Localization of DNA methyltransferase in the chromatin of Friend erythroleukemia cells

F.Creusot* and J.K.Christman*+

*Department of Biochemistry, and +Department of Pediatrics, The Mount Sinai Medical Center, 1 E. 100th Street, New York, NY 10029, USA

Received 25 June 1981

Chromatin fragments released from intact Friend erythroleukemia cell nuclei during limited incubation with micrococcal nuclease, DNase II or DNase I were analyzed to determine the distribution of DNA methyltransferase in chromatin. The enzyme was released in a free form when internucleosomal DNA was digested with micrococcal nuclease but was found associated with Mg^{++} -precipitable polynucleosomes after DNase II digestion. Less than 25% of the enzyme was released from nuclei incubated with DNase I under conditions where transcriptionally active chromatin should have been completely digested. These results indicated that the bulk of DNA methyltransferase was bound to "linker" DNA in condensed regions of chromatin. Preferential rebinding of free enzyme to linker DNA was also demonstrated *in vitro*. The possibility that chromatin proteins play a role in regulating access of DNA methyltransferase to specific sites in DNA is discussed in light of these findings.

INTRODUCTION

There are two lines of evidence indicating that enzymatic methylation of cytosine residues in DNA may influence the expression of genes in vertebrate cells. First, a general correlation exists between the persistence of unmodified methylation sites in or near coding sequences of specific genes and their expression. Such sites are methylated in tissues where the genes are inactive and lack methyl groups in tissues which the genes are expressed. (See ref. 1-4 for reviews). Second, at least two agents which inhibit methylation of cytosine residues in DNA are able to induce stable alterations of gene expression in mammalian cells. L-ethionine, which in the form S-adenosylethionine, acts to inhibit a number of transmethylation enzymes (5), induces erythroid differentiation of Friend erythro-

leukemia (FL) cells and granulocytoid differentiation of human promyeloid (HL-60) cells under conditions where it inhibits DNA methylation (6-8). 5-azacytidine, an inhibitor of both DNA cytosine (9,10) and RNA cytosine (11) methyltransferases induces differentiation of mouse C3H 10T1/2 cells (12), FL cells (10) and HL-60 cells (13).

Although these results imply a causal link between loss of methyl groups at critical sites in the genome and activation of genes during differentiation, it is not clear how regulated loss of methyl groups might be accomplished during normal cellular differentiation. There is no evidence for the existence of enzymes capable of removing 5 methylcytosine residues (5mC) from DNA or of demethylating 5mC residues in DNA. Vertebrate DNA methyltransferases generally serve to maintain a previously established pattern of DNA methylation by transferring methyl groups to C residues in newly synthesized DNA strands at sites opposite methylated sites in the template, i.e. $\text{--}\overset{5\text{mC}}{\text{C}}\text{--}\text{C}\text{--}$ (14). Thus, loss of methyl groups can only occur if methylation of newly synthesized DNA is inhibited. Possible mechanisms for preventing methylation include 1) depletion of the methyl donor, S-adenosyl methionine, 2) direct inhibition or loss of DNA methyltransferase and 3) blockage of access of the methyltransferase to specific sites. For the first two mechanisms to function with the required specificity, it would seem necessary to postulate the existence of a number of different DNA methyltransferases with different recognition sites and perhaps different K_m s for S-adenosylmethionine binding or different sensitivity to inhibitors. Studies of purified mammalian enzymes have not indicated the existence of a large number of highly specific DNA methyltransferases (15).

Direct evidence is also lacking for the third mechanism which would require regulatory molecules capable of blocking methylation at specific sites. However, there have been reports suggesting that chromatin proteins can prevent the methylation of potentially methylatable sites by non-homologous DNA methyltransferases (16,17). Since it is known that DNA methyltransferase is tightly bound to DNA in the nuclei of mammalian cells (18), we reasoned that an examination of the

manner in which the enzyme is distributed in different fractions of chromatin would allow a determination of how chromatin proteins or the ordered packaging of nucleosomes affects the interaction between unmethylated sites in DNA and endogenous DNA methyltransferases.

Here, we report evidence that the bulk of DNA methyltransferase in FL cell nuclei is found in transcriptionally inactive chromatin associated with internucleosomal ("linker") DNA. We also present data indicating that chromatin proteins can prevent methylation of potentially methylatable sites in FL cell DNA by both soluble and chromatin-bound homologous DNA methyltransferase.

MATERIALS AND METHODS

Enzymes and materials

Minimal Eagle's medium was purchased from Gibco (Grand Island, New York). Fetal bovine serum was obtained from Flow Laboratories (Bethesda, MD). Micrococcal nuclease and DNase II were from Worthington Biochemical Corporation (Freehold, N.J.) [³H]-methyl-S-adenosyl methionine (AdoMet, Sp. Act. 7-17 Ci/nmol) was purchased from ICN (Irvine, CA).

Cell culture and preparation of nuclear fractions

FL cells (Strain 745A) kindly provided by Dr. C. Friend, have been maintained in minimal Eagle's medium supplemented with 10% fetal bovine serum, 250 U/ml penicillin and 0.2 mg/ml streptomycin. Nuclei were prepared from cells harvested in logarithmic growth ($8-12 \times 10^{-5}$ /ml) and lysed in RSB-NP40 (Tris-HCl, pH 7.4, 3mM MgCl₂, 0.5% Nonidet P40, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) as described (19).

Nuclease digestion

Micrococcal nuclease digestion of nuclei was performed essentially as described by Giri et al (20). Nuclei were suspended in Tris digestion buffer (Tris-HCl, pH 7.4, 25 mM KCl, 1 mM MgCl₂, 0.25 M sucrose, 15 mM 2-mercaptoethanol and 0.2 mM PMSF). The nuclear suspension (1×10^8 /ml) was made with respect to CaCl₂, warmed for 4 min at 37° and then digested with 30 U/ml of micrococcal nuclease for 2 min at 37°. The reaction was

terminated by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 2 mM and chilling on ice.

For DNase II digestion, nuclei were suspended in MES digestion buffer (15mM 2-(N-morpholino)ethane sulfonic acid (MES), pH 6.4, substituted for Tris) and warmed at 25° for 4 min prior to addition of 100 U/ml DNase II. After 15 min at 25°, the reaction was stopped by raising the pH to 7.5 with 100 mM Tris, pH 10 and chilling on ice.

Micrococcal and DNase II digested nuclei were centrifuged for 10 min at 5,000 x g to give the post-nuclear supernatants designated micrococcal S1 and DNase II S1 respectively.

Nucleosomes released into S1 by nuclease digestion were subsequently fractionated on the basis of their solubility in $MgCl_2$. S1 supernatants were made 8 mM with respect to $MgCl_2$, allowed to sit on ice for 30 min and sedimented at 14,000 x g for 10 min. The Mg^{++} soluble supernatants were removed and saved, the precipitated pellets were suspended in the initial volume of 15 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF by gentle stirring for 15 to 30 min at 4°.

DNase II digestion of chromatin for preparation of "active" and "inactive" fractions was carried out as described by Gottesfeld et al (21). Washed chromatin was suspended in sodium acetate buffer, pH 6.4 (33 A₂₆₀ U/ml), warmed at 25° for 4 min and digested with 100 U/ml DNase II for 15 min at 25°. After adding 100 mM Tris, pH 10 to pH 7.5, undigested chromatin was removed by centrifugation at 14,000 x g for 15 min and the supernatant S1 was made 2 mM with respect to Mg^{++} . Mg^{++} -soluble material (S2) was separated from Mg^{++} -insoluble material (P2) by centrifugation at 14,000 x g for 10 min.

Sucrose gradient sedimentation

Nucleosomal preparations were sedimented through isokinetic gradients of 10 to 30% (w/v) sucrose (22) in Tris digestion buffer with or without Mg^{++} as indicated in the text. Centrifugation was for 20 h at 26,000 rpm in an SW 27.1 rotor. Fractions (0.5 ml) were collected from the top of the gradient using a Buchler auto-densiflow. Aliquots were removed for DNA methyltransferase activity determination.

Analysis of DNA in nucleosome preparations

DNA content of the various nucleosomal preparations and gradient fractions was determined either by UV absorption at 260 nm (measured in 0.9 M NaOH) or fluorometrically after binding 4', 6-diamidino-2-phenylindole (23). Agarose gel electrophoresis of nucleosomal DNA was performed as has been described (19).

Preparation of DNA methyltransferase from FL cells

DNA methyltransferase was prepared from FL nuclei by extraction of chromatin with 0.3 M NaCl (7). The salt extract was dialyzed against 2 changes of buffer (20 mM Tris-HCl, pH 7.4, 20 mM NaCl, 0.5 mM DTT and 0.2 mM PMSF, 30% sucrose) for 4 hr. This preparation is referred to as 0.3 M NaCl extract.

Determination of DNA methyltransferase activity

DNA methyltransferase activity of nucleosomal fractions, free enzyme (0.3 M NaCl extract) and gradient fractions were determined by transfer of methyl groups from [³H]-methyl-S-Ado-Met to an excess of hypomethylated DNA substrate (7). One unit of activity is defined as the amount of enzyme that transfers one pmole of methyl group/15 min to hypomethylated DNA isolated from FL cells grown in the presence of 4 mM L-ethionine for 4 days. This DNA contains both hemimethylated and completely unmethylated sites; the enzyme preferentially transfers methyl groups to the hemimethylated sites.

Results

1. Demonstration that DNA methyltransferase activity can be measured directly in chromatin fractions

Since we intended to fractionate chromatin on the basis of its susceptibility to digestion by various nucleases, it was necessary to determine whether the enzyme could be detected directly in the crude fractions and whether residual nuclease in the fractions interfered in any way with the assay. As shown in Fig. 1, there was a 5-7 fold range for each chromatin fraction, within which a linear relationship existed between the amount of chromatin fraction and the rate at which methyl groups were transferred to DNA. The amount of active DNA methyltransferase estimated by summing the activity of each

chromatin fraction, extrapolated from the linear range of the assay, closely approximated the total DNA methyltransferases activity in the nucleus, i.e., the activity found in a 0.3 M NaCl extract. (The range was 90-120% in 12 separate experiments). The data indicated that methyltransferase activity could be accurately measured in crude chromatin fractions and that, under our assay conditions (pH 7.5, mM EDTA), nucleases did not interfere with DNA methylation. This conclusion was further substantiated by the finding that 17.8 U of enzyme activity could be detected in a reaction mixture containing chromatin fraction (S1) with 9.6 U of enzyme and 8.7 U of 0.3 M NaCl extracted enzyme.

Although DNA methyltransferase in the chromatin fractions retained its ability to transfer methyl groups to added hypomethylated DNA, it was relatively inefficient in methylating endogenous DNA in nucleosomal complexes. A mononucleosomal fraction containing enzyme was incubated with isolated DNA of high molecular weight (>15kb) and [³H]-methyl-S-AdoMet. As shown in Fig. 2, even though equal amounts of high molecular weight DNA and endogenous mononucleosomal DNA were present in the reaction mixture, more than 95% of the transferred radiolabeled methyl groups were associated with the high molecular weight DNA. This demonstrated that the observed incorporation did not represent a stimulation of methylation of endogenous nucleosomal DNA by the addition of purified DNA. The absence of degradation products of the added DNA, detectable either as radiolabeled or ethidium bromide stained material, was a further indication that nucleases were inhibited under our assay conditions.

2. Demonstration that DNA methyltransferase is associated with DNA in the internucleosomal region of chromatin

The results shown in Table 1 demonstrated that release of DNA methyltransferase from intact nuclei required cleavage of chromatin. Almost no DNA or active enzyme was detected in the supernatant (S1) of FL cell nuclei incubated in the absence of added nuclease. In the presence of micrococcal nuclease, active enzyme release was dependent on the time of incubation and the extent of digestion as indicated by the

Fig. 1. DNA methyltransferase activity in chromatin fractions. FL cell nuclei were digested with micrococcal nuclease as described in Methods. After digestion the nuclear suspension was clarified by centrifugation to give the supernatant S1. The nuclear pellet was lysed in 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF. The supernatant, (S2) was separated from debris and undigested chromatin (P) by centrifugation. Extract or chromatin fraction prepared from the indicated number of cells was assayed for DNA methyltransferase activity as described in Methods. Incorporation of [3 H]-methyl groups into protease and alkali resistant, acid precipitable material catalysed by 0.3 M NaCl extracts of whole nuclei (X) or chromatin fractions (S1 o, S2●, and P▲). The solid line indicates reaction mixtures containing hypomethylated DNA, the dotted line reaction mixtures with no added DNA.

Fig. 2. Incorporation of methyl groups into hypomethylated DNA by nucleosome-associated DNA-methyltransferase. Micrococcal nuclease digest S1 prepared by the procedure of Noll and Kornberg (37) was incubated with [3 H]-methyl-S-AdoMet and purified hypomethylated DNA as described in Methods. Agarose gel electrophoresis of DNA from (a) S1 incubated with [3 H]-methyl-S-AdoMet (b) S1 incubated with [3 H]-methyl-S-AdoMet and purified hypomethylated DNA. Incubation was terminated by addition of sarkosyl to 0.4% and proteinase K and the complex mixture electrophoresed in low-melting agar as described in Methods and Ref. 19. The slab was cut into the indicated slices. Each slice was melted in 1 ml 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA and the agar removed by extraction with an equivalent volume of buffer-saturated phenol. The DNA was precipitated from the aqueous phase with 5% TCA and collected on nitocellulose filters for determining radioactivity. Migration of ϕ X 174, Hae III restriction fragments is indicated.

close correlation between the amount of enzyme released and the amount of DNA found in S1.

Under the conditions used for most of the experiments reported here, (30 U micrococcal nuclease /ml, incubation for 2 min at 37°C), approximately 50 to 60% of the DNA methyl-

Table 1. Time course of release of DNA methyltransferase activity during micrococcal digestion of FL cell nuclei

Addition	Time at 37°C (Min)	DNA Content S1 (% of total DNA)	DNA Methyltransferase Activity	
			U/10 ⁸ cells	% total*
micrococcal	0.5	7.6	6.4	22.5
nuclease	1	13.3	12.2	42.8
(30 U/ml)	2	21.0	18.1	63.5
	5	22.0	18.6	65.2
none	5	<1	<0.25	<1

After incubation with micrococcal nuclease for the indicated times, digestion was terminated by addition of EDTA to 2 mM and rapid chilling on ice. Preparation of S1, determination of DNA content and DNA methyltransferase activity are described in Methods. *Total methyltransferase activity extractable from FL cell nuclei with 0.3 M NaCl - average 28.5 U/10⁸ cells (20 separate determinations).

transferase and 20 to 30% of nuclear DNA were found in S1. Analysis by agarose gel electrophoresis (Fig. 3a) revealed that more than 85% of the DNA in S1 was present in mononucleosomes (DNA fragments migrating as a single band with molecular size of 140-175 base-pairs) and the rest in oligonucleosomes (DNA fragments of increasing size with a 190 base pair-periodicity). When S1 was analyzed using sucrose gradients containing less than 1 mM Mg⁺⁺ (Fig. 4a), more than 90% of the active enzyme sedimented in the same area of the gradient as oligonucleosomes and less than 10% sedimented as free enzyme (fractions 5-7) or in association with mononucleosomes. This suggested that the enzyme solubilized by micrococcal nuclease was still bound to linker DNA. Since most nucleosomes are insoluble in the presence of Mg⁺⁺ (24), it seemed likely that bound enzyme would coprecipitate with nucleosomes after raising the Mg⁺⁺ concentration of S1. As seen in Fig. 3, this treatment almost quantitatively precipitated large oligonucleosomes (Fig. 3c) leaving only mono- and small dinucleosomes in the supernatant (Fig. 3b). Seventy five to eighty five percent of the methyltransferase activity remained soluble (Table 3) and on sedimentation through a

Fig. 3. 1.4% agarose gel electrophoretic analysis of nucleosomal DNA released from FL cell nuclei by micrococcal nuclease digestion. (a) Total S1 (b) Mg^{++} -soluble fraction of S1 (c) Mg^{++} -insoluble fraction of S1. The amounts of Mg^{++} -soluble and insoluble fractions in lanes b and c were derived from the same amount of S1 as was run in lane (a). Bands were detected by ethidium bromide staining.

Fig. 4. Sucrose gradient sedimentation of DNA methyltransferase activity released from FL cell nuclei by micrococcal nuclease digestion. (a) S1 sedimented through a 10-30% sucrose gradient in Tris digestion buffer in the absence of Mg^{++} . (—) μg DNA/ml fraction. (-----) $[^3H]$ -methyl, cpm, transferred to hypomethylated DNA by 100 μl of gradient fraction (Methods). (b) 8 mM Mg^{++} -soluble fraction of S1 sedimented through a 10-30% sucrose gradient in Tris digestion buffer containing 8 mM Mg^{++} . The DNA methyltransferase activity recovered from the sucrose gradients was greater than 75% of the loaded activity in all experiments. The arrow indicates the position of free enzyme (0.3 M NaCl extract). Fraction 1 is at the top of the gradient.

sucrose gradient containing 8 mM Mg^{++} , was found to sediment with the same velocity as enzyme extracted from chromatin with 0.3 M NaCl (Fig. 4b). These data are consistent with the interpretation that micrococcal nuclease, by preferentially digesting linker DNA, released DNA methyltransferase in a free form and that, depending on the Mg^{++} concentration, the enzyme remained free or rebound to available linker DNA.

A study of the distribution of enzyme in chromatin fragments released by DNase II digestion of intact nuclei supported the supposition that DNA methyltransferase is bound to linker DNA. DNase II digestion released 10-15% of chromatin DNA and 20-35% of DNA methyltransferase. As shown in Fig. 5b, the electrophoretic pattern of released DNA indicated that under our digestion conditions, DNase II cut linker DNA less efficiently than did micrococcal nuclease. Most of the DNA was found in high molecular weight fragments of heterogeneous length with less than 10% of the DNA present in mononucleosomal DNA. However, as was found after micrococcal nuclease digestion, in low concentrations Mg^{++} (<1mM), all of the released enzyme activity cosedimented with heavy chromatin fragments in sucrose gradients (Fig. 6). In contrast to enzyme released by micrococcal nuclease, little of the enzyme released from nuclei by DNase II remained soluble in the presence of 8 mM Mg^{++} (Table 2). Although accurate quantitation was precluded by variability in the extent of resolubilization of the Mg^{++} precipitate, significant DNA methyltransferase activity was found in this fraction. The enzyme bound to these polynucleosomes could, however, be solubilized by micrococcal nuclease digestion of internucleosomal DNA (Table 2). Even though most of the mono and dinucleosomes produced by such a double digestion (Fig. 5e) remain Mg^{++} precipitable (Fig. 5g) approximately 65% of the formerly chromatin bound enzyme became Mg^{++} -soluble after digestion of linker DNA (Table 2).

Thus, in a DNase II digest, the DNA methyltransferase was still bound to linker DNA and precipitated along with oligonucleosomes in 8 mM Mg^{++} . By digesting the linker

Fig. 5. 1.4% agarose gel electrophoresis of DNA from chromatin fragments released from FL cell nuclei by DNase II digestion. (a) S1 from micrococcal nuclease digestion of nuclei shown as control. (b) DNase II S1 (c) Mg^{++} -soluble and (d) Mg^{++} -insoluble material from DNase II S1. (e) nucleosomes obtained by micrococcal digestion of high molecular weight chromatin fragments present in DNase II S1 (f) Mg^{++} -soluble and (g) Mg^{++} -insoluble material from (e).

regions of these oligonucleosomes with micrococcal nuclease, the enzyme was released and no longer coprecipitated with nucleosomes in 8 mM Mg^{++} . The data also indicated that 8 mM Mg^{++} alone was not sufficient to release the enzyme from its native association with linker DNA.

3. Evidence that free DNA methyltransferase can rebind to any nucleosomal species under in vitro conditions

The difference in Mg^{++} solubility of DNA methyltransferase present in micrococcal and DNase II digests suggested that two types of interaction existed between this enzyme and nucleosomes. The following experiments showed that Mg^{++} sensitive association of the enzyme with chromatin occurred after re-binding of free enzyme to nucleosomes while binding that was unaffected by Mg^{++} indicated an undisturbed native association.

Fig. 6. Sucrose gradient sedimentation of DNA methyltransferase activity released from FL cell nuclei by DNase II digestion. The supernatant (S1) was sedimented through a 10-30% sucrose gradient in Tris digestion buffer in the absence of Mg^{++} . (—) μg DNA/ml fraction, (-----) $[^3H]$ -methyl, cpm. all details as in Fig. 4. The arrow shows the position of the free enzyme.

Fig. 7. Binding of free DNA methyltransferase to nucleosomes. Free DNA methyltransferase (0.3 M NaCl extract) was added to a micrococcal nuclease digest S1 containing both mono and oligonucleosomes and to a Mg^{++} -soluble fraction of S1. Distribution of DNA methyltransferase activity was analyzed by sedimentation through a 10-30% sucrose gradient in Tris digestion buffer in the absence of Mg^{++} . (a) endogenous DNA methyltransferase in S1, (b) DNA methyltransferase (0.3 M NaCl extract) added to (a), (c) endogenous DNA methyltransferase in the Mg^{++} -soluble fraction of S1 and (d) DNA methyltransferase added to (c). (—) μg DNA/ml fraction, (-----) $[^3H]$ -methyl, cpm, all details as in Fig. 4. The arrow shows the position of free enzyme.

The sedimentation behavior of free DNA methyltransferase (0.3 M NaCl extract) was not detectably affected by changing Mg^{++} concentration in the range 0-10 mM nor did such changes cause the enzyme to aggregate. However, when free enzyme was mixed with a micrococcal nuclease digest of chromatin containing both mono- and oligonucleosome and sedimented through

Table 2. Mg^{++} solubility of DNA Methyltransferase Activity Released from FL cell nuclei by DNase II digestion: Dependence on Integrity of Linker DNA

Fraction	DNA Content (% of S1)	DNA Methyltransferase Activity U/10 ⁸ cells	% of S1
DNase II S1	100	5.6	100
Mg^{++} - soluble	0.7	1.1	19.6
Mg^{++} - insoluble	87	1.6	28.5
DNase II S1 Digested with micrococcal nuclease	71	5.3	95
Mg^{++} - soluble	10	3.5	63
Mg^{++} - insoluble	48	0.65	11.6

Nuclei were incubated with DNase II (see Methods) releasing 8.5% of input DNA nuclear and 19% of total DNA methyltransferase activity into the post nuclear supernatant (DNase II S1). An aliquot of DNase II S1 was made 0.2 mM with respect to Ca^{++} , warmed 4 min at 37°C and digested with 30 U/ml of micrococcal nuclease for 5 min at 37°C. The reaction was terminated by adding EDTA to 2 mM. A small amount of precipitate was removed by centrifugation at 4,000 x g for 10 min. Mg^{++} -soluble and insoluble fractions were prepared from the supernatants as described in Methods.

a sucrose gradient in the absence of Mg^{++} , most of the added free enzyme, along with endogenous enzyme present in the chromatin fraction, cosedimented with oligonucleosomes. Very little enzyme cosedimented with mononucleosomes or as free enzyme (Fig. 7a,b). When only mononucleosomes were present both endogenous enzyme released by micrococcal nuclease digestion and added free enzyme sedimented primarily at the leading edge ("heavy" side) of the mononucleosome peak (Fig. 7c,d). Since the observed binding of enzyme to oligonucleosomes indicated a preference for binding to linker DNA, the finding that enzyme mixed with mononucleosomes sedimented slightly more rapidly than the bulk of mononucleosomes suggested that it was bound to a minor fraction of mononucleosomes that retained linker DNA. No evidence was found to support this supposition. As shown in Fig. 8, DNA isolated from the "heavy" side of the mononucleosomal peak is indistinguishable in size from DNA isolated from the "light" side of the peak. Further evidence for absence of linker DNA, was the

Fig. 8. 1.4% agarose gel electrophoresis analysis of DNA from mononucleosomes. Aliquots of equal volume from fractions 5 through 14 of the gradient shown in figure 7c were loaded onto agarose gel (Methods). The number of each lane corresponds to the number of the gradient fractions. DNA was stained with ethidium bromide.

complete lack of detectable histone H1 in these mononucleosomes. This histone, which is predominantly associated with linker DNA and which is released when the linker region is shorter than 20 nucleotides long (for review see 25,26), was precipitated quantitatively with oligonucleosomes (Fig. 9).

Thus, in vitro, the enzyme preferentially bound to oligonucleosomes with linker DNA but in their absence was capable of binding to linker-free mononucleosomes. However, when the Mg^{++} concentration was raised to 8 mM, the association between enzyme and either mono- or polynucleosomes, was disrupted (Table 3, Fig. 4). Mg^{++} had the same effect on the association between added free enzyme and oligonucleosomes released by DNase II digestion. In low Mg^{++} free enzyme bound to oligonucleosomes. Subsequent increase of the Mg^{++} concentration to 8 mM resulted in release of DNA methyltransferase activity approximately equivalent to that of the added free enzyme but did not change the amount of enzyme bound to and precipitable with oligonucleosomes (Table 3). These data indicated 1) that the in vitro association between added DNA methyltransferase and mono- or oligonucleosomes was sensitive to changes in Mg^{++} concen-

Fig. 9. Polyacrylamide gel electrophoretic analysis of histones released from FL cell nuclei by micrococcal nuclease digestion. Histones were extracted from the digests with 0.4 M sulfuric acid (37). Ten to 30 μ g of extracted histones were analyzed by electrophoresis on 15% polyacrylamide gel as described by Panyin and Chalkey (38). (a) histones in S1, (b) histones in Mg^{++} -soluble and (c) insoluble fractions of S1.

tration, 2) that the in vitro association between free enzyme and nucleosomes differed in its sensitivity to Mg^{++} from the "native" association of enzyme with linker DNA found in DNase II digests and 3) that the association observed between enzyme and mono- or oligonucleosomes after micrococcal nuclease digestion represented a rebinding of freed enzyme.

4. Evidence that DNA methyltransferase is preferentially associated with inactive chromatin

The results presented in Tables 2 and 3 demonstrated that most of the DNA methyltransferase released by exposed of nuclei to DNase II was tightly bound to and precipitated with Mg^{++} -insoluble nucleosomes. Since Gottesfeld et al (21) have reported that Mg^{++} insoluble nucleosomes released by DNase II digestion of chromatin were derived from non-transcribed regions of the genome, this result suggested that DNA methyl-

Table 3. Effect of Mg^{++} on the Binding of DNA Methyltransferase to Nucleosomes

Fraction	DNA Content (as % of S1)	DNA Methyltransferase Activity U/10 ⁸ cells	Activity % of S1
Micrococcal Digestion			
<u>S1</u>	100	9.6	100
Mg^{++} -soluble	57.3	8.0	83.0
Mg^{++} -insoluble	43.1	0.1	1.2
<u>S1 + DNA Methyltransferase</u>			
	100	17.8*	
Mg^{++} -soluble	57.3	16.1	
Mg^{++} -insoluble	41.5	0.96	
DNase II Digestion			
<u>S1</u>	100	6.3	100
Mg^{++} -soluble	1.0	0.85	13.5
Mg^{++} -insoluble	58.4	2.9	46.0
<u>S1 + DNA Methyltransferase</u>			
	100	15.4*	
Mg^{++} -soluble	1.5	11.2	
Mg^{++} -insoluble	95.4	2.4	

The micrococcal nuclease digest S1 contained 13% of input nuclear DNA and 33% of total DNA methyltransferase activity. The DNase II digest S1 contained 15% of input nuclear DNA and 22% of total DNA methyltransferase activity. * 8.7 U of DNA methyltransferase (0.3 M NaCl extract) were added to micrococcal and DNase II S1 prior to raising the Mg^{++} concentration to 8 mM for preparation of Mg^{++} -soluble and insoluble fractions.

transferase was primarily associated with inactive chromatin. However, our conditions for DNase II digestion of nuclei were markedly different from those employed by Gottesfeld. Thus, we reinvestigated the distribution of DNA methyltransferase activity using chromatin fractions prepared as described by these authors.

As shown in Table 4, approximately 20% of the DNA methyltransferase was released from chromatin into S1 under these conditions. When nucleosomes in S1 were fractionated into "activity transcribed" and "inactive" fractions by increasing the Mg^{++} concentration to 2 mM, only 16.4% of the

Table 4. Distribution of DNA Methyltransferase Activity in "Active" and "Inactive" Chromatin Fractions

Fraction	DNA Content		DNA Methyltransferase Activity		
	% of total	% of S1	U/10 ⁸ cells	% of total	% of S1
Total Chromatin	100	--	25.8	100	--
S1	24.8	100	5.8	22.3	100
Mg ⁺⁺ -soluble (S2)	13.6	54.0	.95	3.7	16.4
Mg ⁺⁺ -insoluble (P2)	8.5	34.2	2.5	9.8	43.0

Isolated chromatin was digested with DNase II. "Active" and "Inactive" chromatins were fractionated in S1 supernatant on the basis of their differential solubility with regard to Mg⁺⁺ as described (21) (see Methods).

DNA methyltransferase activity in S1 remained with the nucleosomes derived from the actively transcribed chromatin (S2). The recovery of 43% of the enzyme from the Mg⁺⁺ precipitate containing inactive chromatin (P2), represented a minimal value for the enzyme content, since this fraction could not be completely solubilized. This result indicated that only a small proportion of DNA methyltransferase (<4%) was associated with actively transcribed chromatin. Additional, circumstantial evidence supporting this conclusion was our finding that less than 1% of total DNA methyltransferase was released from FL cell nuclei during micrococcal nuclease digestion under conditions reported to preferentially release transcribed chromatin (27). We also found that digestion of intact nuclei with DNase I to the point where 12-15% of the DNA was rendered acid soluble and where actively transcribed chromatin should have been completely degraded (28,29), released no more than 15-25% of the DNA methyltransferase. Although this data could reflect a random distribution of the enzyme, it ruled out the possibility that the enzyme was concentrated in actively transcribed regions. With more limited digestion (7-8% of the DNA solubilized), less than 4% of the enzyme was released. It has been shown that coding regions of active genes are most sensitive to DNase I digestion but that 7-8 kb of DNA on either side of genes is also digested

(30). Thus, the data are compatible with our other results which indicated that most DNA methyltransferase is bound to inactive chromatin.

5. Evidence for an effect of chromatin protein on DNA methylation

To determine whether chromatin proteins could prevent methylation of potentially methylatable sites in DNA by homologous DNA methyltransferases, we compared the efficiency with which the enzyme methylated DNA in chromatin and purified DNA.

As shown in Table 5, FL cell DNA methyltransferase transferred 6-8 times more methyl groups to purified DNA than to DNA still associated with chromatin proteins indicating that some potentially methylatable sites in the DNA were blocked. The chromatin proteins were also effective in preventing the access of homologous methyltransferase to sites which had become hypomethylated during the process of differentiation. As we have previously reported (6,7), DNA isolated from FL cells undergoing differentiation induced by Me_2SO , is a 3-5

Table 5. In Vitro Methylation of FL Cell Chromatin and Nucleosomal DNA

Addition to incubation mixture	Treatment of cells prior DNA isolation	
	None	Me_2SO (240mM)
	cpm/ A_{260}	
Purified DNA + DNA Methyltransferase	59,925	173,780
Nucleosomal DNA + DNA Methyltransferase	58,750	174,530
Chromatin + DNA Methyltransferase	14,890	13,010
Nucleosomes + DNA Methyltransferase	15,000	13,300
Chromatin	3,700	2,720
Nucleosomes	11,000	10,900

Nucleosomes were purified from an S1 micrococcal nuclease digest by sucrose gradient. DNA was isolated from chromatin and nucleosomes by banding in CsCl gradient. Incorporation of $[^3\text{H}]$ -methyl groups was performed as described in Methods. Each assay contained 0.2 A_{260} U of DNA. Incubation in the presence or absence of added enzyme (40U) was for 1 h at 37°. Incorporation of radio-label was not increased by adding more enzyme or extending the incubation period.

fold better methyl acceptor in vitro than is DNA purified from uninduced cells. However, DNA in chromatin or nucleosomes isolated from Me_2SO induced cells did not accept more methyl groups from either added or endogenous methyltransferase than did DNA in chromatin or nucleosomes from untreated FL cells. The increase in methylatable sites which occurred during differentiation could be detected only after the DNA had been freed of chromatin proteins.

DISCUSSION

Our aim in the work described here was to elucidate the structural localization of DNA methyltransferase in the chromatin of FL cells. The evidence presented above indicates that the enzyme is primarily associated with internucleosomal DNA in the more condensed, transcriptionally inactive regions of chromatin.

The conclusion that the enzyme is normally bound to linker DNA of inactive chromatin in intact nuclei is based on the following observations:

- 1) Almost all of the active enzyme released from nuclei by limited digestion with DNase II remains tightly bound to oligonucleosomes which contain linker DNA and can be precipitated with Mg^{++} .

- 2) When linker DNA is degraded by micrococcal nuclease digestion most of the enzyme is released in a form no longer coprecipitable with oligonucleosomes in the presence of Mg^{++} .

- 3) Enzyme stripped from chromatin by 0.3 M NaCl preferentially rebinds to oligonucleosomes when exposed to a mixture of mono- and oligonucleosomes.

It is assumed that the native association between DNA methyltransferase and chromatin is not sensitive to disruption by Mg^{++} while the association which occurs when the enzyme rebinds to chromatin in vitro is, an assumption supported by the observation that 0.3 M NaCl extracted enzyme rebound to oligonucleosomal fragments can be solubilized with 8 mM Mg^{++} while enzyme associated with large chromatin fragments resulting from DNase II digestion cannot.

It should be noted that our results suggest that in vitro

binding of enzyme to nucleosomes may not require linker DNA even though the enzyme-monomonucleosome complex sediments more rapidly than most mononucleosomes in a sucrose density gradient (Fig. 7c,d). The enzyme will bind almost quantitatively to Mg^{++} -soluble mononucleosomes when they are the only species present. However, even though the DNA isolated from such mononucleosomes is heterogenous in length, we found no significant difference in length distribution over the mononucleosome peak indicative of an enrichment of linker-containing mononucleosomes at the heavy side of the peak nor have we been able to detect any histone H1 associated with these mononucleosomes. Thus, the enzyme appears able to bind to mononucleosome cores equivalent to those described by Levy-Wilson et al (MN1-ref 30) and Todd-Garrard (M1, M2-ref 32). From studies of DNA methyltransferase partially purified from different cell types it appears that at low ionic strength the enzyme has a tendency to form multimers with MW 100,000 - 200,000 (33-35). Thus, association of one or several such multimers with a nucleosome core would be sufficient to increase the sedimentation coefficient of the resulting nucleoprotein particle as observed.

Giri et al (20) have reported that poly (ADP-ribose) polymerase is associated with a subset of mononucleosomes that retain linker DNA and histone H1. We cannot rule out a similar association of DNA methyltransferase with a minute fraction of such nucleosomes in our preparations. However, either a direct interaction with the DNA exposed on the surface of the nucleosome or with part of a specific binding site remaining on the core nucleosome seems more likely. Preferential association of the enzyme with oligonucleosomes, even in vitro, however suggests that full linker DNA and/or a higher order of chromatin structure is required to insure strong enzyme-chromatin interactions.

The results reported here further indicate that the bulk of DNA methyltransferase is not associated with actively transcribed regions of the genome. No more than 3-4% of total DNA methyltransferase activity is found with the Mg^{++} -soluble "active" nucleosomes released by DNase II digestion of chromatin

as described by Gottesfeld et al (21). Even this low proportion of activity may represent the result of degradation of some linker DNA in inactive regions rather than a release from active chromatin. In addition, under conditions which should preferentially degrade "active" chromatin, DNase I (28,29) and micrococcal nucleases (27) failed to release a substantial proportion of the DNA methyltransferase activity from nuclei as would be expected if the enzyme was preferentially associated to active genes. This result is not inconsistent with the proposal that DNA methylation is involved in control of gene expression. If, as postulated, lack of methylation of specific sites is of some importance in allowing gene expression, it can be assumed that modification or maintenance of a specific DNA methylation pattern must be set before transcription starts.

Our in vitro results indicate that sites within core nucleosome are not readily accessible to enzyme and that the enzyme is located on linker DNA. If, as suggested by Cantor (36), nucleosomes move along DNA by a "rolling" mechanism, a piece of DNA may be covered by core histones at one time and be exposed in linker regions at another. This may mean that DNA methyltransferase is able to methylate cytosine in DNA only when a potentially methylatable site becomes exposed in linker regions. Methylation by such a mechanism would allow chromosomal proteins that alter chromatin configuration to play a role in regulating DNA methylation. In this light, it is of interest that although the overall distribution and amount of DNA methyltransferase in FL cells undergoing induced differentiation does not differ markedly from that of untreated FL cells, the enzyme is much less readily released from nuclei of differentiating cells by micrococcal nuclease digestion (data not shown). Whether this indicates subtle changes in enzyme-chromatin interaction are occurring as a result of association of the enzyme with regulatory proteins or indicates a sequestering of the enzyme in tightly condensed chromatin, leading to the hypomethylation of newly synthesized DNA we have observed during FL cell differentiation or whether it simply reflects unrelated

changes in chromatin configuration occurring as these cells mature remains to be determined.

Acknowledgements: We are deeply grateful to Dr. G. Acs for his helpful discussions during the progress of this work. We also thank Dr. G. Vorsanger whose initial experiments with FL cell chromatin led to the work described here and to Mrs. N. Schneiderman for her excellent technical assistance. This work was supported in part by NIH grants R01 CA 16890 and R01 CA 25985. FC is an Attachée de Recherche de la Recherche Scientifique (FR). JC is a Senior Investigator of the N.Y. Heart Association.

REFERENCES

1. Burdon, R.H. and Adams, R.L.P. (1980) *TIBS* 5, 294-297.
2. Drahovsky, D. and Boehm, T.J.L. (1980) *Int. J. Biochem.* 12, 523-528.
3. Ehrlich, M.E. and Wang, R.H. (1981) *Science* 212, 1350-1357.
4. Razin, A. and Riggs, A. (1980) *Science* 210, 604-610.
5. Cantoni, G.L., Richards, H.H. and Chiang, P.K. (1978) in *Transmethylation* (Usdin, E., Borchardt, R.T. and Creveling, C.R., Eds.) Elsevier/North Holland pp 155-169.
6. Christman, J.K., Price, P., Pedrinan, L. and Acs, G. (1977) *Eur. J. Biochem.* 81, 53-61.
7. Christman, J.K., Welch, N., Schoenbrun, B., Schneiderman, N. and Acs, G. (1980) *J. Cell. Biol.* 86, 366-370.
8. Mendelsohn, N., Mickl, J., Gilbert, H.S., Acs, G. and Christman, J.K. (1980) *Cancer Res.* 40, 3206-3210.
9. Friedman, S. (1979) *Biochem. Biophys. Res. Commun.* 89, 1328-1333.
10. Creusot, F., Herzog, D., Acs, G. and Christman, J.K. (1981) *Proc. Amer. Assoc. Cancer Res.* 22, 48; *J. Biol. Chem.* (submitted).
11. Lu, L.J. and Randerath, K. (1980) *Cancer Res.* 40, 2701-2705.
12. Jones, P.A. and Taylor, S.M. (1980) *Cell* 20, 85-93.
13. Mendelsohn, N., Herzog, D. and Christman, J.K. (1981) *Proc. Amer. Assoc. Cancer Res.* 22, 48.
14. Bird, A.P. (1978) *J. Mol. Biol.* 118, 49-60.
15. Browne, M.J. and Burdon, R.H. (1977) *Nucleic Acids Res.* 4, 1025-1037.
16. Doenecke, D. and McCarthy, B.J. (1976) *Eur. J. Biochem.* 64, 405-409.
17. Bloch, S. and Cedar, H. (1976) *Nucleic Acids Res.* 3, 1507-1518.
18. Sheid, B., Srinivasan, P.R. and Borek, E. (1968) *Biochemistry* 7, 280-285.
19. Creusot, F. and Christman, J.K. (1980) *Anal. Biochem.* 103, 343-349.
20. Giri, C.P., West, M.H.P., Ramirez, M.L. and Smulson, M. (1978) *Biochemistry* 17, 3504-3505.
21. Gottesfeld, J.M., Garrard, W.T., Baji, G., Wilson, R.F. and Bonner, J. (1974) *Proc. Natl. Acad. Sci USA* 71, 2193-2197.

22. Simpson, R.T. (1978) *Biochemistry* 17, 5524-5531.
23. Brunk, C.F., Jones, K.C. and James, T.W. (1979) *Anal. Biochem.* 92, 497-500.
24. Bonner, J. (1979) in *Chromatin Structure and Function*, Pt. A ed. C.A. Nicolini (Plenum Press) p. 15-23.
25. Kornberg, R.D. (1977) *Ann. Rev. Biochem.* 46, 931-954.
26. Allan, J., Hartman, P.G., Crane-Robinson, C. and Aviles, F.X. (1980) *Nature* 288, 675-679.
27. Dimitriadis, G.J. and Tata, J.R. (1980) *Biochem. J.* 187, 467-477.
28. Weintraub, H. and Groudine, M. (1976) *Science* 193, 848-856.
29. Garel, A. and Axel, R. (1976) *Proc. Natl. Acad. Sci. USA* 73, 3966-3970.
30. Stalder, J., Larsen, A., Engel, J.D., Dolan, M., Groudine M. and Weintraub, H. (1980) *Cell* 20, 451-456.
31. Levy-Wilson, B. and Dixon, G.H. (1979) *Proc. Natl. Acad. Sci. USA* 76, 1682-1686.
32. Todd, R. and Garrard, W.T. (1979) *J. Biol. Chem.* 254, 3074-3083.
33. Turnbull, J.F. and Adams, R.L.P. (1976) *Nucleic Acids Res.* 3, 677-695.
34. Adams, R.L.P., McKay, E.L., Craig, L.M. and Burdon, R.H. (1979) *Biochim. Biophys. Acta* 561, 345-357.
35. Sneider, T.W., Teague, W.M. and Rogachevsky, L.M. (1975) *Nucleic Acids Res.* 2, 1685-1700.
36. Cantor, C.R. (1976) *Proc. Natl. Acad. Sci. USA* 73, 3391-3393.
37. Noll, M. and Kornberg, R.D. (1977) *J. Mol. Biol.* 109, 393-404.
38. Panyin, S. and Chalkey, R. (1969) *Arch. Biochem. Biophys.* 130, 337-346.