proteins are expressed (25). Six of these are expressed from the latency promoter in the *Bam*HI-C fragment of the viral genome, referred to as C_P. In tumor cell lines that preserve the very restricted pattern of viral antigen expression characteristic of the primary tumor, the viral episome is hypermethylated in the vicinity of C_P and elsewhere (19, 20). In contrast, in lymphoblastoid cell lines, the viral genome is hypomethylated. Treatment of a Burkitt's lymphoma cell line with 5-azacytidine, an irreversible inhibitor of DNA methyltransferase, has been reported to lead to demethylation of the viral genome and transcriptional activation of the latency promoters (17).

* Corresponding author. Mailing address: Johns Hopkins Oncology Center, 418 N. Bond St., Baltimore, MD 21231. Phone: (410) 955-5617. Fax: (410) 550-6802.

In the present investigation, we set out to characterize the mechanism by which 5-azacytidine leads to upregulation of the EBV C_P. Our findings suggest that demethylation is an important event in this process and that methylation of a particular CpG site in the C_P inhibits the formation of a sequence-specific binding complex important for transcription.

MATERIALS AND METHODS

Cell culture, reagents, and RT-PCR amplification. Cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1 mM L-glutamine. Stock 10 mM solutions of 5-azacytidine and 6-azacytidine (Sigma, St. Louis, Mo.) were freshly prepared in deionized water and filter sterilized immediately before use. They were diluted to a final concentration of 5 μm in the experiments described below. Reverse transcription (RT)-PCR amplification was performed with rTth polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), according to the manufacturer's instructions. Primers used were as described by Qu and Rowe (22). The RT reaction was carried out at 60°C for 15 min. Amplification conditions were as follows: initial denaturation at 95°C for 1.5 min and 35 cycles each at 95°C for 30 s, at 60°C for 60 s, and at 70°C for 60 s.

DNA methyltransferase assay. The levels of DNA methyltransferase were assayed as described by Issa et al. (10). Briefly, cells were diluted to 3×10^5/ml, allowed to grow overnight, washed, and resuspended in 500 μl of lysis buffer (50 mM Tris [pH 7.8], 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1% sodium azide, 6 mg of phenylmethylsulfonyl fluoride per ml, 10% glycerol, 1% Tween 80). The suspension was then passed through a 22-gauge needle twice and subjected to freeze-thawing at −70 and 37°C, respectively, three times, and the protein concentration was determined. For the DNA methyltransferase assay, 5 μg of cellular protein was mixed with 0.5 μg of poly (dI-dC) and 3 μCi of *S*-adenosyl-L-[methyl-^{3}H]methionine (Amersham) in a total volume of 20 μl for 2 h at 37°C. Three hundred microliters of stop solution (1.0% sodium dodecyl sulfate [SDS], 2.0 mM EDTA, 3% 4-aminosalicylate, 5% butanol, 0.25 mg of calf thymus DNA per ml, 1.0 mg of proteinase K per ml) was added, and the mixture was incubated at 37°C for 30 min. This solution was then extracted once with phenol-chloroform and ethanol precipitated. The pellet was resuspended in 40 μl of 0.3 N NaOH and incubated at 37°C for 45 min. This solution was then spotted onto a Whatman GF/C filter, dried at 80°C for 5 min, and washed with 5% trichloroacetic acid and 70% ethanol, and then the radioactivity was determined.

Genomic sequencing. For genomic sequencing, the bisulfite modification method described by Frommer et al. was used (5). Briefly, 10 μg of genomic DNA was digested with *Eco*RI, alkali denatured, neutralized, and precipitated.

TABLE 1. Sequences of oligonucleotides and primers used in this study

Oligonucleotide or primer	Sequence
Competitor oligonucleotides	
CBF2	
Top strand	5′-GATCTAAAAATTTATGGTTCAGTGCGTCGAGTGCTG-3′
Bottom strand	5′-GATCCAGCACTCGACGCACTGAACCATAAATTTTTA-3′
Double-methyl competitor	
Top strand	5′-GATCTAAAAATTTATGGTTCAGTGC^{ME}GTC^{ME}GAGTGCTG-3′
Bottom strand	5′-GATCCAGCACTC^{ME}GAC^{ME}GCACTGAACCATAAATTTTTA-3′
Single-methyl competitor	
Top strand (CpG site 1)	5′-GATCTAAAAATTTATGGTTCAGTGC^{ME}GTCGAGTGCTG-3′
Bottom strand (CpG site 1)	5′-GATCCAGCACTCGAC^{ME}GCACTGAACCATAAATTTTTA-3′
Top strand (CpG site 2)	5′-GATCTAAAAATTTATGGTTCAGTGCGTC^{ME}GAGTGCTG-3′
Bottom strand (CpG site 2)	5′-GATCCAGCACTC^{ME}GACGCACTGAACCATAAATTTTTA-3′
Sequencing primers for:	
Bisulfite-treated DNA	
Upstream	5′-CGTAGGATCCCTTAAACTCTCTTATTAACTATA-3′ (*Bam*HI)
Downstream	5′-TCGAAGATCTAATGTGTTTTAATTAGAAATTT-3′ (*Bgl*II)
Untreated DNA	
Upstream	5′-TCGAAGATCTAATGTGTCCCAATTAGAAACCC-3′ (*Bgl*II)
Downstream	5′-AGCTGGATCCTTGAGCTCTCTTATTGGCTATA-3′ (*Bam*HI)

The denatured DNA was then incubated in a total volume of 1.2 ml of a solution of 3.1 M sodium bisulfite–0.5 mM hydroquinone (pH 5.0) at 50°C for 16 h under a layer of mineral oil. This solution was then dialyzed against two 1-liter changes of (i) 5 mM sodium acetate–0.5 mM hydroquinone (pH 5.2), (ii) 0.5 mM sodium acetate (pH 5.2), and (iii) deionized water, all at 4°C. The dialyzed solution was dried under vacuum and resuspended in 100 μl of 10 mM Tris–1 mM EDTA (TE) (pH 8.0), and NaOH was added to a final concentration of 0.3 M for 10 min at room temperature. Ammonium acetate (pH 7.0) was added to a final concentration of 3.0 M, and the DNA was precipitated and resuspended in 100 μl of TE (pH 8.0). This solution (2 μl) was used for PCR with primers specific for the top strand (Table 1). The conditions used were initial denaturation at 95°C for 1.5 min and 35 cycles each at 95°C for 30 s, 58°C for 60 s, and at 70°C for 60 s. The reaction mixtures were phenol extracted, precipitated, and digested with *Bam*HI and *Bgl*II, whose sites were incorporated at the end of the PCR primers. These digests were then gel purified and cloned into the polylinker of pGH56, a pUC derivative. The sequence of the region was also determined without bisulfite modification by using the same PCR procedure followed by cloning. The region of EBV amplified with these sets of PCR primers for genomic sequencing is present within the *Bam*HI C fragment at B95-8 nucleotides 10839 to 11091 (253 bp) and contains the CBF1- and CBF2-binding sites of the C promoter.

Plasmids. Plasmid pPDL63 contains sequences from −330 to −430 relative to the Cp start site with the *Bgl*II (−430) and *Bam*HI (−330) linkers added during PCR and cloned into the pUC-based vector pGH56 with these sites (16) (Fig. 1). We refer to this 100-bp region as the Cp EBNA-2 response element. Plasmid pPDL84A contains the Cp EBNA-2 response element, multimerized eight times in the chloramphenicol acetyltransferase (CAT) expression plasmid, pGH262, which contains an E1b TATA box minimal promoter. The plasmid referred to as small C_P-CAT contains EBV nucleotides 10312 to 11336 cloned into the pCAT-Basic vector (Stratagene) and was referred to previously as pPDL5 (16) (Fig. 1). The plasmid large C_P-CAT contains EBV nucleotides 7448 to 11337 cloned into a pSV0CAT-derived vector and was a gift from Cliona Rooney (Fig. 1) (24). The single and double point mutations at the CpG site within the CBF2 binding site (−348) were created by synthesizing overlapping 63-mers from EBV nucleotides −330 to −386 and −373 to −430, which contained CpG to TpA or TpG mutations. One primer contained a *Bgl*II site (−373 to −430), while the complementary strand contained a *Bam*HI site (−330 to −386). These oligonucleotides were annealed and extended with Klenow DNA polymerase under standard conditions, cleaved with *Bgl*II and *Bam*HI, and cloned into pGH56 to create pKR1-8 and pKR22-5. The sequence of the mutants was confirmed by dideoxy sequencing. The mutant Cp EBNA-2 response elements were then multimerized eight times and cloned into pGH262 to create pKR4B and pKR46-1. The EBNA-2 expression vector, pPDL66A, has been previously described (16).

EMSA. Wild-type or mutant 100-bp fragments were used as probes. The fragments were cut from their respective plasmids with *Bgl*II and *Bam*HI, gel purified, and labeled with Klenow DNA polymerase by standard methods. For each electrophoretic mobility shift assay (EMSA) reaction, 20,000 cpm of probe was added, with 2 μg of poly (dI-dC), 2 μl of 10× shift-up buffer (10 mM HEPES [*N*-2-hydroxylethylpiperazine-*N*′-2-ethanesulfonic acid; [pH 7.5], 50 mM KCl, 1.0 mM EDTA, 0.1 mM dithiothreitol, 0.1% Triton X-100, 5% glycerol), and 1 μl of nuclear extract (prepared from CA46 cells as described previously [16]) in a total volume of 20 μl. This reaction mixture was incubated at room temperature for 30 min and then loaded onto a 4.5% nondenaturing acrylamide gel run in HEE buffer {10 mM HEPES (pH 7.5), 1.0 mM EDTA, 0.5 mM EGTA [ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid]} at 200 V. For competition experiments, the volume of water was adjusted to account for the cold competitor oligonucleotide, which was added to the reaction mixture before the labeled probe. Gel shift competitor oligonucleotides were synthesized at the Johns Hopkins University DNA Synthesis facility and annealed. Methylated oligonucleotides were synthesized with 5-methylcytosine. Competitor oligonucleotides for the CBF2 binding site are shown in Table 1. The amounts of the competitors in each EMSA ranged from 0.1 to 100 ng.

Transfections. Generally, either 3 or 5 μg of CAT plasmid was cotransfected with an equimolar amount of EBNA-2 expression vector (pPDL66A) or its parent plasmid pSG5 (Stratagene) by the DEAE dextran method (26). After 48 h, cell extracts were prepared by standard methods and used to determine CAT expression levels. All quantitation of EMSA and CAT assays was performed on a Molecular Dynamics PhosphoImager system.

In vitro DNA methylation. Bacterial CpG methylase (M.*Sss*I), *Hpa*II methylase, and *Hha*I methylases were used according to the manufacturer's instructions (New England Biolabs, Beverly, Mass.). Reaction products were phenol extracted and precipitated, and the completeness of the reaction was tested by digesting an aliquot with an appropriate methylation-sensitive restriction enzyme (*Hpa*II or *Hha*I) and running the digest on an agarose gel. Complete protection from digestion indicated that the methylation reaction went to completion. After the methylation reaction, plasmids were extracted with phenol-chloroform and ethanol precipitated. These plasmids were then used for transfections. For EMSA the methylated plasmids were digested with *Bgl*II and *Bam*HI, and the 100-bp fragment was gel purified before being labelled.

DNase I footprinting. After Klenow fill-in labeling at the *Bgl*II site followed by *Pvu*II cleavage, 25,000 cpm of the gel-purified probe was added to a mixture containing 1 μg of poly (dI-dC), 25 μl of binding buffer (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 12.5 mM $MgCl_2$, 1.0 mM EDTA, 20% glycerol, 1.0 mM dithiothreitol) and 10 μl of nuclear extract in a total volume of 50 μl. This mixture was incubated on ice for 10 min, 50 μl of Ca^{2+}-Mg^{2+} solution (5.0 mM $CaCl_2$, 10 mM $MgCl_2$) was added, and the mixture was allowed to incubate at room temperature for 1 min. DNase I (0.01 U) was then added and gently mixed. After 2 min, 90 μl of the stop solution (200 mM NaCl, 30 mM EDTA, 1.0% SDS, 100 μg of yeast tRNA per ml) was added. After phenol-chloroform extraction (equilibrated in 50 mM Tris-HCl [pH 8.0], 1.0 mM EDTA, 15 mM β-mercaptoethanol) and ethanol precipitation, the samples were washed once with 70% ethanol, dried, resuspended in loading dye, and electrophoresed on a 6% denaturing polyacrylamide sequencing gel. Samples were visualized by autoradiography.

RESULTS

5-Azacytidine brings about demethylation of EBV genomes in a Burkitt's lymphoma cell line in the vicinity of C_P and activates transcription from this promoter. Following treat-

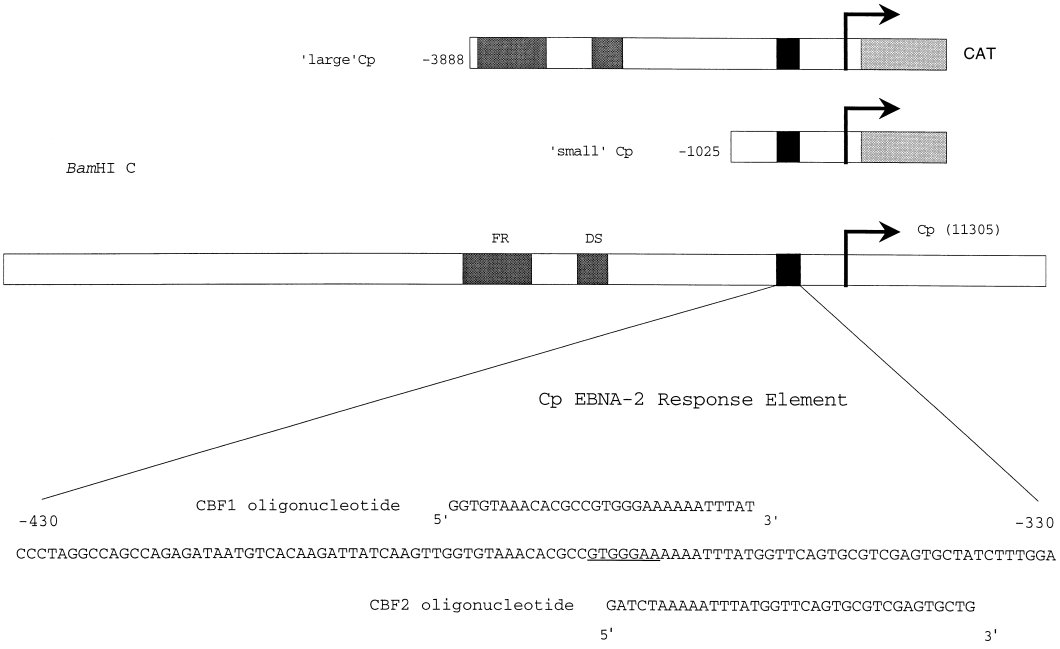


FIG. 1. Schematic drawing of EBV genomic region containing C_P and upstream sequences. All numbering in this diagram is relative to the C_P start site (base 11305 in the B95-8 EBV genome), which is denoted by a bent arrow. The family of repeats (FR) and the region of dyad symmetry (DS) which constitute the origin of plasmid replication are indicated by stippled boxes. The boundaries of the large and small C_P regions and of the EBNA-2 response element included in various reporter plasmids are indicated. The sequences of the EBNA-2 response element and the competitor oligonucleotides for CBF1 and CBF2 binding are shown, and the core binding motif for CBF1 is underlined.

ment of the Rael cell line, derived from an EBV Burkitt's lymphoma, with 5 μM 5-azacytidine, DNA methyltransferase activity fell to an undetectable level (Fig. 2). This was accompanied by demethylation of adjacent *Hpa*II sites, as determined by Southern blot hybridization (data not shown); the appearance of transcripts from C_P, as detected by reverse PCR (Fig. 3), and the appearance of EBNA-2, as detected by immunohistochemistry (data not shown). In contrast, treatment with a control reagent, 6-azacytidine, known not to inhibit DNA methyltransferase activity, had no effect on the methylation status, transcription from C_P, or antigen expression (Fig. 2 and 3).

Impact of methylation on transcription. Next, we sought to determine whether demethylation of the C_P in treated cells might be an epiphenomenon that correlated with transcriptional activity or whether demethylation might play a direct role in activating transcription. We investigated the effects of in vitro methylation with CpG methylase on expression from three reporter plasmids transfected into untreated Rael cells which do not express EBNA-2 (Fig. 1 and 4). Two recombinant plasmids contained the C_P and approximately 4,000 bp (large C_P-CAT) or 1,000 bp (small C_P-CAT) of the upstream sequence. The third recombinant plasmid contained eight tandem copies of the 100-bp EBNA-2-responsive region from C_P driving an E1b promoter-CAT (8× C_P E2RE-CAT). The large

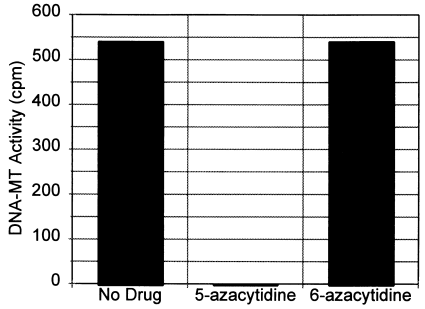


FIG. 2. Inhibition of DNA methyltransferase (DNA-MT) activity in a Burkitt's lymphoma cell line (Rael) treated with various agents. The DNA methyltransferase activity in 5 μg of whole cell extract was determined as the incorporation of C^3H_3 into the poly (dI-dC) substrate. The averages of two separate measurements are shown. Cells were untreated (no drug) or treated with 5-azacytidine (5 μm) or 6-azacytidine (5 μm).

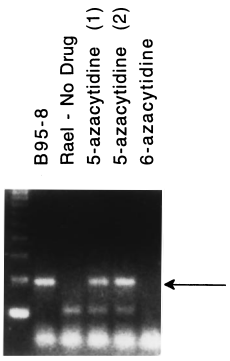


FIG. 3. Reverse PCR demonstrates transcriptional activation of the C_P by inhibitors of DNA methyltransferase. The Rael cell line was not treated (no drug) or was treated with 5-azacytidine (5 μm) or 6-azacytidine (5 μm). The treatment with 5-azacytidine was repeated, i.e., two separate experiments (labeled 1 and 2). After 72 h, RNA was extracted, and reverse PCR amplification was performed with primers designed to amplify any member of the family of appropriately spliced C_P transcripts (22). The products of reverse PCR were electrophoresed in agarose. B95-8, an EBV lymphoblastoid cell line with the C_P constitutively active, served as a positive control. An arrow indicates the amplified band corresponding to the C_P in the ethidium bromide-stained gel. A 123-bp DNA ladder (Bethesda Research Laboratories, Gaithersburg, Md.) is shown in the first lane.

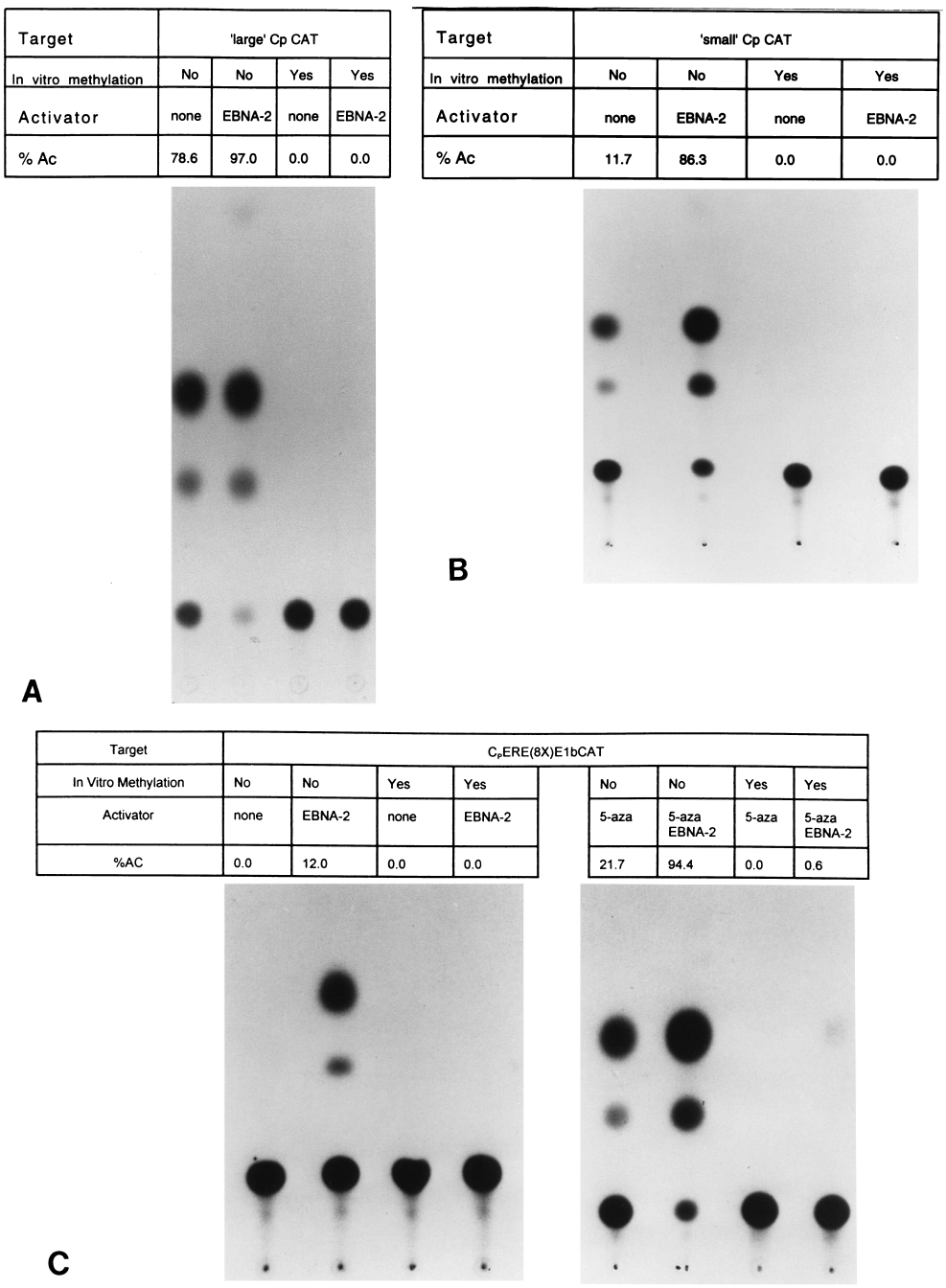


FIG. 4. Expression from CAT reporter plasmids containing elements of C_P is methylation sensitive. Basal and EBNA-2 induced activity associated with transfection of the large (A) and small (B) C_P-CAT reporter plasmids into Rael cells are abolished by in vitro methylation with CpG methylase. (C) EBNA-2-induced activity associated with transfection of the 8× C_P E2RE-CAT reporter plasmid into Rael cells is also abolished by in vitro methylation with CpG methylase. In addition, transcriptional activation associated with 5-azacytidine pretreatment is abolished by in vitro methylation. The methylation status of the target plasmid, the activator (EBNA-2 expression vector, 5-azacytidine [5-aza], or both), and the percent conversion of [^{14}C]chloramphenicol (% Ac) is indicated above the lanes.

C_P-CAT showed strong basal activity, probably attributable to the presence of the origin of latency replication with its tandem EBNA-1 binding sites (23) but minimal increase in activity with EBNA-2 cotransfection (Fig. 4A). The small C_P-CAT and 8× C_P E2RE-CAT showed little and no basal activity, respectively, but both showed strong activity with cotransfection of the EBNA-2 expression vector (Fig. 4B and C). In vitro CpG methylation abolished CAT activity of all three plasmids, even in the setting of EBNA-2 cotransfection. CpG methylation similarly inhibited transcription of plasmids transfected into the B95-8 and Raji cell lines, two EBV lymphoblastoid cell lines (Fig. 5).

5-Azacytidine treatment does not activate a transcriptional pathway that can overcome methylation. One possible explanation for the activation of the C_P following 5-azacytidine treatment was that 5-azacytidine might lead to the expression of cellular proteins that would lead to transcription of C_P without regard to the methylation status of C_P. In order to be

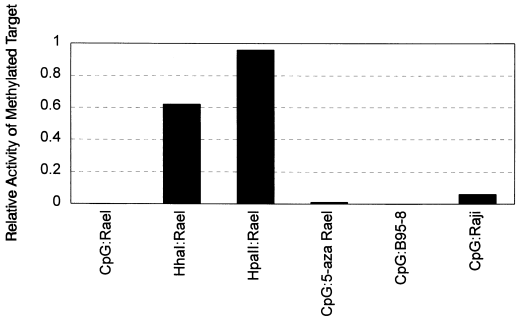


FIG. 5. CpG methylase, but not *Hha*I or *Hpa*II methylase, abolishes EBNA-2-activated transcription in a variety of cell types. In each experiment, the target plasmid was transfected with or without in vitro treatment with a bacterial methylase. Cell lines were transfected with the 8× C_P E2RE-CAT target. RaeI cells were cotransfected with an EBNA-2 expression vector. The bacterial methylase and target cell line are indicated on the *x* axis (bacterial methylase:target cell line). The ratios of the percentages of [^{14}C]chloramphenicol conversion (methylase treated/untreated) are shown on the *y* axis.

sure that demethylation of elements in the target promoter was important in the activation of the C_P in 5-azacytidine-treated cells, we transfected the 8× C_P E2RE-CAT plasmid into 5-azacytidine-treated RaeI cells without the EBNA-2 expression vector. Strong CAT activity resulted (Fig. 4C). In contrast, even in 5-azacytidine-treated cells there was no transcription of the 8× C_P E2RE-CAT plasmid treated with CpG methylase although in cells cotransfected with EBNA-2 after treatment with 5-azacytidine, a trace of CAT activity was detected.

The importance of particular CpG sites within the EBNA-2 response element is suggested by the use of other methylases and by genomic sequencing. The effects of two other methylases on the target plasmid were also assessed. In contrast to the complete inhibition of EBNA-2-activated transcription resulting from treatment with CpG methylase, *Hpa*II methylase had virtually no effect and *Hha*I methylase had a modest effect (Fig. 5). These last two methylases have no recognition sites in the 100-bp C_P EBNA-2 response element, whereas there are four recognition sites for the CpG methylase. To determine whether the methylation status of one or more of these particular CpG sites did indeed change with 5-azacytidine treatment, we performed genomic sequencing. DNA extracted from RaeI cells with and without 5-azacytidine treatment and from the B95-8 lymphoblastoid cell line was reacted with bisulfite under conditions in which cytosine is converted to uracil but 5-methylcytosine is nonreactive. The top strand of the reacted DNA was amplified with strand-specific PCR primers and cloned (5). As shown in Fig. 6, dideoxy sequencing of three clones derived from untreated RaeI cells indicated that all CpG sites in the 100-bp EBNA-2-responsive region were methylated. This is shown by the presence of a band in the lane labeled "C" where a CpG is found in the sequence. This result

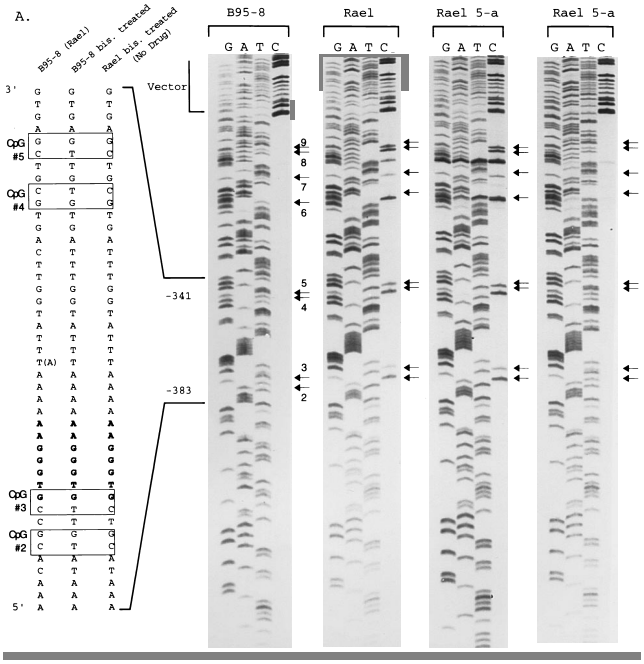
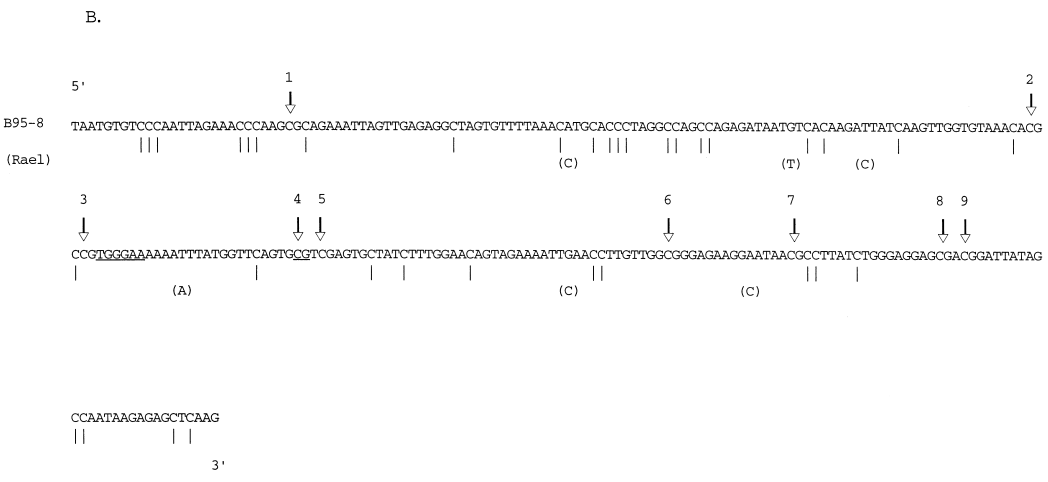


FIG. 6. Strand-specific genomic sequences from the C_P region. (A) Autoradiograph showing sequences of representative clones from the indicated cell lines. Representative sequences from B95-8 and the untreated RaeI cell line are shown. The patterns of methylation were identical clone to clone. Two clones from the 5-azacytidine-treated RaeI cells (RaeI 5-a) are shown. One shows complete CpG methylation, and one shows an absence of CpG methylation. No clones with mixed patterns of CpG methylation were identified. Dark arrows indicate the location of CpG dinucleotides within the C_P sequence (only 2 to 5 shown). The vector-insert boundary and relevant portion of the EBNA-2-responsive region are shown. CpG numbers correspond to those in panel B. bis., bisulfite. (B) Summary of genomic sequence data. Arrows above the sequence indicate cytosine residues 1 to 9 not converted to thymine by bisulfite treatment in RaeI cells and therefore identified as methylcytosines. Vertical lines below the sequence indicate cytosine residues converted to thymine and therefore identified as unmethylated. Bases in parentheses below the B95-8 sequence indicate sites where the RaeI sequence deviates.



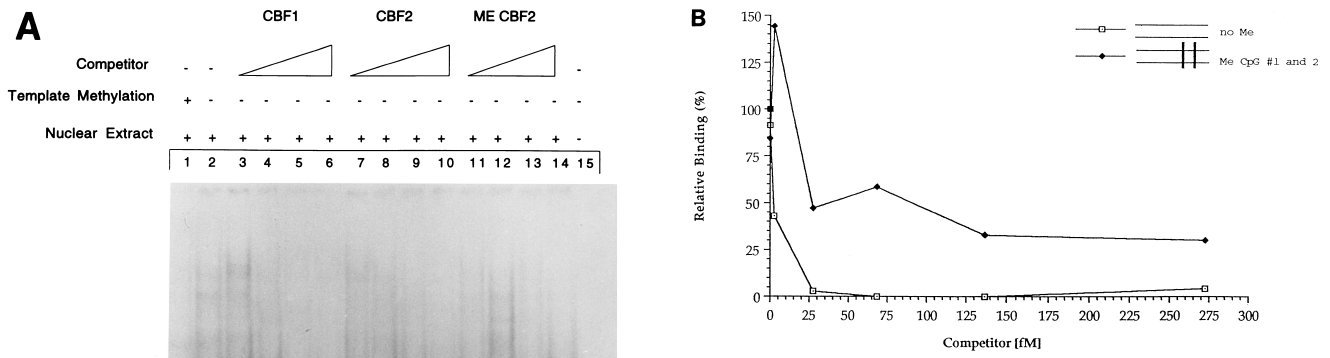


FIG. 7. CBF2 binding activity is sensitive to methylation of DNA. (A) Methylated and unmethylated 100-bp DNA probes (−430 to −330) containing the EBNA-2-responsive region from the C_P were bound to a nuclear protein extract and analyzed by EMSA. The methylated DNA probe yielded only a CBF1 complex (lane 1), while the unmethylated probe yielded CBF1 and CBF2 complexes (lane 2). The complexes were identified by their disappearance when increasing amounts of unlabeled competitor oligonucleotides were included in the binding reaction. CBF1 competitor oligonucleotides were included in lanes 3 to 6, and CBF2 competitor oligonucleotides were included in lanes 7 to 10. An oligonucleotide methylated at two CpG sites within the CBF2 binding region was much less effective as a competitor (lanes 11 to 14). (B) Quantitative comparison of CBF2 binding to probe in competition assays using unmethylated and methylated synthetic oligonucleotide competitors containing the CBF2 binding region. The first five points in each titration correspond to lanes in panel A. Me, methyl.

corresponds with the results of Southern blot hybridization with a *Bam*HI-C EBV probe, indicating that nearby *Hpa*II sites are also methylated (data not shown). Sequencing of four clones derived from 5-azacytidine-treated Rael cells revealed a mixed population with these same CpG sites which were not methylated in two clones but still methylated in two others. This result corresponds with the results of Southern blot hybridization with a *Bam*HI-C EBV probe showing demethylation of nearby *Hpa*II sites. Sequencing also showed that on a given strand, demethylation in response to 5-azacytidine treatment was an all-or-none effect, i.e., each of the strands sequenced either was methylated at every CpG site or was not methylated at any site. Sequencing of three clones derived from B95-8 cells, which have a pattern of gene expression similar to that of 5-azacytidine-treated Rael cells, also showed no methylation of these CpG sites (lack of bands in the lane labeled "C"), a result that also corresponds with Southern blot hybridization data. Thus, the four CpG sites in the EBNA-2-responsive region are methylated in untreated Rael cells but demethylate in at least a major subpopulation of molecules following 5-azacytidine treatment. The genomic sequencing data and differences between Rael and B95-8 sequences are summarized in Fig. 6B.

Impact of methylation on DNA binding activities. Could the inhibitory effects of CpG methylation on transcription be mediated by direct effects on the binding of cellular proteins to

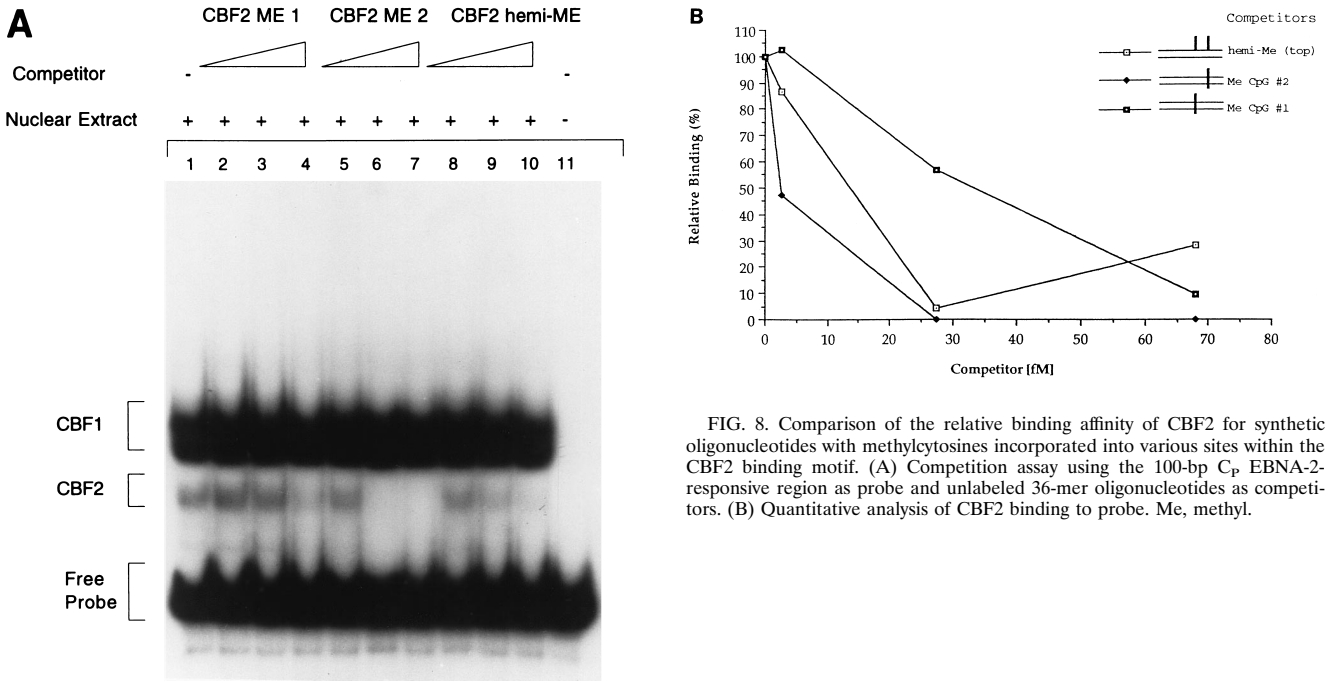


FIG. 8. Comparison of the relative binding affinity of CBF2 for synthetic oligonucleotides with methylcytosines incorporated into various sites within the CBF2 binding motif. (A) Competition assay using the 100-bp C_P EBNA-2-responsive region as probe and unlabeled 36-mer oligonucleotides as competitors. (B) Quantitative analysis of CBF2 binding to probe. Me, methyl.

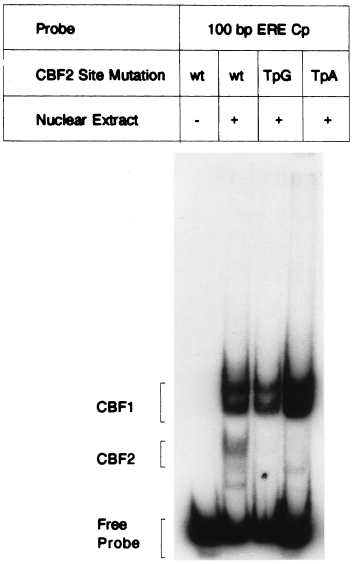


FIG. 9. Inhibition of binding by C→T transition mutations. Mutant 100-bp C_P probes incorporating either TpG or TpA substituted for CpG site 1 in the CBF2 binding region were studied by EMSA. Both mutations abolished formation of the CBF2 complex. wt, wild type.

the EBNA-2-responsive region of C_P? Two major binding activities have previously been identified in the EBNA-2 response region by an EMSA (16). One of these activities, referred to as CBF1, binds to sequences containing the motif GTGGGAA and in an EMSA is supershifted by EBNA-2 synthesized in vitro (6, 8, 15, 16, 29, 30). This binding activity corresponds to a previously identified cellular protein (4). The other binding activity, also cellular in origin, is referred to as CBF2 and is less well characterized. We used an EMSA with both the methylated and nonmethylated 100-bp EBNA-2 response region to investigate the impact of methylation on both CBF1 and CBF2 binding activities. As shown in Fig. 7, the methylated probe yields only one major binding complex (lane 1), whereas the unmethylated probe yields two major binding complexes (lane 2). The position of these binding complexes suggested that they corresponded to CBF1 and CBF2 binding activities, respectively. The identities of the complexes were confirmed by the addition of increasing amounts of previously defined unlabeled competitor oligonucleotides (16). Thus, formation of the CBF2 but not CBF1 binding complex was inhibited by methylation. Parallel EMSA experiments showed similar binding activities with the same patterns of methylation sensitivity in DG75, Rael, and Raji Burkitt's lymphoma cell lines (data not shown).

Two CpG sites are located within the CBF2 binding region as defined by the competitor oligonucleotide (Fig. 1). We assessed the effect of methylation of these CpG sites on the ability of a CBF2 competitor oligonucleotide to competitively inhibit binding to the ^{32}P-labeled 100-bp EBNA-2 response element probe. As shown in Fig. 7, lanes 11 to 14, an oligonucleotide incorporating methylcytosine at both sites within the CBF2 competitor oligonucleotide was a poor competitor in comparison with the matched unmethylated oligonucleotide. In further experiments, the effects of methylation at each of the sites separately were compared. A synthetic oligonucleotide incorporating methylcytosine at the first CpG site in the CBF2 binding region (hereafter referred to as site 1) was a weak competitor, whereas an oligonucleotide incorporating methylcytosine at the second CpG site (hereafter referred to as site 2) competed much more efficiently (Fig. 8, lanes 2 to 4 and 5 to 7). Thus, full methylation of site 1 inhibits CBF2 binding, but full methylation of site 2 has little or no effect. A hemimethylated oligonucleotide incorporating methylcytosine in the top strand of both CpG sites was intermediate in competitive strength (lanes 8 to 10), suggesting that CBF2 binding is sensitive to methylation of site 1 on either strand.

Inhibition of binding by a transition mutation. Having shown that incorporation of methylcytosine in the first CpG site within the CBF2 region resulted in a loss of affinity for CBF2, we wondered whether substitution of C with T might similarly reduce CBF2 binding since both nucleotides position a methyl group in the major groove of DNA. Substitution of the C in only the upper strand results in TpG while substitution of the C in both the upper and lower strands results in TpA. Mutant 100-bp fragments were constructed as described in Materials and Methods. In an EMSA, the mutant 100-bp fragments yielded CBF1 complexes but no CBF2 complexes (Fig. 9). A DNase I footprint assay was used to compare binding with the wild-type, methylated, and TpA-mutated 100-bp DNA probes (Fig. 10). The probes were end labeled at the −430 terminus, incubated with nuclear extract, and subjected to digestion with DNase I. A region from −343 to −380 of the C_P was protected from nuclease in the wild-type fragment, while in the methylated or mutated fragments the region from −343 to −366 (overlying the CBF2-binding region) was nuclease sensitive.

Effects of mutation on expression. These experiments established that mutation of the first CpG in the CBF2 region reduced binding affinity in vitro in a fashion parallel to that associated with methylation of this same CpG site. However, the transcriptional significance of these mutations remained to be shown. Methylation of CpG sites on an entire plasmid was associated with inhibition of transcription but whether alterations in the CBF2-binding region in particular could be implicated in this inhibition was not clear. We cloned 8× oligomers of the mutant 100-bp EBNA-2 response fragments into E1b-CAT expression plasmids. In transient transfection assays, no basal activity was detected with wild-type (CpG) or mutant (TpG and TpA) plasmids (Fig. 11). The wild type was strongly activated by cotransfection with EBNA-2, but the mutants were only minimally activated.

DISCUSSION

EBV-associated malignancies that arise in hosts that are not profoundly immunocompromised generally show a restricted pattern of viral latency antigen expression. Thus, whereas the infection of primary B cells in vitro results in the outgrowth of an EBV-transformed lymphoblastoid cell line expressing at least nine viral proteins, Burkitt's lymphoma cells generally express only a single viral protein, EBNA-1. This protein is necessary for maintenance of the viral episome. Other EBV-associated tumors including nasopharyngeal carcinoma, mixed cellularity Hodgkin's disease, and undifferentiated gastric carcinoma also show highly restricted patterns of viral antigen expression. In contrast, EBV-associated lymphomas which arise in highly immunocompromised populations commonly express the full spectrum of latency antigens expressed by lymphoblastoid cell lines. At one level, these empiric observations may be explained by selection. In healthy EBV-seropositive volunteers, a potent response to EBV latency antigens is chronically maintained and estimates of the frequency of cytotoxic T-cell precursors specific for EBV antigens run as high as 1/400. Most of the response appears to be directed against the EBNA family of antigens (EBNA-2, -3A, -3B, and -3C),

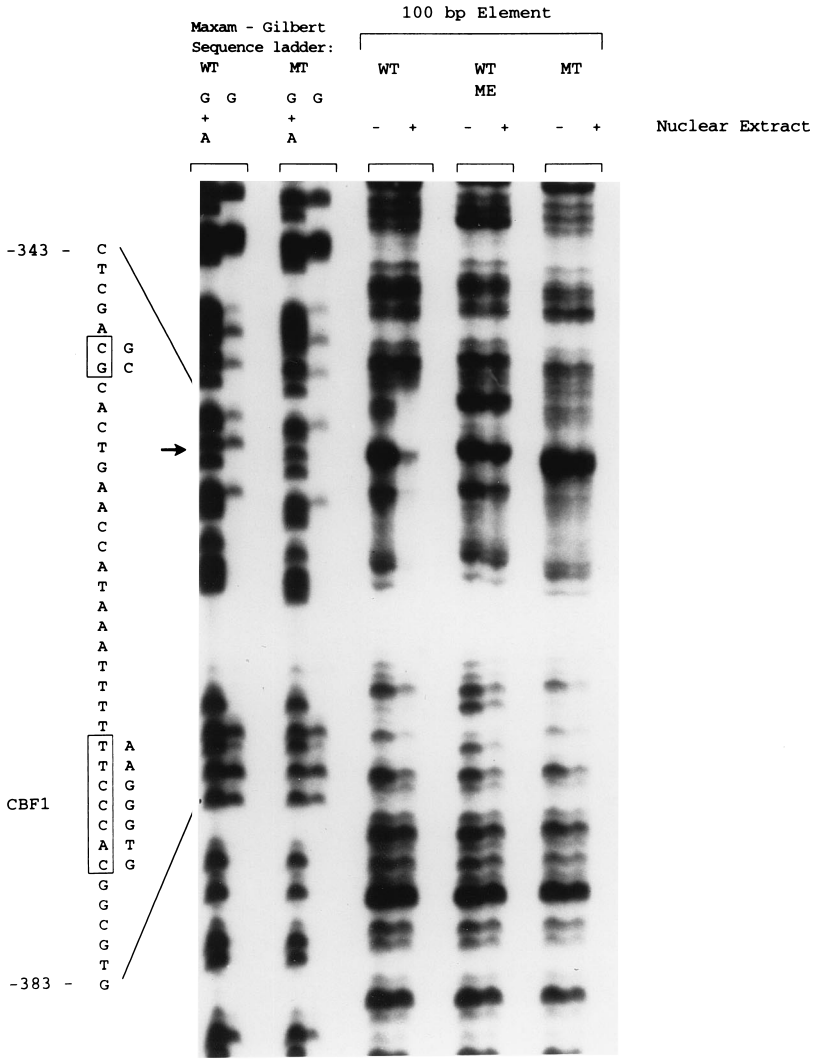


FIG. 10. DNase I footprint of unmethylated, methylated, and mutant C_P. Nuclear extract was added to end-labeled C_P probes (−330 to −430) subsequently digested with DNase I. On the left, Maxam-Gilbert sequencing of the wild-type and mutant fragments and the sequence position relative to the C_P mRNA start site are shown. On the right, DNase I digestion of the wild type (WT), CpG methylase-treated wild type (ME-WT), and TpA mutant (MT) probe in the absence (−) and presence (+) of nuclear extract is shown. The arrow indicates the position in the sequence ladder that corresponds to G in the wild type and A in the mutant.

although EBNA-1 itself is apparently not recognized by $CD8^+$ cytotoxic T cells.

Very little is known of the mechanism by which expression of the EBV antigens is suppressed in tumor cells. Several investigators have reported that there is an inverse correlation between patterns of latency gene expression and methylation of the viral episome in cell lines and in primary tumor tissue. The argument that methylation was important in suppressing transcription was strengthened when it was reported that treatment of a Burkitt's cell line with 5-azacytidine reversed viral episome methylation and resulted in expression of latency and lytic genes. However, these experiments raised a host of other questions.

The first question raised was whether the effect of 5-azacytidine in this system related to direct effects on the viral episome or to the activation of cellular genes that, in turn, mediated activation of latency promoters. Many cellular genes have been shown to be induced by 5-azacytidine, generally in a tissue-specific fashion (12). These include gamma globin in erythroid precursors (9) and *myoD* in 10T1/2 mouse fibroblasts (13). We focused our investigations on the C_P because it is central to EBV latency gene expression in that it drives expression of all of the EBNAs including EBNA-2. EBNA-2 feeds back to upregulate C_P and is associated with transcriptional activation of viral promoters for LMP-1 and LMP-2 as well as the cellular promoter for CD23 (3, 15, 18, 28). Methylation of CAT reporter plasmids in vitro with CpG methylase was associated with inhibition of transcription of the C_P in a variety of cell lines, including 5-azacytidine-treated Rael cells. Inhibition of expression of reporter plasmids by in vitro methylation has been previously reported in many systems including EBV-derived plasmids in B lymphocytes (19). Our studies differed from those of previous investigators in that we specifically demonstrated that in vitro methylation blocked transcription in 5-azacytidine-treated cells.

We also found dramatic differences in the impact of methylation by various methylases. *Hpa*II and *Hha*I methylases were associated with less than a 2-fold inhibition of CAT activity, while CpG methylase was associated with more than a 100-fold inhibition of CAT activity. One interpretation of these findings

Target: Cp (8X) ERE/E1b CAT	wt CBF2 CpG		mt CBF2 TpG		mt CBF2 TpA	
Activator	none	EBNA-2	none	EBNA-2	none	EBNA-2
% Ac	0.0	65.0	0.1	1.2	0.0	2.0

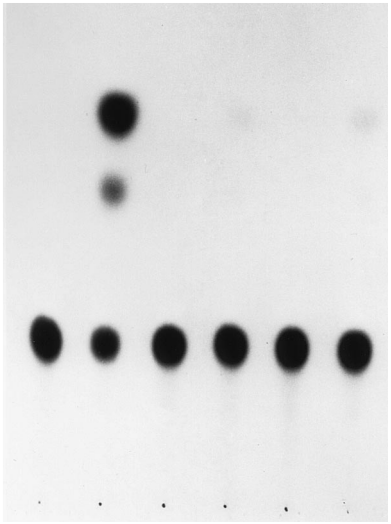


FIG. 11. EBNA-2 transactivates a target reporter plasmid with eight copies of the wild-type (wt) EBNA-2 100-bp response region more effectively than similar reporter plasmids with eight copies of a 100-bp response region with mutant (mt) CBF2 binding sites. In the mutant plasmids a CpG has been replaced with a TpA or TpG in the CBF2 binding region. As shown in Fig. 9, these mutations inhibit CBF2 binding. % Ac, percent conversion of [^{14}C]chloramphenicol.

is that the density but not the particular sequence of methyl-CpG's is crucial for gene regulation and that CpG methylase recognizes at least 10-fold more sites than either *Hpa*II or *Hha*I and as a result is more effective at suppressing transcription. An alternate interpretation invokes some sequence specificity in the inhibition of transcription by methylation. This possibility was particularly relevant because the 100-bp EBNA-2 response element in the reporter construct lacked sites for either *Hpa*II or *Hha*I methylase whereas it contained four CpG sites. Previous investigators assessed viral methylation with methylation-sensitive restriction enzymes, but in order to determine whether the methylation status of the CpG sites within the EBNA-2 response element was important, it was necessary to know whether these sites were methylated in untreated Rael cells and demethylated in 5-azacytidine-treated Rael cells.

This was accomplished by genomic sequencing by the bisulfite technique (5, 21). This technique relies on preferential deamination of cytosine, but not methylcytosine, to uracil by bisulfite in single-stranded DNA. Following PCR amplification, cloning, and conventional dideoxy sequencing, methylcytosine residues are identified as cytosine and unmethylated cytosine residues are identified as thymine in the sequence ladder. In Rael cells, each of the CpG sites in question was consistently methylated before treatment with 5-azacytidine. Nearby CpG sites were also methylated. In contrast, in B95-8 cells, none of these CpG sites were methylated.

Our analysis of methylation following 5-azacytidine treatment revealed completely methylated and completely demethylated strands but no strands that were partially demethylated. This all-or-none response to 5-azacytidine with regard to the failure to perpetuate methylation of a particular strand is consistent with the processive character of DNA methyltransferase (7).

EMSA studies indicated the possible functional importance of methylation of the CpG sites in the EBNA-2-responsive sequence in that methylation of this fragment abolished formation of the CBF2-binding complex. In contrast, methylation had no apparent effect on CBF1 binding, and in other experiments (data not shown), methylation had no effect on the binding of EBNA-1. Competition studies with synthetic oligonucleotides methylated at particular sites showed that the methylation status of a particular CpG site was crucial to CBF2 binding. It was not possible to test the consequences of methylation at a single site for transcription, so we studied the binding properties of mutant plasmids with T substituted for C at the CpG sites of interest. EMSA and DNase I protection assays confirmed that the alterations in binding following methylation and C→T transition are parallel. Therefore, it seemed appropriate to evaluate possible transcriptional consequences of failure to form the CBF2 complex as a result of methylation by studying the C→T transition mutants in reporter assays. A single C→T transition was associated with a marked reduction (>50-fold) but not complete abolition of transcriptional activity.

Other transcriptional factors whose binding is methylation sensitive have been recognized. Several groups of investigators have reported that CpG methylation appears to block binding of E2F to the adenovirus E2 and major late promoters, binding of ATF/CREB-like proteins to the cyclic AMP response element (CRE), and binding of AP-2 to its binding site in the proenkephalin promoter (2). Undoubtedly, more are waiting to be discovered. In addition, in many systems, methyl-CpG-binding proteins may repress transcription directly (27). The reduction in transcription of plasmids following in vitro methylation with *Hpa*II or *Hha*I methylase, which do not alter CBF2 complex formation, points to a possible contribution of methyl-CpG-binding proteins or other factors not related to CBF2 complex formation to repression of transcription. The more

C_p:		AAAAATTTATGGTTCAGTGCGTCGAGTGCTATCTT
LMP-1:		AGGAAATGGAAAGGCAGTGCGGCAATCAGAAGGGG
LMP-2A:		CCAGGTGACAGCAGCAGTGTGTGAAGATTGTCACA
CD23	(1) 'distal'	ATAGTGGTATGATTCAGTGTGCAGTAACAGTGGTT
	(2) 'proximal'	GGGGGTGGGGGCCGCAGTGTGGACAGAATCTCGAG

FIG. 12. CBF2 binding site sequence alignment with elements upstream of other EBNA-2-responsive viral and cellular promoters. CD23 promoter elements are indicated as proximal and distal to the promoter start site.

complete inhibition of transcription associated with in vitro treatment with CpG methylase than that associated with C→T transition at the first CpG site in the CBF2 binding region similarly points to the importance of other factors. Nonetheless, our experiments make clear that CBF2 complex formation is methylation sensitive and is a major determinant of transcriptional activity at the C_P.

The evidence presented here suggests that CBF2 binding is crucial to EBNA-2 activation of the C_P and is consistent with analyses of the C_P reported by Jin and Speck (11). Viral and cellular promoters other than C_P are also transcriptionally activated by EBNA-2 expression or in Rael cells following 5-azacytidine treatment. CBF1 binding sites have previously been recognized in the LMP-1, LMP-2A, and CD23 promoters. We call attention to the fact that these promoters also contain sequence elements resembling the CBF2 binding site and note that these CBF2-like binding sites appear to be transcriptionally important (Fig. 12) (14, 18). It is of some interest in this regard that the CpG site whose methylation status we have demonstrated is critical for activation of the C_P is replaced by a TpG in the LMP-2A and CD23 sequences. In summary, methylation of a particular CpG site appears to block sequence-specific binding of a cellular factor(s) and thereby to inhibit expression of a family of viral genes.

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