The Combination of DNA Methylation and H1 Histone Binding Inhibits the Action of a Restriction Nuclease on Plasmid DNA*

(Received for publication, November 1, 1990)

Masao Higurashi and R. David Cole

From the Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

To investigate the potentials of DNA methylation and H1 histone in regulating the action of DNA binding proteins, well ordered complexes were formed by slow salt gradient dialysis of mixtures of H1 histone with either methylated or nonmethylated DNA. The sites methylated in the plasmids were CCGG. Methylation of cytosine in this site protects the DNA against HpaII endonuclease but not against MspI. However, when the methylated DNA was complexed to H1, it was protected against MspI. The protection was only effective for a subset of the MspI restriction sites.

The protection of DNA afforded by the combination of H1 binding and DNA methylation did not apply to EcoRI, PstI, or BamHI sites and so did not seem to be due to aggregation of the DNA by H1 histone. Gel retardation assays indicated that the affinity of H1 for methylated DNA was not detectably different from its affinity for nonmethylated DNA. Probably methylated DNA when bound to H1 is in a conformation that is resistant to MspI endonuclease. Such conformational changes induced by DNA methylation and H1 binding might affect the action of other DNA binding proteins, perhaps in chromatin as well as in H1-DNA complexes.

It is widely accepted that chromatin consists of nucleosomes which are formed by DNA wound around a complex of four kinds of core histone (1, 2) and that H1 histone which is bound to the outside of the nucleosome (3-5) condenses chromatin to form higher order structures (6-10). In chemical cross-linking studies of chromatin, H1 histone homopolymers have been obtained (3), and it is evident (11, 12) that H1 histones are tandemly arrayed head to tail in the native chromatin fiber. As H1 is essential to the formation of the 30-nm chromatin fiber, the H1 array is thought to stabilize the 30-nm filament in chromatin by cross-linking nucleosomes in a salt-dependent manner (5). The 30-nm filament is generally pictured as some kind of solenoid (for review, see Ref. 13). The effects of modifications of the DNA and of histones on the fine structure and properties of nucleosomes and higher order structures is an open question.

DNA methylation which occurs in almost every higher organism is supposed to be a component in the regulation of vertebrate gene expression (for reviews, see Refs. 14-16), possibly through its effect on histone-DNA interactions. Certain unmethylated exogenous DNAs transfected into specific cultured cells are continuously transcribed in nuclei and even in cytoplasm to some extent, but when the DNA is methylated at cytosine residues in vitro before transfection, the gene expression is markedly reduced. The notion that methylation inhibits transcription is further supported by the report that the treatment of certain cell types with 5-azacytidine, which results in undermethylated DNA, also induces the activation of certain genes. Thus, it is now evident that the methylation of DNA inhibits the transcription of several genes. The mechanism of inhibition, however, is poorly understood, although it is assumed that DNA methylation affects protein-DNA interaction and induces some conformational changes in the gene (17). Such conformational changes could affect the accessibility of regulatory proteins to the control regions of certain genes. It is reasonably supposed that the higher order structure of chromatin changes when DNA is methylated because transcriptionally active chromatin is DNase I hypersensitive, whereas the same chromatin inactivated by in vitro DNA methylation is resistant to DNase I (17). Since H1 histone is involved in chromatin condensation to form higher order structures, H1 might be concerned in the control of gene expression by DNA methylation mediating a conformation change in the DNA or the chromatin.

The effects of H1 histone on DNA conformation have been studied by use of H1-DNA complexes formed in vitro, which have a cable-like appearance similar to a chromatin fiber. These complexes change their conformations depending on ionic strength (18, 19) as does native chromatin. Moreover, just as in chromatin, H1 homopolymers were observed in cross-linking experiments in H1-DNA complexes and it was found that the N-terminal tail and the globular domain of H1 were protected from cleavage by protease in H1-DNA complexes (20). Therefore, H1-DNA complexes formed in vitro seem to mimic, at least some H1-DNA interactions of native chromatin.

In the present study H1-DNA complexes were used to study the effects of DNA methylation on H1-DNA interactions. We first formed complexes between H1 histone and linear DNA by salt gradient dialysis and characterized the complexes at different ionic strengths by sedimentation analysis, circular dichroism, and electron microscopy to show that H1 DNA complexes formed under certain experimental conditions had a definite structure. Next, we investigated the effect of DNA methylation on the H1-DNA interaction in such H1-DNA complexes by using endonuclease protection assays. Methylated DNA when it was complexed to H1 histone was quite resistant to digestion by the restriction nuclease MspI even if treated with excess enzyme while naked methylated DNA was readily digested. In contrast, unmethylated DNA was easily digested by MspI whether or not the DNA was complexed by H1 histone.

MATERIALS AND METHODS

Preparation of DNA—In the preparation of unmethylated DNA, two strains of Escherichia coli, TG-1 (dam", dcm") and JM110 (dam", 8619)
DNA Methylation and H1 Binding Inhibit Nuclease

dcm−) were used as host cell for the plasmid (pc-1, in which about 3800 base pairs of calf DNA had been inserted into the vector pTZ19R). Plasmid DNA was isolated from E. coli by an alkaline lysis method (21). The nucleic acid preparation treated with RNase was integrity of 2.5×106 polyethylene glycol 8000 in 0.5 M NaCl for 1 min on ice, and the mixture was centrifuged at 12,000 × g for 15 min at 4°C. The plasmid DNA was extracted from the pellet by two treatments with one volume of phenol saturated with 0.1 M Tris, pH 8.0, and 1 volume of chloroform-isooamyl alcohol (24:1) and one treatment with chloroform-isooamyl alcohol; it was further purified in a cesium chloride density gradient containing 0.6 mg/ml ethidium bromide as described previously (21). To obtain linear DNA, purified superhelical DNA was treated with the restriction endonuclease XhoI.

For labeling plasmid DNA with [3H]thymidine, E. coli was grown in M9 medium supplemented with 0.1% casamino acids, 0.001% vitamin B6, 0.01% leucine, 0.01% proline, 0.5 mM mg/ml ampicillin, and, in the while the logarithmic phase of growth (A600nm = 0.7–0.8), chloramphenicol was added to the culture at 25 μg/ml; 50 min later [H]thymidine (specific activity, 77 Ci/mmol: Amersham Corp.) was added at 1 μCi/ml, and the culture was incubated overnight at 37°C with vigorous shaking.

Oligodeoxynucleotides (CCGCGG)12 and (C5CGG)12 were synthesized by the GIBA-synthesis technique. Oligo-C5-deoxythymyl 5-methyl-2'-deoxycytidine phosphoramidate (Glen Research) was used for the synthesis of CCGCGG12. For de-salting, samples were absorbed onto C-18 Sep-Pak cartridges (Waters Associates) and eluted with 30% acetonitrile in 50 mM triethylammonium acetate, 0.1% TFA. To wash the column, was added 20 volumes of distilled water. The desired full-length oligonucleotides were purified on a 12% polyacrylamide gel (3 mm thick) containing 7 M urea (21).

The desired histone DNA complexes were formed at various ionic strengths and characterized by sedimentation velocity analysis with metrizamide density gradients (5–20%) containing 5 mM Tris, pH 7.5, 1 mM EDTA, 15 M NaCl with 0.4 mM of [(5% methylated) histone DNA complexes essentially as described previously (24). Fractions of eight drops were collected from the bottom of the tubes, and the radioactivities of the fractions were measured with a liquid scintillation counter. Centrifuge tubes (polyethylene) were washed by rinsing in a 5% solution of dichlorodimethylsilane in chloroform.

Electron Microscopy—Two different methods of specimen preparation for electron microscopy were used. A droplet technique was used as follows: soluble materials were adsorbed to a formvar/carbon coated grid which had been made hydrophilic by glow-discharge according to the method described previously (25). For aggregated material the Miller spreading technique modified by Labhart and Koller (26) was used. Samples in a solution of 1% formaldehyde, 0.1 M sucrose, 0.5 mM sodium borate, pH 8.5, in centrifuge chambers were centrifuged onto copper grids for 20 min at 3,500 × g at 4°C using a Sorvall HB-4 rotor. Samples were washed with distilled water and stained with a 1% solution of uranyl acetate for 1 min. H1-DNA complex for Miller spreading were formed in a buffer of 5 mM triethanolamine, 1 mM EDTA, pH 7.5, instead of 5 mM Tris-HCl, 1 mM EDTA, pH 7.5.

Determination of DNA and Protein Concentration—The DNA concentration was determined by absorption at 260 nm, assuming EG39 = 1.0. The protein concentration was determined by the method of Lowry et al. (27) calibrated with a standard of bovine serum albumin. The concentrations of oligo(CCGG) and (C5CGG) were determined by absorbance, assuming ε260 = 0.2 N NaOH to be 7,300, 5,700, 11,400, 14,200 for DNA, dCp, dCp, and dGp, respectively.

Circular Dichroism—Samples were diluted 10-fold with the buffer used for dialysis and circular dichroism spectra were measured at 20°C on a Jasco spectrophotometer using a 1-cm cuvette.

Agarose Gel Electrophoresis—Agarose gel electrophoresis and polyacrylamide gel electrophoresis for DNA were performed in either buffer I (2 mM EDTA, 80 mM Tris, 67 mM phosphoric acid, pH 7.8) or buffer II (2 mM EDTA, 89 mM Tris, 89 mM boric acid, pH 7.8), respectively, as described previously (21). The gels were stained with 0.5 μg/ml ethidium bromide. Autoradiograms were made from dried gels, exposing them to Kodak XAR-5 films. Band intensities were obtained from densitometric analysis of the autoradiograms by 2292 ULTRASCAN laser densitometer (LKB Broma).

Digestion of H1-DNA Complexes with Restriction Endonuclease—H1-DNA complexes in 10 μl of the buffers in which they were prepared were diluted 2-fold with 20 mM MgCl2, 15 mM Tris, 2 mM dithiothreitol, pH 7.5, containing certain concentrations of NaCl depending on a desired final NaCl concentration, and incubated with various amounts of enzyme at 37°C for 1 h. Digested DNA was extracted with phenol-chloroform/isooamyl alcohol once and chloroform-isooamyl alcohol once after addition of 1/20 volume of 0.5 M EDTA and 1/20 volume of 10% sodium dodecyl sulfate.

RESULTS

Characterization of H1 Histone-Linear DNA Complexes—To test the effects of DNA methylation we studied well defined complexes of DNA and H1 histone. The H1-DNA complexes were formed at various ionic strengths and characterized by sedimentation velocity analysis with metrizamide density gradient. Also, by electron microscopy. For the formation of H1-DNA complexes, mixtures of purified [3H]histone H1 from HeLa cells and double-stranded linear plasmid [3H]DNA (see Fig. 1) were dialyzed overnight against a buffer (5 mM Tris, 1 mM EDTA, pH 7.5)

FIG. 1. Partial restriction map of pc-1. Numbering is from XhoI site. The heavy line corresponds to the insert of calf DNA.
DNA Methylation and H1 Binding Inhibit Nuclease

containing 0.6 M NaCl and further dialyzed against the same buffer but with stepwise decreases in salt concentration to 0.15 M NaCl as described previously (23). As shown in Fig. 2 (top), H1-DNA complexes sedimented to a cushion of 75% metrizamide at the bottom of the gradient, whereas free DNA entered the gradient to a level of about 8% metrizamide in all cases when various H1:DNA input ratios were used. The absence of complexes intermediate between naked DNA and fully complexed DNA indicated that H1 histone had bound to linear DNA cooperatively under the experimental conditions used. It has been shown that all the H1 is bound to DNA under these conditions (24). The distribution of the DNA between rapidly sedimenting H1-DNA complexes and free DNA was proportional to the H1:DNA weight ratio (input) at a salt concentration of 0.15 M NaCl (Fig. 2, bottom). The H1:DNA ratio of the complexes that sedimented to the bottom of the gradient was calculated to be 0.47 by extrapolation along the line shown in Fig. 2 (bottom). As discussed later, these results are consistent with previous reports (20, 24).

The H1-DNA mixtures containing free DNA and complexes showed negative molar ellipticity from about 290 nm at least through 250 nm at 0.15 M NaCl, although naked DNA shows a modestly positive ellipticity at 270 nm (Fig. 3). Under these salt conditions, the H1-DNA mixtures gave negative molar ellipticities at 270 nm essentially in proportion to the input H1:DNA ratio. Since the amount of H1-DNA complex was proportional to the H1:DNA ratio, H1-DNA complexes must have produced substantially more negative molar ellipticity than the mixtures. As reported previously (23) the negative molar ellipticities from about 280 through 250 nm changed markedly depending on ionic strength. CD spectra of H1-DNA complexes that were formed at an input H1:DNA weight ratio of 0.4 were recorded at various NaCl concentrations and molar ellipticities at 270 nm are plotted as a function of NaCl concentration in Fig. 4. At 0.6-0.4 M NaCl the molar ellipticities of H1-DNA mixtures at 270 nm were approximately +8000 deg. cm².dmoll⁻¹, like that of naked DNA. As the concentration of NaCl was decreased, the positive molar ellipticity was eliminated and the molar ellipticities became increasingly negative to a negative extreme at about 0.25-0.20 M NaCl. The absolute magnitude of [θ]₂₇₀ at the negative extreme varied depending on the size and concentration of the DNA used in any particular experiment. With further decreases in the concentration of NaCl, molar ellipticities increased progressively until the same CD spectrum as free DNA was obtained at 0-0.5 M NaCl. It was shown previously (28) for H1-DNA complexes that the gradual change of molar ellipticity

![Fig. 2. Sedimentation velocity profiles of complexes of H1 histone and linear plasmid DNA. Top, [¹³C]H1 histone from HeLa cells and [³²P]DNA complexes were formed by salt gradient dialysis at different H1/DNA weight ratios and analyzed in 5-20% metrizamide density gradient on top of cushion of 75% metrizamide. Sedimentation was from right to left. a, H1/DNA = 0.15; b, H1/DNA = 0.26; c, H1/DNA = 0.42; d, H1/DNA = 0.57. Bottom, the percentage of the DNA sedimented onto a cushion at the bottom of each tube was plotted against H1/DNA weight ratio. The weight ratios were calculated from the radioactivities of samples after dialysis.](image)

![Fig. 3. Circular dichroism of complexes. H1-DNA complexes were formed at different H1/DNA weight ratios, and CD spectra were measured at DNA concentrations of 5-10 µg/ml. H1/DNA ratios were 0.16, 0.26 and 0.40 respectively, top to bottom.](image)

![Fig. 4. Change in CD spectra of H1-DNA complexes by increasing ionic strength. CD spectra of H1-DNA complexes were measured in different NaCl concentrations, and the CD parameter θ₂₇₀ was plotted as a function of NaCl concentration.](image)
ellipticity induced by increasing the NaCl concentration from 0 to 0.30 M was not directly related to the amount of aggregation ($A_{260}$), and therefore the salt concentration must have affected the structural nature of the complexes as well as the amount of aggregation. The negative molar ellipticities from about 250 to 300 nm have been postulated to result from side-to-side DNA interactions (29, 30).

To examine the relation between the structure of H1-DNA complexes and the negative molar ellipticity induced by moderate concentrations of NaCl, H1-DNA complexes were formed at various ionic strengths as described above and then analyzed by electron microscopy using the Miller spreading technique. At 0.35 M NaCl H1-DNA complexes appeared to be balls of tangled nucleoprotein fibers. With progressive decreases of the ionic strength, the H1-DNA complexes became rod-like, with small branches and whiskers extending from the rod. The rods became longer and more slender as the NaCl was lowered from 0.25 to 0.20 M to 0.15 M and the branches more numerous. When the salt concentration was lowered to 0.10 M, the dispersal of the rod-like form into thinner branches was dramatically increased so that the thin entwined branches completely dominated the electron micrographs of these H1-DNA complexes. The typical appearance of the complexes at 0.10 M NaCl shown by electron microscopy using the Miller spreading technique was a condensed fiber which had a thickness approximately 50–70 nm and a length more than 1,000 nm (Fig. 5). Moreover, the many whiskers which protruded from the condensed part of the fiber appeared to be single filaments of double-stranded DNA, but it is not clear whether this DNA had H1 histone bound to it. Judging from the length of condensed fiber, it must have been constructed by overlapping the ends of some DNA fibers in addition to packing them side-by-side. At 0.05 M NaCl, H1 DNA complexes had a still thinner fiber-like appearance that did not appear to be single filaments of DNA by binding H1 to it in direct mixing at the weigh ratio of 0.4 was small relative to the distortion observed when the H1-DNA complexes were formed by direct mixing or dialysis from 0.60 M NaCl to the terminal concentration (data not shown). Since the circular dichroism depends on regular periodic binding of H1 histone to linear DNA. The complexes of DNA by binding H1 to it in direct mixing at the weigh ratio of 0.4 was small relative to the distortion observed when the H1-DNA complexes were formed by stepwise dialysis from 0.60 M NaCl to the terminal concentration (data not shown). The distortion of the CD spectrum of DNA by binding H1 to it in direct mixing at the weigh ratio of 0.4 was small relative to the distortion observed when the H1-DNA complexes were formed by direct mixing or dialysis from 0.05 M NaCl which produced structures much like those seen in Fig. 5e, although the fiber was shorter than that of the latter complexes and thicker. The structure changes of H1-DNA complexes which are dependent on ionic strength might be fully reversible if the salt conditions were optimized. When the NaCl concentration of the sample solution was increased from 0 to 0.15 M rapidly by adding concentrated NaCl solution, negative molar ellipticities from 250 nm through to 300 nm were not observed.

We conclude that H1-DNA complexes formed by stepwise dialysis under experimental conditions described above have a definite structure with variable H1:DNA ratio, independent of the H1:DNA input ratio probably because of the cooperative binding of H1 histone to linear DNA. The complexes have a specific regular structure at any particular salt concentration as evidenced by CD and electron microscopy, but to prepare relatively homogeneous populations of those structures, the H1-DNA complexes need to be formed by stepwise dialysis rather than direct mixing.

**The Effect of DNA Methylation on H1-DNA Interactions—** To investigate the effect of cytosine methylation on the H1-DNA interaction, complexes were formed by stepwise salt gradient dialysis of mixtures of purified mouse liver H1 histones and linear plasmid DNA which was either untreated or treated with HpaII methylase (designated (u)DNA), was tested it was found to be sensitive to both MsPI and HpaII endonucleases. As controls, naked DNA was tested (Fig. 6) in both its methylated and unmethylated forms. First, when DNA, untreated with HpaII methylase (designated (u)DNA), was tested it was found to be sensitive to both MsPI and HpaII endonuclease. Then, naked DNA methylated in vitro with DNA Methylation and H1 Binding Inhibit Nuclease
explained by a change in the conformation of the DNA or by
 Thus, the protection of the CCGG site against the action of
MspI requires two conditions: methylation of the site and
MspI/HpaII sites in H1. DNA complexes. Analysis by circular
dichroism and
MspI endonuclease, even when much aggregation is
shown in Fig. 2, when H1 histone and linear DNA were mixed
and H1DNA complexes were formed under appropriate ex-
perimental conditions, a part of the DNA formed complexes
with H1 histone in 0.60 M NaCl containing buffer at an H1/DNA ratio
that had been methylated with HpdI methylase and with H1
binding inhibit nuclease.

**Fig. 6.** Agarose gel electrophoresis of naked DNA methylated in vitro or unmethylated DNA was digested with 1 unit of HpaII or
MspI in low salt buffer (10 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM
dithiothreitol) for 60 min. Lanes 1 and 2, untreated DNA; lanes 3 and
4, methylated DNA; lanes 1 and 3, HpaII digests; lanes 2 and 4, Msp I
digests; lane m, DNA size markers (ΔDNA digested with BstEII). b, DNA was digested with MspI in high salt buffer (100 mM
NaCl, 10 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol) for 1 h.
Lanes 1–6, methylated DNA; lanes 7–12, untreated DNA; lanes 1
and 7, 0 min; lanes 2 and 8, 0.5 min; lanes 3 and 9, 1 min; lanes 4
and 10, 2 min; lanes 5 and 11, 4 min; lanes 6 and 12, 8 min. c, time
course of MspI digestion in low salt buffer. Lanes 1–6, methylated
dNA; lanes 7–12, untreated DNA; lanes 1 and 7, 0 min; lanes 2 and
5, 5 min; lanes 3 and 9, 10 min; lanes 4 and 10, 20 min; lanes 5 and
11, 40 min; lanes 6 and 12, 80 min. d, HpaII digestion in low salt
buffer for 60 min. Lanes 1–5, methylated DNA; lanes 6–8, untreated
DNA; lanes 1 and 6, 0 min; lanes 2 and 7, 1 min; lanes 3 and 8, 2
units; lanes 4 and 9, 4 units; lanes 5, 8 units.

HpaII methylase (designed (m)DNA) was tested, and although
(m)DNA was still quite sensitive to MspI endonuclease digestion,
even though the two restriction enzymes are both specific for the
sequence of CCGG. The all or none protection by methylase
shows that the internal cytosines of all the HpaII sites
(CCGG) in the plasmid DNA (see Fig. 1) must have been
completely methylated after treatment with HpdI. The methy-
alization with HpaII methylase clearly interfered with HpaII
digestion, but it was ineffective for protection (Fig. 2).

**Fig. 7.** Digestion of H1-DNA complexes with MspI. H1-
DNA complexes were formed by salt gradient dialysis and digested
with different amounts of enzymes. a: lanes 1–6, (m)DNA-H1 com-
plexes; lanes 8–11, (u)DNA-H1 complexes; lanes 1 and 7, 0 min;
lanes 2 and 8, 4 units; lanes 3 and 9, 8 units; lanes 4 and 10, 16 units; lanes
5 and 11, 22.5 units; lane 6, 55 units. b: lanes 1, plasmid DNA,
untreated; lanes 2, 4, and 6, H1-(m)DNA complexes; lanes 3, 5, and
7, naked DNA; lanes 2 and 3, EcoRI digest with 10 units in a 100 mM
NaCl; lanes 2, 6, and 7, BamHI digest with 10 units in a 50 mM NaCl.

H1-DNA complexes were formed and digested with MspI.
Unlike naked (m)DNA, H1-(m)DNA complexes were resis-
tant to MspI digestion as well as to HpaII nuclease digestion as
shown in Fig. 7a. In contrast, the DNA in H1-(u)DNA complexes
was digested completely by MspI. Digestion of H1-(u)DNA complexes showed that complex formation did not interfer-
completely with digestion of the DNA by MspI. Some
MspI/HpaII sites in H1-(m)DNA complexes were protected
from MspI even when a great excess (55 units) of enzyme was
added. As little as 8 units of enzyme was enough for the
complete digestion of 1 µg of (u)DNA in H1-DNA complexes.
Thus, the protection of the CCGG site against the action of
MspI requires two conditions: methylation of the site and
interaction between the DNA and H1 histone. Neither of the
two conditions is sufficient alone. The protection could be
explained by a change in the conformation of the DNA or by
a masking of the site either through the direct binding of H1
or through aggregation of many nucleoprotein fibers that
contain the sequences. Analysis by circular dichroism and
electron microscopy failed to detect any difference between
H1-(m)DNA complexes and H1-(u)DNA complexes (data not
shown) in the amounts or the nature of the structures they
formed. Aggregation thus seems to be ruled out as an expla-
nation for the protection of the methylated sites, especially
since methylated DNA-H1 complexes were not protected
against other nuclease.

**Competition Experiment between Methylated and Unmethy-
alized Oligonucleotides—**An experiment was designed to test
the possibility that the methylated MspI site binds H1 histone
much more strongly that does the unmethylated site. As
shown in Fig. 2, when H1 histone and linear DNA were mixed
and H1-DNA complexes were formed under appropriate ex-
perimental conditions, a part of the DNA formed complexes
and the rest of the DNA remained free. In other words, H1
histones bind to DNA cooperatively. If H1 histone binds
preferentially to methylated sites of DNA, methylated DNA
would preferentially form H1-DNA complexes. Therefore,
if limited amounts of H1 were available in a mixture of (m)DNA
and (u)DNA, the H1-DNA complexes formed would be en-
riched in (m)DNA, whereas the free DNA would be enriched in
(u)DNA. Unmethylated DNA was mixed (20:1) with DNA
that had been methylated with HpaII methylase and with H1
histone in 0.60 M NaCl containing buffer at an H1/DNA ratio
of 0.2. H1-DNA complexes were formed by decreasing the
ionic strength through stepwise dialysis to 0.15 M NaCl. The
H1-DNA complexes and the free DNA were separated by
metrizamide density gradient centrifugation. The distribution
of methylated DNA was determined from its radioactivity since it had been end-labeled with $^{32}$P]ATP using polynucleotide kinase. As shown in Fig. 8, H1-DNA complexes sedimented at fractions 2-4 and the free DNA sedimented at fractions 17-23. The methylated DNA was distributed between H1-DNA complexes and naked DNA fractions in parallel with the distribution of the nonradioactive nonmethylated DNA. Therefore, if (m)DNA binds H1 more strongly than (u)DNA does, the ratio of affinities is substantially less than 20:1 (including the cooperativity factor).

Since it was possible that nonspecific binding of H1 to the very large surface of general DNA could obscure the specific binding of H1 to the CCGG site, a different experiment was done that eliminated this possibility. (CmCGG)-polymer was synthesized, and its interaction with H1 was compared with the interaction of H1 and a CCGG polymer. Double-stranded (CCGG) dodecamer (24 base pairs) and H1 histone were mixed in a buffer of 0.10 M NaCl, 5 mM Tris, 1 mM EDTA, 0.5 mg/ml bovine serum albumin and incubated 5 h before analysis by a gel retardation assay. As shown in Fig. 9, when the H1/DNA ratio was increased, the electrophoretic band at the position of free DNA gradually disappeared, suggesting that H1 histone was progressively bound to oligonucleotide. Assuming that only one H1 molecule could bind to each oligonucleotide, because the oligonucleotide was smaller than the expected size of the H1 binding site, binding constants were calculated after densitometry of the autoradiograms as indicated in Fig. 2. The DNA in major peak fractions was extracted from each fraction and analyzed for radioactivity by a gel retardation assay. As shown in Fig. 9, when the H1/DNA ratio was increased, the electrophoretic band at the position of free DNA gradually disappeared, suggesting that H1 histone was progressively bound to oligonucleotide. The result means that methylation does not increase the affinity of H1 for CCGG site substantially. A competition experiment using a small amount of (CmCGG)$_{12}$ labeled with $^{32}$P, and a large amount of unlabeled (CCGG)$_{12}$ also did not indicate any significant difference between the two oligonucleotides (data not shown). Probably then, methylation of the CCGG site and its interaction with histone H1 change the conformation so that it is no longer susceptible to MspI.

**DISCUSSION**

**Characterization of H1-DNA Complexes**—The present investigation of H1-DNA complexes has confirmed that H1-DNA complexes have a definite structure appearing as a thick fiber or cable, which seems to represent the side-by-side aggregation of nucleoprotein fibers. It is also clear that the structure of H1-DNA complexes varied depending on ionic strength, DNA size, DNA concentration, and method of formation. When H1-DNA complexes were formed by direct mixing the circular dichroism spectrum of the DNA was distorted less than in the case of H1-DNA complexes formed by dialysis, indicating a more ordered structure in the latter case. In the present study, H1-DNA complexes that had been formed by the dialysis method were characterized by sedimentation velocity analysis, circular dichroism, and electron microscopy, which demonstrated that H1-DNA complexes formed under particular experimental conditions have a specific character and appearance. As shown in Fig. 2, when the H1-DNA ratio was less than that required to saturate the DNA, the H1 bound to only a fraction of the DNA, forming stable H1-DNA complexes, whereas the rest of the DNA remained free; that is, H1 histone bound to linear DNA cooperatively, as consistent with previous work (20, 24, 31, 32). Furthermore, electron microscopy (Fig. 5) revealed that the H1-DNA complexes prepared by the dialysis method were more regularly packed and more homogeneous in form than those prepared by the direct mixing method, probably because H1-DNA complexes were formed gradually, step-by-step, minimizing more random (and relatively irreversible) aggregation.

**Effect of DNA Methylation on H1-DNA Complexes**—In Fig. 7, we demonstrated that when the DNA was associated with H1 histone CCGG sites methylated in vitro with HpaII methyltransferase were rendered resistant to MspI digestion, whereas unmethylated CCGG sites were not. The protection of CmCGG sites with H1 histone against MspI digestion is not likely due to the general aggregation of H1-DNA fibers, since EcoRI sites, PstI sites, and BamHI sites in methylated DNA-H1 complexes were digested as well as in unmethylated DNA-H1 complexes (Fig. 7b). Neither was the protection due to
increased masking of CCGG sites by tighter binding of H1 histones, since the affinity of H1 toward methylated DNA was not greater than that toward unmethylated DNA; in fact, ρ was less. Therefore, it is suggested that the conformation of DNA must have been different in H1-DNA complexes when the cytosine residue of CCGG in DNA was methylated and associated with H1 than when the DNA in the complex was unmethylated. Although both naked (m)DNA and naked (u)DNA could be fully digested by MspI, the digestion of (m)DNA was slower than that of (u)DNA (Fig. 6b). Such would be the case if (m)DNA were in equilibrium between two conformational states, one of which was nuclease-resistant. Stabilization of both conformations by H1 binding could slow down the equilibrium enough to prevent effective conversion of the resistant conformation to the susceptible one during the digestion under the conditions used here. In the experiment of Fig. 7, the digestion of H1-mlDNA complexes was observed to be partial, and even when the amount of enzyme was increased from 8 to 55 units, no detectable increase of digestion resulted. This suggests that if there are two conformations the rate of their interconversion is rate limiting during nuclear digestion.

Now, it is widely accepted that DNA methylation, especially cytosine methylation itself is sufficient for the inactivation of particular genes in certain cell types. Recent studies reveal that cytosine methylation of DNA at the promoter region of gene is critical for gene inactivation. Although the mechanism of inactivation of methylated DNA is poorly understood, it is clear that the repression of the methylated genes requires the formation of chromatin (33). It was reported that methylated DNA in nuclei (or chromatin) which was transcriptionally inactive was relatively resistant to DNase I treatment compared with transcriptionally active unmethylated DNA (17). Similarly, methylated HpaII/MspI sites in nuclei are more resistant to MspI digestion than the unmethylated sites (34). This indicates that the accessibility of methylated DNA to some enzymes in nuclei is hindered. The present results reveal that H1 histone has a potential for a supporting role in the repression of methylated genes. In chromatin as well as in H1-DNA complexes, H1 histone may induce or stabilize a conformational change in methylated DNA that is protective against the association of some DNA binding proteins, perhaps including transcription factors as well as certain nuclease.

REFERENCES