Organization of 5-Methylcytosine in Chromosomal DNA†

Adinah Solage and Howard Cedar*

ABSTRACT: The 5-methylcytosine residues of L-cells have been labeled with [*methyl*-^{3}H]-L-methionine and their chromatin localization studied using deoxyribonucleases. The kinetics of micrococcal nuclease digestion showed that the methylated cytosine residues are concentrated within regions resistant to nuclease digestion and preferentially missing from those regions between nucleosomes which are nuclease sensitive. Using DNA hybridization kinetic analysis, it is shown that 5-methylcytosine is abundant in highly repeated sequences but is also present in middle repetitive and unique sequence DNA.

Although DNA methylation in bacteria has been fairly well studied, the biological significance of most of these methyl groups is still obscure. Only in some cases is the function of methylated bases known. Thus, methylation is known to be involved in restriction modification (Arber & Linn, 1969) and may have a role in bacterial DNA replication (Lark, 1968; Billen, 1968). In the bacteriophage ϕX 174, viral DNA methylation plays a role in the final steps of virus maturation (Razin et al., 1975). A single m^{5}Cyt residue in the phage DNA seems to serve as a recognition site for a specific endonuclease which is necessary to process the newly replicated DNA into viral DNA of one genome length.

In eukaryotes only cytosine occurs in a methylated form and the biological role of this base has not been well studied. One aspect which could shed light on the function of this modified base in eukaryotes concerns its localization with respect to particular DNA sequences and its distribution with respect to the chromatin proteins. Previous work has demonstrated that, in both of these cases, the methyl groups are distributed nonrandomly. Thus, using cesium chloride gradient analysis (Salomon et al., 1969; Harbers et al., 1975) and in situ immunofluorescence (Miller et al., 1974), it has been shown that 5-methylcytosine is concentrated in highly repetitive satellite sequences. By employing mass spectrometric analysis we have previously demonstrated that these methyl moieties are selectively protected from digestion by micrococcal nuclease (Razin & Cedar, 1977), indicating that there is a specific relation between chromatin proteins and these DNA methyl groups. In this paper we attempt to further define the distribution of methylcytosine with regard to DNA sequences and the localization of these groups with respect to the structure of chromatin.

Materials and Methods

Materials. [^{3}H]Thymidine (42 Ci/mmol), [^{3}H]cytidine (20 Ci/mmol), and [*methyl*-^{3}H]-L-methionine (45 Ci/mmol) were obtained from New England Nuclear Corp. Purified micrococcal nuclease and RNase free of DNase were purchased from Worthington Biochemical Co. Pancreatic deoxyribonuclease I (DNase I) was obtained from Sigma, Proteinase K from E. Merck, and trifluoroacetic acid (Reagent 99%+) from Pierce Chemical Co.

Growth and Labeling of L-Cells. Mouse L-cells were the kind gift of Dr. David Paretsky. They were grown either in suspension or in monolayer in Eagle's minimum essential medium supplemented with 5% fetal calf serum in the presence of antibiotic. For the purpose of labeling, cells were washed once in the above medium and transferred to a suspension culture, containing Eagle's minimum essential medium with 10% the normal amount of methionine and supplemented with 7% fetal calf serum (Harbers et al., 1975). To each 50 mL of suspension culture (5×10^6 cells/mL) was added either 50 μCi of [^{3}H]thymidine, 50 μCi of [^{3}H]cytidine, or 1 mCi of [*methyl*-^{3}H]-L-methionine, and the incubation was continued with gentle rotary shaking for a period of 4 h, after which they were washed and stored frozen at −20 °C. During the labeling period, cell growth continues at the same rate as in the normal medium.

Preparation of Nuclei and Purified DNA. Nuclei were isolated essentially as described by Weintraub & Groudine (1976). Cells were suspended in RSB (0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, 3 mM $MgCl_2$) containing 0.5% Nonidet P-40, disrupted by homogenization with a Dounce homogenizer (A pestle), and washed several times in RSB. In order to obtain DNA, nuclei were dissolved in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.9, 5 mM EDTA, 1% sodium dodecyl sulfate and treated with proteinase K (50 μg/mL) at 37 °C for 1 h. The DNA was then extracted once with buffer-saturated phenol and once with chloroform–isoamyl alcohol (24:1) and precipitated with ethanol. The ethanol precipitate was dissolved in 0.1 M NaCl, 10 mM Tris-HCl, pH 7.9, 5 mM EDTA, incubated at 37 °C for 1 h with 25 μg/mL RNase A, extracted with chloroform–isoamyl alcohol, and reprecipitated with ethanol. The DNA was dissolved in 0.1 mM EDTA to the desired concentration. DNA obtained from [^{3}H]thymidine-labeled cells had a specific activity of 3000 cpm/μg. When extracted from [*methyl*-^{3}H]-L-methionine-labeled cells it had a specific activity of 50 cpm/μg. DNA prepared from whole undigested nuclei was sonicated in order to obtain DNA with an average size of 300 nucleotide pairs, suitable for hybridization experiments.

DNA extracted from cells labeled with [*methyl*-^{3}H]-L-methionine was digested to bases by treatment with trifluoroacetic acid and analyzed by two-dimensional, thin-layer chromatography (Razin et al., 1970). By this criterion 85% of the radioactivity was found in m^{5}Cyt with the rest of the counts distributed in the standard nucleotides as well as in some unidentified substances. This percentage, however, is not a constant feature of every fraction of the DNA. Thus, digestion or hybridization of the DNA might reveal DNA fractions in which the fraction of counts in m^{5}Cyt is not the same as in total

† From the Department of Molecular Biology, Hebrew University Medical School, Jerusalem, Israel. *Received January 17, 1978.* This research was supported by U.S. Public Health Service Grant GM20483 and U.S.-Israel Binational Research Foundation Grant 1140.

0006-2960/78/0417-2934$01.00/0

DNA. In order to avoid having to perform chromatographic analysis for every measurement, we developed a technique for normalizing our data to correct for those counts which are not in m^5Cyt. In all of the experiments performed in this work, the fate of m^5Cyt was compared with that of total DNA labeled either with [^{3}H]thymidine or [^{3}H]cytidine.

Let c_m be the amount of DNA radioactivity due to [*methyl*-^{3}H]-L-methionine labeling and c_t be the DNA radioactivity due to [^{3}H]thymidine labeling. We now define:

$$r = c_m/c_t$$

If f is the fraction of methionine counts which are not in m^5Cyt, then R, the ratio of counts in m^5Cyt to counts in thymidine, is:

$$R = \frac{c_m - fc_m}{c_t} = r - rf$$

Although f is not known, the quantity rf must remain constant if we assume that the radioactivity which is not in m^5Cyt behaves identically with the general DNA (represented by c_t). Thus $rf = r_0f_0$, where r_0 is the ratio of counts in untreated DNA and f_0 is the fraction of methionine counts in untreated DNA which are not in m^5Cyt. In our case f_0 is 0.15. Thus

$$R = r - r_0f_0$$

All of the data presented in this paper have been corrected using this simple formula. Since 85% of [*methyl*-^{3}H]-L-methionine enters the m^5Cyt moiety of the DNA, this correction was usually small.

Digestion of Nuclei and DNA by Deoxyribonucleases. Nuclei were suspended in RSB at a concentration of 300 μg/mL nuclear DNA. This concentration was determined by dissolving a small aliquot of the suspension in 5 M urea, 2 M NaCl and measuring the optical density at 260 nm. Nuclei were digested with 10 μg/mL micrococcal nuclease or 10 μg/mL pancreatic DNase I at 37 °C for the times indicated. Digestion by micrococcal nuclease was carried out in the presence of RSB plus 0.1 mM $CaCl_2$. The digestion of nuclei labeled either with [^{3}H]thymidine or [*methyl*-^{3}H]-L-methionine was followed by procedures previously described (Bloch & Cedar, 1976). Using this method [^{3}H]DNA was purified from labeled RNA and protein. The reaction mix was brought to 10 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 0.5% sodium dodecyl sulfate, and 1 mM EDTA and treated with 50 μg/mL proteinase K for 1 h at 37 °C. After extractions by phenol and chloroform–isoamyl alcohol (24:1), the DNA was precipitated with trichloroacetic 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 of 10% trichloroacetic acid. The precipitates were collected on glass fiber filters and washed six times with 3 mL of 5% trichloroacetic acid and dried, and the radioactivity was determined by liquid scintillation counting. It should be noted that 100% of the radioactivity recorded by this technique is sensitive to DNase I digestion, indicating that the counts are not due to labeled RNA or protein. Undigested nuclear DNA was lightly sonicated before treatment with proteinase K in order to permit easy handling of the DNA during the subsequent purification procedures. In some cases the degree of digestion was determined by measuring the acid soluble optical density at 260 nm after precipitation of an aliquot with cold 1 M perchloric acid, 1 M NaCl (Panet & Cedar, 1977). Digestion of purified DNA was carried out in RSB containing 4 μg/mL micrococcal nuclease and 0.3 μg/mL DNA.

DNA Hybridization. Kinetic hybridization was carried out at 68 °C in 20–100 μL containing 1 mM Tris, pH 7.0, 0.4 M NaCl, and 60–1000 μg of DNA. At various time intervals, aliquots were removed and assayed for double-stranded DNA by S_1 nuclease treatment (Weintraub & Groudine, 1976). When radioactivity was used to follow the degree of hybridization, trichloroacetic acid precipitable material was determined by scintillation counting on glass fiber filters, as described above. When the degree of hybridization was determined using unlabeled DNA, the amount of double-stranded DNA was measured by precipitation of S_1-resistant DNA using trichloroacetic acid precipitation. The precipitated DNA was eluted from the glass fiber filter with 1 N NaOH and the amount measured by determination of the optical density at 260 nm. Recovery of the DNA from the filters by this technique is reproducible and greater than 90%.

Determination of 5-Methylcytosine. The content of m^5Cyt in various DNA samples was measured using high resolution mass spectrometry (Deutsch et al., 1976). Samples of DNA or deoxynucleoside monophosphates were dried over concentrated sulfuric acid and solid KOH in vacuo and then treated with trifluoroacetic acid in sealed ampules at 180 °C for 30 min. Trifluoroacetic acid was removed by vacuum and the samples dissolved in H_2O. An equivalent of 1–20 μg of material was injected into the gold cup of the direct inlet and heated to 100 °C to evaporate the H_2O. After lowering the temperature to 20 °C, the sample was inserted into the direct inlet of the ion source. The amount of 5-methylcytosine was determined using the molecular ion peak of thymine as an internal standard. The peak matching unit was set so that the reference thymine peak at *m/e* 126 was placed on one channel and the mass ratio adjusted so that the *m/e* 125 peak fell into the second channel. The two channels were alternately scanned using the peak matcher to flip between the two channels at a slow speed, and results of the scans were fed into a fast response chart recorder. The temperature programmer was then used to automatically raise the temperature so that the bases could be distilled off. The peak heights were plotted as a function of temperature and the area under the curves was measured. The m^5Cyt/Cyt molar ratio was calculated using the base composition data obtained by native and melting spectral analysis (Hirschmann & Felsenfeld, 1966). In all DNA samples used in this paper, the GC content was about 42%.

S_1 resistant DNA was prepared for mass spectrometric analysis by treatment of the S_1 reaction mix with chloroform–isoamyl alcohol (24:1) and ethanol precipitation. The products of S_1 digestion were separated from undigested DNA by trichloroacetic acid precipitation followed by recovery of the nucleotides by absorption to charcoal. Recovery by this technique was greater than 80%.

Results

Digestion of Nuclear m^5Cyt by Micrococcal Nuclease. Extensive treatment of nuclei with micrococcal nuclease results in the digestion of 50% of the nuclear DNA. Using mass spectrometry to determine the amount of m^5Cyt, we have previously shown that the DNA remaining after micrococcal nuclease digestion of chromatin from various sources contains 70% of the nuclear m^5Cyt (Razin & Cedar, 1977). Since mass spectrometry is not suitable for the analysis of the kinetics of this digestion, we have used L-cell nuclei which are radioactively labeled in m^5Cyt. The digestion of m^5Cyt is compared with that of nuclear DNA labeled with [^{3}H]thymidine in Figure 1. Total thymidine-labeled DNA is rapidly digested and at the limit 50% remains resistant to nuclease treatment. In contrast to this, m^5Cyt is digested at a much slower rate and

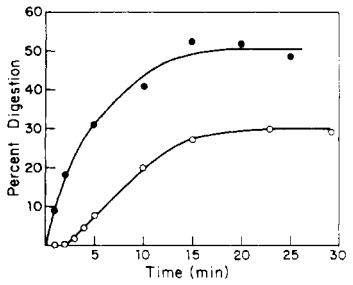

FIGURE 1: Micrococcal nuclease digestion of nuclear m^{5}Cyt. Labeled L-cell nuclei were digested as described in Materials and Methods, using 0.3 mg/mL of nuclear DNA and 10 μg/mL micrococcal nuclease. Cells were labeled either with [^{3}H]thymidine (●) or [^{3}H]methionine (○). The undigested aliquots used in this experiment contained 90 000 cpm of [^{3}H]thymidine or 8000 cpm of [^{3}H]methionine.

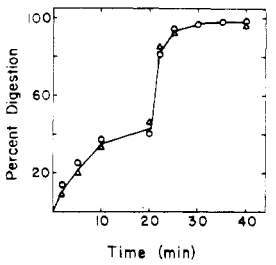

FIGURE 2: Micrococcal nuclease digestion of DNA. Purified DNA (0.3 mg/mL) containing either [^{3}H]thymidine (○) or [^{3}H]m^{5}Cyt (△) was digested with 4 μg/mL micrococcal nuclease. After 20 min of incubation, an additional 8 μg/mL of enzyme was added. Digestion was assayed by trichloroacetic acid precipitation of 50-μL aliquots.

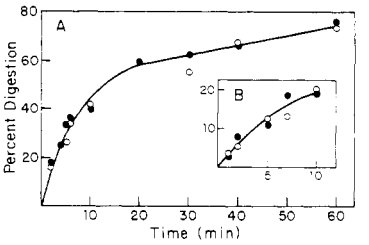

FIGURE 3: DNase I digestion of nuclear m^{5}Cyt. Labeled L-cell nuclei were digested as described in Materials and Methods, using 0.3 mg/mL nuclear DNA and 10 μg/mL DNase I (A) or 2 μg/mL DNase I (B). Cells were labeled either with [^{3}H]thymidine (●) or [^{3}H]methionine (○).

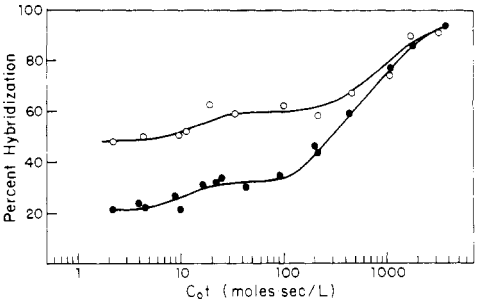

FIGURE 4: Hybridization of m^{5}Cyt. Self-hybridization of DNA was carried out as described in Materials and Methods, using purified DNA. Unless otherwise noted, hybridization was assayed by S_1 digestion. [^{3}H]Thymidine-labeled L-cell DNA (●); [^{3}H]methionine-labeled L-cell DNA (○).

after extensive nuclease action, 70% remains undigested. In addition, the digestion of m^{5}Cyt does not begin until over 15% of the total DNA has been digested. This lag is quite reproducible and has been verified by experiments in which early digestion has been magnified by the use of lower concentrations of enzyme. Similar results were obtained either when the concentration of enzyme or the concentration of nuclei was varied. The observation that very little, if any, m^{5}Cyt is digested at early times was confirmed by mass spectrometric analysis of the nucleoside monophosphates released during digestion. Whereas total L-cell DNA had a m^{5}Cyt/Cyt molar ratio of 0.042, nucleotides released early in digestion (20% of total nuclear DNA) had an m^{5}Cyt/Cyt molar ratio of 0.004. This is ten times less than that expected if digestion of m^{5}Cyt was random with respect to the rest of the DNA.

In order to show that the digestion of [^{3}H]thymidine is representative of the digestion of total DNA, we have assayed total DNA digestion using both optical density at 260 nm and [^{3}H]cytidine as markers. In both cases the kinetics of digestion were identical with that obtained with [^{3}H]thymidine. The fact that cytidine is digested at the same overall rate as thymidine indicates that our results with m^{5}Cyt are not due to any base preference of micrococcal nuclease.

Confirmation of the idea that the specificity of micrococcal nuclease toward m^{5}Cyt is due to the conformation and localization of chromatin proteins is demonstrated in Figure 2. Naked DNA was extracted and purified from L-cell nuclei and subjected to treatment with micrococcal nuclease. In this case, both [^{3}H]thymidine and [^{3}H]m^{5}Cyt were digested at the same rate. The addition of higher concentrations of enzyme after 20 min of digestion shows that, at the limit, almost 100% of both thymidine and m^{5}Cyt are converted to acid soluble material. Thus the digestion pattern observed in Figure 1 is due to the specific distribution of m^{5}Cyt within the chromatin protein network and is not the result of substrate specificity of the enzyme.

In order to further characterize the location of m^{5}Cyt with respect to chromatin proteins, we have studied the kinetics of digestion of m^{5}Cyt using the enzyme deoxyribonuclease I. This enzyme has been shown to digest DNA within nucleosomes and has some specificity for the active regions of the chromosome (Weintraub & Groudine, 1976; Garel & Axel, 1976). As demonstrated in Figure 3, using this nuclease m^{5}Cyt was digested at the same rate and to the same extent as total [^{3}H]thymidine-labeled DNA.

Hybridization of 5-Methylcytosine. Other investigators have shown that satellite sequences from various sources are enriched in m^{5}Cyt. It is not known, however, if these 5-methylcytosines are indeed part of the satellite repeating sequences or if m^{5}Cyt is also found in intermediate repeated sequences or in unique sequences. To answer these questions we have isolated [^{3}H]m^{5}Cyt-labeled DNA from L-cells and have analyzed the pattern of self annealing as compared with [^{3}H]-thymidine-labeled DNA. The results are shown in Figure 4. The hybridization of [^{3}H]thymidine-labeled DNA is consistent with the pattern of hybridization described in the literature (Britten & Kohne, 1968) and is identical with the hybridization kinetics of mouse liver DNA obtained using optical density at 260 nm to assay the formation of double-stranded DNA (Figure 4). The hybridization pattern of m^{5}Cyt demonstrates that over 50% of these nucleotides are indeed present in satellite sequences which represent only 10–20% of the DNA. The rest of the m^{5}Cyt is distributed among the intermediate repeated sequences and the unique sequences in the same proportion as the total DNA. We have also analyzed the hybridization of m^{5}Cyt using hydroxylapatite chromatography to assay the formation of hybrids. The results obtained by this assay (Figure 4) were very similar to those obtained using the enzyme S_1. Since the average size of the DNA used in the hydroxylapatite

TABLE I: Mass Spectrometric Analysis of m^{5}Cyt in Hybridized DNA.[a]

DNA	C_0t	% DNA hybridized	m^{5}Cyt (mol % of Cyt) S_1 resistant	m^{5}Cyt (mol % of Cyt) S_1 sensitive	% m^{5}Cyt hybridized
calf thymus	1.5	45	6.0		75
calf thymus	35.0	48	7.4	1.3	92
calf thymus	87.0	44	6.9		87
mouse liver	50.0	33	5.7		48

[a] Hybridization was performed and followed as described in Materials and Methods, and the S_1-resistant and -sensitive DNA were isolated and analyzed for m^{5}Cyt content by mass spectrometry. Total calf thymus DNA and mouse liver DNA had an m^{5}Cyt content of 4.1 mol % Cyt.

experiments was about 600 nucleotides in length, we can conclude that those 5-methylcytosines which are not located in repeated sequences are also not adjacent to these repeats.

The results of these hybridization experiments have been confirmed using mass spectrometric analysis to assay the presence of m^{5}Cyt in hybrids. In these experiments (Table I), DNAs from various sources were hybridized to certain C_0t values. After digestion of the DNA by the single-strand specific enzyme S_1, the acid soluble (nonhybridized material) and the acid insoluble (hybridized material) fractions were isolated and subjected to mass spectrometric analysis. About 50% of the sequences in calf thymus DNA are repeated to a large degree. Thus, at C_0t values ranging from 1.5 to 87, we found that 45–50% of the DNA is already hybridized (Britten & Kohne, 1968). When this rapidly hybridizing fraction of the DNA was analyzed by mass spectrometric analysis, it was found that it was considerably enriched in m^{5}Cyt. Whereas the content of m^{5}Cyt in total DNA has been determined as 4.1 mol % of cytidine, the repeated fraction contained between 6 and 7.4 mol %. Since this fraction contains about 50% of the DNA, we can calculate that 75–90% of all m^{5}Cyt in calf thymus DNA is concentrated in the repeated fraction.

In one instance we have analyzed the m^{5}Cyt content of those sequences which are not hybridized at these low C_0t values and which are therefore rendered acid soluble after S_1 digestion. This fraction corresponds to the nucleotides comprising the unique DNA. As expected, this fraction is relatively poor in m^{5}Cyt, consistent with the observation that most of the m^{5}Cyt is located in the repeated fraction. A similar analysis has been done on mouse liver DNA. At a C_0t of 50, 33% of the mouse DNA is already self-hybridized (see Figure 4). These repeated sequences are also enriched in m^{5}Cyt and a simple calculation reveals that about 50% of the m^{5}Cyt is associated with this fraction. This is consistent with the results obtained using [^{3}H]m^{5}Cyt-labeled L-cell DNA.

Since most of the chromatin m^{5}Cyt is resistant to micrococcal nuclease digestion, it was of interest to determine whether this is due to a general enrichment of m^{5}Cyt in this fraction or to an enrichment of m^{5}Cyt containing satellite sequences. To this end, we have isolated this resistant fraction (covered regions) and have analyzed its pattern of self-hybridization. The results (Figure 5) indicated that the covered region m^{5}Cyt has the same annealing pattern as total chromatin DNA; i.e., 50% of the m^{5}Cyt in this fraction is from satellite sequences. The remainder of the m^{5}Cyt is distributed between intermediate repeated and unique sequences as it is for total DNA.

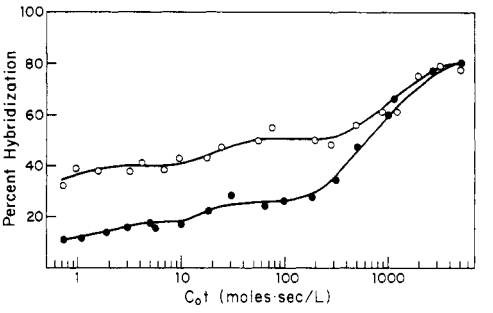

FIGURE 5: Hybridization of covered region DNA. Self-hybridization of DNA was carried out as described in Materials and Methods and assayed by S_1 digestion. [^{3}H]Thymidine-labeled L-cell "covered region" DNA (●). [^{3}H]Methionine-labeled L-cell "covered region" DNA (○).

Discussion

In this paper we have analyzed the distribution of m^{5}Cyt with reference to other DNA sequences and with reference to chromatin structure. Although several workers have shown that satellite sequences from a number of sources are enriched in m^{5}Cyt (Salomon et al., 1969; Harbers et al., 1975; Miller et al., 1974), there is no systematic study of the distribution of m^{5}Cyt in other fractions of the DNA. We have examined this distribution by analyzing the pattern of self-hybridization obtained with [^{3}H]m^{5}Cyt-labeled DNA. Our results indicate that in L-cells 50% of the m^{5}Cyt is localized to satellite sequences, 10% to intermediate repeated sequences and the remainder is found in unique sequences. These results were confirmed by mass spectrometric analysis of the hybrids. In calf thymus over 85% of the m^{5}Cyt is in highly repeated sequences, whereas unique sequence DNA was deficient in m^{5}Cyt. Although it has been previously demonstrated that rapidly annealing sequences in Chinese hamster ovary cells are enriched in m^{5}Cyt (Schneiderman & Billen, 1973), our data represent conclusive evidence that m^{5}Cyt sequences are also found in middle repetitive and unique sequences. Similar results have been obtained by Smirnov et al. (1977) using rat liver DNA. Whatever the significance of methylated cytosine residues in eukaryotic DNA, any hypothesis must take into account the fact that these sequences are distributed throughout the DNA.

We have used deoxyribonucleases to probe the localization of m^{5}Cyt with respect to chromatin proteins. The action of micrococcal nuclease on nuclear DNA has been well characterized in many systems (Sollner-Webb & Felsenfeld, 1975; Axel, 1975). These data demonstrate that mild nuclease digestion of nuclei results in the liberation of a series of nucleoprotein particles, ν bodies, containing DNA fragments whose molecular weights are all multiples of a unit fragment 150–200 base pairs in length. Further digestion results in rapid preferential degradation of spacer DNA, leading, in the case of monomer, to formation of a relatively stable nucleosome "core" containing the histone octamer and a DNA segment 140 base

pairs in length. Subsequent studies have revealed that this "core" represents a transient intermediate in the digestion process and that cleavage of DNA within the nucleosome results in the generation of a true limit digest reflecting the internal structure of the monomeric subunit.

Our results using micrococcal nuclease indicate that methylcytosine is nonrandomly distributed with respect to the nucleosome structure. During early digestion times, when 15–20% of the total nuclear DNA is digested, the m^5Cyt remains blatantly resistant to digestion. This result suggests that very few m^5Cyt moieties are located in the nuclease-sensitive spacer regions between nucleosome cores. Since at 20% digestion most of the DNA is found in monomers or core particles (as shown by gel electrophoresis in our laboratory), we can conclude that a very high percentage of the methyl groups are preferentially located in cores.

As digestion of core DNA proceeds, some m^5Cyt is digested, but the DNA remaining after limit digestion is markedly enriched for methylcytosine. This enrichment of methyl groups in "covered regions" is not due to the presence of a specific type of m^5Cyt containing sequence. When "covered region" DNA was self-hybridized, m^5Cyt was distributed in satellite, intermediate repeated, and unique DNA much the same as in total DNA. This indicates that methyl groups from all these fractions are specifically nuclease resistant, and suggests that it is some property of the reaction between m^5Cyt (or a special m^5Cyt containing sequence) and chromatin proteins which is conferring this protection. The relative resistance of m^5Cyt to micrococcal nuclease treatment is a constant feature of the mammalian cell. Thus, we have observed this phenomenon in calf thymus nuclei and nuclei from various tissues of the chicken (Razin & Cedar, 1977) as well as in unsynchronized dividing L-cells. Furthermore, the m^5Cyt of L-cells which were synchronized by serum depletion behaved similarly, as did pulse-labeled (15 min) m^5Cyt (unpublished results).

This phenomenon of m^5Cyt resistance to nuclease digestion may be explained in several ways. One possibility is that m^5Cyt becomes protected by certain proteins very quickly after the addition of the methyl group to the cytosine, during S phase, and that this association continues throughout the life cycle of the cell. In order to determine whether chromatin proteins preferentially bind to methylated regions of the DNA, we have studied the sensitivity of methyl moieties to micrococcal nuclease in native and reconstituted chromatin. The results indicate that the specificity is retained in soluble chromatin, but that proteins rearrange randomly with respect to m^5Cyt during reconstitution (unpublished results and Razin & Cedar, 1977). Thus, under these in vitro conditions we did not detect specific protein binding to these regions. Some specificity may, however, be inherent in the in vivo nucleosome assembly mechanism. A second possibility is that methylation occurs after the placement of chromatin proteins on the newly replicated DNA but preferentially in nuclease-resistant regions of the chromatin. The m^5Cyt may in fact be protected by its own methylase. In contrast to these theories, one might suggest that m^5Cyt is indeed distributed randomly with respect to nucleosome organization, but the presence of this methyl group may alter the nucleosome structure, making it relatively resistant to digestion.

Adams et al. (1977) have analyzed the distribution of m^5Cyt in the chromatin of Chinese hamster ovary cells. In contrast to our results, they find that m^5Cyt is evenly distributed throughout the nucleosome structures. We have no simple explanation for this discrepancy. However, these authors admit that, even if all of the methyl groups were present in core particles, their methods are not reliable enough to detect the enrichment.

Although the results of Lacy & Axel (1975) indicated that the nucleosome structure as probed by micrococcal nuclease is present on all types of cellular sequences including those for active genes, pancreatic DNase I has been found to preferentially digest active regions of nuclear chromatin (Weintraub & Groudine, 1976; Garel & Axel, 1976; Panet & Cedar, 1977). Our results with DNase I indicate that m^5Cyt is not selectively digested by this enzyme. This, of course, does not rule out the possibility that methyl groups may be involved in the process of transcription or transcriptional control.

Acknowledgments

We thank Dr. Aharon Razin for his help and encouragement, Dr. J. Maio for providing us with his results prior to publication, and Drs. A. Panet and R. Axel for their critical reading of the manuscript and many helpful suggestions.

References

Adams, R. L. P., McKay, E. L., Douglas, J. T., & Burdon, R. H. (1977) *Nucleic Acids Res. 4,* 3097–3108.

Arbor, W., & Linn, S. (1969) *Annu. Rev. Biochem. 38,* 467–500.

Axel, R. (1975) *Biochemistry 14,* 2921–2925.

Billen, D. (1968) *J. Mol. Biol. 31,* 477–486.

Bloch, S., & Cedar, H. (1976) *Nucleic Acids Res. 3,* 1507–1519.

Britten, R. J., & Kohne, D. E. (1968) *Science 161,* 529–540.

Deutsch, J., Razin, A., & Sedat, J. (1976) *Anal. Biochem. 72,* 586–592.

Garel, A., & Axel, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 3966–3970.

Harbers, K., Harbers, B., & Spencer, J. H. (1975) *Biochem. Biophys. Res. Commun. 66,* 738–746.

Hirschmann, S. Z., & Felsenfeld, G. (1966) *J. Mol. Biol. 16,* 347–358.

Lacy, E., & Axel, R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3978–3982.

Lark, C. (1968) *J. Mol. Biol. 31,* 389–399.

Miller, O. J., Schnedl, W., Allen, J. & Erlanger, B. F. (1974) *Nature (London) 251,* 636–637.

Panet, A., & Cedar, H. (1977) *Cell 11,* 933–940.

Razin, A., & Cedar, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2725–2728.

Razin, A., Sedat, J. W., & Sinsheimer, R. L. (1970) *J. Mol. Biol. 53,* 251–259.

Razin, A., Goren, D., & Friedman, J. (1975) *Nucleic Acids Res. 2,* 1967–1974.

Salomon, R., Kaye, A. M., & Herzberg, M. (1969) *J. Mol. Biol. 43,* 581–592.

Schneiderman, M. H., & Billen, D. (1973) *Biochim. Biophys. Acta 308,* 352–360.

Smirnov, V. G., Romanov, G. A., & Vanyushin, B. F. (1977) *Dokl. Akad. Nauk SSSR 232,* 961–964.

Sollner-Webb, B., & Felsenfeld, G. (1975) *Biochemistry 14,* 2915–2920.

Weintraub, H., & Groudine, M. (1976) *Science 193,* 848–856.