Methylation of chromatin DNA

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ABSTRACT

E. coli DNA methylase has been used to methylate chromatin DNA *in vitro*. At saturation only 50% of the chromatin DNA becomes methylated. The methylated regions of chromatin correspond to that fraction of the chromatin which is sensitive to staphylococcal nuclease. Using *in vitro* methylated chromatin followed by nuclease digestion movement of chromatin proteins along the DNA can be detected. By this criterion, sonication of chromatin or precipitation with $MnCl_2$ causes 10% of the previously uncovered methylated regions to become covered by protein. Reconstitution of methylated chromatin results in the randomization of the chromatin proteins. Using nuclei which were methylated *in vitro* we have demonstrated that a small degree of protein sliding does occur during the preparation of chromatin from nuclei. Finally, we have prepared open region DNA by polylysine titration. This procedure does not cause displacement of chromatin proteins.

INTRODUCTION

In order to better understand the process of transcriptional control *in vivo*, many laboratories have concentrated on studying the properties of chromatin *in vitro*. Chromatin seems to maintain many of the properties of the *in vivo* substrate. Axel *et al.* (1) and Gilmour and Paul (2) have demonstrated that hemoglobin RNA can be synthesized *in vitro* from reticulocyte chromatin, but not from chromatin isolated from other tissues. Furthermore, Axel *et al.* (3) have also shown that the localization of the chromatin proteins in the region of the hemoglobin gene is altered in tissues which express the gene. Although it is clear from these experiments that the chromatin proteins affect gene expression the relationship between the structure and function remains a mystery. One of the key questions in this problem is whether chromatin proteins are free to move around on the DNA, and whether protein movement affects gene expression. In order to study this problem we have developed an assay for measuring the movement of chromatin proteins along the DNA.

In previous studies, Clark and Felsenfeld (4) have measured the accessibility of the DNA in chromatin to various chemical probes. About half the DNA

is accessible to titration with divalent cations, histones, or polylysine and to digestion by staphylococcal nuclease. The DNA resistant to digestion consists principally of a series of double stranded fractions of discrete lengths ranging between about 150 and 45 base pairs (5). Using DNA methylase from E. coli we can selectively label those parts of the chromatin which are relatively free of protein and completely digestible by nuclease. Any movement of protein along this specially labelled chromatin will result in the conversion of the labelled DNA from nuclease sensitive to nuclease resistant. Using this assay we have shown that laboratory handling of chromatin does not cause a large amount of protein movement.

MATERIALS AND METHODS

a) Materials - (^3H)-S-adenosylmethionine (7.5 Ci/mmole) and (^3H)-thymidine (30 Ci/mmole) were purchased from New England Nuclear Corp. Poly-D-lysine hydrobromide (PDL) was obtained from Sigma. It had an average molecular weight of 70,000. Purified staphylococcal nuclease and RNase free of DNase was purchased from Worthington Biochemical Co. and Pronase B was from Calbiochem. E. coli exonuclease I was a gift from A. Razin.

b) Preparation of DNA and chromatin - Chicken erythrocyte chromatin was prepared from Triton and salt washed nuclei by a step-wise reduction in ionic strength as previously described (1). The final preparation was sheared to an average DNA molecular weight of $6-8 \times 10^6$ in a Waring blender (1 min at 90 V). Chromatin prepared in this way had a protein/DNA ratio of 1.3 g/g and was stable for up to 6 weeks at 4°C . DNA was prepared from isolated nuclei as described (1).

c) DNA methylase - DNA methylase was extracted from E. coli C grown to mid log phase and partially purified according to the method of Marinus and Morris (6). The enzyme was prepared from frozen cells by alumina grinding and stored at -70°C in 10% glycerol at a concentration of 20 mg/ml protein. Methylation was carried out in 250 λ containing 50 mM Tris-HCl (pH 7.9), 5 mM dithiothreitol, 2 μCi (^3H)-S-adenosylmethionine (7.5 Ci/mmole), DNA or chromatin and 0.5 to 1 mg DNA methylase. In some experiments non-radioactive S-adenosylmethionine was added to a concentration of 0.1 mM. In order to determine the extent of methylation of DNA, the reaction mixtures were treated in order to remove RNA and protein. To this end 200 μg carrier DNA was added; the reaction mix was brought to 8% sodium dodecylsulfate and heated at 60°C for 10 min. After adding NaClO_4 (0.5 M final concentration) the samples were brought to 2.0 ml, extracted with an equal volume of chloroform: isoamylalcohol (24:1), and the aqueous phase

precipitated with 2 volumes of 0.5 M perchloroacetic acid. The precipitate was taken up in 0.5 ml of 0.5 M NaOH and heated at 60°C for 10 min in order to digest any RNA and the remaining DNA was precipitated by the addition of 2 ml 10% trichloroacetic acid. The precipitate was collected on glass fiber filters and washed 6 times with 3 ml 5% trichloroacetic acid, dried and the radioactivity determined by liquid scintillation counting.

When methylated chromatin was digested by staphylococcal nuclease, the reaction mix was brought to 1 ml containing 10 mM Tris-HCl (pH 7.9), 0.1 mM CaCl_2 . Staphylococcal nuclease (10 μg) was added and the reaction carried out at 37°C for 1.5 hrs. Under these conditions 50% of the chromatin DNA was digested as determined by the method of Clark and Felsenfeld (4). For determination of the amount of methylated DNA remaining after digestion, the nuclease assay mix was brought to 8% sodium dodecylsulfate and treated as described above.

RESULTS

Methylation of DNA and chromatin - When *E. coli* DNA methylase is incubated with DNA from another source in the presence of (^3H)-S-adenosylmethionine the methylation of DNA proceeds until saturation is reached (7). Using chicken DNA we find that at saturation, 9.5 out of 10,000 nucleotides in DNA have undergone methylation (Table 1). That this was indeed the maximum amount of methylation was shown by the fact that the addition of twice the amount of enzyme or the addition of one half the amount of substrate did not influence the density of methylation. All of the label incorporated into the DNA was shown to be in 5-methylcytosine and 6-methyladenine by chromatography of the bases produced by trifluoroacetic acid treatment of the DNA (8).

When DNA is titrated with poly-D-lysine, the areas of DNA covered by this polypeptide are protected from digestion with staphylococcal nuclease. Thus, if 50% of the DNA phosphates are covered by poly-D-lysine, 50% of the DNA is rendered nuclease resistant. When these poly-D-lysine complexes are treated with *E. coli* DNA methylase under saturating conditions, only a limited amount of DNA is available for methylation. The degree of methylation is proportional to the amount of DNA which is not covered and therefore sensitive to nuclease digestion (Table 1). Chromatin which is 50% digestible by staphylococcal nuclease, has 50% as many available sites for methylation as native DNA. Thus only half of the DNA in chromatin is available to react with DNA methylase even under saturating conditions.

The fact that there are half as many sites for methylation on chromatin

TABLE 1

Substrate	DNA Methylation (per 10,000 nucleotides)	DNA Methylation (% of DNA)	Nuclease Digestibility (%)
DNA	9.5	100	100
Chromatin	4.3	44	48
DNA-PDL ₁	7.3	80	81
DNA-PDL ₂	6.3	70	68
DNA-PDL ₃	5.0	58	55

Methylation of DNA and chromatin - Methylation was carried out for 120 min at 37°C as described in Materials and Methods using 0.1 mM S-adenosyl-methionine. Each reaction mix contained either 25 µg or 50 µg of substrate and 1 mg of methylase. Similar results were obtained with both substrate concentrations and the average is shown in the table. Under these conditions methylation of each substrate reaches saturation and incubation for longer time or the addition of more enzyme did not increase the amount of methylation. DNA-PDL complexes were prepared and tested for nuclease digestibility as described by Clark and Felsenfeld (4). Incorporation of (³H)-S-adenosyl-methionine in the absence of added DNA was less than 1% of the incorporation in the presence of 50 µg of DNA.

as compared to DNA could also be shown by kinetic experiments. When the initial rate of methylation was measured at various concentrations of DNA and chromatin, typical Michaelis-Menten kinetics were obtained (Fig. 1). Whereas DNA had a K_m of 55 µg/ml, the K_m for chromatin was 120 µg/ml, or about twice. The V_{max} for both substrates is equal, but twice as much chromatin as DNA is needed to obtain the same rate of methylation. This is consistent with the suggestion that only half of the chromatin DNA is available to react with the enzyme.

It should be pointed out that although the methylation reaction probably occurs at specific sequences in the DNA these sequences seem to be randomly distributed along the DNA. Thus, when in vitro methylated DNA or chromatin was digested with staphylococcal nuclease, a kinetic analysis showed that total DNA and labelled DNA were digested at the same rate. When methylated DNA was digested with exonuclease I from E. coli the rate of disappearance of methyl groups was the same as that for the total DNA, indicating that the methyl groups are equally distributed along the length of the DNA molecules. Finally, using a

Figure 1. Lineweaver-Burke plot of methylation. Methylation was determined as described in Materials and Methods using 0.1 mM S-adenosylmethionine. Incubation was for 1 hr, during which time the reaction was linear. DNA (O); chromatin (●)

single strand specific nuclease (1) we have shown that the methylation does not occur specifically on single stranded regions of the DNA.

Digestion of methylated chromatin - Since the amount of methylation was proportional to the amount of unprotected DNA, it was of interest to see if methylation occurs on the same regions of the DNA which are nuclease sensitive. As shown in Table 2, only 1.2% of the methyl label remained after digestion of chromatin with staphylococcal nuclease. Thus, despite the fact that only 50% of the chromatin DNA is digested, virtually all of the methylated DNA is digested. We thus conclude that only the DNA in the open regions of chromatin are subject to methylation, while the covered regions of chromatin do not undergo methylation.

Using this fact, we can now employ methylated chromatin as a tool for measuring the movement of chromatin proteins along the DNA. Thus, if proteins move from the covered regions to the labelled open regions, a portion of the open regions will now be rendered resistant to nuclease digestion. The effects of various treatments which could cause movement of chromatin proteins are

TABLE 2

Treatment	Nuclease Resistance (%)
None	1.2
Shearing	1.5
Sonication I	4.4
Sonication II	5.8
MnCl ₂ (2 mM)	12.0

Nuclease digestion of methylated chromatin - 100 µg chromatin was labelled with (³H)-S-adenosylmethionine to a specific activity of 500 cpm/µg as described in Materials and Methods. The reaction mix was then diluted to 1 ml containing 10 mM Tris-HCl (pH 7.9), 0.1 mM Ca Cl₂, and subjected to various treatments. Shearing was done with a Sorvall mixer for 90 sec at a speed of 170/220 volts. Sonication was performed with a Braun Sonifier at a setting of 35 with the 2 mm probe either for 6 ten-second intervals (I) or for 2 min continuously (II). Chromatin was precipitated with 2 mM MnCl₂ and the pellet containing 95% of the chromatin DNA was suspended in nuclease buffer. Digestion by staphylococcal nuclease was performed as described in Materials and Methods. For all samples, 48-52% of the chromatin DNA was nuclease sensitive.

shown in Table 2. When chromatin was sheared by means of a Waring blender, a technique frequently used to solubilize chromatin, there was virtually no detectable exchange. Sonication of chromatin either for 2 min continuously or at 10 sec intervals for a period of 2 min resulted in about 5% exchange of proteins. The largest extent of protein movement was observed when chromatin was precipitated with 2 mM MnCl₂.

Chromatin reconstitution - When chromatin is placed in 2 M NaCl, 5 M Urea all of the proteins are disassociated from the DNA. Using a system of step-wise dialysis into lower salt and urea, one can reconstitute chromatin. This reconstituted chromatin retains many of the properties of the native chromatin (9,10). Using chromatin specifically labelled by methylation in the open regions we can ask whether chromatin proteins return to their original location on the DNA during the reconstitution procedure. If the proteins indeed return to specific regions, then one would expect methylated chromatin to remain 100% digestible even after reconstitution. As can be seen from the data in Table 3,

TABLE 3

Substrate	Total DNA Digestibility (%)	[³ H]-methy DNA Digestibility (%)
Native Chromatin	54	99
Reconstituted Chromatin	55	51

Distribution of (³H)-methyl DNA in reconstituted chromatin - Chromatin was labelled with (³H)-S-adenosylmethionine to a specific activity of 500 cpm/μg, and the DNA was purified free of the chromatin proteins. 100 μg of this DNA was mixed with 1.6 mg of unlabelled chromatin in a volume of 2 ml. To this was added 900 mg urea (ultra pure from Schwartz Mann) and 350 mg NaCl at 0°C. The resulting solution (2.7 ml) was subjected successively to dialysis in 100 volumes of 5 M urea, 20 mM β-mercaptoethanol containing 1.2 M, 1.0 M, 0.6 M, and 0 M NaCl. Each dialysis was for 1 hr at 4°C. Finally, the chromatin was dialyzed against 0.1 mM EDTA for 1 hr and then again for 16 hr. The digestibility of the total chromatin DNA (OD₂₆₀) was determined as described by Clark and Felsenfeld (4).

the proteins return in a random fashion, rendering the labelled portions of the DNA 50% digestible.

Titration of chromatin with poly-D-lysine - The synthetic polycation, polylysine, may be used to titrate accessible phosphodiester groups on the DNA of chromatin. Titration of chromatin with polylysine reveals that about half the phosphodiester groups of DNA are free to react. Beyond this point further added polylysine fails to react (3). As can be seen in Figure 2, twice as much poly-D-lysine is needed to protect methylated DNA as compared with methylated chromatin, indicating that poly-D-lysine reacts with only 50% of the total chromatin DNA.

The regions accessible to polylysine can be isolated from this chromatin-polylysine complex. If this complex is treated with Pronase, the chromatin proteins are hydrolyzed, but the poly-D-lysine is resistant, leaving only poly-D-lysine bound to regions of DNA with which it was able to react. This poly-lysine-DNA complex is now subjected to nuclease digestion and the DNA which resists digestion (50%) is purified free of polylysine. We refer to the product as "open DNA." In order to determine if there is any protein migration

Figure 2. Titration with poly-D-lysine. DNA and chromatin were methylated as described in Materials and Methods and diluted to 60 $\mu\text{g/ml}$ in 1 mM Tris-HCl (pH 7.9). Titration with poly-D-lysine was accomplished by the slow addition of 35 $\mu\text{g/ml}$ poly-D-lysine (4). Aliquots of poly-D-lysine complex were diluted to 10 $\mu\text{g/ml}$ in 10 mM Tris-HCl (pH 7.9), 0.1 mM CaCl_2 and digested with 10 $\mu\text{g/ml}$ staphylococcal nuclease. DNA (○) ; Δ chromatin

occurring during the preparation of open DNA, we have prepared open DNA from methylated chromatin. As can be seen from the data in Table 4, the nuclease resistance of polylysine-chromatin complexes is the same before and after Pronase treatment, indicating that neither the polylysine nor the chromatin proteins have moved to any considerable extent during the preparation of open DNA.

Methylation of nuclear DNA - In order to study the extent of protein movement occurring during the preparation of chromatin from nuclei, we have methylated nuclear DNA in vitro. This was accomplished by incubating washed nuclei with *E. coli* DNA methylase and S-adenosylmethionine. Incorporation of (^3H)-S-adenosylmethionine into nuclear DNA was about 30% as rapid as incorporation into soluble chromatin. When labelled nuclei were digested with staphylococcal nuclease, 95% of the methylated DNA was rendered acid soluble (Table 5),

TABLE 4

Nuclease Resistance (%)	
Before Pronase	After Pronase
14	14
52	62
85	80

Isolation of open regions from methylated chromatin - 600 μ g Chromatin labelled with (3 H)-S-adenosylmethionine to a specific activity of 500 cpm/ μ g was diluted to 60 μ g/ml in 1 mM Tris-HCl (pH 7.9) and titrated with 35 μ g/ml poly-D-lysine as described by Clark and Felsenfeld (4). Aliquots containing various ratios of poly-D-lysine/DNA were incubated with 0.04 μ g/ml Pronase B for 3 hr at 37°C and then for 16 hr at 4°C (3). Nuclease resistance was determined both before and after treatment with Pronase.

TABLE 5

Substrate	Nuclease Resistance (%)
Nuclei	5.9
Chromatin	11.8

Methylation of nuclear DNA - Erythrocyte nuclei were prepared by breaking cells with a Dounce homogenizer in 10 mM Tris-HCl (pH 7.9), 1 mM CaCl_2 , 0.25 M Sucrose. The nuclei were centrifuged and washed once with 0.5% Triton X-100 in the same buffer and twice with 10 mM Tris-HCl (pH 7.9), 0.1 mM CaCl_2 , 0.25 M Sucrose, 5 mM dithiothreitol and finally resuspended in this buffer at a concentration of 80 μ g/250 μ l. To this suspension was added 1 mg DNA methylase and 2 μ Ci (3 H)-S-adenosylmethionine (7.5 Ci/mmol) and the reaction incubated at 37°C for 1.5 hr. Nuclease digestion of nuclei was accomplished after diluting the reaction mix 1 to 5 in Tris-sucrose buffer, containing 0.1 mM CaCl_2 and incubating at 37°C for 1.5 hr, in the presence of 10 μ g/ml staphylococcal nuclease. Chromatin was prepared from methylated nuclei and digested as described in Materials and Methods. The kinetics of digestion of nuclei was the same as that for chromatin (11).

whereas 50% of the total nuclear DNA was digestible (11). These labelled nuclei were then used as a source for the preparation of chromatin and the resulting product was again subjected to nuclease digestion. As shown in Table 5, 12% of the labelled regions of this chromatin was resistant to nuclease. Thus, there was some migration of chromatin proteins during the process of chromatin preparation.

DISCUSSION

The amount of DNA which is covered by protein and the amount which is free DNA has long been a matter of contention, and depends on the nature of the probe used to assay this property. Thus, using staphylococcal nuclease, Clark and Felsenfeld (4) demonstrated that 50% of the chromatin DNA is susceptible to nuclease digestion. Other experiments using titration with poly-D-lysine (4), Mn^{2+} (12) or DNA ligase activity (13) have confirmed the idea that 50% of the DNA phosphates are tightly covered with protein, while the other 50% are relatively free to react with these probes. In this paper we have demonstrated that 50% of the chromatin DNA is available as a substrate for DNA methylase. This was shown both by saturation experiments (Table 1) and by initial rate kinetics (Fig. 1). We have also presented evidence indicating that the same regions of chromatin which are available to DNA methylase are also sensitive to staphylococcal nuclease and titratable by polylysine. Thus DNA methylation occurs exclusively in the open regions of the chromatin.

Since all of the methyl groups added to chromatin are digestible by staphylococcal nuclease, methylated chromatin is a good probe for measuring the movement of proteins along the DNA. Under conditions of 10 mM Tris-HCl (pH 7.9) and 0.1 mM divalent ion concentration there is only a minimal amount of methylated DNA which resists digestion (Table 2). When chromatin is treated with 2 mM $MnCl_2$ about 12% of the methylated regions become nuclease resistant, despite the fact that the chromatin DNA remains 50% digestible. Sonication of chromatin, a treatment frequently used to solubilize chromatin, caused a considerable amount of protein exchange. On the other hand, shearing had only a slight effect on protein movement.

In order to examine protein movement during chromatin preparation, we selectively labelled nuclear DNA by incubating nuclei with endogenous *E. coli* methylase and (3H)-S-adenosylmethionine. This labelled template behaved like chromatin in regard to digestion by staphylococcal nuclease and 95% of the label was digestible. When chromatin was prepared from these nuclei only 12% of the label became nuclease resistant. Thus, the procedures used for preparing

chromatin, including a 0.25 M NaCl wash and subsequent shearing, do cause some degree of protein sliding.

It should be noted that this method of measuring protein exchange is more sensitive than other techniques previously used. Clark and Felsenfeld (4) measured protein exchange by adding radioactively labelled naked DNA to chromatin and measuring the conversion of the DNA from nuclease sensitive to nuclease resistant. Their technique as well as that of Varshavsky and Georgiev (14) and Hancock (15) measures exchange of proteins from one molecule of DNA to another, whereas our technique also detects the sliding of proteins along the same DNA molecule.

Recently, Doenecke and McCarthy (16) have labelled chromatin and nuclei using a restriction modification methylase and have shown that shearing causes considerable protein sliding. Although we find that reshearing of chromatin does not cause protein movement (Table 2), some protein rearrangement was detected after shearing of nuclei labelled chromatin (Table 3). These results are consistent with those of Noll *et al.* (17) who demonstrated that shearing of large but not small DNA leads to changes in the chromatin-DNA-protein interactions.

Reconstituted chromatin has many of the properties of native chromatin. When digested with staphylococcal nuclease, for example, it is 50% digestible and the resulting protected DNA has the same gel electrophoresis pattern as digests of native chromatin (5). Furthermore, in the case hemoglobin synthesis transcriptional specificity is retained after reconstitution (9,10). Despite the preservation of functional specificity, the majority of the proteins seem to be reconstituted at random locations on the DNA. Zimmerman and Levin (13) have obtained similar results using DNA ligase activity as a label for open region DNA. These results suggest that transcriptional specificity is not determined by the exact physical location of most of the chromatin proteins. Thus functional specificity may be controlled by a small number of non-histone proteins whose localization is not detected by our assay. Alternatively, the specificity may not be determined by any specific protein localization but rather by factors which control the tertiary structure of the chromatin.

In initial studies of the accessibility of DNA in chromatin to a variety of chemical and biological probes, it was found that about half of the DNA in chromatin is susceptible either to attack by staphylococcal nuclease or to titration by the polycation, poly-D-lysine (4). These studies demonstrate that the DNA of chromatin can be structurally divided into two distinct classes: one which is tightly bound to protein (covered DNA) and another which is either

free or associated with chromatin protein in such a way as to be chemically reactive (open DNA). By preparing open region DNA from methylated chromatin we have demonstrated that there is no appreciable migration of either chromatin proteins or poly-D-lysine during this procedure. Axel *et al.* (3) showed that although most sequences of the duck genome are common to both open and covered regions of reticulocyte chromatin, portions of the hemoglobin genes are lacking in the open regions. Their data suggests that the bulk of the chromatin proteins are distributed randomly along the DNA backbone whereas a small number of proteins are located specifically. Our results add credence to this hypothesis by showing that this effect cannot be due to a redistribution of the proteins during the preparation of open DNA.

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