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Mapping Promoter Regions That Are Hypersensitive to Methylation-Mediated Inhibition of Transcription: Application of the Methylation Cassette Assay to the Epstein-Barr Virus Major Latency Promoter

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Methylation-associated transcriptional repression is recognized in many settings and may play a role in normal differentiation and in tumorigenesis. Both sequence-specific and nonspecific mechanisms have been elaborated. Recently, we have presented evidence that methylation-associated inhibition of the Epstein-Barr virus (EBV) major latency promoter (*Bam*HI C promoter or Cp) in Burkitt's lymphoma and Hodgkin's disease may play an important role in the pathogenesis of these tumors by protecting them from $CD8^+$ cytotoxic T-cell immunosurveillance. The mechanism of transcriptional repression may relate to specific inhibition of the binding of a cellular transcription factor by methylation. To dissect the viral promoter with regard to transcriptional sensitivity to methylation, we have devised an assay that allows the methylation of discrete regions of reporter plasmids. During the course of the assay, methylation patterns appeared to be stable; there was no evidence of either spread or reversal of the imposed methylation pattern. Application of the assay to the 3.8-kb region upstream of the major EBV latency promoter with natural Cp reporter plasmids showed that sensitivity to methylation is not homogeneously distributed but is concentrated in two discrete regions. The first of these methylation-hypersensitive regions (MHRI) is the previously identified EBNA-2 response element, which includes the methylation-sensitive CBF2 binding site. The second (MHRII) is a sequence further downstream whose potential role in methylation-mediated transcriptional repression had been previously unsuspected. In chimeric enhancer/promoter plasmids, methylation of this downstream region was sufficient to virtually abolish simian virus 40 enhancer-driven transcription. Further dissection indicated that methylation of the EBNA-2 response element (MHRI) was sufficient to abolish EBNA-2-mediated Cp activity while methylation of a region including the EBNA-2 response element and downstream sequence (MHRI and MHRII) was sufficient to abolish all Cp-mediated reporter activity, including that driven by the EBNA-1-dependent enhancer in the origin of plasmid replication, *oriP*.

In peripheral blood lymphocytes and in tumors, Epstein-Barr virus (EBV) persists in the face of intense cytotoxic T-cell immunosurveillance. This persistence is attributable, at least in part, to the restricted patterns of viral latent gene expression in normal lymphocytes and in many tumors. In particular, the major latency promoter, the *Bam*HI C promoter or Cp, is silent and the family of immunodominant EBNAs is not expressed (1, 17, 22, 28, 39). We have shown that the sequence-specific binding of a cellular factor, CBF2, approximately 310 bp upstream of the Cp TATAA box is inhibited by CpG methylation and that the binding sequence is consistently hypermethylated in EBV-associated Burkitt's lymphoma, Hodgkin's disease, and the peripheral blood lymphocytes of healthy seropositive individuals (34, 35). However, recent evidence has shown that EBNA-2 expression is not required for Cp expression in reporter plasmids which include other regulatory elements present in the natural promoter, such as the EBNA-1-dependent enhancer located nearly 3.5 kb upstream of the Cp in the latent origin of plasmid replication, *oriP* (30). Indeed, Cp is expressed in recombinant viruses in which the EBNA-2 response element has been deleted (3, 27, 38). Thus, the question arises whether methylation of the EBNA-2 response element is relevant to transcriptional activation of the Cp and whether regions other than the EBNA-2 response element may also be important in this regard. To address this question, we devised an approach to assay the effect of methylation of a discrete region of a reporter plasmid on reporter activity. This report focuses on the application of this approach to the Cp and upstream regulatory elements.

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MATERIALS AND METHODS

Plasmids. Recombinant PCR (rPCR) mutagenesis (2) was used to create unique restriction sites for use in the methylation cassette assay in the previously described plasmid pPDL5 (18). Conditions for rPCR were as follows: 95°C for 1.5 min (1 cycle) followed by 95°C for 0.5 min, 60°C for 1.0 min, and 70°C for 2.0 min for 35 cycles. PCR was carried out with a high-fidelity thermal polymerase (Vent DNA polymerase; New England Biolabs) in buffer provided by the manufacturer in a 50-μl reaction volume with 350 ng of each primer and 200 μM each deoxynucleoside triphosphate. The primers used for the rPCR are shown in Table 1. Mutations are indicated in boldface type, and restriction sites are underlined. An *Xho*I site was introduced at +8 (relative to the Cp TATAA box

TABLE 1. Oligonucleotides used in this study

Oligonucleotides	Sequence[a]
Primers for rPCR	
5′ /*Xho*I/+8	ACAAAAACTCGAGGTTCTGCTGCAAATTTTAGATC
3′ /*Xho*I/+8	CAGCAGAACCTCGAGTTTTTGTAGTGGGACCTTAG
5′ /*Spe*I/−302	ATCTTTGGACTAGTAGAAAATTGAACCTTGTT
3′ /*Spe*I/−302	TTTTCTACTAGTCCAAAGATAGCACTCGACGC
5′ /MluI/−376	TAATGTCACGCCGTTTATCAAGTTGGTGTAAACACG
3′ /*Mlu*I/−376	TTGATAAACGCGTGACATTATCTCTGGCTGGCCTA
5′ /*Nhe*I/−581	GCTCGGCTAGCGAGGGCGGCCTTGCGAACAAT
3′ /*Nhe*I/−581	GCCGCCCTCGCTAGCCGAGCCGCCCGGCTCCC
Primers for bisulfite sequencing	
5′ / RA 389	CGATAGATCTCAAAAATACACTAACTATTTACTC (*Bgl*II)
3′ / RA 390	GCTAGGATTCTTAAAATTTGTAGTAGAATATAGAG (*Bam*HI)

[a] Mutations are in boldface type. Restriction sites are underlined.

at position 11305 of the B95-8 genome) to create pKR129. This plasmid was digested with *Kpn*I and *Xho*I, and the EBV insert was cloned into the same sites of pBSKSII (Stratagene) to create pKR142. The *Spe*I site at position −302 was introduced into pKR142 to create pKR143. The *Mlu*I site at −376 was introduced into pKR143 to create pKR144. The *Nhe*I site at −581 was introduced into pKR144 to create pKR145. The *Kpn*I-*Xho*I insert from pKR145 was then cloned back into pKR129 at the same sites to create p(−997)CpCAT. Following each rPCR step, amplified material was used to transform DH5α *Escherichia coli*. Plasmids recovered from colonies were screened for the presence of the new restriction enzyme site. p(−997)CpCAT was tested for chloramphenicol acetyltransferase (CAT) activity against the wild-type parent, pPDL5, and the activity was unaffected by the mutations introduced by rPCR. The p(−997)CpCAT plasmid was also sequenced in the vicinity of the EBNA-2 responsive region and over the new restriction site positions and found to be wild type. The *Nsi*I-*Kpn*I fragment which contains the EBV latency origin of replication (*oriP*) from pSL93 (29) was blunted with T4 DNA polymerase and cloned into the blunted *Kpn*I site of p(−997)CpCAT to create p(−3862)CpCAT. All the other restriction enzyme sites described occurred naturally. Plasmid FR(−221)CpCAT was made by subcloning the *Sac*I-*Eco*RV fragment containing the region with the family of EBNA-1 binding sites from p(−3862)CpCAT (blunted with T4 DNA polymerase) into the *Sma*I site of pBSKSII to create pKR252. The family-of-repeats (FR) region was then excised from pKR252 with *Bam*HI and *Hin*dIII and cloned into the same sites of p(−221)CpCAT. p(−221)CpCAT was made by digesting p(−400)CpCAT with *Mlu*I and *Sac*I, blunting the ends with T4 DNA polymerase, and ligating the *Xho*I-*Sac*I polylinker region (also blunted) of pBSKSII at this position to facilitate further cloning. p(−25)CpCAT was constructed in a similar manner, except that the region from the *Mlu*I-to-*Bsu*36I sites of p(−400)CpCAT was deleted. Plasmid (−400)CpCAT was constructed as follows: p(−997)CpCAT was digested with *Eco*RI and blunted with T4 DNA polymerase to yield a vector fragment. Separately, p(−997)CpCAT was digested with *Avr*II and *Eco*RI (blunted with T4 DNA polymerase) and the Cp-containing insert was isolated and cloned into the p(−997)CpCAT *Eco*RI vector fragment. A recombinant plasmid with the correct insert orientation was identified as pKR236. Blunting at the *Eco*RI site resulted in loss of the site and change of reading frame of the CAT gene. This was repaired by cloning the wild-type *Xho*I-*Hin*dIII insert fragment from p(−997)CpCAT into the same sites of the vector portion of pKR236 to create p(−400)CpCAT. pSV(−221)CpCAT was created by first subcloning the *Hin*dIII-*Acc*I (blunted) fragment from pSV2CAT containing the simian virus 40 (SV40) enhancer/promoter region into the *Sma*I site of pBSKSII to create pKR250. The SV40 enhancer region was then excised as a *Hin*dIII-*Bam*HI fragment from pKR250 and cloned into the same sites of p(−221)CpCAT to create pSV(−221)CpCAT. The plasmid gal4(−221)CpCAT was made by cloning the *Hin*dIII-*Eco*RI fragment from p(gal4)$_1$E1bCAT (provided by G. S. Hayward) containing one gal4 binding site into the same sites of p(−221)CpCAT to create pgal4(−221)CpCAT. The gal4-VP16 fusion protein expression vector was provided by G. S. Hayward (36), and the EBNA-2 expression vector pPDL66A has been described previously (18).

Generation of cassettes and transfection. Twenty micrograms of plasmid DNA was digested with the two desired restriction enzymes overnight in duplicate as specified by the manufacturer. The products of one reaction were phenol-chloroform extracted, precipitated, and then methylated overnight with CpG methylase (New England Biolabs) as specified by the manufacturer. The completeness of the methylation reaction was monitored by digestion of an aliquot with *Hpa*II. The products of both reactions were then precipitated, resuspended in loading buffer, and electrophoresed on a 1 to 1.5% agarose gel. Cassette and background fragments (methylated and unmethylated) were isolated by gel purification with the Qiaex II gel extraction kit (Qiagen) as specified by the manufacturer. Approximately 2 μg of cassette and background fragments were ligated in the desired combination in a 1:1 molar ratio overnight at 16°C with 10 U of T4 DNA ligase (Boehringer Mannheim) in a 20-μl volume. A schematic diagram summarizing the methylation cassette assay is shown in Fig. 1. A ligation involving the corresponding unmethylated cassette in an unmethylated background was performed in parallel for every region to control for ligation efficiency and possible loss of DNA due to the above manipulations, and the results of most reporter assays are expressed relative to the corresponding unmethylated control cassette (relative activity). Ligation reactions were monitored by agarose gel electrophoresis. Usually greater than 90% of the DNA was ligated as determined by visual inspection of the ethidium bromide-stained agarose gel. The ligation mixture was transfected by the DEAE-dextran method into the Rael cell line for all regions tested. Many of the constructs were also evaluated in other cell lines, including DG75, Akata, Raji, CA46, and BJAB, and similar methylation sensitivities were observed. The EBNA-2 expression vector pPDL66A or its parent pSG5 (Stratagene) was cotransfected into the EBNA-2 negative cell lines (Rael, Akata, CA46, and BJAB) to equalize DNA content. In some cases, genomic DNA was also isolated by standard procedures after the 2-day incubation period. At 2 days after transfection, the cells were harvested for CAT assay (33). Quantitation was performed with a Molecular Dynamics PhosphorImager.

Cell lines, tissue culture, and drug treatment. The cell lines Rael, Akata, and Raji are EBV-positive Burkitt's lymphoma cell lines. CA46, BJAB, and DG75 are EBV-negative lymphoma cell lines. Rael and Akata do not express EBNA-2. All lines were maintained in RPMI 1640 supplemented with 100 mM L-glu-

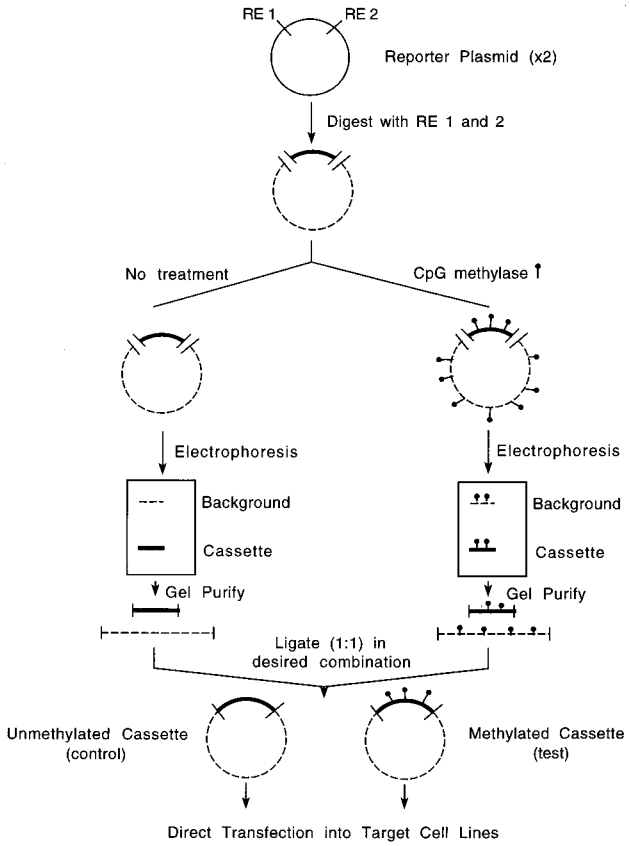


FIG. 1. Methylation cassette assay. As described in Materials and Methods, the reporter plasmid is digested in duplicate reactions with two restriction enzymes (RE 1 and RE 2). The products of one reaction are treated with CpG methylase. The resultant methylated DNA is indicated diagramatically by ●. Background (dashed line) and cassette (thick line) fragments were separated by gel electrophoresis and extracted. The desired combinations of background and cassette were incubated with ligase and transfected directly into cells.

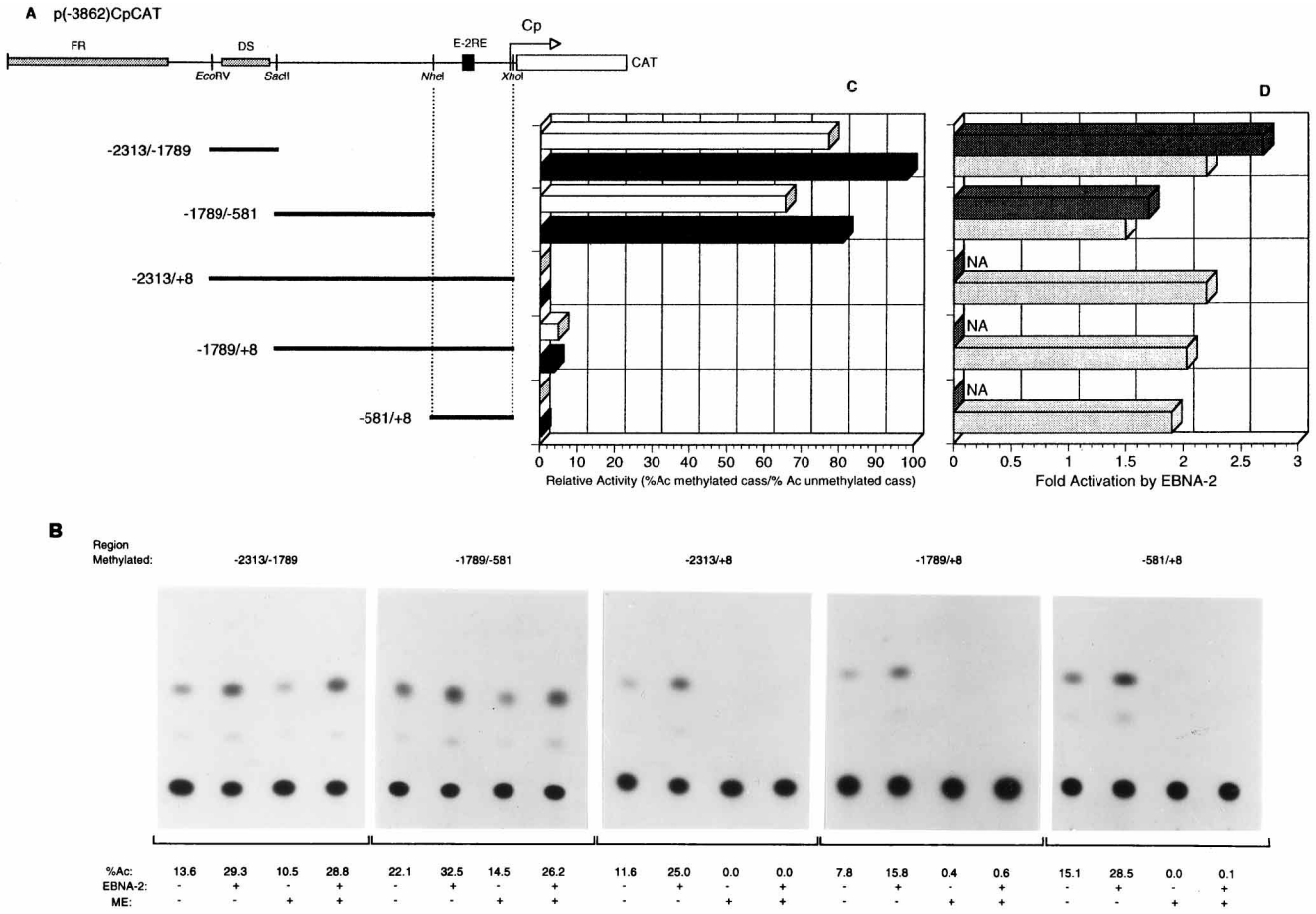


FIG. 2. Results of the methylation cassette assay using p(−3862)CpCAT. (A) Schematic diagram of the reporter construct. E2-RE is the EBNA-2 response element; FR and DS indicate the family of repeats and the dyad symmetry regions, respectively, of the EBV origin of latency replication, *oriP*. Numbering is relative to the Cp TATAA box. (B) Representative thin-layer chromatography and autoradiography with the region tested for methylation sensitivity indicated at the top and the percent acetylation (%Ac), presence of EBNA-2, and methylation status indicated at the bottom. (C) Results of duplicate transfections into the Rael cell line expressed as the relative activity (percent acetylation with the methylated cassette divided by the percent acetylation with the corresponding unmethylated control cassette). Transfections including an EBNA-2 expression vector are indicated by the solid bars and transfections in the absence of EBNA-2 (pSG5 parent control) are indicated by the open bars. (D) Effect of methylation on EBNA-2 transactivation. The results are presented in terms of fold activation in the presence of EBNA-2. Dark bars indicate experiments with methylated cassettes, and light bars indicate experiments with unmethylated cassettes. The fold activation calculation was not applicable (NA) to transfections where no acetylation was observed in the absence of the EBNA-2 expression vector. Vertical dashed lines in panel A represent the minimal MHR.

tamine. Rael cells were treated with freshly prepared 5-azacytidine (Sigma) at 4 μM for 72 h.

Bisulfite genomic sequencing. The bisulfite reaction and subsequent PCR and cloning have been described previously (5, 33). The primer sequences are listed in Table 1 and were specific for the bottom strand. The 5′ and 3′ primers reside at positions 10519 and 11337, respectively, in the B95-8 genome. The positions of *Bgl*II and *Bam*HI sites are indicated in boldface type and were used for cloning of the PCR product into the pUC derivative pGH56 for subsequent dideoxy sequencing.

Southern blotting. Genomic DNA samples (5 μg) isolated after transfection were digested in parallel with 50 U of *Hpa*II or *Msp*I, electrophoresed on a 1.5% agarose gel, transferred to nylon membrane (Biotrans; ICN), and probed with the *Eco*RI vector fragment of pPDL5 to assess the methylation status of vector sequences and then stripped in boiling 0.1% SDS and reprobed with the insert region being tested (defined by the two restriction sites) in the specific methylation cassette experiment to assess the methylation status of the insert. DNA extraction, Southern transfer, probe labeling, and hybridization solutions were as previously described (32, 33).

RESULTS

The cassette assay reveals a discrete region of hypersensitivity to methylation-associated transcriptional repression. Plasmid p(−3862)CpCAT extends 3,862 bp upstream of the Cp TATAA box and includes the transcriptional control element located furthest upstream of the Cp known to affect Cp activity, the FR located within the origin of plasmid replication (30). It also contains restriction sites introduced to facilitate the isolation of discrete fragments or cassettes. These cassettes can be ligated back into the parental background plasmid and the ligation mixture can be transfected directly into target cells (Fig. 1). The target cell line, Rael, is an EBV^+ Burkitt's lymphoma line in which the endogenous Cp is silent and hypermethylated and EBNA-2 is not constitutively expressed (33). This cell line was chosen because the methylation patterns and EBV gene expression patterns of the endogenous virus closely resemble those seen in most EBV-associated tumors (34). Three nonoverlapping promoter region cassettes of 524, 1,208, and 589 bp can be isolated from p(−3862)CpCAT, as illustrated in Fig. 2A. In addition, several overlapping permutations of these cassettes can be isolated. Autoradiographs showing the acetylation associated with transfection of each of the cassette ligation mixtures is shown in Fig. 2B (first lane of each series of four assays). The basal activity reflects activation of the FR enhancer associated with constitutive expression of EBNA-1 in Rael cells and is consistent with activity associated

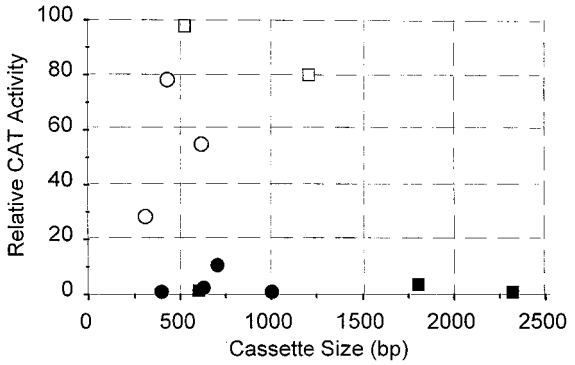


FIG. 3. Relative activity (percent acetylation with the methylated cassette divided by the percent acetylation with the corresponding unmethylated control cassette) of reporter plasmids with methylation cassettes as a function of the number of base pairs methylated. Plasmids (−3862)CpCAT and (−997)CpCAT are indicated by boxes and circles, respectively. Solid symbols indicate plasmids with methylation cassettes extending into the EBNA-2 response element region.

with the parent plasmid in conventional reporter assays. Cotransfection of an EBNA-2 expression vector resulted in a 1.5- to 2.2-fold increase in reporter activity (Fig. 2B, second lane of each series, and Fig. 2D, grey bars). Again, the magnitude of this increase in activity is similar to that reported by others with similar plasmids in conventional reporter assays (3, 27). However, because this ligation-direct transformation technique allows for the combination of a methylated cassette with an unmethylated background in the ligation mixture (Fig. 1), it was possible to assess the effect of methylation of a discrete region of the promoter on promoter activity. This assessment could be made in the absence or presence of EBNA-2 (Fig. 2B, third and fourth lanes of each panel, respectively). Ligation of a methylated cassette into an unmethylated background was associated with only a modest reduction in overall activity when the methylated cassette did not include the region extending from the EBNA-2 response element to the TATAA box (cassettes −2313 to −1789 and −1789 to −581 in Fig. 2). As the size of the methylated cassette increased, there was a slight decrease in reporter activity consistent with a methyl binding protein or chromatin structure-type effect, as reported by others (13). Ligation of a methylated cassette into an unmethylated background did not alter the transactivating effects of EBNA-2 when the methylated cassette did not extend into the EBNA-2 response element. However, when the region extending from just upstream of the EBNA-2 response element through the Cp TATAA box was included in the methylated cassette (e.g., cassette −581 to +8 in Fig. 2), activity in the presence or absence of EBNA-2 was virtually abolished. Even though the methylated cassette was short, the effect on reporter activity could not be differentiated from that associated with methylation of nearly the entire viral insert, since both abolished Cp activity. Thus, −581 to +8 mark the boundaries of a region that is methylation hypersensitive (dashed lines in Fig. 2A). By taking the ratio of the activity with the methylated cassette to the activity with the corresponding unmethylated cassette, it was possible to generate an estimate of the effect of methylation on reporter activity. We refer to this as relative activity, as illustrated graphically in Fig. 2C. In terms of absolute or relative activity (Fig. 2B and C), the effect of methylation of cassettes including the EBNA-2 response element through the Cp TATAA box on reporter expression was much greater than that of methylation of the adjacent nonoverlapping upstream cassettes. This effect was out of proportion to the size of the cassette (Fig. 3). Methylation of sequences from −581 to +8, which completely suppressed Cp activity, encompass one of the known Cp regulatory elements, the EBNA-2 response element.

To further refine the boundaries of the region of methylation hypersensitivity, we engineered a reporter plasmid, (−997) CpCAT, which included more unique restriction enzyme sites in the identified hypersensitive region but less upstream sequence (Fig. 4). Methylation of a cassette extending from −997 to −581 had only a modest effect on reporter activity, as was anticipated from the previous analysis. Similarly, extending the length of the cassette 205 bp (to −376) had little additional effect on reporter activity. However, a further extension of the cassette by 74 bp (to −302), such that the EBNA-2 response element was now included in the methylated region, markedly reduced reporter activity (Fig. 4C). This was consistent with our previous experiments indicating that the EBNA-2 response element was hypersensitive to methylation-mediated inhibition (33). A series of cassettes that included the EBNA-2 response element as well as downstream sequence were associated with virtually a complete abolition of reporter activity, as seen with the cassette extending from −376 to +8. When the cassette did not include the EBNA-2 response element but did include downstream sequences (−302 to +8), significant activity was restored (Fig. 4C).

Effect of methylation on the EBNA-2 response. For many of the plasmids tested, EBNA-2 increased reporter activity. However, the effect of methylation expressed as the relative activity of the methylated versus the unmethylated control remained nearly constant in the absence or in the presence of EBNA-2, as can be seen by comparing the open and solid bars in Fig. 2C and 4C. However, methylation of cassettes that did or did not include the EBNA-2 response element had different effects on EBNA-2-mediated transactivation of the Cp (Fig. 2D, 4D, and 5C). When cassettes containing the EBNA-2 response element were not methylated, activation was always between 1.5- and 2.7-fold (shaded bars in Fig. 2D, 4D, and 5C). This is consistent with previous reports indicating that deletion of the EBNA-2 response element results in a two- to fivefold decrease in Cp activity rather than complete inactivation (27). In contrast, when the EBNA-2 response element and downstream sequences (to the TATAA box) were included in the methylated cassette, chloramphenicol acetylation could not be detected in the absence of EBNA-2. Even in the presence of EBNA-2, acetylation was always less than 0.2%. These values precluded calculation of a fold activation ratio as a function of EBNA-2 (see NA in Fig. 2D, 4D, and 5C). The single exception was the −997 to −302 cassette, which included the EBNA-2 response element but not the downstream sequences (Fig. 4D, asterisk). In this instance, acetylation actually decreased in the presence of EBNA-2. Similarly, when methylated cassettes did not include the EBNA-2 response element, activation was always between 1.4- and 2.5-fold (solid bars in Fig. 2D, 4D, and 5C). Thus, methylation of the EBNA-2 response element inhibits EBNA-2 activation of Cp, whereas methylation of adjacent sequences has no consistent effect on EBNA-2 activation, although it does affect the overall activity. The relative effect of cassette methylation of the sequences downstream of the EBNA-2 response element appeared to be independent of the presence of EBNA-2. We hereafter refer to the EBNA-2 response element as methylation-hypersensitive region I (MHRI).

Identification of a second methylation-hypersensitive region of the Cp. To investigate the downstream region identified with p(−997)CpCAT (−302 to +8) in more detail, a smaller Cp reporter plasmid, (−400)CpCAT, was constructed for use in the methylation cassette assay. This plasmid contained unique

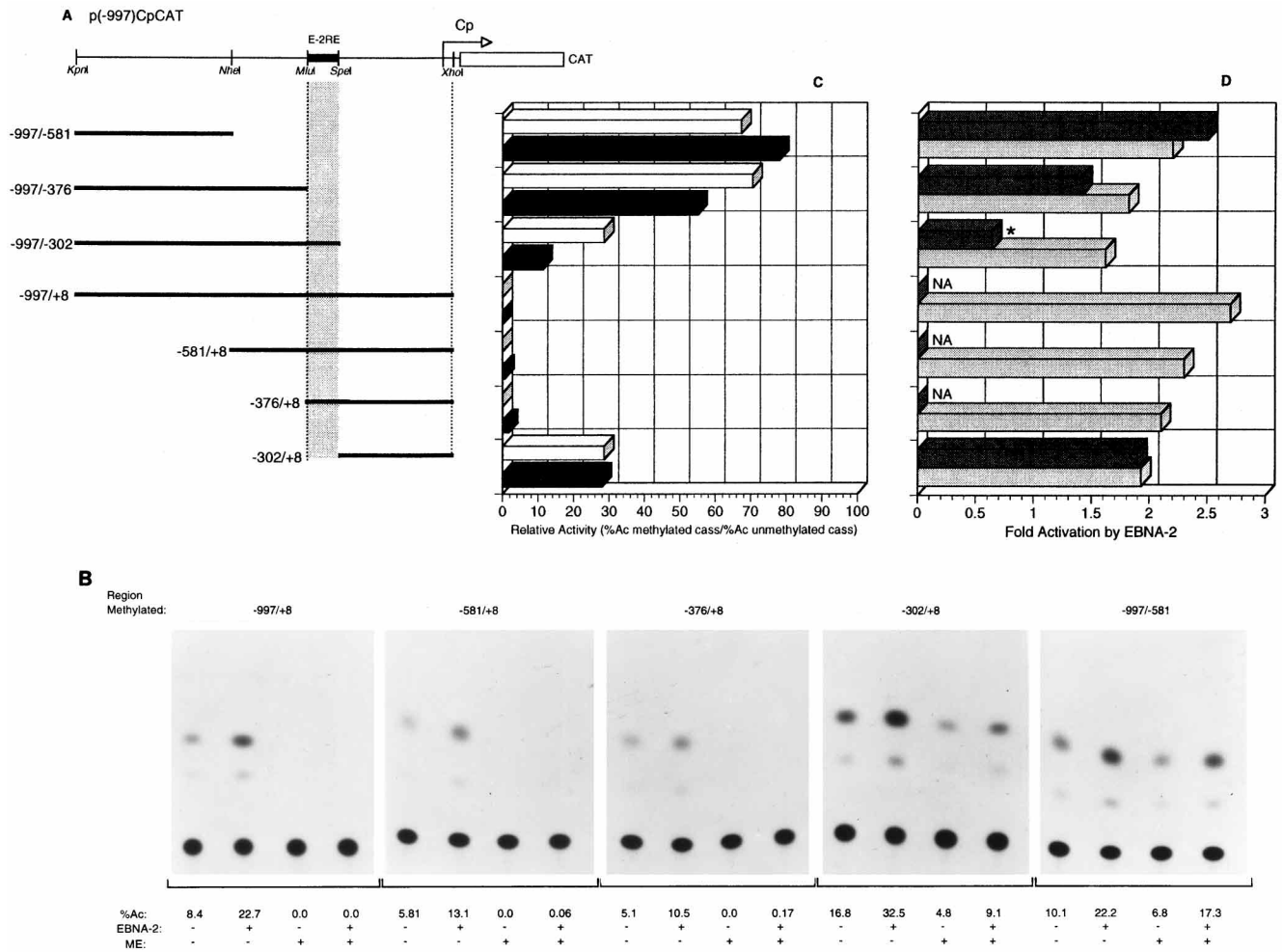


FIG. 4. Results of the methylation cassette assay with p(−997)CpCAT. (A) Schematic of the reporter construct used with the EBNA-2 response element indicated by the shaded region. (B to D) Same as in Fig. 2 but with p(−997)Cp CAT. The asterisk in panel D indicates the only plasmid cassette in which the EBNA-2 response element was methylated and for which an activation ratio could be calculated.

restriction enzyme sites which allowed regional methylation and the assessment of the effect of methylation on reporter activity in the region just downstream of the EBNA-2 response element. The results with p(−400)CpCAT are summarized in graphical form in Fig. 5. Methylation of a cassette including nearly the entire EBV insert (−376 to −25) resulted in almost complete repression in the presence and absence of EBNA-2 in accordance with previous results obtained with p(−997) CpCAT. Methylation of downstream cassettes that did not include the EBNA-2 response element but extended from −302 to −25 and −221 to −25 resulted in a nearly complete abolition of Cp-mediated reporter activity (Fig. 5B). Thus, we recognized a second methylation hypersensitive region (MHR-II) lying downstream (at −25 to −221) of the previously identified EBNA-2 responsive element (MHRI). Methylation of this region was associated with transcriptional repression in the presence or absence of EBNA-2.

MHRII is a potent transcriptional repressor when methylated. To investigate the repressive effects of methylation of MHRII (−221 to −25), recombinant reporter plasmids were constructed with heterologous enhancers (SV40 or a gal4 binding site targeting a gal4/VP16 fusion protein) adjacent to MHR-II in its natural context of the Cp. The SV40 enhancer has previously been shown to partially overcome the effect of regional methylation (16). Figure 6 shows the results of using the methylation cassette assay to selectively methylate MHRII in the context of the heterologous enhancer. Methylation of MHR-II adjacent to the SV40 enhancer resulted in an approximately 25-fold reduction in reporter activity. Methylation of MHRII adjacent to the gal4 binding site activated by a gal4/VP16 fusion protein expression vector similarly resulted in nearly a 10-fold reduction in Cp-mediated reporter activity. Thus, the presence of a strong enhancer did not overcome the effect of regional methylation. In contrast, selective methylation of the SV40 enhancer was associated with only a twofold reduction in reporter activity, a finding in agreement with previous results indicating that the SV40 enhancer is relatively resistant to the effects of methylation (8).

MHRII and Cp activity. What is the role of MHRII in Cp activity? This region contains the Cp CCAAT box and has been reported to contribute to basal promoter activity (23, 27). To further define the contribution to basal promoter activity, a series of deletions of the Cp were made which extended into MHRII. Figure 7 summarizes the results, expressed as the percent acetylation. Deletion of all sequences upstream of MHRII (−221) results in approximately a 10-fold reduction in

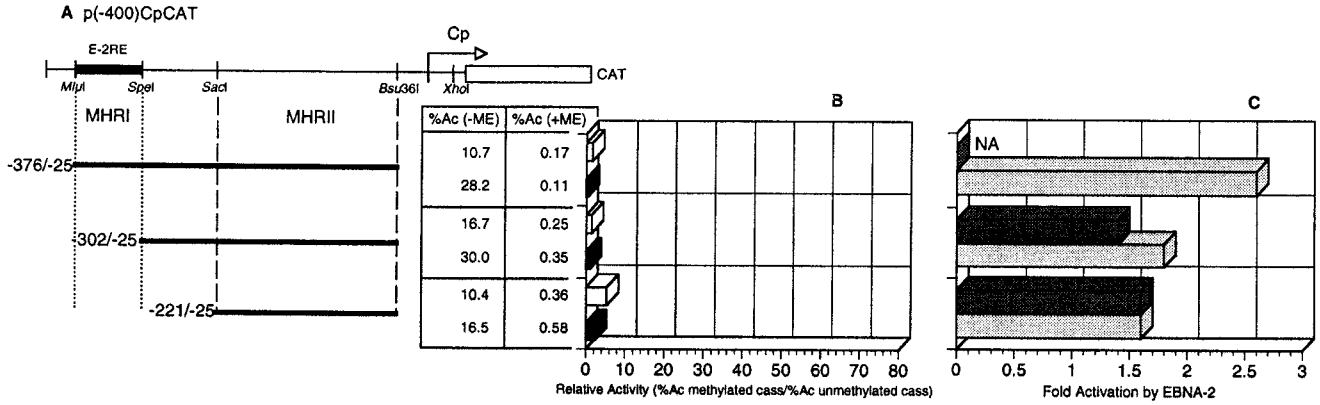


FIG. 5. Identification of a second methylation hypersensitive region of the Cp, using p(−400)CpCAT and the methylation cassette technique. (A) Schematic of the reporter construct used with the EBNA-2 response element indicated (E-2RE). (B) Results of duplicate transfections into the Rael cell line expressed as the relative activity (percent acetylation with the methylated cassette divided by the percent acetylation with the corresponding unmethylated control cassette). Transfections including an EBNA-2 expression vector are indicated by the solid bars, and transfections in the absence of EBNA-2 (pSG5 parent control) are indicated by the open bars. The locations of MHRI and MHRII are indicated by the two sets of dashed lines in panel A. The percent acetylation (%Ac) values used to calculate the relative activities are summarized in table form next to the graph. −ME and +ME are the unmethylated and the CpG-methylated cassette values, respectively. (C) Effect of methylation on EBNA-2 transactivation. Calculations are as described in the legend to Fig. 2D. Note that percent acetylation values below 0.2% were considered zero and thus the calculation was not applicable to these situations (NA).

Cp activity, while deletion of MHRII (all sequences upstream of −25) results in a further approximately 10-fold reduction in Cp activity.

MHRII is methylated in vivo. Transient transfections with in vitro-methylated reporter plasmids indicated that methylation of MHRII could repress transcription driven by potent enhancers such as the SV40 enhancer, but is MHRII methylated in vivo? To address this question, bisulfite genomic sequencing was used. In this procedure, genomic DNA is reacted with sodium bisulfite, which effectively converts unmethylated cytosines to uracil. After PCR with strand-specific primers (Table 1), cloning, and dideoxy sequencing, unmethylated cytosines will be read as thymine and methylated cytosines will be read as cytosine in the sequence ladder. Representative genomic sequence of MHRII (Fig. 8) from an EBV cell line with a constitutively active Cp (B95-8) revealed that all CpG sites were unmethylated, while in a Cp-negative cell line (Rael), nearly all CpG sites were methylated. Treatment of the Rael cell line with 4 μM 5-azacytidine for 3 days resulted in complete demethylation of all CpG sites within MHRII. Two or more clones were sequenced for each cell line, and the methylation patterns were identical in each (data not shown). The CpG sites analyzed by bisulfite sequencing are also shown, relative to other Cp regulatory elements, in Fig. 11.

Does methylation affect transcriptional activation by the FR region of the Cp? The FR region, which consists of approximately 20 copies of a 30-bp imperfect repeat, has been recognized as having enhancer activity in the presence of EBNA-1 (30). To determine the effect of methylation on this region of the Cp, two plasmids were created with the FR (−2313 to −3862) driving a Cp TATAA-containing reporter plasmid. The plasmids were identical except for the orientation of the FR relative to the Cp. The presence of the FR in the parent plasmid resulted in a three- to fivefold increase in reporter activity in an EBV-positive cell line (data not shown) over the identical reporter construct lacking the FR region. Cassettes containing the FR were methylated, and the ligation mixtures were transfected into the Rael cell line that expresses EBNA-1

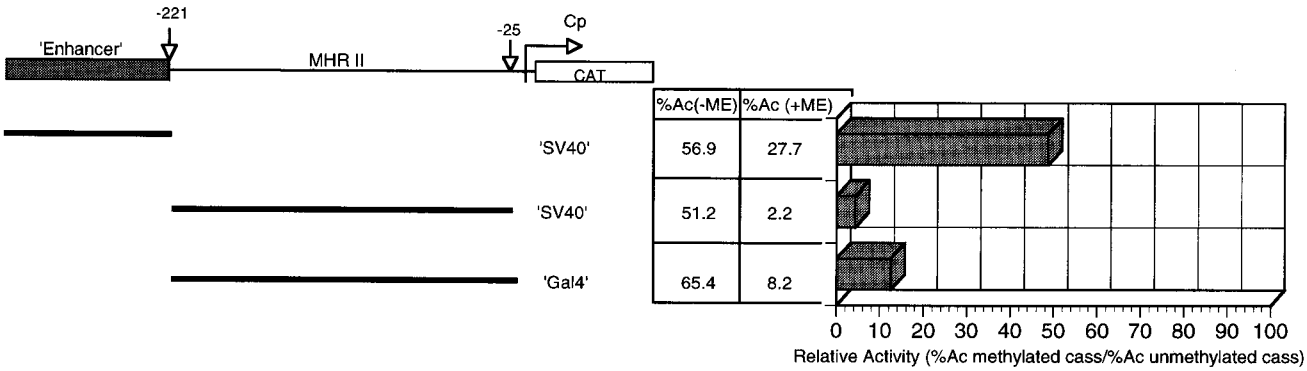


FIG. 6. MHRII is a transcriptional repressor when methylated. The methylation cassette assay was used to regionally methylate only the SV40 enhancer region of pSV(−221)CpCAT (topmost bar) or MHRII in the context of the SV40 enhancer (middle bar) or MHRII in the context of a gal4 binding site in the presence of a gal4/VP16 expression vector (bottom bar). The horizontal lines under the schematic of the reporter construct indicate the region being methylated. Results are expressed as the relative activity (percent acetylation observed with the methylated cassette divided by the percent acetylation observed with the corresponding unmethylated control cassette) after transfection into the Rael cell line in the absence of the EBNA-2 expression vector. SV40 and Gal4 represent the SV40 enhancer and a gal4 binding site, respectively. Numbering is relative to the Cp TATAA box. The percent acetylation (%Ac) values used to calculate the relative activities are summarized in table form next to the graph. −ME and +ME are the unmethylated and the CpG-methylated cassette values, respectively.

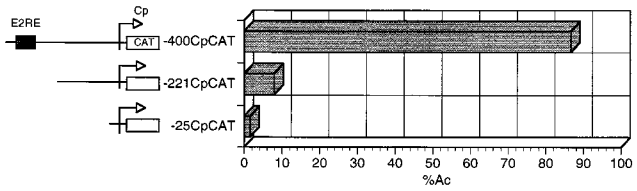


FIG. 7. Deletion analysis of MHRII. The schematic on the left indicates the extent of each of the three Cp reporter constructs tested (coordinates are relative to the Cp TATAA box [bent arrow]), while the graph indicates the reporter activity (expressed as the percent acetylation) observed after transfection into the Rael cell line in the presence of the EBNA-2 expression vector. E2RE is the EBNA-2 response element.

constitutively. Methylation of FR increased reporter activity 4.3-fold in one orientation and 4.7-fold in the other (Fig. 9). In contrast, parallel experiments with the SV40 enhancer cloned at the same position as the FR showed that methylation decreased reporter activity approximately twofold in either orientation (Fig. 6).

Stability of methylation of input DNA in the methylation cassette assay. Interpretation of the results of the methylation cassette assay depends on knowledge regarding the stability of methylation of input DNA. In stably transfected cell lines, regional methylation of transfected DNA can spread into unmethylated regions (10, 12, 19, 21, 24, 25). Alternatively, replication of input DNA might be associated with loss of methylation. The latter possibility is of special concern since p(−3862)CpCAT includes the EBV origin of latency replication (37). To investigate the stability of methylation during the course of short-term assays, DNA was isolated from transfected cell lines at the end of the 2-day assay period and the methylation status was determined by digestion with methylation-sensitive or methylation-insensitive restriction enzyme isoschizomers, followed by Southern transfer. Blots were hybridized with probes derived from cassette or background fragments so as to detect any changes in methylation. Plasmids with and without the EBV origin of replication were transfected into EBV^+ and EBV^- cell lines that do and do not support the replication of plasmids containing the EBV latency origin, respectively. In every case, Southern blot hybridization showed that methylation patterns of input DNA were preserved without change. The results are shown for methylation cassettes extending from −2313 to +8 and −1789 to +8 in the context of p(−3862)CpCAT (Fig. 10). Similar results were demonstrated with methylation cassettes extending from −997 to +8 and −581 to +8 in p(−997)CpCAT (data not shown).

DISCUSSION

Analyses of deletions, mutations, and substitutions in reporter plasmids in short-term transfection assays have played an important role in identifying a multitude of specific transcriptional regulatory elements. In contrast, investigations of epigenetic modifications such as DNA methylation have been relatively limited, in part because methods to regionally modify reporter plasmids have been unavailable or cumbersome. CpG methylation of entire plasmids often results in abolition of activity and thus does not allow for functional dissection. We have developed a cassette assay that facilitates regional modification. This assay was used to analyze almost 4 kb of sequence upstream of the EBV Cp. Investigations show that the pattern of methylation of input DNA is preserved during the short-term assay and that sensitivity to methylation-mediated repression is concentrated in two discrete and relatively small stretches of sequence that we refer to as MHRI and MHRII. Further dissection of MHRI indicated that the previously described EBNA-2 response element was a locus of hypersensitivity. Our previous studies had suggested that methylation of the EBNA-2 response element might be important in the regulation of the Cp. In particular, we had confirmed the observations initially reported by others that treatment of a Burkitt's cell line with 5-azacytidine led to upregulation of the Cp (20, 33). In addition, we showed that methylated DNA did not form a CBF2 binding complex (33). The present experiments clearly demonstrate that the methylation status of the EBNA-2-responsive region in its natural context is important for Cp activity. Methylation of MHRII in both its natural context driven by Cp enhancers and adjacent to heterologous enhancers revealed that this region acted as a transcriptional repressor when methylated. The locations of MHRI and MHRII relative to each other and other Cp-regulatory elements are summarized in Fig. 11.

The assay described here is conceptually similar to several previously described techniques to methylate discrete regions of DNA. Some of these relied on manipulations of single-stranded bacteriophage DNA to create a double-stranded patch on a single-stranded background which allowed selective methylation of patches, polymerase fill-in, and transfection (13, 14). Others involved less efficient in vitro methylation schemes and were assayed in *Xenopus* oocytes (7). Our technique eliminates the need for manipulation of phage or phagemids, allows evaluation of the effect of methylation in a variety of cell lines, and allows for fine mapping, with unique restriction sites

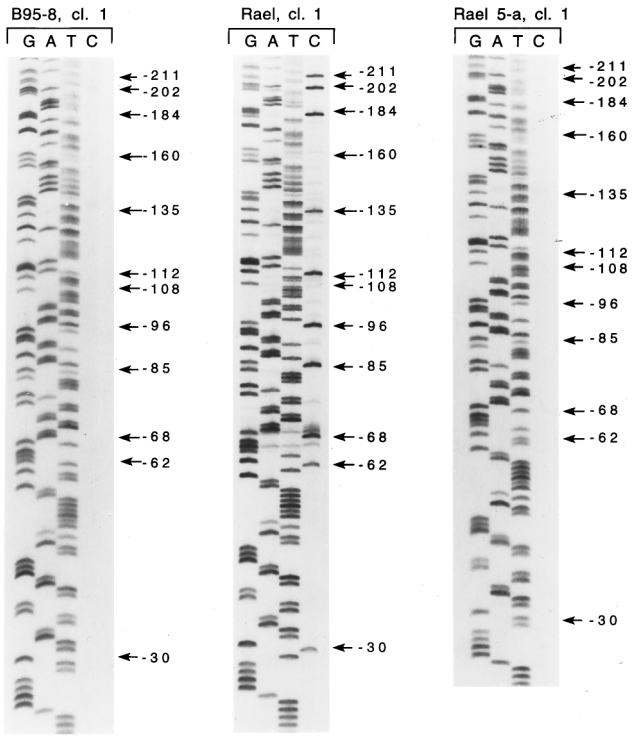
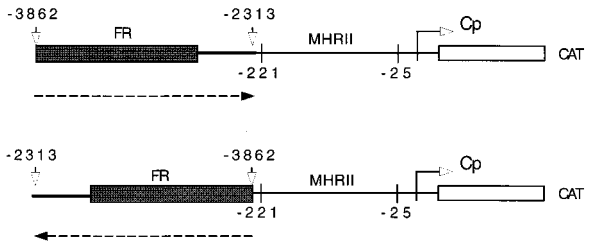


FIG. 8. Representative bisulfite genomic sequence of MHRII. Patterns of methylation in the Rael cell line, the Rael cell line treated with 5-azacytidine (Rael 5-a), and the B95-8 cell line are shown. Arrows indicate the positions of CpG dinucleotides in the native sequence (relative to the Cp TATAA box). The presence of a band in the C lanes is indicative of methyl-C in the native DNA. Two cloned inserts were sequenced for each and found to be identical. Only clone 1 (cl. 1) is shown.



	%Ac, -ME	%Ac, +ME	Fold Change
	3.8	17.7	4.7
	2.7	11.7	4.3

FIG. 9. The methylation cassette assay indicates that transcription driven by the FR enhancer of the Cp is not repressed by methylation. The orientation of the region methylated (−2313 to −3862) is indicated by the dashed line with an arrowhead under the schematic of the reporter construct, while the table on the right shows the result after transfection into the Rael EBV$^+$ cell line as the percent acetylation (%Ac). All numbering is relative to the Cp TATAA box at position 11305 of the B95-8 genome.

being the only requirement. Flexibility in terms of cells assayed is important, and it should be noted that the major findings reported here have been confirmed in the EBV$^-$ lymphoma cell lines DG75, CA46, and BJAB, as well as the EBV$^+$ Burkitt's cell lines Akata and Raji. The previously described techniques have been applied to only a few promoters and have revealed in them a homogeneous distribution of sensitivity to methylation-associated transcriptional repression. Synthetic methylated oligonucleotides with random sequences have been ligated into reporter plasmids by Levine et al. and used to characterize the effect of the position of the methylated patch relative to the TATAA box on transcriptional activity. These investigators showed that methylation near the TATAA box inhibited the formation of the preinitiation complex (16). In contrast, our technique identified specific regions and sequences that were disproportionately sensitive to methylation. Even more important than the technique, however, is that a thorough and systematic analysis of the effects of methylation on a large region of an intact natural promoter has never before been carried out.

We have previously presented evidence that methylation of CpG sites in the Cp plays a critical role in suppression of transcription from this promoter (33). It is also clear that the requisite transcription factors are present in cell lines in which the endogenous Cp is silent because superinfection with virus or transfection with Cp reporter plasmids is associated with activity of the input nonmethylated promoter (4, 33). We have argued that inhibition of the sequence-specific binding of CBF2 in the Cp EBNA-2 response element might play an important role in mediating methylation-associated repression; however, conventional mutagenesis did not allow this hypothesis to be tested directly in the context of a natural Cp reporter plasmid. Instead, we had previously shown that mutation of the crucial cytidine to a thymidine, thus placing a methyl group in the major groove like 5-methylcytidine, was associated with reduced binding and reduced reporter activity. The cassette assay described here allowed this hypothesis to be tested directly with the CBF2 binding site in its natural context of the Cp.

MHRI identified with p(−997)CpCAT corresponds to the EBNA-2 response element (Fig. 11). EBNA-2 response elements have been recognized in a number of viral and cellular genes. These have in common a binding site for CBF1 (also known as RBP-Jκ) (9, 11, 40). We have studied the binding of CBF1 to DNA in previous work and found no evidence that the interaction of CBF1 with DNA was affected by methylation (33). In contrast, immediately downstream of the CBF1 binding site is a second site protected by nuclear extracts from uninfected lymphocytes. This binding activity, CBF2, is methylation sensitive. CBF2 will not footprint a methylated DNA template, and in electrophoretic mobility shift competition assays, methylated oligonucleotides are only weak competitors in comparison with unmethylated oligonucleotides (33). The results of the cassette assay presented here indicate that methylation of the EBNA-2 response element completely suppresses EBNA-2-mediated transactivation of the Cp (Fig. 4D)

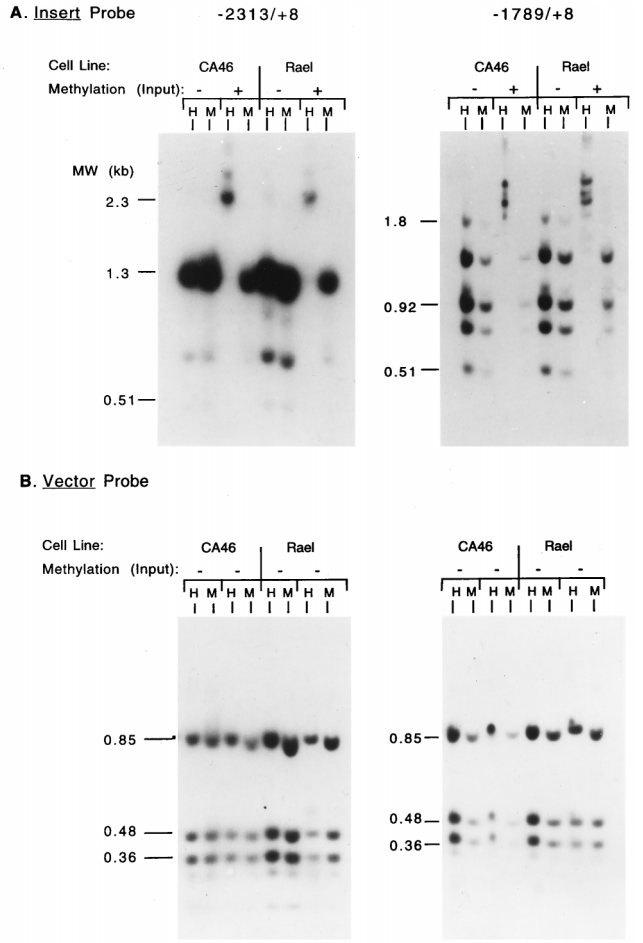


FIG. 10. Southern blot demonstrates the stability of the imposed methylation patterns in the methylation cassette assay. For p(−3862)CpCAT, the stability of methylation in regions from −2313 to +8 and −1789 to +8 was tested in both the Rael and CA46 cell lines. DNA samples were digested in parallel with *Hpa*II (H) or *Msp*I (M). The methylation status of the input ligation reaction is indicated with a + or − for both the cassette (A, Insert) and the background (B, Vector) regions. (A) The blot is probed with the respective cassette (−2313 to +8 or −1789 to +8) and indicates that the cassette remains methylated after the 2-day transfection period if the input DNA was methylated. (B) The same blot in which background sequences were probed and seen to remain unmethylated even if the Cp cassette was methylated.

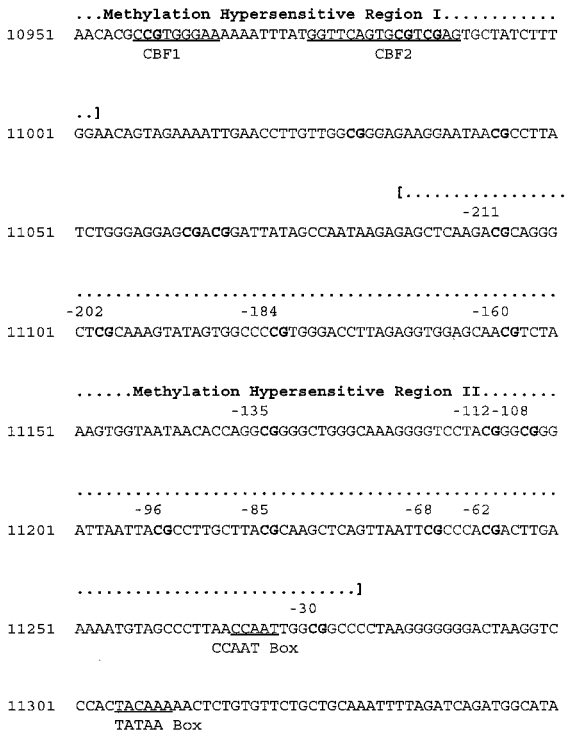


FIG. 11. Sequence of the Cp including the EBNA-2 response element (MHRI), MHRII, CCAAT box, and TATAA box. Potential methylation sites (CpG dinucleotides) are indicated in boldface type and numbered as in Fig. 8. CBF1 and CBF2 sites as defined by nuclease protection studies are underlined. Numbering on the left of the figure corresponds to the sequence of B95-8.

but does not result in complete loss of promoter activity. This is consistent with a report showing that EBV lacking a functional EBNA-2 response element will still transform B cells, giving rise to lymphoblastoid cell lines with Cp activity (3, 27). Clearly, the inhibition of CBF2 binding is an important methylation-sensitive control point, but additional control mechanisms probably exist to maintain complete Cp silence in vivo in tumors.

Evidence for such a second control element was seen when the −302 to +8 region of p(−997)CpCAT was methylated and resulted in a significant degree of repression (a reduction to approximately 30% or less) in an EBNA-2-independent manner (Fig. 4C). Methylation of both the EBNA-2 response element and sequences downstream, to the Cp TATAA box, was sufficient to shut off the promoter, even in the presence of activation of the Cp through binding of EBNA-1 to the FR region. This downstream region contains the Cp CCAAT box, which has been shown by another group to be important for Cp activity (27). Our limited deletion studies of sequences including MHRII confirm the importance of this region for Cp activity (Fig. 7). Using p(−400)CpCAT, we identified MHRII, a region extending from −25 to −221 relative to the Cp TATAA box (Fig. 11). Levine et al. (16) previously reported that methylation in the preinitiation domain could significantly repress promoter activity. The mechanism of this repression has not been defined; however, it is known that the binding of the basal transcription components TFIIA and TFIID are unaffected by methylation. Levine et al. suggested that the repression may be mediated by a protein factor possibly interfering with the binding of TFIIA and TFIID, and this may be the explanation for the repression associated with methylation of MHRII as well (16). That the repression observed when MHRII (−221 to −25) was methylated in the context of p(−400)CpCAT resulted in a greater degree of repression than when a similar region (−302 to +8) was methylated in the context of p(−997) CpCAT may indicate that sequences upstream of −400 contain transcriptionally important sequences or are able to mitigate the effects of MHRII methylation.

The finding of increased reporter activity in association with methylation of the FR was unexpected (Fig. 9). Situations in which methylated genes are transcribed have been recognized (6, 26), but to the best of our knowledge, direct evidence that methylation actually increases transcription has not been previously presented. Alternative explanations for increased reporter activity are that methylation of the FR stabilized transfected DNA and did not directly affect transcription, that methylation interfered with the binding of a cellular protein competing with EBNA-1 for the EBNA-1 binding sites in FR, or that methylation of sequences downstream of FR included in the reporter construct used for these studies influenced overall reporter activity, although no important regulatory elements have been demonstrated in this region.

A second unexpected finding was the observation that EBNA-2 cotransfection in association with methylation of the EBNA-2 response element consistently decreased reporter activity associated with a variety of reporter plasmids (Fig. 4D). This finding, which was also noted in the context of methylation of a cassette extending from −376 to −221 of p(−400) CpCAT (data not shown), does not simply reflect promoter competition, since this was controlled for by cotransfection of expression vector DNA including the promoter but lacking the EBNA-2 coding region. Alternatively, it may point to other protein-protein interactions involving EBNA-2. A whole series of cellular and viral protein interactions either directly with EBNA-2 or with CBF1, which mediates EBNA-2 transcriptional transactivation, have recently been recognized (15, 31, 41). Although recent studies have indicated that deletion of the EBNA-2 response element results in a two- to fivefold reduction in Cp activity and that recombinant virus lacking a functional CBF1 binding site can still immortalize B cells in vitro and possess Cp activity, methylation of this region in our studies has a significant repressive effect on the Cp, reducing Cp activity in the presence of EBNA-2 and inhibiting all EBNA-2 responsiveness. Thus, in terms of methylation, the EBNA-2 response element may be more important than that observed with deletion and mutagenesis studies described previously and may be attributable to the highly complex regulation of this element. The crucial difference between the two methods of studying promoter function is that in our assay, all *cis* elements and potential protein binding sites are preserved and the only difference is the addition of methyl groups to select regions.

In conclusion, these experiments have demonstrated that methylation of Cp regulatory sequences is sufficient to completely inhibit Cp-driven reporter expression even in the presence of both EBNA-1 and EBNA-2 transcriptional transactivators. The effect of methylation of various Cp sequences and regulatory upstream elements is not homogeneous. Methylation of the FR was associated with modestly enhanced reporter activity, while methylation of approximately 2 kb immediately downstream of FR was associated with modestly decreased reporter activity. Methylation of the region encompassing the EBNA-2 response element through the Cp TATAA box abolished the EBNA-2 response and all basal transcription. Methylation of only the EBNA-2 response element abolished EBNA-2 transactivation and was associated with a small but consistent downregulation of expression in the presence of

EBNA-2, while methylation of the region downstream of the EBNA-2 response element inhibited activity but did not affect the EBNA-2 transactivation of the Cp. Finally, these experiments demonstrate the utility of the methylation cassette assay for mapping regions of methylation hypersensitivity.

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