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Cytosine methylation does not affect binding of transcription factor Sp1

(DNA methylation/metallothionein gene expression/"GC box")

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ABSTRACT **DNA methylation may be a component of a multilevel control mechanism that regulates eukaryotic gene expression. We used synthetic oligonucleotides to investigate the effect of cytosine methylation on the binding of the transcription factor Sp1 to its target sequence (a G+C-rich sequence known as a "GC box"). Concatemers of double-stranded 14-mers containing a GC box successfully competed with the human metallothionein IIA promoter for binding to Sp1 in DNase I protection experiments. The presence of 5-methylcytosine in the CpG sequence of the GC box did not influence Sp1 binding. The result was confirmed using double-stranded 20-mers containing 16 base pairs of complementary sequence. Electrophoretic gel retardation analysis of annealed 28-mers containing a GC box incubated with an Sp1-containing HeLa cell nuclear extract demonstrated the formation of DNA–protein complexes; formation of these complexes was not inhibited when an oligomer without a GC box was used as a competitor. Once again, the presence of a 5-methylcytosine residue in the GC box did not influence the binding of the protein to DNA. The results therefore preclude a direct effect of cytosine methylation on Sp1–DNA interactions.**

There is strong experimental support for the proposal that DNA methylation may be a component of a multilevel control mechanism through which the expression of certain eukaryotic genes is regulated (1). Inhibition of genomic methylation by 5-azacytidine can result in reactivation of genes on the transcriptionally inactive X chromosome (2, 3), induction of tissue-specific gene expression (4, 5), and expression of differentiated phenotypes in cultured cells (6–8). Introduction of *in vitro* hypermethylated sequences into recipient cells can preclude gene expression (9, 10). Despite these correlations the relationship between methylation patterns and expression of some genes remains unclear (11) and it is not known how changes in the methylation patterns of certain genes alter expression while the activities of other genes remain unaffected.

Recent reports strongly suggest that cytosine methylation may be involved in the structural ordering of chromatin (12, 13). Methylation of phage M13 constructs before transfection into eukaryotic cells targets the assimilated DNA into DNase I-insensitive conformations. Buschhausen *et al.* (13) showed that reconstitution of methylated thymidine kinase DNA with histone octamers prior to microinjection resulted in biological inactivity even in the absence of integration into the host genome. In contrast, mock-methylated constructs actively expressed thymidine kinase and the inhibitory effect of methylation could be reversed by treatment with 5-azacytidine. These observations suggest that positioning of structural nuclear proteins (histones, high-mobility group proteins, or matrix proteins) may be involved in transducing the methylation signal. What remains unclear, however, is whether methylation also alters the direct binding of specific transcription factors to defined promoter elements.

Tjian and coworkers originally described (14–16) and subsequently purified from HeLa cell nuclear extracts (17) a transcription factor, Sp1, that specifically recognizes the simian virus 40 early promoter. Subsequent studies revealed the decanucleotide consensus Sp1 binding sequence to be 5′ $\overset{GC}{TA}GGCG\overset{GGGC}{TAAT}$ 3′ (the "GC box"). GC boxes have been identified in the 5′ region of several eukaryotic genes, including the human metallothionein (hMT) IIA, IA, and IB genes (18–20); the Harvey *ras-1* protooncogene (21); the epidermal growth factor receptor gene (22); and the human immunodeficiency virus long terminal repeat (23). The observations that Sp1 interacts with GC-box sequences in the major groove (16), that the GC box contains a potential methylation site (CpG), and that the methyl group of 5-methylcytosine extends into the major groove suggested that the protein might be able to sense the presence or absence of the methyl group. There is also evidence that both the mouse metallothionein I (24) and the hMT-1B (20) genes might be sensitive to methylation control. We therefore measured the effect of cytosine methylation on the interaction of Sp1 with its target sequence.

The hMT-IIA promoter region was selected as a substrate for Sp1 binding because the region contains at least five distinct control elements in the 5′ flanking region (Fig. 1) (18, 25). Recently, Lee *et al.* (26) showed that the GC-box element in coordination with the basal-level elements (BLEs) regulate basal levels of hMT-IIA transcription *in vitro*, and Karin *et al.* (25) showed that the same elements are required for optimal expression *in vivo*. The well-characterized nature of the promoter region therefore provided an ideal system in which to study the effect of DNA sequence modification on the binding of Sp1.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Synthetic oligodeoxyribonucleotides were prepared with an Applied Biosystems oligonucleotide synthesizer in the Microchemical Core Laboratory at the University of Southern California Cancer Center. Oligonucleotides were purified by gel electrophoresis (7 M urea/20% acrylamide/1% *N,N′*-methylenebisacrylamide in 89 mM Tris/89 mM boric acid/2 mM EDTA buffer, pH 8.2) and concentrated by passage through Sep-Pak C_{18} cartridges (Waters Associates), and organic contaminants were removed by Sephadex G-25 (Pharmacia) column chromatography. The phosphoramidite of 5-methylcytosine was obtained from Applied Biosystems (Foster City, CA) and the occurrence of the modified base in the synthetic oligonucleotides

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Abbreviations: hMT, human metallothionein; BLE, basal-level element.

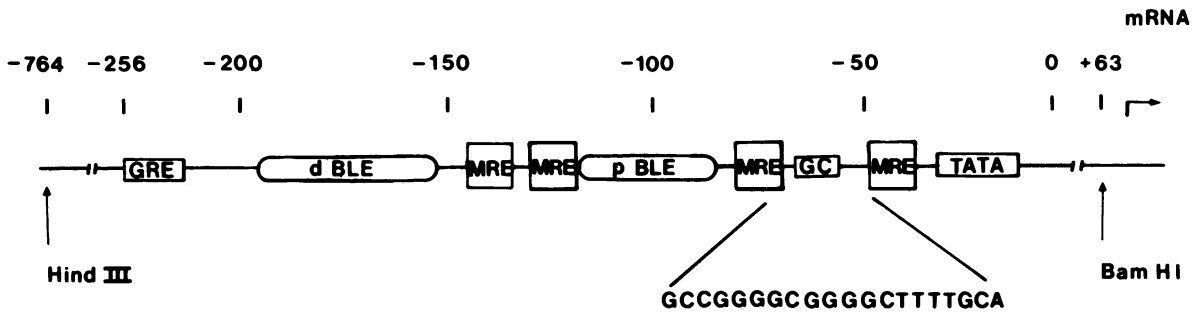

FIG. 1. Map of the hMT-IIA promoter region. The start site for transcription is indicated by the horizontal arrow. dBLE, distal basal-level element; pBLE, proximal basal-level element; GC, GC-box element; MRE, metal-responsive element; GRE, glucocorticoid-responsive element; TATA, "TATA box."

was verified by hydrolysis with formic acid followed by HPLC (27).

Fig. 2 shows the oligomers used to investigate Sp1 binding. In addition, control experiments with the following annealed 26-mers were performed: 5′ GATCCCATGATTGTGATTCTCACGAG 3′ annealed to 5′ GATCCTCGTGAGAATCACAATCATGG 3′.

Annealing Conditions. Oligonucleotides were combined in 10 mM Tris·HCl/1 mM EDTA, pH 8.0, containing 0.3 M NaCl. Mixtures were heated to 90°C for 3 min and transferred to a 65°C water bath, which was slowly brought to room temperature. Annealed oligonucleotides were then purified on Sephadex G-25 columns and electrophoresed in 10% polyacrylamide gels (acrylamide/methylene-bisacrylamide weight ratio 29.5:1) in an 89 mM Tris borate/1 mM EDTA buffer system.

HeLa Cell Nuclear Extracts. HeLa cell nuclear extracts were prepared according to the method of Dignam *et al.* (28). In brief, 3×10^9 HeLa cells were pelleted by centrifugation, washed in phosphate-buffered saline, resuspended in 10 mM Hepes, pH 7.9/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride, and homogenized in a Dounce homogenizer by 10 passes of the tight plunger. Nuclei were pelleted and homogenized in 20 mM Hepes, pH 7.9/25% (vol/vol) glycerol/0.42 M NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride. Insoluble debris was removed by centrifugation, and the supernatant was dialyzed against TM buffer (50 mM Tris·HCl, pH 7.9/20% glycerol/12.5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol) containing 0.1 M KCl. The dialyzate was centrifuged to remove insoluble debris, and the supernatant was applied to a heparin-agarose (Bio-Rad) column. Bound material was eluted in a stepwise manner with TM buffer containing 0.1, 0.2, and 0.4 M KCl, in 0.5-ml fractions. The 0.4 M KCl fractions were pooled and approximately 9 mg of protein was recovered. Preliminary studies revealed that 4 μl of extract (8 μg) adequately prevented DNase I digestion of the GC box in the hMT-IIA promoter (data not shown).

a. 5′ ACGTTGCAGCCGGGGCGGGGCTTCTGCA 3′

b. 5′ ACGTTGCAGAAGCCCCGCCCCGGCTGCA 3′

c. 5′ ACGTTGCAGCCGGGGC^{m}GGGGCTTCTGCA 3′

d. 5′ ACGTTGCAGAAGCCCC^{m}GCCCCGGCTGCA 3′

FIG. 2. Sequences of oligomers. Three series of oligomers containing 28 bases (28a–d), 20 bases (20a–d, underlined), or 14 bases (14a–d, overlined and missing the TTC and GGC residues indicated) were synthesized. Annealed oligomers were used in all experiments and are designated in the text as pairs (e.g., 28a·b is oligomer 28a annealed to 28b). Oligomers c and d contained single 5-methylcytosine residues, as indicated (m, methyl).

DNase I Protection Assays. The plasmid pH S1 (25) (see Fig. 1) was cut with *Bam*HI restriction endonuclease and labeled at the 3′ end by using the Klenow fragment of *Escherichia coli* DNA polymerase (United States Biochemical, Cleveland, OH) and [α-^{32}P]dNTPs or at the 5′ end by using calf intestinal alkaline phosphatase (Boehringer Mannheim) followed by bacteriophage T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. After digestion with *Hin*dIII (Boehringer Mannheim), the hMT-IIA promoter region (nucleotides −764 to +63) inserts were purified by gel electrophoresis. Individual assay mixtures contained 2 ng of labeled DNA (8000–10,000 cpm), 8 μg of nuclear extract (based on protein), 2% (wt/vol) polyvinyl alcohol, 1 μg of poly(deoxyinosinate-deoxycytidylate [poly(dI-dC), alternating copolymer], and the indicated concentrations of unlabeled annealed synthetic oligonucleotides. Assay mixtures were incubated on ice for 15 min prior to the addition of DNase 1 [Cooper, Malvern, PA; 4×10^{-4} units for 90 sec at room temperature (22°C)] and reactions were terminated by the addition of 100 μl of a solution containing 200 mM NaCl, 20 mM EDTA, 1% $NaDodSO_4$, and 250 μg of yeast tRNA per ml. Samples were extracted with phenol/chloroform (1:1, vol/vol) and ethanol-precipitated overnight. The precipitates were lyophilized, resuspended in 5.0 μl of sequencing sample buffer (29), and heated to 65°C for 5 min. Samples were then loaded onto sequencing gels (29), and following electrophoresis, gels were exposed to x-ray film (Kodak XAR-5) at −80°C.

Gel Retardation Assays. Oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase according to supplier specifications (New England Biolabs) and annealed as described above. Individual binding assays were carried out in 10-μl reaction mixtures containing 1 μg of HeLa cell nuclear extract, 1–2 fmol of synthetic oligonucleotide, and 260 ng of *Msp* I-digested pBR322 DNA in 25 mM Hepes, pH 7.5/1 mM EDTA/5 mM dithiothreitol/10% glycerol/150 mM NaCl. Reaction mixtures were incubated for 30 min at room temperature. Samples in a buffer containing 5% glycerol, 10 mM dithiothreitol, and 0.05% bromophenol blue were applied to 7.5% polyacrylamide gels (acrylamide/methylenebisacrylamide ratio 55:1) (30). Gels were run at 8–10 V/cm in a buffer (pH 8.0) containing 50 mM Tris and 0.38 M glycine (31). Gels were dried and exposed to x-ray film (Kodak, XAR-5) with intensifying screens at −80°C. Preliminary studies revealed that the binding reac-

tions were at equilibrium within 30 min and that all available specific binding sites were saturated (data not shown).

RESULTS

DNase I Protection Analysis. We first used DNase I protection assays to determine whether annealed double-stranded 14-mers with or without 5-methylcytosine in the CpG sequence would compete for Sp1 binding with the GC box contained in the hMT-IIA promoter. These experiments (data not shown) failed to demonstrate competition by any of the 14-mers for Sp1 binding even at a 1000-fold molar excess, presumably due to the small size of the competitors. Individual 14-mers were therefore phosphorylated, annealed, and ligated to form concatemers. Addition of a 10- or 100-fold molar excess of any of the concatemerized 14-mers resulted in inhibition of protection of the GC-box region as well as the weak Sp1 site in the proximal BLE (Fig. 3). Control experiments showed that the presence of 200 ng of a human collagen type I gene, which does not contain a GC box, did not compete for Sp1 binding (data not shown). The competition was therefore sequence-specific, and methylation of the CpG sequence in the GC box did not substantially affect Sp1 binding by the concatemers.

These results were checked by synthesizing 20-mers (Fig. 2) and testing their annealed forms for competition (Fig. 4). All 20-mers tested competed with the end-labeled hMT-IIA promoter region for Sp1 binding to the GC box and the weak site in the BLE (Fig. 4). The competition was dose-dependent (30- to 500-fold excess) and was independent of cytosine methylation. The ability of the double-stranded 20-mers to compete for Sp1 binding to the GC box was not unique to the hMT-IIA promoter, since in similar assays conducted with a hMT-IB promoter fragment, both forms of the double-stranded 20-mer successfully competed for Sp1 binding (data not shown). Results of these studies therefore revealed that synthetic fragments containing 16 complementary base pairs facilitated Sp1 binding and confirmed the earlier results that cytosine methylation of the GC box did not appear to markedly alter Sp1 binding. Although partially purified extracts were used as a source of Sp1, homogeneous purified Sp1 gave an identical protection pattern over the GC box (26). In addition, we know of no other protein in these extracts that can bind to the GC box of the hMT-IIA gene (M.I. and M.K., unpublished results). Therefore the use of partially purified extracts and the hMT-IIA GC box to monitor the effects of cytosine methylation on Sp1 binding was valid.

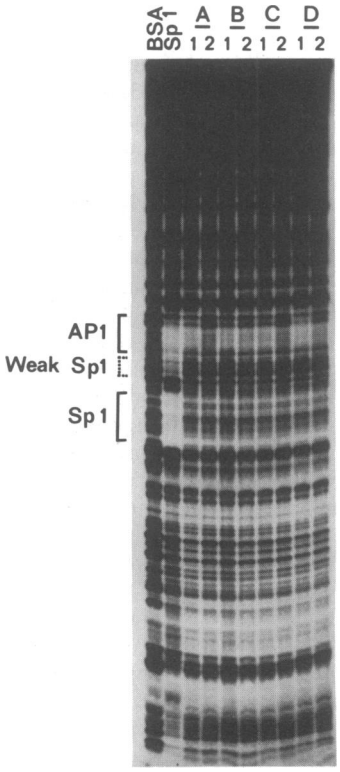

FIG. 3. Inhibition of Sp1 binding by concatemerized annealed 14-mers. Noncoding-strand probe was prepared by labeling the *Bam*HI cleavage site in pHSI, using [γ-^{32}P]ATP. AP1 refers to the binding site of activator protein 1. (*A*) Nonmethylated competitor (14a·b concatemer). (*B*) Hemimethylated competitor (14a·d concatemer). (*C*) Hemimethylated competitor (14b·c concatemer). (*D*) Methylated competitor (14c·d concatemer). Lanes: BSA, pattern of protection in presence of bovine serum albumin; Sp1, pattern of protection in presence of 8 μg of nuclear extract; 1 and 2, pattern of protection in presence of nuclear extract plus 10- or 100-fold molar excess of competitor, respectively.

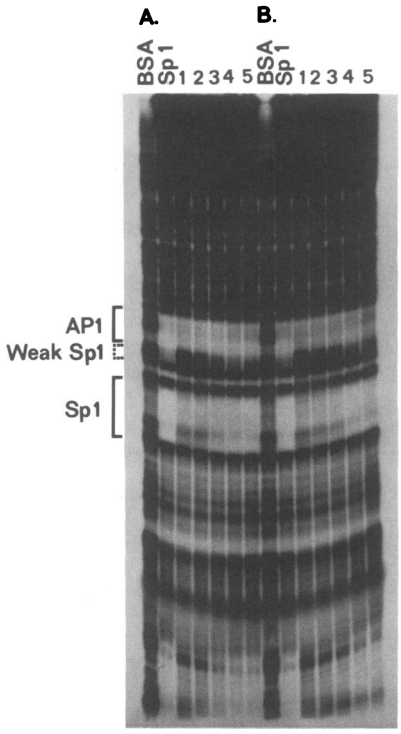

FIG. 4. Inhibition of Sp1 binding by double-stranded 20-mers. Coding-strand probe was prepared by labeling the *Bam*HI cleavage site in pHSI with [α-^{32}P]dNTPs and Klenow fragment of *E. coli* DNA polymerase I. (*A*) Nonmethylated competitor (20a·b). (*B*) Methylated competitor (20c·d). Lanes: BSA, pattern of protection in presence of bovine serum albumin; Sp1, pattern of protection in presence of 8 μg of nuclear extract; 1–5, pattern of protection in presence of nuclear extract plus 500-, 250-, 125-, 62.5-, or 31-fold molar excess of appropriate competitor.

Gel Retardation Assays. Gel retardation assays (30, 31) were conducted to assess more quantitatively the effect of cytosine methylation on the ability of Sp1 to bind to the GC-box consensus sequence. Although the DNase I protection assays suggested that the double-stranded 20-mers contained a sufficiently stable binding site for Sp1, we experienced difficulty in obtaining reproducible results on gel retardation analysis. Consequently, 28-mers containing 8 additional bases (ACGTTGCA) attached to the 5′ end of each form of the single-stranded 20-mers were synthesized (Fig. 2). When the nonmethylated form of the double-stranded 28-mer was used as a binding substrate, the majority of radiolabeled probe entered the gel unretarded in the absence of protein (Fig. 5). Addition of nuclear extract resulted in formation of complexes that were resolved into

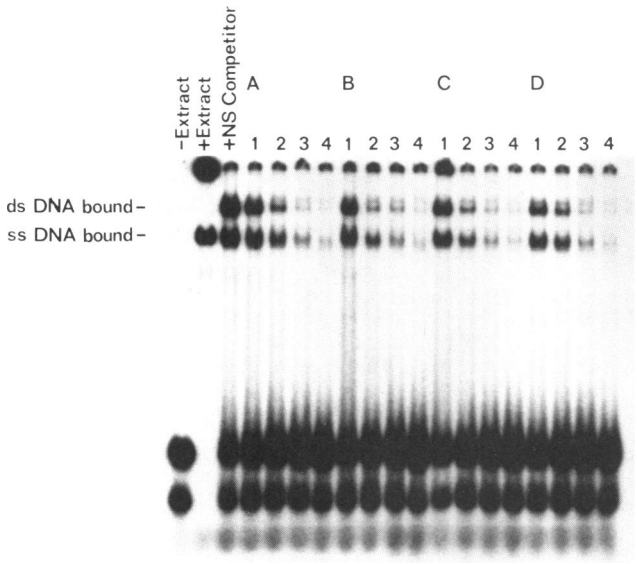

FIG. 5. Gel retardation analysis of Sp1 binding to 28-mers. Binding reactions were conducted using radiolabeled, annealed, nonmethylated 28-mers as substrates. Reactions also contained 1 μg of protein extract and 260 ng of *Msp* I-digested pBR322 DNA as a nonspecific competitor. The competitor 28-mers were nonmethylated, 28a·b (*A*); hemimethylated, 28b·c (*B*); hemimethylated, 28a·d (*C*); and fully methylated, 28c·d (*D*). Lanes: − Extract, probe alone; + Extract, probe plus 1 μg of extract; + NS competitor, probe plus nonspecific competitor with 1 μg of extract; 1–4, appropriate unlabeled competitor 28-mer (1, 3, 10, or 30 ng, respectively). ds, Double-stranded; ss, single-stranded.

two bands following the addition of nonspecific competitor DNA. The upper band represents double-stranded 28-mer complexed with protein; this band was resolved into two separate bands under some conditions, most likely due to the presence of both the 95-kDa and the 105-kDa forms of Sp1 (17). The lower band was probably due to the presence of single strands in the reaction, since its mobility was exactly that found for single-stranded oligomers incubated with the extract (data not shown).

The nonmethylated (28a·b, Fig. 5*A*) and the methylated (28c·d, Fig. 5*D*) annealed oligomers competed for complex formation in a concentration-dependent manner with the end-labeled, non-methylcytosine-containing fragment. In Fig. 5*B*, the double-stranded 28-mer competitor (28b·c) contained a single 5-methylcytosine residue, whereas in Fig. 5*C* the competitor contained a single modified base residue in the opposite strand (28a·d). Both of these hemimethylated constructs were able to compete for Sp1 binding within the same range (1–30 ng) as either the fully methylated or nonmethylated constructs. Similar results were obtained in competition assays when the radiolabeled double-stranded 28-mer contained 5-methylcytosine in both strands (data not presented). The data varied slightly from experiment to experiment but several gel retardation/competition assays showed that the presence of 5-methylcytosine in the GC-box consensus sequence did not reproducibly affect Sp1 binding. Under the conditions employed in this assay system the inhibition constants (K_i), which approximate the dissociation constants for the protein–competitor DNA complexes, are equivalent to the competitor concentration that resulted in a 50% decrease in complex formation. The values obtained when the methylated probe was end-labeled were 2.5 nM for the methylated competitor and 3.1 nM for the nonmethylated competitor. Similar values were obtained with the nonmethylated probe: 1.3 nM for the nonmethylated competitor and 1.5 nM for the methylated competitor. The effect of cytosine methylation on the internal CpG dinucleotide in the GC box had no measurable effect on Sp1 binding in this assay. However, the results do not preclude the possibility that methylation might alter which amino acid residues in Sp1 contact the target DNA sequence.

The specificity of competition on the gel retardation assays was checked in the control experiment shown in Fig. 6. An unlabeled annealed 26-mer that did not contain a GC box was not effective in competing for Sp1 binding with the labeled 28-mer. Thus the gel retardation assays were specific for GC-box-binding protein, providing further evidence that methylation of the CpG sequence did not substantially alter Sp1 binding.

DISCUSSION

The mechanisms by which transcription-controlling proteins modulate eukaryotic gene expression remain unknown. One function involved is the recognition of specific target sequences within upstream promoter regions. The consensus binding sequence for the transcription factor Sp1 has been found in a variety of eukaryotic cellular genes, protooncogenes, and viral genomes. These include several "housekeeping" genes, some of which are known to be subject to X chromosome inactivation, an effect thought to be mediated by CpG methylation (2, 3). Divergence from the consensus sequence outside the core hexanucleotide sequence does not preclude binding but instead alters the affinity of Sp1 for the site (32). The *in vitro* studies presented here show that methylation of the internal cytosine residue does not substantially alter the *in vitro* binding properties of Sp1 to the hMT-IIA promoter. Although the assays we used were shown to be sequence-specific, they may not have been sensitive enough to detect small differences in binding coefficients between methylated and nonmethylated targets that might still be biologically significant.

In addition to Sp1, the control region of the hMT-IIA gene is recognized by several other transcription factors such as AP1 (33, 34) and AP2 (40, 41). Although we only examined

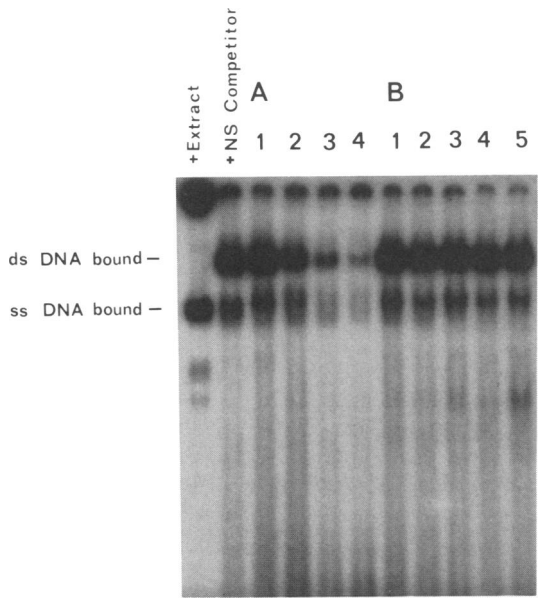

FIG. 6. Oligomers without a GC box do not compete for Sp1 binding. Binding reactions were carried out with radiolabeled, annealed, nonmethylated 28a·b. Lanes: + Extract, probe plus 1 μg of extract; + NS competitor, probe plus 1 μg of extract plus 260 μg of *Msp* I-digested pBR322 DNA. (*A*) Unlabeled 28a·b competitor (4, 8, 15, and 30 μg in lanes 1–4). (*B*) Unlabeled 26-mer without GC box (see text for sequence) as competitor (4, 8, 16, 33, and 66 ng in lanes 1–5). ds, Double-stranded; ss, single-stranded.

the effect of CpG methylation on the binding of Sp1, we do not expect that methylation will affect metallothionein gene expression by interfering with the binding of either AP1 or AP2, since neither the AP1 nor the AP2 consensus sequence contains a potential methylation site. Furthermore, *in vitro* (26) and *in vivo* (25) experiments indicate that Sp1 and the GC-box element exert the greatest effects on hMT-IIA expression.

The lack of a marked effect of methylation of the CpG dinucleotide in the GC-box consensus sequence on Sp1 binding is consistent with results of *in vivo* studies recently published. Fully methylated copies of the simian virus 40 genome that contain multiple GC boxes retain their methylation status and are fully competent with respect to early gene expression when microinjected into TC7 African green monkey cells (39). Recently, Buchanan and Gralla (36) showed that monkey cells contain a factor that binds to viral GC-box elements in a manner analogous to that observed for human Sp1. These data provide indirect *in vivo* evidence suggesting that cytosine methylation of the CpG dinucleotide within the GC box does not affect the functional activity of bound Sp1. Whether cytosine methylation of GC boxes within eukaryotic promoters will lead to an alteration in promoter functioning *in vivo* is unclear.

How alterations in the methylation pattern of certain genes alter expression while the activity of other genes remains unaffected is unknown. The presence of CpG-enriched domains in the promoter regions of numerous eukaryotic genes (35) suggests that binding of transcription-dependent protein factors could be affected. Recently, Kovesdi *et al.* (37) reported a reduction in host-cell-factor binding and functional activity of the adenovirus E1A enhancer when a cytosine residue in the internal portion of the binding site was methylated. The identification of a protein(s) that preferentially binds to methylated sequences by Huang *et al.* (38) provides an indirect mechanism through which methylation may prevent gene expression. Thus, while the binding and functional activity of positive-acting transcription factors, such as Sp1, may be indifferent to changes in methylation patterns, the presence of proteins that preferentially bind to methylated sequences may prevent binding of the specific regulatory molecules. In conjunction with our data, these specific examples demonstrate that methylation of certain CpG dinucleotides may have no effect on, decrease, or in fact target the binding of certain DNA-binding proteins.

In addition to potential effects on the binding and/or activity of regulatory proteins, the effects of methylation on the binding of proteins involved in chromatin structure need to be considered. Work by Keshet *et al.* (12) and Buschhausen *et al.* (13) is quite provocative and shows that the effects of cytosine methylation on processes that are known to be involved in regulating gene expression involve a temporal component. This suggests that the methylation signal is only transduced after the formation of an appropriate chromatin structure. Methylated sequences were transcriptionally active *in vivo* initially, but the activity eventually diminished and the transient activity in cells was eliminated by preincubating the methylated constructs with histone octamers (13). These data imply that binding of chromatin-associated proteins and/or chromatin conformation is affected by methylation. *In vivo* studies employing methylated GC-box constructs will be necessary to determine (*i*) whether the functional activity of Sp1 is retained, (*ii*) whether this change is sufficient to direct the transfected DNA into an inactive conformation, and (*iii*) whether potential protein–protein interactions with other required trans-acting factors are affected.

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