MOLECULAR AND CELLULAR BIOLOGY, Jan. 1991, p. 117–125 Vol. 11, No. 1
0270-7306/91/010117-09$02.00/0
Copyright © 1991, American Society for Microbiology

Binding of the Transcription Factor EBP-80 Mediates the Methylation Response of an Intracisternal A-Particle Long Terminal Repeat Promoter

MIRIAM FALZON* AND EDWARD L. KUFF

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received 18 July 1990/Accepted 12 October 1990

Intracisternal A-particle (IAP) expression in mouse cells has been correlated with hypomethylation of *HhaI* and *HpaII* sites in proviral long terminal repeats (LTRs). In a previous study, in vitro methylation of three *HhaI* sites in the U3 region of the LTR from the cloned genomic IAP element, MIA14, was shown to inhibit promoter activity in vivo. In this study, we found by site-directed mutagenesis that the two more downstream *HhaI* sites within this LTR were responsible for the methylation effects on promoter activity in vivo; methylation of the other (5′) *HhaI* site, which lies within a putative SP1 binding domain, did not affect promoter activity. Methylation of the *HhaI* sites also inhibited promoter activity of the LTR in a cell-free transcription system. Exonuclease III footprinting demonstrated methylation-induced changes in protein binding over the region encompassing the downstream *HhaI* site, designated the Enh2 domain. The protein that interacts specifically with this domain, EBP-80, was characterized in a previous study (M. Falzon and E. L. Kuff, J. Biol. Chem. 264:21915–21922, 1989). We show here that the presence of methylcytosine in the *HhaI* site within the Enh2 domain inhibited binding of EBP-80 in vitro. The methylated MIA14 LTR construct was much less responsive to added EBP-80 in an in vitro transcription system than was the unmethylated construct. These data suggest that CpG methylation within the Enh2 domain may exert its effect on transcription in vivo by altering the interaction between EBP-80 and its cognate DNA sequence.

Site-specific promoter methylation causes gene inactivation in a number of viral and nonviral eucaryotic systems (for reviews, see references 10, 11, and 36). For example, in vitro methylation at specific sites interferes with expression of the α-globin gene (7, 37), the herpes simplex thymidine kinase gene (5), and the adenine phosphoribosyltransferase gene (26). DNA methylation has been implicated in the regulation of intracisternal A-particle (IAP) gene expression. Expression of IAP proviral elements in various normal and transformed mouse cells is inversely correlated with their methylation state (22, 29, 34) and specifically with the methylation state of their 5′ long terminal repeats (LTRs) (15, 35). In vitro CpG methylation at *HhaI* or *HpaII* sites in the 5′ LTR of the mouse proviral IAP clone MIA14 resulted in a marked decrease in promoter activity, as measured by transient expression assays with a chloramphenicol acetyltransferase (CAT) reporter gene in COS7 cells (15) or 293 cells (13). The 5′ and 3′ LTRs from MIA14 each contain three *HhaI* sites (Fig. 1). We show here by site-directed mutagenesis studies that only two of these sites (sites *b* and *c*; Fig. 1) are involved in the methylation effect.

Methylation may influence LTR function by altering the interaction between one or more regulatory proteins and their binding sites. In this study, we show that the exonuclease III footprint of the *HhaI*-methylated LTR in the presence of crude nuclear extract is perturbed over one of the methylation sites (site *c*; Fig. 1). We also show that a purified DNA-binding protein (EBP-80) specific for the LTR region containing this site fails to bind to an oligonucleotide containing the methylated form of the *HhaI* site *c*. The MIA14 LTR methylated at the *HhaI* site *c* is a less effective promoter of transcription in vitro than is its unmethylated counterpart and responds poorly to added EBP-80. These results provide evidence that methylation-sensitive binding of EBP-80 is important in regulating the activity of IAP LTRs in vivo.

* Corresponding author.

MATERIALS AND METHODS

Cell lines and nuclear extracts. Nuclear extract was prepared from the 293 cell line, which is an adenovirus type 5-transformed human kidney cell line (18), by the method of Parker and Topol (40) as modified by Ohlsson and Edlund (39). Whole-cell extracts for in vitro transcription assays were prepared from 293 and HeLa cells as described by Manley (32). The cells were grown in spinner flasks in Joklik modified Eagle medium supplemented with 10% fetal bovine serum and 1 mM glutamine. Cells were harvested during the log phase (4×10^5 to 6×10^5 cells per ml). Transient expression assays were carried out using 293 cells (see below).

Protein purification. Nuclear extract from 293 cells was fractionated by chromatography on heparin-Sepharose and DNA affinity columns as previously described (13, 25).

Plasmids and oligonucleotides. The expression plasmid pMIAcat-3′L, which contains the 3′ LTR from the mouse genomic IAP element MIA14 (31), as well as mutant versions of pMIAcat-3′L (described below), and pMIAcat-3′(del) (13) were used for transient transfection assays. These constructs contain the CAT gene downstream of the IAP sequence and were prepared by cloning the 3′ LTR-containing DNA fragment from the mouse IAP gene MIA14 into plasmid pSV0cat (17). The constructs (linear or circular) were also used in in vitro transcription assays. When circular DNA was used as a template, the hybridization probe used

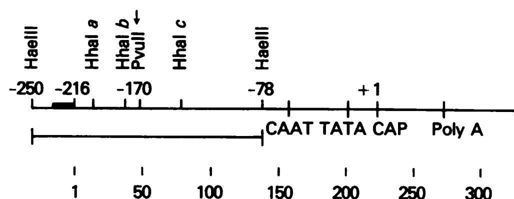

FIG. 1. Schematic representation of the 3′ LTR and immediate upstream region of the mouse IAP clone, MIA14. Numbers above the sequence represent nucleotide positions with respect to the RNA start site; numbers below represent nucleotide positions in the LTR. ▬, Position of a polypurine stretch; ——, the probe used for exonuclease III footprinting (a 173-bp *Hae*III-*Hae*III fragment). The three *Hha*I sites (*a*, *b*, and *c*) used to assess the methylation-induced effects in vivo and in vitro are indicated. The *Pvu*II site, which marks the 5′ end of the construct pMIAcat-3′L(del), is indicated by an arrow.

prior to S1 nuclease digestion was an *Eco*RI fragment from the respective construct. *Eco*RI fragments were used as templates in runoff transcription assays. A DNA fragment containing a region (−367 to +33) from the adenovirus major late promoter (MLP) cloned in pUC18 was used as internal control in the in vitro transcription assays. A *Bgl*I fragment of this construct was used as hybridization probe prior to S1 nuclease digestion. The MLP construct was provided by J. Brady, National Cancer Institute, National Institutes of Health.

A 173-bp *Hae*III-*Hae*III fragment extending from nucleotide 33 upstream of the LTR to nucleotide 139 within the LTR (Fig. 1) was used as a probe for exonuclease III footprinting. This fragment was cloned into pUC13 and has been described elsewhere (12). Oligonucleotides representing the Enh2 domain were synthesized as self-complementary single-stranded molecules and were annealed prior to use as probes or competitors. The annealed Enh2 oligonucleotide cloned in pUC13 was used as a probe for methylation interference experiments. For synthesis of methylated oligonucleotides, 5-methylcytidine phosphoramidite replaced cytidine phosphoramidite at the positions indicated in Fig. 4 and 5b. These oligonucleotides were kindly synthesized by Michael Brownstein, National Institute of Mental Health. The 173-bp *Hae*III-*Hae*III fragment and oligonucleotides were 5′ end labeled as previously described (12).

In vitro methylation of DNA fragments. Plasmids pMIAcat-3′L (and mutants thereof) and pMIAcat-3′L(del) were methylated with *Hha*I methylase as previously described (15). The purified end-labeled 173-bp LTR fragment was methylated with *Hha*I methylase under the same conditions. To check whether the methylation was complete, circular DNA was first linearized with *Bam*HI. The linear methylated DNA was then digested with *Hha*I and examined by gel electrophoresis (followed by autoradiography for labeled fragments). Mock-methylated constructs were prepared by treatment with *Hha*I methylase in the absence of *S*-adenosylmethionine.

In vitro transcription assays. Transcription reaction mixtures typically contained 40 to 100 μg of 293 or HeLa whole-cell extract protein. Reactions were carried out in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–50 mM KCl–6 mM $MgCl_2$–50 μM EDTA–1 mM dithiothreitol–8% glycerol in a final volume of 15 μl. ATP, CTP, GTP, and [^{32}P]UTP were each added in a final concentration of 1 mM. EBP-80 or the appropriate buffer was added to the template DNA before the whole-cell extract, and the mixture was incubated at 30°C for 15 min. Following addition of transcription extract, transcription reaction mixtures were incubated at 30°C for 60 min, and reactions were stopped by addition of 285 μl of a solution containing 0.05% sodium dodecyl sulfate, 150 mM NaCl, and 10 mM Tris hydrochloride (pH 7.5). The transcription reaction mixtures were phenol-chloroform and chloroform extracted and ethanol precipitated. Transcripts from run-off transcription experiments were analyzed directly on a 5% polyacrylamide gel containing 8 M urea. When circular DNA was used as the template, the transcription reaction mixtures were first treated with DNase I (32) and then analyzed by hybridization and S1 nuclease digestion as previously described (31, 32).

Gel retardation and footprinting assays. Gel retardation assays and binding competition experiments were carried out as previously described (12, 13). The exonuclease III assay was also carried out as previously described (12, 48), using the amounts of extract and exonuclease III specified in the legend to Fig. 3. The probe was the 173-bp *Hae*III-*Hae*III fragment from the LTR of MIA14 (Fig. 1). G ladders used to calibrate exonuclease III footprints were generated by the method of Maxam and Gilbert (33). Methylation interference assays were carried out as described by Speck and Baltimore (43), using as a probe an oligonucleotide representing the Enh2 domain cloned in pUC13.

Nitrocellulose filter binding assays. Nitrocellulose filter binding assays were carried out as originally described by Riggs et al. (42). Typical binding reaction mixtures (25 μl) contained 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol (binding buffer), 100 μg of bovine serum albumin per ml, 4.55 fmol of probe, and the amount of EBP-80 indicated in Fig. 6. Binding was allowed to proceed for 30 min at 25°C. The reaction mixture was then diluted with 1 ml of binding buffer and applied to a nitrocellulose filter (Millipore type HA; 0.45-μm pore size; wet in water and prewashed with binding buffer). The filter was washed with three 1-ml portions of binding buffer and dissolved in Piersolve (Pierce, Rockford, Ill.). Retained radioactivity was measured by liquid scintillation counting.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis was used to generate mutants (44, 45). A 1.0-kb *Hind*III fragment from pMIAcat-3′L (3′ LTR from MIA14) (31) was cloned into M13mp18. Oligonucleotides complementary to regions encompassing each of the three *Hha*I sites were used to generate mutants. The sequences of the oligonucleotides are as follows:

*Hha*I a AATGTGGCCGCGGCTCCC
*Hha*I b TGTCACCGCCATCTTGTA
*Hha*I c GGCATATCCGCAGATTAT

Double mutants were generated by using the single-site mutant as the template to anneal to the second oligonucleotide. The oligonucleotide-directed in vitro mutagenesis system from Amersham was used. Plaques were isolated and screened by sequencing, using the Sequenase kit (U.S. Biochemicals). The entire sequence of the insert in positive clones was confirmed by sequence analysis.

The mutant fragments were excised from M13mp18 and cloned into the expression plasmid pSV0cat (17) for use in expression studies in vivo.

Transfection procedures and CAT assay. Transfections of 293 cells were carried out as previously described (15), using 5 μg of DNA per 6 × 10^5 cells in a 100-mm plate. Cells were

TABLE 1. Effect of methylation on the promoter activity of the MIA14 3′ LTR[a]

Construct	*HhaI* site present	Relative CAT activity	
		Unmethylated	*HhaI* methylated
pMIAcat-3′L	*a, b, c*	100	30
HhaI a mut	*b, c*	35	6
HhaI b mut	*a, c*	90	22
HhaI c mut	*a, b*	50	20
HhaI a,b mut	*c*	33	9
HhaI a,c mut	*b*	20	9
HhaI b,c mut	*a*	50	48
pMIAcat-3′L(del)	*c*	40	10

[a] Construct pMIAcat-3′L contains the 3′ LTR from MIA14. *HhaI* mutants (mut) were prepared by site-specific mutagenesis of pMIAcat-3′L. In these constructs, one or two of the *HhaI* sites (GCGC recognition sequence) are no longer present, and therefore that site is no longer a substrate for *HhaI* methylase. In pMIAcat-3′L(del), the region upstream of the *PvuII* site in pMIAcat-3′L (Fig. 1) has been deleted. Extracts from 293 cells transfected with unmethylated or *HhaI* methylase-treated plasmids were assayed for CAT activity. Activity is shown as arbitrary units relative to that of the parent pMIAcat-3′L construct. Values are the means of three different experiments using different plasmid preparations; 100% activity represents a conversion of parent chloramphenicol to its acetylated forms of 20 pmol/h/μg of protein.

harvested and extracts were prepared after 48 h. Assays for CAT activity were carried as previously described (15).

RESULTS

Effect of methylation of individual *HhaI* sites within the MIA14 3′ LTR on in vivo promoter activity. The construct pMIAcat-3′L contains three methylatable *HhaI* sites (*a* to *c*; Fig. 1). Previous studies have shown that treatment of this construct with the enzyme *HhaI* methylase results in a decrease in promoter activity following transfection into COS7 or 293 cells (13, 15). In this study, we assessed the contribution of the three individual *HhaI* sites to the overall methylation-induced reduction in promoter activity. The *HhaI* recognition sequence GCGC was altered by oligonucleotide-directed mutagenesis to GCGG, a sequence no longer recognized by *HhaI* methylase. Table 1 shows the effect of this base change itself on promoter activity. Mutagenesis of *HhaI* sites *a* and *c* caused three- and two-fold decreases in CAT activity, respectively, but mutagenesis of *HhaI* site *b* had little effect. When only one of the three *HhaI* sites was mutagenized, methylation of the remaining two sites still resulted in decreases in CAT activity (Table 1). Simultaneous mutagenesis of two *HhaI* sites allowed definition of the role of each remaining methylatable site. After alteration of sites *b* and *c*, methylation of site *a* had no effect on promoter activity (Table 1). However, methylation of either site *b* or site *c* resulted in considerable loss of promoter activity (Table 1). Further, methylation of construct pMIAcat-3′L(del), which lacks the upstream portion of the MIA14 LTR and *HhaI* sites *a* and *b* and contains site *c*, caused a fourfold decrease of promoter activity similar to the reduction observed after methylation of the double *HhaI* mutant lacking sites *a* and *b* (Table 1). These results demonstrate the importance of site *c* in controlling the promoter activity of the IAP LTR.

Methylation of *HhaI* sites within the LTR inhibits promoter activity in a cell-free transcription system. Construct pMIAcat-3′L (Fig. 1 and 2a) was treated with the enzyme *HhaI* methylase, and its promoter activity was compared with that of the unmethylated and mock-methylated constructs in a cell-free transcription system, using extracts

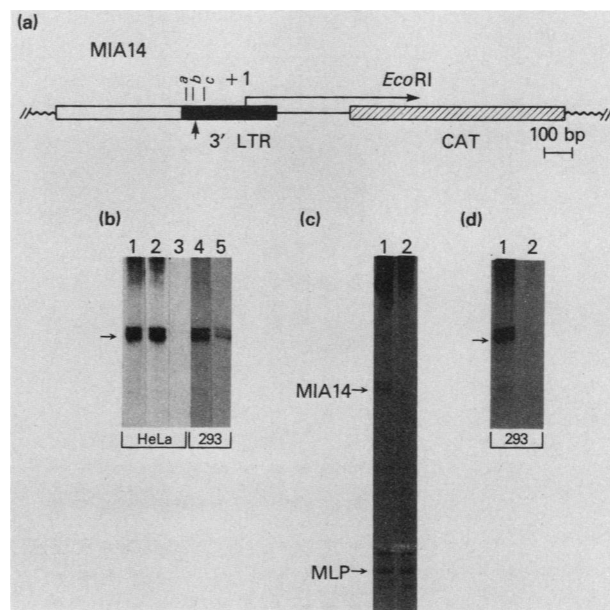

FIG. 2. Inhibition of cell-free transcription by sequence-specific methylation of the 3′ LTR from MIA14, using the indicated whole-cell extract protein (HeLa or 293). (a) Schematic representation of the constructs used, containing the 3′ LTR and flanking sequences from MIA14 cloned into plasmid pSV0cat. *a*, *b*, and *c* indicate the positions of the three *HhaI* sites within the LTR. The arrow indicates the position of the *PvuII* site which marks the 5′ end of the LTR sequence within construct pMIAcat-3′L(del). The open bar and thin line represent sequences 5′ and 3′, respectively, of the LTR. The major transcription start site is indicated (there is also a minor start site 10 bp upstream of the indicated +1 [31]). The probe used for S1 nuclease analysis was an *EcoRI* fragment extending from a site within the pBR322 vector (not shown) to a site within the CAT gene (shown). The size of the transcript is 613 bp. (b to d) Assays of promoter activity. (b) The template DNA was pMIAcat-3′L, containing the entire MIA14 LTR. Lanes: 1 and 4, unmethylated DNA; 2; mock-methylated DNA; 3 and 5, DNA treated with *HhaI* methylase. (c) Two templates were included in the same reaction mixture, pMIAcat-3′L and a construct containing the region (−367 to +33) from the MLP. pMIAcat-3′L was unmethylated (lane 1) or methylated (lane 2). MLP was unmethylated in both instances. (d) The template DNA was pMIAcat-3′L(del), containing the region of the LTR downstream of the *PvuII* site. Lanes: 1, unmethylated DNA; 2, *HhaI* methylase-treated DNA. All plasmid constructs were added in closed circular form. Transcripts are indicated by arrows.

from HeLa and 293 cells. Provided that the DNA template was in closed circular form, methylation of the three *HhaI* sites in the LTR of pMIAcat-3′L greatly reduced the promoter activity obtained with either 293 or HeLa extracts (Fig. 2b; compare lanes 1 and 4 with lanes 3 and 5). Mock methylation had no effect on promoter activity (Fig. 2b; compare lanes 1 and 2). A control plasmid containing a region from the adenovirus MLP was transcribed to the same extent in transcription reaction mixtures containing either the methylated or unmethylated form of pMIAcat-3′L (Fig. 2c; compare lanes 1 and 2). The templates were examined by gel electrophoresis following preincubation with EBP-80 and during the course of the transcription reaction. Both the unmethylated and methylated templates remained in a closed circular conformation (data not shown).

We also studied transcription from construct pMIAcat-3′L(del), in which the region upstream of *PvuII* (containing

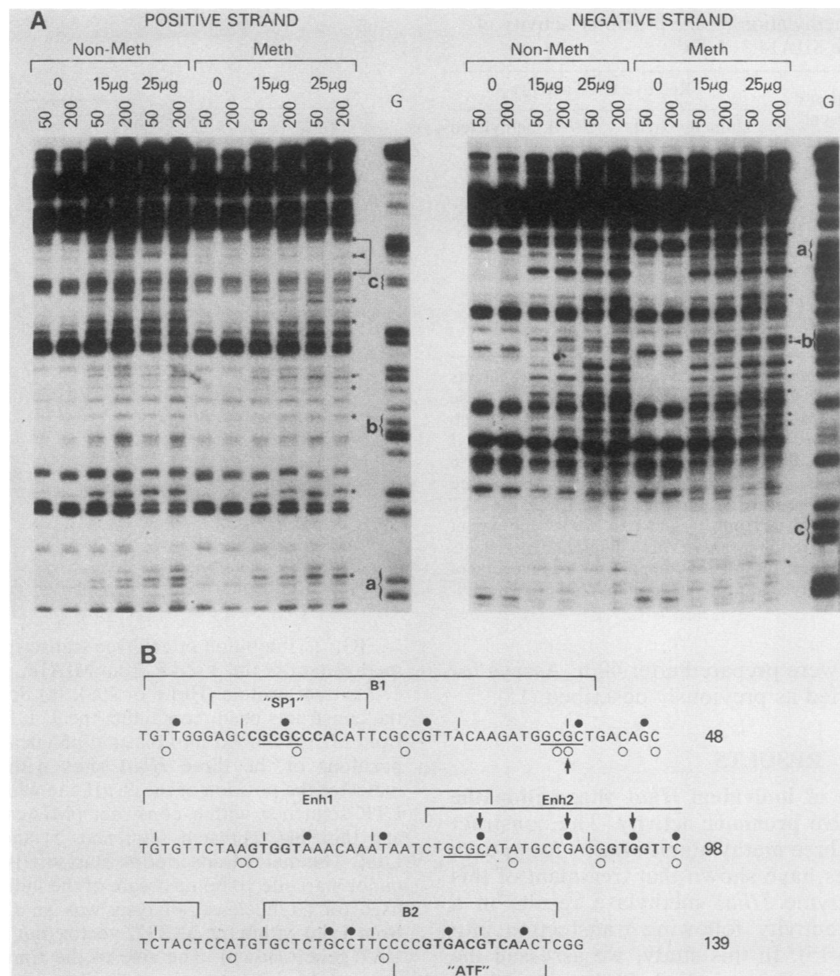

FIG. 3. (A) Effect of *Hha*I methylation on the exonuclease III footprint of the 3′ LTR from MIA14. a, b, and c indicate the positions of the three *Hha*I sites in the *Hae*III-*Hae*III fragment used as a probe. The binding reaction mixture contained the indicated amount of crude nuclear protein from 293 cells; 50 or 200 U of exonuclease III was used. Lane G represents the Maxam-Gilbert sequence ladder (33). On the positive strand, the bracket marks the sequence that is no longer protected following methylation, with the arrow pointing to the position where the most marked change occurs; on the negative strand, the arrow points to a position of altered exonuclease III sensitivity following methylation. (B) Sequence of the DNA fragment used as a probe, showing the exonuclease III stop sites (●, positive strand; ○, negative strand). The three *Hha*I sites are underlined. SP1 (4) and ATF (30) represent sequences with homology to the consensus sequences of the respective transcription factors. A 5-bp sequence common to the Enh1 and Enh2 domains, and forming part of the enhancer core sequence (47), is shown in bold lettering. The arrows point to exonuclease III stop sites that are altered following methylation. They appear as a diminution or disappearance on the positive strand and an enhancement on the negative strand.

*Hha*I sites *a* and *b*) has been deleted (Fig. 1 and 2a). Methylation of the single remaining *Hha*I site *c* in this construct also resulted in a strong decrease in promoter activity (shown for 293 extracts in Fig. 2d; compare lanes 1 and 2). This result is consistent with the inhibitory effect of methylation of this site on in vivo promoter activity of the IAP LTR (Table 1; 13).

The same series of experiments was carried out using linear forms of the LTR-containing constructs. Transcription was generally much less efficient with the linearized forms, and methylation had no significant effect on the promoter activity over a range of extract protein and DNA concentrations (data not shown). Therefore, the inhibitory effect of methylation on transcriptional activity appears to depend critically on conformation of the template in this system.

Methylation-induced changes in the exonuclease III footprint of the MIA14 LTR. Exonuclease III footprinting was used to map the sites of interaction of nuclear extract proteins from 293 cells with the unmethylated or *Hha*I-methylated 173-bp *Hae*III-*Hae*III fragment from the MIA14 LTR (Fig. 1). In the presence of crude nuclear extract, the LTR sequences in the unmethylated and methylated constructs shared a number of novel or greatly accentuated exonuclease III stops, indicating multiple protein-DNA interactions (Fig. 3). In addition, *Hha*I methylation produced several specific changes in the exonuclease III footprint. On the negative strand of the LTR, methylation resulted in decreased sensitivity to exonuclease III over *Hha*I site *b* (Fig. 3). On the positive strand of the LTR, three exonuclease III stops between positions 80 and 94 were no longer apparent when methylated DNA was used in the binding reaction (Fig. 3). These three altered contact points lie within the protein-binding domain which includes *Hha*I site *c*.

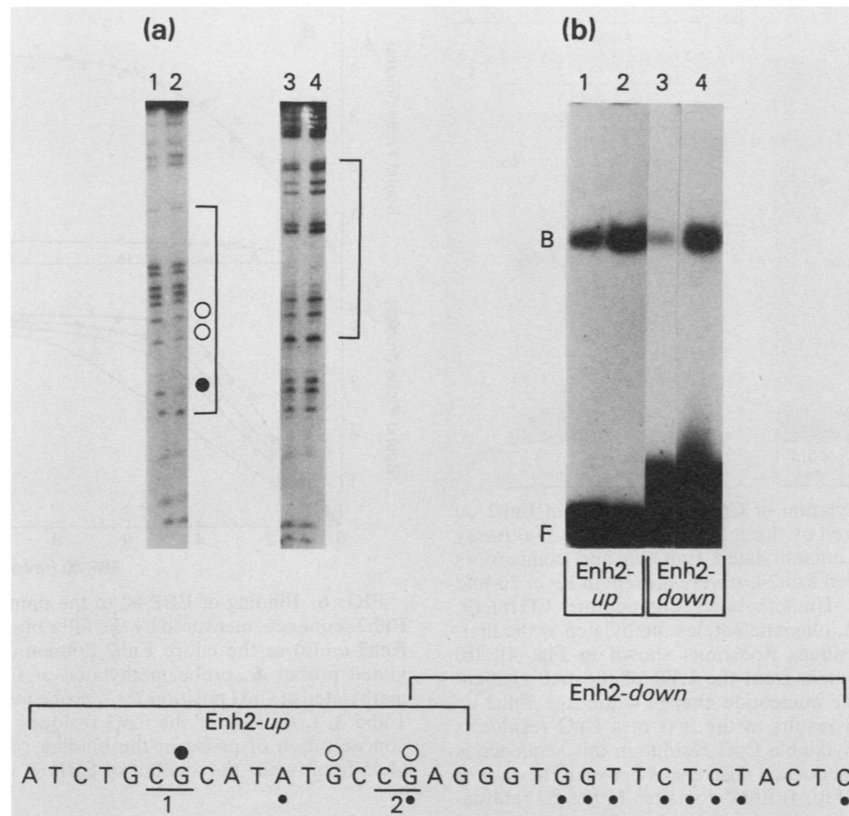

FIG. 4. (a) Determination of the region of interaction of EBP-80 with the Enh2 domain by methylation interference. The probe was an oligonucleotide representing the Enh2 domain. Lanes: 1 and 2, positive strand; 3 and 4, negative strand. Lanes 1 and 3 represent the G ladder obtained from free probe; lanes 2 and 4 represent the G ladder obtained from the protein-DNA complex. The position of G residues whose methylation interferes with the protein-DNA interaction is shown below the footprint (●, strong interference; ○, weak interference). The two methylatable CpG residues (designated 1 and 2) are underlined in the sequence. The two half-site oligonucleotides from the Enh2 domain, Enh2-*up* and Enh2-*down*, are indicated in the sequence. Dots below the sequence indicate differences between the Enh2 domains of MIA14 (shown here) and rc-*mos* (Fig. 5b). (b) Gel retardation assay using the oligonucleotides Enh2-*up* and Enh2-*down* as probes (2 ng in lanes 1 and 3 and 4 ng in lanes 2 and 4) in the presence of 0.5 ng of EBP-80. F, Free probe; B, protein-DNA complex.

In a previous study (13), this protein-binding domain was designated Enh2. A protein fraction, EBP-80, was isolated by affinity chromatography on an oligonucleotide corresponding to this domain from the MIA14 LTR. EBP-80 was shown to interact preferentially with Enh2 and several other oligonucleotides whose only shared sequence was the simian virus 40 enhancer core motif.

The following experiments were carried out to determine whether the reduction in LTR promoter activity following methylation at *Hha*I site *c* might be caused by altered interaction of EBP-80 with target sites within the Enh2 domain.

EBP-80 binds to subregions of the Enh2 domain. The MIA14 LTR enhancer core homology (GTGGT) is located in the downstream portion of the Enh2 domain (Fig. 3B), identifying this region as a site of interaction with EBP-80. However, when methylation interference studies were carried out to identify EBP-80 contact points more precisely, the guanine residues whose methylation interfered with binding of EBP-80 were located within the alternating purine-pyrimidine tract in the upstream portion of Enh2 (Fig. 4a). These observations prompted us to ask whether binding of EBP-80 might involve interactions with separable components of the Enh2 domain. Accordingly, we synthesized oligonucleotides corresponding to the upstream (containing the purine-pyrimidine tract) and downstream (containing the enhancer core motif) portions of the Enh2 domain (Enh2-*up* and Enh2-*down*; Fig. 4) and used them as probes in the gel retardation assay. Enh2-*up* and Enh2-*down* could both bind to EPB-80 independently of one another (Fig. 4b). The two oligonucleotides shown in Fig. 4 shared 3 bp from the middle of the Enh2 region. To rule out the possibility that these 3 bp were responsible for the binding, we synthesized Enh2-*up* and -*down* oligonucleotides that lacked the 3-bp overlap. These oligonucleotides also bound efficiently to EBP-80 (data not shown).

Methylation of CpG sites in the Enh2 domain inhibits binding of EBP-80. The Enh2 domain of the MIA14 LTR contains two methylatable CpG residues, both of which (underlined in the sequence shown in Fig. 4) are included in the Enh2-*up* oligonucleotide. We used the gel retardation assay to assess the effect of cytosine methylation on the interaction of EBP-80 with the upstream portion of the Enh2 domain.

In the assay shown in Fig. 5a, the probe was unmethylated Enh2-*up*, and competitors were present in 10- and 20-fold molar excess. As expected, unmethylated Enh2-*up* (self) was an effective competitor. In contrast, the Enh2-*up* oligonucleotide methylated at CpG position 1 (within the *Hha*I site) competed poorly, indicating that methylation at this site

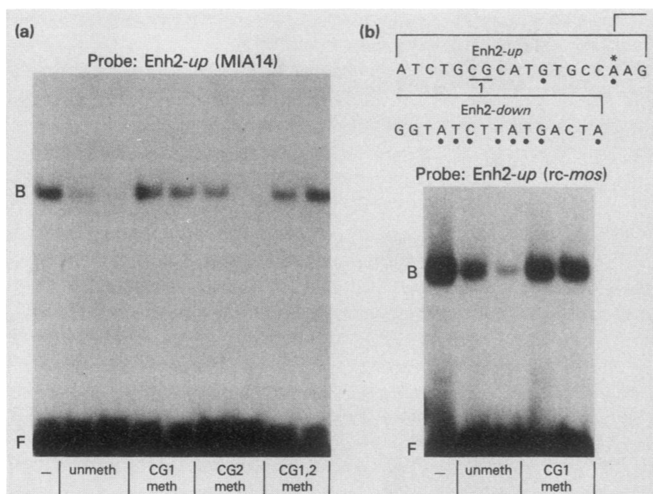

FIG. 5. Effect of methylation of CpG residues within Enh2 on binding of EBP-80, measured by the gel retardation assay. (a) Assay in which the probe was unmethylated Enh2-*up* and competitors (unmethylated or methylated Enh2-*up*) were present in 10- or 20-fold molar excess. Unmeth, Unmethylated competitor; CG1meth, CG2meth, and CG1,2meth, oligonucleotides methylated at the first, second, or both CpG residues (locations shown in Fig. 4). (b) Sequence of the Enh2 domain from the LTR of the IAP element rc-*mos*. The position of the nucleotide change within the Enh2-*up* region of this LTR which results in the loss of a CpG residue is marked (*). The one methylatable CpG residue in this sequence is underlined. Dots indicate sequence differences between the rc-*mos* (shown here) and MIA14 (Fig. 4) Enh2 domains. In the gel retardation profile, the probe was unmethylated Enh2-*up* from rc-*mos*, and competitors (unmethylated or methylated) were present as for panel a.

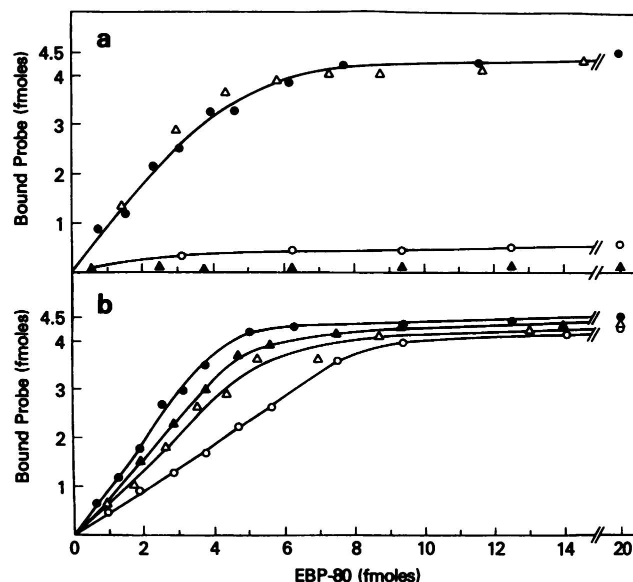

FIG. 6. Binding of EBP-80 to the unmethylated and methylated Enh2 sequence, measured by the filter binding assay. The probe was Enh2-*up* (a) or the entire Enh2 domain (b). Symbols: ●, unmethylated probe; ▲, probe methylated at CpG position 1; △, probe methylated at CpG position 2; ○, probe methylated at CpG positions 1 and 2. Locations of the CpG residues are shown in Fig. 4. The concentration of probe in the binding reaction was kept constant (4.55 fmol) while the amount of EBP-80 was varied.

inhibited binding to EBP-80. The oligonucleotide methylated at both position 1 and position 2 also failed to compete (Fig. 5a). The oligonucleotide singly methylated at CpG position 2 competed effectively, though to a slightly lesser extent than the unmethylated competitor. The inhibition of binding following methylation at CpG position 1 in the Enh2-*up* sequence is consistent with the methylation interference data presented in Fig. 4a, which indicate that the guanine residue at this position is involved in formation of the Enh2/EBP-80 complex.

The Enh2 domain in the 5′ LTR of another cloned IAP element, rc-*mos*, contains a single CpG located at position 1 (underlined in Fig. 5b). When the Enh2-*up* oligonucleotide from rc-*mos* was used as a probe for EBP-80 binding in a gel retardation assay, the CpG-methylated form was a poorer competitor than the unmethylated sequence (Fig. 5b). This observation confirms that the interaction of EBP-80 with the upstream portion of Enh2 is sensitive to the methylation state of the CpG residue at position 1 (*Hha*I site *c*).

The reduced affinity to EBP-80 for the methylated Enh2-*up* oligonucleotide from MIA14 was confirmed by the filter binding assay (Fig. 6a). Binding to the doubly methylated Enh2-*up* or to the oligonucleotide methylated at CpG position 1 alone was 10% or less that obtained with the unmethylated oligonucleotide. On the other hand, methylation of Enh2-*up* at CpG position 2 had no effect on its binding to EBP-80.

CpG methylation also reduced binding of EBP-80 to the whole Enh2 sequence of MIA14, as measured by the filter binding assay, although the effect was less pronounced than that seen with the half-site oligonucleotide. The doubly methylated Enh2 probe required twice as high a level of EBP-80 for half saturation as did the unmethylated form (Fig. 6b). The binding profiles of Enh2 oligonucleotides singly methylated at either CpG position 1 or 2 were intermediate between the curves for the unmethylated and fully methylated forms (Fig. 6b).

CpG methylation at position 2 had no quantitative effect on binding to the Enh2-*up* oligonucleotide but significant effect on binding of EBP-80 to the whole Enh2 sequence (compare Fig. 6a and b). A protein interaction that spans CpG position 2 in the whole Enh2 sequence may be more sensitive to conformational changes caused by methylation at this position than is an interaction with the half-site oligonucleotide, where CpG 2 is in a near-terminal location.

Methylated and unmethylated LTR constructs respond differently to purified EBP-80 in the in vitro transcription system. We tested the ability of EBP-80 to stimulate in vitro transcription from the unmethylated and methylated LTR constructs. EBP-80 increased transcription from the unmethylated construct in a concentration-dependent manner (Fig. 7a; compare lane 1 with lanes 2 to 5). The LTR methylated at all three *Hha*I sites had lower promoter activity than did the unmethylated construct (compare lanes 1 and 6 in Fig. 7a; see also Fig. 2). Addition of EBP-80 also increased transcription from the methylated template (Fig. 7a; compare lane 6 with lanes 7 to 11); however, a higher threshold concentration of EBP-80 was required. In addition, the maximum transcript level from the methylated template was only 50% that of the unmethylated template. A graphic representation of these data is presented in Fig. 7b. The difference in transcriptional activity between the unmethylated and methylated constructs (shown by the broken line in Fig. 7b) represents the methylation-sensitive portion of

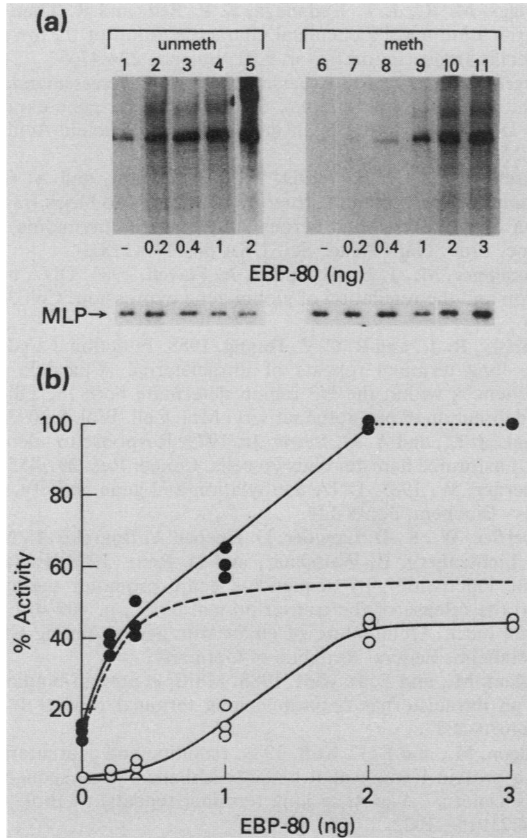

FIG. 7. Stimulation of in vitro transcription of the unmethylated or methylated MIA14 LTR by EBP-80. (a) Assay in which the reaction mixtures contained 50 μg of HeLa whole-cell extract in the presence of the indicated amount of EBP-80. The template (closed circular form) was unmethylated (lanes 1 to 5) or *Hha*I methylated (lanes 6 to 11). The amount of EBP-80 added to the reaction mixture is indicated below each lane. Transcripts were detected by hybridization followed by S1 nuclease digestion. MLP represents the transcript from a construct containing the region (−367 to +33) from the adenovirus MLP, which was included as an internal control. (b) Graphic representation of the data in panel a. Symbols: ●, unmethylated construct; ○, methylated construct. One hundred percent activity represents the maximal activation of the unmethylated construct by 2 ng of EBP-80. The last point in the unmethylated series (connected to the rest of the curve by a dotted line) was obtained from a separate experiment in which the amount of EBP-80 required for maximal activation was determined. The broken line between the two curves represents the difference in percent activity between the unmethylated and methylated constructs.

the LTR response, which clearly saturated at a much lower level of added EBP-80 than did the methylation-resistant component of LTR activity.

DISCUSSION

EBP-80 binds to the Enh2 domain within IAP LTRs and stimulates transcription in a cell-free system (13, 14). In this study, we show that site-specific methylation within the Enh2 domain of an IAP LTR interferes with its in vivo and in vitro promoter activities. Methylation at this same site also inhibits the in vitro interaction of EBP-80 with its cognate sequence. This finding suggests a mechanism for the regulation of expression of these endogenous retroviruses.

Previous work showed that the enzymatic methylation of all three *Hha*I sites in the U3 region of an IAP LTR reduced promoter activity in vivo (13, 15). Here, we have used site-directed mutagenesis of the LTR to assess the effects of methylation at individual *Hha*I sites. Methylation of *Hha*I site *a* had no apparent effect on LTR promoter activity in vivo in comparison with the unmethylated construct. This site lies in a protein-binding region that bears homology to the consensus sequence for the transcription factor SP1 (4). Our results are consistent with reports that binding of SP1 to its cognate sequence and activation of transcription are both insensitive to methylation of CpG residues in the recognition site (3, 20, 23).

Methylation of either *Hha*I site *b* or *c* in the IAP LTR caused a marked loss of promoter activity in vivo. Both of these sites are located in protein-binding domains defined by DNase I and exonuclease III footprinting (12) and shown to be important in regulating the promoter activity of IAP LTRs (8). Protein interactions at the site *b*-inclusive domain have not been studied. However, *Hha*I site *c* lies within the LTR binding region that we have designated Enh2, for which a cognate transcription factor, EBP-80, has been isolated (13). EBP-80 binds to and enhances transcription from IAP LTRs in a cell-free system (13, 14).

Methylation of cytosine residues in the human immunodeficiency virus LTR (1), the Moloney murine leukemia virus genome (19), the E1A promoter of adenovirus type 12 (28), and the late promoter of the E2A region of adenovirus type 2 (9) has been shown to inactivate transcription. Suggested mechanisms (not mutually exclusive) for these effects include induction of altered chromatin structure over methylated DNA (5, 6, 26) and direct inhibition of binding of transcription factors necessary for the initiation of transcription. Examples of the latter include the adenovirus type 2 transcription factors E2F (27) and MLTF (46) and the factor binding to the late E2A promoter (21), as well as the transcription factor binding to the cyclic AMP-responsive element (24). Inhibition of binding may occur as a result of steric hindrance of protein contacts by methyl groups in the DNA major groove or conformation changes in DNA induced by the presence of the methylated bases. However, in no case has the methylation effect been elucidated at a detailed molecular level. In this study, we have shown that the methylation-induced inhibition of EBP-80-mediated transcription could be reproduced in a cell-free system, in which chromatin structure probably does not play a role under the assay conditions used. Therefore, it is likely that methylation directly alters the interaction between EBP-80 and its cognate sequence, as also suggested by the in vitro binding data.

EBP-80-supported transcription in vitro is sensitive to site-specific methylation within the Enh2 domain provided that the LTR-containing template is presented in closed circular rather than linear form. This requirement suggests that changes in DNA configuration have a significant role in the methylation-induced inhibition of transcription, possibly in conjunction with steric hindrance of specific protein-DNA contacts by the introduced methyl group. Methylation of the *Hha*I recognition sequence GCGC is known to cause localized changes in helix geometry by altering phosphodiester bond orientation 5′ to the methylated cytosine (16). Of possibly greater importance, *Hha*I site *c* is part of a highly conserved 11-nucleotide tract of regularly alternating purine and pyrimidine residues. When placed under superhelical constraint, this type of purine-pyrimidine run has the potential to assume a left-handed (Z-DNA) configuration at physiological salt concentrations (38, 41). This tendency is en-

hanced by cytosine methylation of the contained *Hha*I site (2). Thus, methylation of *Hha*I site *c* could inhibit transcription from supercoiled templates by inducing a DNA configuration that is incompatible with effective binding of EBP-80.

If this concept is correct, linear oligonucleotides may have limited usefulness in studying the effects of methylation on EBP-80 binding. Nevertheless, results obtained with the two half-domain oligonucleotides indicate that EBP-80 can bind independently to sequence elements in both parts of the Enh2 domain and that interaction with the more 5′ portion is highly sensitive to methylation at the CpG residue in *Hha*I site *c*. The effect of methylation on EBP-80 binding to the whole (linear) Enh2 sequence was less dramatic: binding was sensitive to methylation at each of the two CpG positions in Enh2, but even the doubly methylated oligonucleotide had appreciable affinity for EBP-80. Interaction with the 3′ portion of Enh2 would be expected to contribute to the methylation-resistant factor binding. However, the situation is probably complex and may involve cooperativity in binding of EBP-80 to the two portions of the Enh2 domain.

EBP-80 stimulates transcription from the unmethylated and methylated MIA14 LTR in a cell-free system. However, the methylated template requires at least 10-fold higher concentrations of EBP-80 for activation and reaches a level of transcription only half that of the unmodified template (Fig. 7). The nature of the methylation-resistant component is not clear. Since EBP-80 can interact independently with both half-sites of the Enh2 domain, high levels of EBP-80 may stimulate transcription from the methylated LTR as a consequence of binding to Enh2-*down*. Transcription from the methylated construct might also reflect binding of EBP-80 to the Enh1 region of the LTR (12). In any event, the in vitro data indicate that very high levels of EBP-80 can partially overcome the effects of methylation, but whether these levels are attained in vivo is not known.

EBP-80 is a cellular transcription factor to which IAP LTRs happen to respond. The protein also interacts with oligonucleotides containing the enhancer core sequences from simian virus 40, polyomavirus, and the murine sarcoma virus LTR (13; unpublished observations). As part of its role as a cellular transcription factor, EBP-80 may be involved in the regulation of a number of genes. Transcriptional activity mediated by EBP-80 binding may be methylation sensitive if the recognition site happens to contain a CpG residue in a crucial position. Future studies will address the function of EBP-80 in normal cell regulation and the causes and consequences of elevated levels in transformed cells.

ACKNOWLEDGMENTS

We thank C. Klee, K. K. Lueders, and S. H. Wilson for critical reading of the manuscript, E. Englander and S. G. Widen for helpful discussions, M. Brownstein for oligonucleotide synthesis, J. Brady for the adenovirus MLP construct, and May Liu for preparation of the manuscript.

REFERENCES

1. **Bednarik, D. P., J. A. Cook, and P. M. Pitha.** 1990. Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency. EMBO J. **9**:1157–1164.
2. **Behe, M., and G. Felsenfeld.** 1981. Effect of methylation on a synthetic polynucleotide: the B-Z transition in poly(dG-m^{5}dC) · poly(dG-m^{5}dC). Proc. Natl. Acad. Sci. USA **78**:1619–1623.
3. **Ben-Hattar, J., and J. Jiricny.** 1988. Methylation of single CpGs within the second distal promoter element of the *Herpes simplex* virus *tk* gene reduces its transcription in vivo. Gene **65**:219–227.
4. **Briggs, M. R., J. T. Kadonaga, S. P. Bell, and R. Tjian.** 1986. Purification and biochemical characterization of the promoter-specific transcription factor, SP1. Science **234**:47–52.
5. **Buschhausen, G., M. Graessmann, and A. Graessmann.** 1985. Inhibition of herpes simplex thymidine kinase gene expression by DNA methylation is an indirect effect. Nucleic Acids Res. **13**:5503–5513.
6. **Buschhausen, G., B. Wittig, M. Graessmann, and A. Graessmann.** 1987. Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **84**:1177–1181.
7. **Busslinger, M., J. Hurst, and R. A. Flavell.** 1983. DNA methylation and the regulation of globin gene expression. Cell **34**:197–206.
8. **Christy, R. J., and R. C. C. Huang.** 1988. Functional analysis of the long terminal repeats of intracisternal A-particle genes: sequences within the U3 region determine both the efficiency and direction of promoter activity. Mol. Cell. Biol. **8**:1093–1102.
9. **Cook, J. L., and A. M. Lewis, Jr.** 1979. Response to adenovirus 2-transformed hamster embryo cells. Cancer Res. **39**:1455–1461.
10. **Doerfler, W.** 1983. DNA methylation and gene activity. Annu. Rev. Biochem. **52**:93–124.
11. **Doerfler, W., K.-D. Langner, D. Knebel, A. Hoeveler, U. Müller, U. Lichtenberg, B. Weisshaar, and D. Renz.** 1988. Eukaryotic gene inactivation by sequence-specific promoter methylation and the release of the transcriptional block, p. 409–417. *In* G. Kahl (ed.), Architecture of eukaryotic genes. Verlag Chemie, Weinheim, Federal Republic of Germany.
12. **Falzon, M., and E. L. Kuff.** 1988. Multiple protein-binding sites in an intracisternal A particle long terminal repeat. J. Virol. **62**:4070–4077.
13. **Falzon, M., and E. L. Kuff.** 1989. Isolation and characterization of a protein fraction that binds to enhancer core sequences in intracisternal A-particle long terminal repeats. J. Biol. Chem. **264**:21915–21922.
14. **Falzon, M., and E. L. Kuff.** 1990. A variant binding sequence for transcription factor EBP-80 confers increased promoter activity on a retroviral long terminal repeat. J. Biol. Chem. **265**:13084–13090.
15. **Feenstra, A., J. Fewell, K. Lueders, and E. Kuff.** 1986. *In vitro* methylation inhibits the promoter activity of a cloned intracisternal A-particle LTR. Nucleic Acids Res. **14**:4343–4352.
16. **Fox, K. R.** 1986. The effect of *Hha*I methylation on DNA local structure. Biochem. J. **234**:213–216.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
18. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
19. **Harbers, K., A. Schnieke, H. Stuhlmann, D. Jähner, and R. Jaenisch.** 1981. DNA methylation and gene expression: endogenous retroviral genome becomes infectious after molecular cloning. Proc. Natl. Acad. Sci. USA **78**:7609–7613.
20. **Harrington, M. A., P. A. Jones, M. Imagawa, and M. Karin.** 1988. Cytosine methylation does not effect binding of transcription factor Sp1. Proc. Natl. Acad. Sci. USA **85**:2066–2070.
21. **Hermann, R., A. Hoeveler, and W. Doerfler.** 1989. Sequence-specific methylation in a downstream region of the late E2A promoter of adenovirus type 2 DNA prevents protein binding. J. Mol. Biol. **210**:411–415.
22. **Hojman-Montes de Oca, F., L. Dianoux, J. Peries, and R. Emanoil-Ravicovitch.** 1983. Intracisternal A particles: RNA expression and DNA methylation in murine teratocarcinoma cell lines. J. Virol. **46**:307–310.
23. **Höller, M., G. Westin, J. Jiricny, and W. Schaffner.** 1988. SP1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev. **2**:1127–1135.
24. **Iguchi-Ariga, S. M. M., and W. Schaffner.** 1989. CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev. **3**:612–619.

25. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83**:5889–5893.
26. **Keshet, I., J. Yisraeli, and H. Cedar.** 1985. Effect of regional DNA methylation on gene expression. Proc. Natl. Acad. Sci. USA **82**:2560–2564.
27. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1987. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. Proc. Natl. Acad. Sci. USA **84**:2180–2184.
28. **Kruczek, I., and W. Doerfler.** 1983. Expression of the chloramphenicol acetyltransferase gene in mammalian cells under the control of adenovirus type 12 promoters: effect of promoter methylation on gene expression. Proc. Natl. Acad. Sci. USA **80**:7586–7590.
29. **Lasneret, J., M. Canivet, F. Hojman-Montes de Oca, J. Tobaly, R. Emanoil-Ravicovitch, and J. Peries.** 1983. Activation of intracisternal A particles by 5-azacytidine in mouse Ki-BALB cell line. Virology **128**:485–489.
30. **Lin, Y. S., and M. R. Green.** 1988. Interaction of a common cellular transcription factor, ATF, with regulatory elements in both Ela- and cyclic AMP-inducible promoters. Proc. Natl. Acad. Sci. USA **85**:3396–3400.
31. **Lueders, K. K., J. W. Fewell, E. L. Kuff, and T. Koch.** 1984. The long terminal repeat of an endogenous intracisternal A-particle gene functions as a promoter when introduced into eucaryotic cells by transfection. Mol. Cell. Biol. **4**:2128–2135.
32. **Manley, J. L.** 1984. Transcription of eukaryotic genes in a whole-cell extract, p. 71–88. *In* B. D. Hames and S. J. Higgins (ed.), Transcription and translation, a practical approach. IRL Press, Oxford.
33. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
34. **Mays-Hoopes, L. L., A. Brown, and R. C. C. Huang.** 1983. Methylation and rearrangement of mouse intracisternal A particle genes in development, aging, and myeloma. Mol. Cell. Biol. **3**:1371–1380.
35. **Morgan, R. A., and R. C. C. Huang.** 1984. Correlation of undermethylation of intracisternal A-particle genes with expression in murine plasmacytomas but not in NIH/3T3 embryo fibroblasts. Cancer Res. **44**:5234–5241.
36. **Murray, E., and F. Grosveld.** 1985. Methylation and γ-globin expression, p. 157–176. *In* G. L. Cantoni and A. Razin (ed.), Biochemistry and biology of DNA methylation. Alan R. Liss, Inc., New York.
37. **Murray, E., and F. Grosveld.** 1987. Site-specific demethylation in the promoter of human γ-globin gene does not alleviate methylation mediated suppression. EMBO J. **6**:2329–2335.
38. **Nordheim, A., and A. Rich.** 1983. Negatively supercoiled simian virus 40 DNA contains Z-DNA segments within transcriptional enhancer sequences. Nature **303**:674–679.
39. **Ohlsson, H., and T. Edlund.** 1986. Sequence-specific interactions of nuclear factors with the insulin gene enhancer. Cell **45**:35–44.
40. **Parker, C. S., and J. Topol.** 1984. A Drosophila RNA polymerase II transcription factor contains a promoter-region-specific DNA-binding activity. Cell **36**:357–369.
41. **Rahmouni, A. R., and R. D. Wells.** 1989. Stabilization of Z DNA in vivo by localized supercoiling. Science **246**:358–363.
42. **Riggs, A. D., H. Suzuki, and S. Bourgeois.** 1970. *lac* repressor-operator interaction. I. Equilibrium studies. J. Mol. Biol. **48**:67–83.
43. **Speck, N. A., and D. Baltimore.** 1987. Six distinct factors interact with the 75-base-pair repeat of the Moloney murine leukemia virus enhancer. Mol. Cell. Biol. **7**:1101–1110.
44. **Taylor, J. W., J. Ott, and F. Eckstein.** 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. **13**:8765–8785.
45. **Taylor, J. W., W. Schmidt, R. Cosstick, A. Okruszek, and F. Eckstein.** 1985. The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA. Nucl. Acids Res. **13**:8749–8764.
46. **Watt, F., and P. L. Molloy.** 1988. Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes Dev. **2**:1136–1143.
47. **Weiher, H., M. König, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219**: 626–631.
48. **Wu, C.** 1985. An exonuclease protection assay reveals heat shock element and TATA box DNA-binding proteins in crude nuclear extracts. Nature (London) **317**:84–87.