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5-Methylcytosine is localized in nucleosomes that contain histone H1

(DNA methylation/chromatin/gene expression/high mobility group proteins/immunoautoradiography)

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ABSTRACT **We have developed a procedure for purifying highly specific polyclonal antibodies against 5-methylcytosine. These antibodies were used to probe the distribution of 5-methylcytosine among fractionated nucleosomes of mouse cell chromatin. Our results demonstrate that at least 80% of the 5-methylcytosine is localized in nucleosomes that contain histone H1. Native nucleosomes that lack histone H1 or possess high mobility group proteins package DNA that is 1.6- to 2.3-fold undermethylated. We suggest that the preferential association of methylated sequences with histone H1 has functional significance because DNA methylation has been linked to gene inactivation and histone H1 is known to promote chromatin condensation.**

Although DNA methylation has been implicated in the epigenetic repression of genetic information in higher eukaryotes (see refs. 1 and 2 for reviews), the mechanism of this repression has not yet been elucidated. In prokaryotes, DNA methylation has been shown to alter the binding affinities of specific proteins for specific DNA sequences (3, 4). If a generally similar (but not necessarily identical) mechanism exists in eukaryotes, then one might predict that methylation either reduces or promotes the binding of specific chromosomal proteins to DNA.

Previous studies on chromatin have suggested that sequences that contain 5-methylcytosine (m^5C) are packaged into nucleosomes (5–7) and may be primarily localized within core particles (7). However, nucleosomes are chemically heterogeneous because of the association of different accessory proteins with histone octamer–DNA complexes. The highly abundant nonhistone proteins, high mobility group (HMG) 14 and 17, are believed to be bound to nucleosomes in transcribed regions of chromatin (8), whereas some evidence suggests that histone H1 may be enriched in inactive chromatin (9, 10). Most nucleosomes that possess HMG proteins seem to lack histone H1 and vice versa (11), indicating that these proteins are nonrandomly distributed, although not every nucleosome that lacks HMG proteins possesses histone H1 (12). Based on stoichiometry estimates, at least 40% of the nucleosomes of a cultured mouse cell line lack histone H1 (13), but H1 content varies between biological systems (13, 14), and estimates of the fraction of nucleosomes that lack H1 *in vivo* are dependent on whether 1 or 2 mol of this protein are bound per nucleosome (15).

The proteins associated with nucleosomes that package methylated DNA have not been identified. We have addressed this question in the present study by fractionating nucleosomes into histone H1-depleted (fraction S1) and histone H1-enriched (fraction S2) components and have further resolved these fractions by gel electrophoresis into either DNA components or discrete nucleosome subsets that possess well-defined protein compositions. By modifying the immunological techniques of Sager and co-workers (16) to probe the m^5C content among these species, we have found that m^5C is localized primarily in nucleosomes that contain histone H1 and is underrepresented in native nucleosomes that lack histone H1 or possess HMG proteins. These findings suggest a general mechanism for the maintenance and propagation of inactive chromatin structures in higher eukaryotic cells.

MATERIALS AND METHODS

Production and Isolation of Antibodies Against m^5C. A m^5C–bovine serum albumin (Sigma fraction V) conjugate, which contained ≈9 mol of m^5C per mol of bovine serum albumin, was prepared as described elsewhere (17). Female New Zealand White rabbits were immunized intramuscularly and were given booster injections 2 wk later with 1 mg of conjugate, by using complete and incomplete Freund's adjuvant, respectively. Subsequent booster injections given at monthly intervals used 0.25 mg of conjugate in incomplete Freund's adjuvant. Immune sera were collected 11–14 days after the booster injections. Immunoglobulin was precipitated with sodium sulfate (18), suspended in 0.5 vol of phosphate-buffered saline (P_i/NaCl)/1 mM phenylmethylsufonyl fluoride ($PhMeSO_2F$), and dialyzed for 16 hr at 4°C against the same buffer. The dialysate (15 ml) was combined with antibody affinity resin (3 g of wet weight; see below) and the slurry was mixed by rotation for 16 hr at 4°C. A column was packed at 24°C and after the effluent had been recycled once, the column was washed with 100 ml of P_i/NaCl/1 mM $PhMeSO_2F$. Antibody [affinity-purified rabbit polyclonal antibodies against m^5C (anti-m^5C)] was eluted with 0.1 M glycine·HCl/0.5 M NaCl, pH 2.5, into tubes that contained sufficient Tris base to neutralize the pH of effluent solutions. Tubes were cooled to 0°C, fractions that contained protein were pooled, and, after dialysis against P_i/NaCl/1 mM $PhMeSO_2F$, aliquots were lyophilized and stored at −70°C. Approximately 6 mg of anti-m^5C was recovered from 30 ml of sera. The antibody affinity resin consisted of cytidine and 5-methyluridine conjugated to keyhole limpet hemacyanin (Calbiochem), which was coupled to Sepharose 4B, washed with 2 vol of 1 mM HCl, and equilibrated with P_i/NaCl/1 mM $PhMeSO_2F$ (17, 19). We emphasize that use of a m^5C–bovine serum albumin–Sepharose 4B column (20) resulted in a lower recovery of antibody, and such preparations exhibited greater crossreactivity with cytosine. Presumably, highly specific anti-m^5C remains bound to m^5C–bovine serum albumin–Sepharose 4B columns under our elution conditions, and therefore we developed the described alternative procedure.

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Abbreviations: anti-DNA, mouse monoclonal antibody against single-stranded DNA; m^5C, 5-methylcytosine; anti-m^5C, affinity-purified rabbit polyclonal antibodies against m^5C; DBM-paper, diazobenzyloxymethyl-cellulose; HMG, high mobility group; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; bp, base pair(s).

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Preparation of Nuclei, Chromatin, and DNA. Mouse mastocytoma (line P815) cells were grown and uniformly labeled as described elsewhere (13). Published techniques were used for nuclei isolation and micrococcal nuclease digestion (21), quantitation of digestion and DNA yields, and DNA purification (22). For preparation of chromatin fractions, digested nuclei were cooled rapidly and centrifuged, yielding the S1 supernatant; the nuclear pellet was then lysed by suspension in 2 mM EDTA and centrifuged, yielding the S2 supernatant and the P pellet fraction, as described in detail elsewhere (23). Calf thymus DNA was from Sigma; restriction endonucleases were from New England BioLabs. *Escherichia coli* K12 strains (GM31 and C600) were kindly provided by Paul L. Modrich.

Gel Electrophoresis, DNA Transfer, and Dot Blots. Purified DNA samples were separated by using 5% acrylamide/0.1% $NaDodSO_4$ slab gels (21); nucleoproteins were resolved by using 3.5% acrylamide/0.5% agarose/30% glycerol slab gels (15). For electroelution of mononucleosomes, ethidium bromide-stained excised bands that had been placed in dialysis bags with nucleoprotein electrophoresis buffer (15) were subjected to electrophoresis while being submerged in the same buffer in an open chamber. Published procedures were employed for denaturing and electrophoretically transferring DNA from gels to diazobenzyloxymethyl-cellulose (DBM-paper) (ref. 21), except that a one-fifth power setting was used for the first hour to improve retention of 121-base-pair (bp) pBR322 fragments. Dot blots were performed essentially as described elsewhere (23).

Fluorography and Immunoautoradiography. Immobilized DNA displays were treated with 10% 2,5-diphenyloxazole in toluene and subjected to fluorography by using preflashed film as described (24). Exposures were for 5–10 days. Fluor was removed by washing for two 30-min periods with acetone, followed by distilled water. Anti-m^5C binding to immobilized DNA was assayed by a published technique (25), except 5% horse serum was substituted for bovine serum albumin. To reduce potential crossreactivity, anti-m^5C (15–20 μg/ml) was preincubated at 24°C for 30 min with 100 μM (each) cytidine and thymidine (unless otherwise indicated in the text). Immobilized DNA displays were reacted with 0.1 ml of primary antibody per cm^2 and 1×10^6 cpm of affinity-purified, ^{125}I-labeled goat anti-rabbit secondary antibody per ml (0.1 ml/cm^2) (ref. 25). After iodination (26), secondary antibody was purified by chromatography over a Sephadex G-50 column in P_i/NaCl/0.25% gelatin. Aliquots stored at −70°C were used within 6–8 wk. Techniques for probing with a mouse monoclonal antibody against single-stranded DNA (H43SC1), a gift of B. David Stollar, are described elsewhere (21). For autoradiography, DBM-paper was blotted dry, wrapped in plastic wrap, and exposed at −70°C to preflashed film, by using a DuPont Cronex Lightning Plus AG intensifying screen. Exposure times ranged from 30 min to 2 hr. Reactions exhibiting linearity with DNA concentration were quantitated by densitometry. For purposes of reprobing, antibody complexes were removed by soaking filters for 16 hr at 37°C in 25 mM Tris/10 mM EDTA/0.1% $NaDodSO_4$/50 μg of proteinase K per ml, pH 7.5, followed by washing for two 1-hr intervals with 0.4 M NaOH at 24°C and then with distilled water.

RESULTS

Antibodies Against m^5C Are Highly Specific. We have developed a procedure, which employs an analog affinity column that contains cytidine and 5-methyluridine, to purify highly specific polyclonal antibodies against m^5C (anti-m^5C). Fig. 1*A* shows that anti-m^5C reacts preferentially with calf or mouse DNA, which contain m^5C, as compared to m^5C-deficient DNA prepared from an *E. coli* mutant. The antibody reaction is inhibited by preincubation with m^5C but not by preincubation with cytidine or thymidine. That the *E. coli* DNA was present and accessible for reaction was demonstrated by reprobing with a mouse monoclonal antibody against single-stranded DNA (anti-DNA).

To assess further the level of sensitivity of the anti-m^5C reaction, we assayed anti-m^5C binding to restriction fragments of pBR322 that differed in m^5C content. The *Bst*N1 sites of pBR322 are methylated in wild-type but not in *dcm*$^-$ *E. coli* K12 (data not shown; ref. 27). Plasmids grown in *dcm*$^+$ and *dcm*$^-$ strains were digested with this enzyme so that each duplex fragment originating from the methylated plasmid would have two m^5C residues (one at each 3′ end). After the electrophoretically resolved cleavage products were transferred to DBM-paper, a pairwise comparison of antibody binding to fragments of identical nucleotide sequence reveals that anti-m^5C, but not anti-DNA, preferentially reacts with those fragments that possess methylated *Bst*N1 sites (Fig. 1*B*). Densitometric analysis indicates that at least 25-fold more anti-m^5C binds to methylated, 121- or 383-nucleotide-long fragments than to unmethylated counterparts. The m^5C content of these fragments is within the range of that found in DNA of higher eukaryotes (2). Thus, taken together with the results shown in Fig. 1*A*, we conclude that anti-m^5C is a highly specific probe for methylated sequences in mammalian DNA.

Finally, because the nucleosomal DNA fragments examined in our study vary over 10-fold in molecular weight, we determined the effect of nucleotide chain length on anti-m^5C binding. DNA prepared from [^3H]thymidine-labeled mouse cells was randomly sheared to various extents and size fractionated, and resulting dot blots were subjected to sequential fluorography and anti-m^5C probing. Comparison of the fluorogram and autoradiogram signals shown in Fig. 1*C* reveals that less anti-m^5C binds to DNA fragments of shorter chain lengths. Thus, in the absence of calibration curves, the absolute m^5C content of fragments that differ significantly in chain length cannot be readily ascertained.

Methylated Sequences Are Organized into Typical Chromatin Structures. Prior to studying the proteins associated with nucleosomes that package methylated DNA, we analyzed the overall chromatin structure of these sequences. Nuclei prepared from [^3H]thymidine-labeled mouse cells were digested either briefly or extensively with micrococcal nuclease. DNA purified from these nuclei, or from soluble (S1 and S2) and insoluble (P) chromatin fractions, was electrophoretically separated and transferred to DBM-paper. Resulting immobilized DNA displays were subjected to fluorography and then probed with anti-m^5C (Fig. 2).

The results shown in Fig. 2 reveal that the chromatin structure of methylated DNA is similar to that of bulk DNA. Thus, methylated sequences: (*i*) are packaged into typical polynucleosomal structures that possess repeat lengths like those of bulk DNA (188 bp); (*ii*) are processed by micrococcal nuclease to mononucleosomes at a rate that is not markedly different from that observed for bulk DNA, particularly when the effect of fragment length on anti-m^5C binding is considered (Fig. 1*C*); (*iii*) are found in nucleosomal DNA fragments as short as 146 bp and thus are not exclusively localized in linker regions between nucleosome cores; and (*iv*) are present in soluble chromatin fractions S1 and S2, which comprise about 5% and 80% of the bulk nuclear DNA, respectively (23), and are not significantly enriched in the insoluble chromatin fraction P. (However, a quantitative analysis of the m^5C content of S1 mononucleosomes reveals the presence of undermethylated DNA; see be-

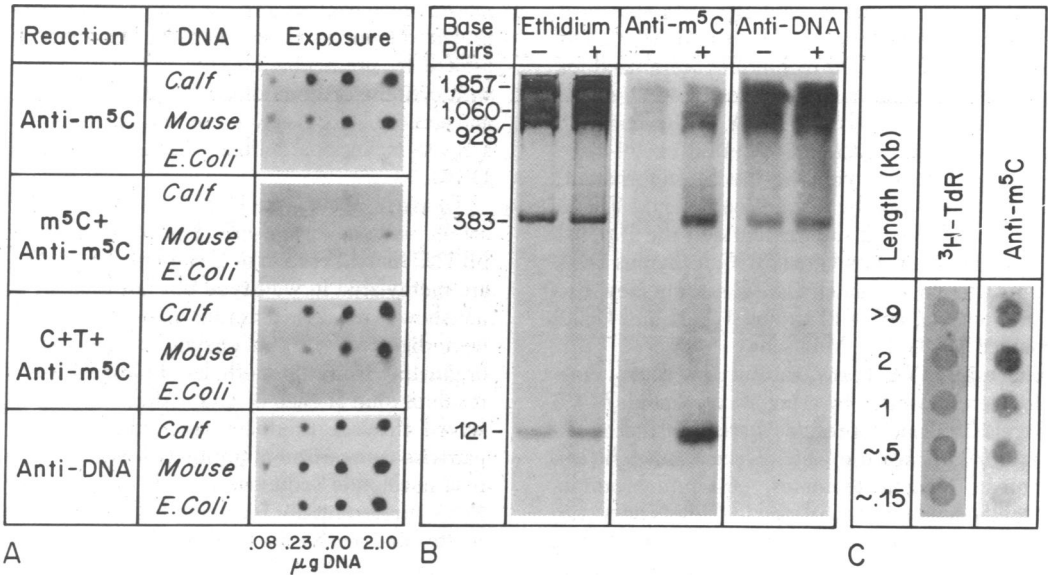

FIG. 1. Immunoautoradiography demonstrates specificity of anti-m^{5}C. (*A*) Purified, denatured DNA of calf thymus, mouse cells (P815), and *E. coli* (GM31) was spotted onto DBM-paper at the indicated concentrations. Immobilized DNA displays were reacted with anti-m^{5}C, in the presence and absence of 0.2 mM m^{5}C or 0.1 mM (each) cytidine and thymidine, and subjected to autoradiography after reacting with ^{125}I-labeled secondary antibody. The upper display was reprobed with anti-DNA (lower display). (*B*) *Bst*N1 restriction fragments of pBR322 with unmethylated (−) or methylated (+) 3′ ends were electrophoretically separated (5 μg per lane), stained with ethidium bromide, and transferred to DBM-paper, and the immobilized DNA was sequentially probed with anti-m^{5}C and anti-DNA. (*C*) Sonicated, [^{3}H]thymidine (^{3}H-TdR)-labeled P815 DNA was size fractionated on agarose gels. Electroeluted, denatured DNA was dot blotted (0.6 μg per spot) onto DBM-paper and subjected to sequential fluorography and anti-m^{5}C probing.

low.) Therefore, at this level of resolution, m^{5}C appears to be uniformly distributed in chromatin.

Methylated Sequences Are Predominantly Localized in Nucleosomes that Contain Histone H1. Previous studies have shown that native nucleosomes that possess DNA and histone octamers can be electrophoretically resolved based on the presence or absence of associated histone H1 and HMG proteins (11, 28). We have taken advantage of this technique to investigate the protein compositions of nucleosomes among soluble chromatin fractions that package methylated sequences. As an initial approach, nuclei prepared from [^{3}H]thymidine-labeled mouse cells were digested to different extents with micrococcal nuclease and the resulting S2 chromatin fractions were resolved electrophoretically. After *in situ* removal of basic proteins (21), DNA components were transferred to DBM-paper for fluorography and anti-m^{5}C probing (Fig. 3).

Close inspection of the data shown in Fig. 3 reveals that significant quantitative differences exist between the distributions of bulk DNA and methylated DNA. Within the dinucleosomal region, anti-m^{5}C preferentially reacts with DNA originating from component D_2 as compared to that of component D_1 (Fig. 3;

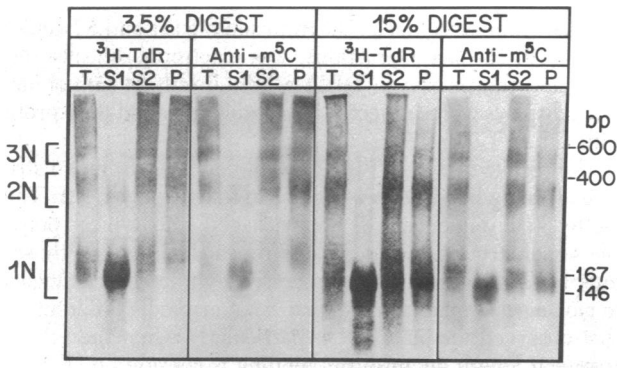

FIG. 2. Methylated sequences are organized into typical nucleosomal arrays. Nuclei prepared from [^{3}H]thymidine (^{3}H-TdR)-labeled P815 cells were digested with micrococcal nuclease to the indicated extents of DNA acid solubility. DNA purified from total nuclei (T) or from S1, S2, and P chromatin fractions was electrophoretically separated (25 μg per lane), transferred to DBM-paper, and immobilized DNA displays were subjected to sequential fluorography and anti-m^{5}C probing. The positions of mono-, di-, and trinucleosomal DNA are indicated (1N, 2N, and 3N, respectively).

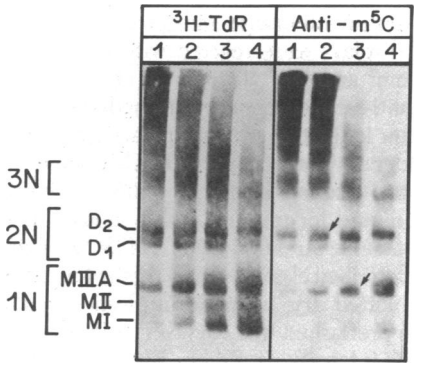

FIG. 3. Methylated sequences are localized nonrandomly among nucleosome classes that possess different accessory proteins. Nuclei prepared from [^{3}H]thymidine (^{3}H-TdR)-labeled P815 cells were digested with micrococcal nuclease to render 3%, 5%, 9%, and 20% of the DNA acid soluble (lanes 1–4, respectively). The S2 chromatin fractions were electrophoretically resolved on a nucleoprotein gel (25 μg per lane), and, after transfer of DNA to DBM-paper, the immobilized DNA was subjected to sequential fluorography and anti-m^{5}C probing. The positions of mono-, di-, and trinucleosomes (1N, 2N, and 3N, respectively) and different electrophoretic classes of mono- (MI, MII, MIIIA) and dinucleosomes (D_1, D_2) are indicated. MI and MII lack histone H1, MIIIA contains histone H1, and D_1 and D_2 possess one and two copies of histone H1 per dinucleosome, respectively (ref. 13; see Table 1 and text for details). D_2 is not processed by micrococcal nuclease digestion to D_1; instead, D_2 is processed directly to MIIIA upon redigestion (12). D_1 may arise, in part, from artifactual histone H1 redistribution (13). Arrows refer to components that react preferentially with anti-m^{5}C.

e.g., lane 2, arrow). Within the mononucleosomal region, the antibody preferentially binds to DNA derived from component MIIIA relative to that of components MII and MI (Fig. 3; e.g., lane 3, arrow). Interestingly, these preferential reactions are localized in nucleosome classes that have previously been shown to contain *stoichiometric* amounts of histone H1 (13); those components that possess undermethylated DNA either lack accessory proteins (MI), possess HMG proteins (MII), or have only one copy of histone H1 per dinucleosome (D_1) (refs. 11 and 13). Moreover, these anti-m^5C binding differences cannot be due to chain length effects (Fig. 1*C*), because different electrophoretic forms of mono- or dinucleosomes possess nearly identical DNA lengths (11, 13). Densitometric analysis indicates that at least 80% of the anti-m^5C binding within the mononucleosome region occurs to DNA derived from the nucleosome class that contains histone H1 (MIIIA), whereas this component comprises no more than 60% of the bulk DNA. Furthermore, DNA derived from nucleosome classes that lack or have reduced amounts of histone H1 is depleted 33–66% in m^5C content relative to that of MIIIA or D_2.

We also analyzed histone H1-deficient nucleosomes of the S1 chromatin fraction that are known to be enriched in HMG proteins and transcribed sequences (data not shown; refs. 10 and 29). Different electrophoretic forms of mononucleosomes were isolated from S1 and S2 chromatin fractions by preparative gel electrophoresis. As shown in Fig. 4, DNA derived from HMG-containing (MII + MIIIB) nucleosomes and HMG-deficient (MI) nucleosomes of the S1 chromatin fraction is depleted about 66% in m^5C content relative to that of histone H1-containing (MIIIA) nucleosomes of the S2 chromatin fraction. The results of these and further analyses are summarized in Table 1 and demonstrate that relative to MIIIA, DNA originating from other mononucleosome classes exhibits a statistically significant 37–56% mean depletion in m^5C content (range: 17–70%). We consider this depletion to be a minimal estimate because of the potential occurrence of internucleosomal protein exchange (11, 13, 30) and because a portion of H1-minus (MI) nucleosomes may be derived from H1-plus (MIIIA) nucleosomes by nuclease-mediated processing (12). Therefore, we conclude that m^5C is localized primarily in nucleosomes that contain histone H1.

DISCUSSION

Utilizing a combination of immunological and electrophoretic techniques, we confirm and extend previous reports that m^5C is predominantly packaged into nucleosomal chromatin (5–7).

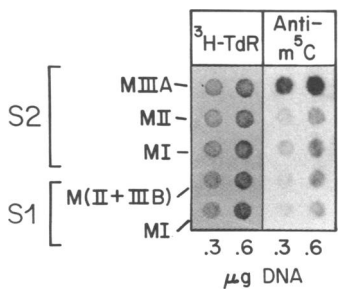

FIG. 4. Methylated sequences are predominantly localized in the mononucleosome class that contains histone H1. Mononucleosomes from the S1 and S2 chromatin fractions of a 15% acid-soluble nuclear digest of [^{3}H]thymidine (^{3}H-TdR)-labeled P815 cells were electrophoretically isolated. Purified, denatured DNA of these components was dot blotted onto DBM-paper at the concentrations indicated below the composite, and subjected to sequential fluorography and anti-m^5C probing. See Table 1 for a description of mononucleosome classes.

Table 1. Distribution of m^5C among different electrophoretic forms of mononucleosomes

Chromatin fraction	Mononucleosome class	DNA length, bp	Major accesory proteins	Relative m^5C content	*P*
S2	MIIIA	160–195	H1	1.00	—
	MII	150–165	HMG-14, -17	0.56 ± 0.13	<0.01
	MI	146–160	None	0.63 ± 0.19	0.03
S1	MII + MIIIB	140–155	HMG-14, -17	0.47 ± 0.20	0.01
	MI	140–150	None	0.44 ± 0.21	0.01

The analysis shown in Fig. 4 was performed in duplicate. Fluorograms and autoradiograms were scanned for each dot and the ratios of these areas were determined. These ratios were normalized to that of MIIIA, yielding the indicated means and standard deviations ($n = 4$). [The absolute content of m^5C in total mononucleosomal DNA, as determined by radioimmunoassay, is close to that found in bulk DNA (data not shown)]. Data were analyzed by a paired Student *t* test, yielding the indicated *P* values. Protein compositions and DNA lengths are from published work (11–13) and data not shown.

Indeed, methylated DNA is organized into chromatin structures that possess many properties that are characteristic of bulk DNA (Fig. 2). Our results also substantiate previous reports that nucleosomes that possess HMG proteins have undermethylated DNA (29, 31, 32). However, the major finding of our study is that m^5C is predominantly localized in native nucleosomes that contain histone H1 and is underrepresented in all other major nucleosome species. As discussed below, this finding may have functional implications with regard to the maintenance and propagation of inactive chromatin structures.

We find that *at least* 80% of the m^5C of soluble chromatin is packaged into nucleosomes that contain histone H1. This association is nonrandom because, as previously demonstrated (13), only 60% of the nucleosomes of soluble chromatin possess histone H1. How might this nonrandom distribution be maintained and propagated? If accessory proteins are in equilibrium between preferred binding sites *in vivo* (11, 30, 32), then methylation might directly increase the binding affinity of histone H1 to nucleosomal DNA or reduce the binding affinities of abundant nonhistone proteins (or both). This could occur by modification of key cytosine residues, either within protein binding sites or in distal regions that may conformationally alter such binding sites. Furthermore, because methylation is symmetric, semiconservative replication coupled with maintenance methylation ensures the faithful inheritance of methyl groups to daughter cells (1, 2). Therefore, if methylation is the signal for selective deposition of histone H1, then the fidelity of propagation of H1-associated chromatin structures is guaranteed.

Where are methyl groups located within the framework of the nucleosomal repeat? Because nucleosome classes that lack histone H1 may have arisen, in part, from the digestion of the H1 binding site external to 146-bp core particle DNA (15), the observed underrepresentation of methyl groups in H1-minus nucleosomes might be explained if methyl groups are preferentially located in nuclease-sensitive linker regions between nucleosome cores. However, Solage and Cedar (7) have shown that m^5C is highly resistant to conversion to acid-soluble nucleotides upon extensive digestion of chromatin with micrococcal nuclease. In addition, we have found that methyl groups are present in 146-bp core particle DNA (Fig. 2) and that dinucleosomes, which still possess intact linkers, differ in m^5C content depending on their H1 content (Fig. 3). Taken together, these findings argue that the association of histone H1 is cor-

related with the presence of methylated sequences within 146-bp nucleosome core DNA.

Dinucleosomes that contain two histone H1 molecules are enriched in methylated sequences relative to dinucleosomes that possess one histone H1 molecule (Fig. 3). This intriguing finding suggests that methylated sequences are localized primarily in nucleosome nearest neighbors along chromatin fibers. Alternatively, the presence of m^5C in a single nucleosome conceivably could initiate a "spreading effect" with respect to histone H1 association via cooperative horizontal templating (33).

It can be estimated that 90–95% of the total m^5C of nuclear DNA is localized in nontranscribed regions of chromatin (31). Our results permit the conclusion that such regions are predominantly associated with histone H1. The preferential location of histone H1 along nontranscribed regions of chromatin is also supported by the results of experiments that have monitored the distribution of expressed sequences among different chromatin fractions (refs. 9, 10, and 23; unpublished data). Furthermore, this protein family possesses several properties that make it an attractive candidate to serve generalized roles in genetic repression: (*i*) H1 seals the two turns of DNA about the histone octamer (15, 34–37); (*ii*) H1 stabilizes nucleosomal DNA to thermal denaturation (34); and (*iii*) H1 promotes the higher order coiling of nucleosomes (35, 37).

The link between m^5C and inactive chromatin is clearly not absolute. Both *Saccharomyces* and *Drosophila* have been reported to lack m^5C (2, 38). In higher eukaryotes, undermethylation alone is not always sufficient to activate gene expression (39), and certain active genes possess detectable and sometimes significant levels of methylation (40). Furthermore, total expressed sequences are depleted only 50–66% in m^5C content (31) and therefore possess a methylation level close to that observed for nucleosomes that lack H1. Thus, only a subset of methyl groups appears to be functionally important. Possibly this subset corresponds to those methyl groups that are phased properly with respect to the path of nucleosomal DNA (41), within 5′ regions of genes (42), to facilitate the association of histone H1 according to the proposals discussed above.

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