Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923–2925.
Sykes, B., & Weiner, J. (1980) in *Magnetic Resonance in Biology* (Cohen J. S., Ed.) Vol. 1, pp 171–196, Wiley, New York.
Takeda, Y., Folkmanis, A., & Echols, H. (1977) *J. Biol. Chem. 252*, 6177–6183.
Takeda, Y., Ohlendorf, D., Anderson, W., & Matthews, B. (1983) *Science (Washington, D.C.) 221*, 1020–1026.
Ulrich, E., John, E., Googh, G., Brunden, M., Gilham, P., Westler, W., & Markley, J. (1983) *Biochemistry 22*, 4362–4365.
von Wilckem-Bergmann, B., & Muller-Hill, B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2427–2431.
Weber, I., McKay, D., & Steitz, T. (1982) *Nucleic Acids Res. 10*, 5085–5102.
Weiss, M., Patel, D., Sauer, R., & Karplus, M. (1984) *J. Am. Chem. Soc. 106*, 4269–4270.

Distribution of 5-Methyldeoxycytidine in Products of Staphylococcal Nuclease Digestion of Nuclei and Purified DNA†

Frederic G. Barr,‡ Michael B. Kastan,§ and Michael W. Lieberman*,‡

Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110
Received August 1, 1984

ABSTRACT: We have compared the distribution of 5-methyldeoxycytidine (m^5dC) between staphylococcal nuclease (SN) sensitive and resistant regions of human diploid fibroblast chromatin to the corresponding distribution in purified DNA. After SN digestion of fibroblast nuclei or purified DNA, nuclease-resistant products were separated from sensitive products by perchloric acid or ethanol precipitation; the radioactively labeled nucleosides were then fractionated by high-performance liquid chromatography and quantitated. Our results indicate that m^5dC is preferentially associated with SN-resistant regions of both chromatin and purified DNA. The magnitudes of these preferences in fibroblast chromatin and DNA are similar; we find that the enrichment of m^5dC content in SN-resistant fractions of nuclei and DNA relative to the corresponding sensitive fractions is approximately 2–3-fold. Therefore, highly methylated regions of DNA have an intrinsic resistance to digestion by SN that is of sufficient magnitude to explain the high degree of nuclease resistance of chromatin containing highly methylated DNA.

One approach to elucidating the biological role of DNA methylation in eukaryotes is to investigate the interaction of methylated and unmethylated regions of the genome with chromosomal proteins. A probe used widely in the study of the interaction of DNA and nucleosomal proteins is the enzyme SN.[1] By preferentially cutting between nucleosome cores, SN facilitates the fractionation of chromatin into a nuclease-resistant, core-enriched fraction and a nuclease-sensitive, linker-enriched fraction (Felsenfeld, 1978). Previous investigations of the distribution of m^5dC between SN-sensitive and -resistant regions of chromatin have led to differing conclusions. Adams et al. (1977) found no significant difference between the m^5dC content of nuclease-resistant chromatin and total DNA of CHO cells and concluded that the distribution was random. Using a variety of cell types, Cedar and collaborators (Razin & Cedar, 1977; Solage & Cedar, 1978) demonstrated an enrichment of m^5dC in SN-resistant chromatin regions and suggested that m^5dC residues are preferentially localized within nucleosomal core particles.

We have reexamined this issue of the distribution of m^5dC between SN-resistant and -sensitive regions of chromatin in human diploid fibroblasts. In order to evaluate whether or not chromosomal proteins are responsible for the specific distribution of m^5dC between resistant and sensitive regions of human fibroblast chromatin, we have studied this distribution in purified human fibroblast DNA. Our findings indicate that m^5dC is preferentially associated with SN-resistant fractions in both chromatin and purified DNA, suggesting that chromosomal proteins are not responsible for the enrichment of m^5dC in SN-resistant regions of chromatin. These results further emphasize the complexity of the action of SN and the difficulty in using this enzyme as a probe in detailed studies of chromatin.

Materials and Methods

Cell Culture. Human diploid fibroblasts (AG1518; Institute for Medical Research) were grown as previously described (Dresler et al., 1982). Cells were labeled during exponential growth with 10 nCi/mL [^{14}C]dC (480 mCi/mmol; Amersham) or 250 nCi/mL [6-^{3}H]dC (5–6 Ci/mmol; Moravek Biochemicals). To reduce the conversion of labeled dC to dT, unlabeled dT (100–400 μM final concentration) was added to the medium. In all experiments, the radioactivity was chased with unlabeled medium after 1 week of labeling, and

†This work was supported by NIH Grant CA 37134 and by the following companies: Brown & Williamson Tobacco Corp.; Philip Morris Inc.; R. J. Reynolds Tobacco Co.; United States Tobacco Co. M.B.K. and F.G.B. are fellows of the Medical Scientist Training Program (5T32 GMO 7200).
‡Present address: Department of Pathology, Fox Chase Cancer Center, Philadelphia, PA 19111.
§Present address: Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

[1] Abbreviations: SN, staphylococcal nuclease; m^5dC, 5-methyldeoxycytidine; dC, deoxycytidine; dT, thymidine; PCA, perchloric acid; HPLC, high-performance liquid chromatography; dG, deoxyguanosine; dA, deoxyadenosine; SEM, standard error of the mean; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-1424$01.50/0

the cells were incubated for at least an additional 1 week. The specific activities of ^{3}H-labeled and ^{14}C-labeled DNA were approximately 4000 and 1000 cpm/μg, respectively.

In double-label experiments, in order to label the methyl group of m^{5}dC, cells were grown for 1 week with 10 μCi/mL L-[*methyl*-^{3}H]methionine (70–90 Ci/mmol; Amersham) in methionine-free medium supplemented with 25 μM methionine. Sodium formate (20 mM final concentration) was added to reduce the random incorporation through the one carbon pool of the label into other nucleosides (Adams et al., 1977). During the labeling week, two additional aliquots of sodium formate (20 mM) and unlabeled methionine (12.5 mM) were added to the medium. The specific activity of ^{3}H-labeled DNA was approximately 500 cpm/μg.

Preparation of Nuclei and Purified DNA. Nuclei were prepared as previously described (Smerdon et al., 1979). DNA was isolated directly from cells by a modification of the method of Davis et al. (1980). Cells were suspended in 100 mM EDTA, 200 mM Tris, pH 8.5, 1% SDS, and 100 μg/mL proteinase K and incubated overnight at 37 °C. Proteins and SDS were precipitated in 1 M potassium acetate at 4 °C and removed by centrifugation (12000*g*, 10 min). RNA was hydrolyzed by incubation with 100 μg/mL RNase A and 350 units/mL RNase T_1 in 10 mM Tris, pH 7.8, at 37 °C for 2 h. RNases and other proteins were removed by several extractions with buffered phenol followed by extraction with 50% buffered phenol, 50% chloroform–isoamyl alcohol (24:1), chloroform–isoamyl alcohol (24:1), and water-saturated ether. The final purified DNA pellet was dissolved in nuclease digestion buffer.

In the double-label experiment, DNA was further purified by two successive bandings in neutral cesium chloride (density = 1.70 g/mL); the sample was centrifuged in a Sorvall TV865 rotor at 50000 rpm and 20 °C for more than 6 h. The peak of radioactivity was collected and dialyzed against nuclease digestion buffer.

Staphylococcal Nuclease Digestion. Nuclei and purified DNA were suspended in nuclease digestion buffer (10 mM Tris, pH 8.0, 0.1 mM $CaCl_2$, 0.25 M sucrose) to a final concentration of about 1×10^7 nuclei/mL and 100 μg/mL, respectively. Staphylococcal nuclease (Sigma) was added to a final concentration of 0.2 unit/μg of DNA in nuclei digestions and to a final concentration of 0.025 unit/μg of DNA in DNA digestions. Under these conditions, the approximate times for 25%, 50%, and 75% solubilization of radioactive counts are 50, 110, and 200 min for nuclei digestions and 14, 28, and 42 min for DNA digestions. After digestion at 37 °C, reactions were terminated by the addition of 3 volumes of ice-cold 95% ethanol and NaCl to a final concentration of 0.15 M or $^1/_{10}$th volume of ice-cold 70% PCA. PCA solutions were incubated at 4 °C for 20 min, and ethanol solutions were incubated on dry ice until frozen. The precipitate was collected by centrifugation for 10–20 min at 12000–15000*g* at 4 °C. The extent of digestion was calculated from the ratio of radioactivity in an aliquot of supernatant from a time point to the radioactivity in a comparable volume of supernatant from a limit digest.

For a limit digest of nuclei, nuclei were first digested in 50 μg/mL proteinase K at 55 °C for at least 1 h; SN was then added to a final ratio of 20 units/μg of DNA, and the mixture was incubated at 37 °C for at least 2 h. For a limit digest of purified DNA, SN was added to a ratio of 2.5 units/μg of DNA and incubated similarly.

HPLC Analysis of SN Digestion Products. DNA was isolated from the SN-resistant fraction of nuclei by the previously described proteinase K–SDS digestion, followed by potassium acetate precipitation, hydrolysis of RNA in 0.3 N NaOH at 37 °C for 2 h, and several ethanol precipitations. Ethanol-soluble fractions were evaporated to dryness in a Speed Vac centrifuge evaporator (Savant) under vacuum. Perchloric acid soluble fractions were neutralized with KOH, followed by centrifugation to remove perchlorate salts and lyophilization of the neutralized supernatant.

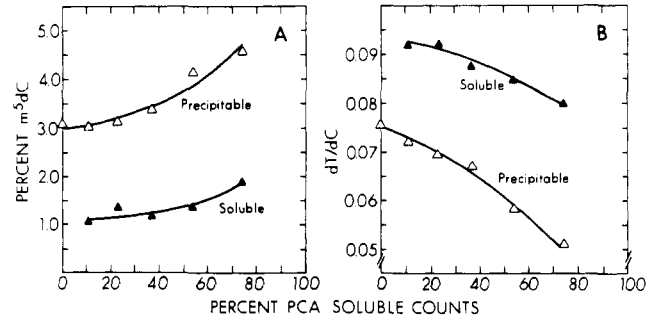

FIGURE 1: Staphylococcal nuclease digestion of human fibroblast nuclei. Perchloric acid soluble (▲) and precipitable (△) fractions of the digestion time points were analyzed by HPLC, followed by liquid scintillation counting. (A) Content of m^{5}dC as a function of extent of digestion. The formula 100(cpm in m^{5}dC)/(cpm in dC + cpm in m^{5}dC) was used to calculate m^{5}dC content. Each point represents a single HPLC determination of the m^{5}dC content. Data on the statistical accuracy of the HPLC determinations is presented in Figure 3. (B) Ratio of dT to dC as a function of extent of digestion. Each point represents a single determination. The percentage of radioactivity in the soluble fraction is used as the measure of extent of digestion. Under our labeling conditions, the majority of the radioactivity is associated with dC. It should be noted that, because of the differential release of nucleotides by SN, the apparent extent of digestion is dependent on the labeled nucleotide.

All samples (containing between 1 and 200 μg of DNA) were resuspended in 0.2 mL of 10 mM Tris, pH 8.0, and 10 mM $MgCl_2$ and then digested to nucleosides by the addition of 20 μL of 480 μg/mL DNase I (Sigma) and incubation at 37 °C for at least 2 h, followed by addition of 5 μL of snake venom phosphodiesterase (Sigma; 1 unit/mL in 0.11 M Tris, pH 8.9, 0.11 M NaCl, and 15 mM $MgCl_2$) and 10 μL of alkaline phosphatase (Sigma, 88 units/mL in 50 mM Tris, pH 8.0) and incubation at 37 °C for at least 4 h. Phosphodiesterase and alkaline phosphatase were added a second time, and the samples were incubated overnight at 37 °C. HPLC analysis of these samples was done as described previously (Kastan et al., 1982; Krawisz & Lieberman, 1984).

RESULTS

To study the m^{5}dC composition of chromatin, nuclei were isolated from human diploid fibroblasts prelabeled with [^{14}C]dC and digested to varying extents with SN. The digested chromatin was separated into soluble and precipitable fractions with PCA, and the m^{5}dC content in each fraction was determined by HPLC fractionation of the labeled nucleosides. At each extent of digestion, the acid-precipitable, SN-resistant fraction has a higher m^{5}dC content than the corresponding acid-soluble, SN-sensitive fraction (Figure 1A). In both the soluble and precipitable fractions, the lowest m^{5}dC content occurs at the lowest extent of digestion, and with increasing digestion, the m^{5}dC content steadily increases. All of these observations are consistent with the conclusion that m^{5}dC is preferentially associated with nuclease-resistant regions of human fibroblast chromatin.

Because some of the labeled dC precursor is metabolized by the cell to labeled dT, HPLC fractionation of nucleosides also allows comparison of the relative release of dT and dC by SN from human fibroblast chromatin (Figure 1B). At each

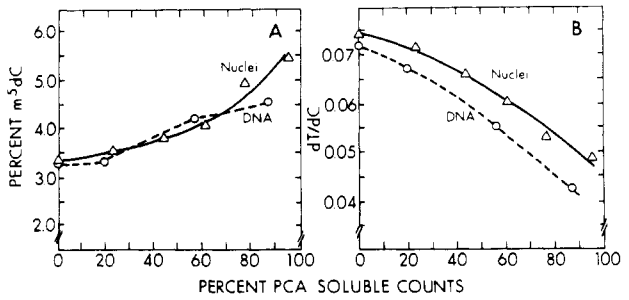

FIGURE 2: Staphylococcal nuclease digestion of nuclei (Δ) and purified DNA (O) from human fibroblasts. Perchloric acid precipitable fractions of the digestion time points were analyzed by HPLC for dC, m⁵dC, and dT content. (A) Content of m⁵dC as a function of extent of digestion. (B) Ratio of dT to dC as a function of extent of digestion. Each point represents a single HPLC determination.

extent of digestion, the soluble fraction has a higher dT/dC ratio than the precipitable fraction, and with increasing extents of digestion, the dT/dC ratios of both soluble and precipitable fractions decrease. These observations lead to the conclusion that dT residues are preferentially released from chromatin by SN or that, relative to dT, dC is preferentially associated with nuclease-resistant regions of chromatin.

Staphylococcal nuclease sensitivity and resistance are most commonly interpreted in terms of the preference of SN to digest linker DNA over nucleosome core DNA. The preference of SN for linker DNA is however not absolute. Other factors also influence the action of SN. Several laboratories (Von Hippel & Felsenfeld, 1964; Wingert & Von Hippel, 1968; Nelson et al., 1979; Dingwell et al., 1981) have described a preference of SN for dA·dT-rich regions relative to dC·dG-rich regions in purified DNA; this DNA sequence preference is unrelated to a relative protection of regions by nucleosomal proteins.

To determine whether the relative SN resistance of m⁵dC in human fibroblast chromatin results from preferential protection by nucleosomal proteins, m⁵dC release during SN digestion of purified DNA was compared to the release from intact chromatin. In this experiment, the prelabeled fibroblasts were divided into two groups; from one group, nuclei were isolated and digested with SN, and from the second group, DNA was isolated and then digested with SN. Following the SN digestion, nucleosides were prepared from the acid-precipitable fractions and analyzed by HPLC (Figure 2). For both nuclei and purified DNA, the m⁵dC content of the nuclease-resistant fraction increased with increasing extent of digestion. The preferential association of m⁵dC with SN-resistant regions therefore is also seen in the absence of chromosomal proteins. Furthermore, the magnitudes of the preferences in chromatin and purified DNA, as judged by the slopes of the two curves, are similar.

The relative SN sensitivity of dT and dC was also examined in purified DNA. In accord with the results from other laboratories (Von Hippel & Felsenfeld, 1964; Wingert & Von Hippel, 1968; Nelson et al., 1979; Dingwell et al., 1981), dT is released from purified DNA faster relative to dC (Figure 2B). The magnitudes of the preferences for dT in chromatin and purified DNA are also similar.

To demonstrate that the preferential association of m⁵dC with SN-resistant regions of purified DNA is not an artifact of our experimental protocol, we examined several variables, including the possible presence of endogenous nucleases, the method of precipitation of nuclease resistant DNA, and the method of analysis of m⁵dC content. First, incubation of nuclei or purified DNA for several hours in the absence of SN does not release acid-soluble radioactivity (data not shown). The

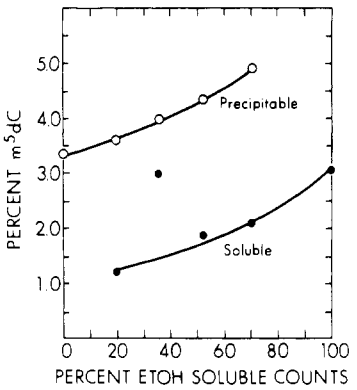

FIGURE 3: Staphylococcal nuclease digestion of purified DNA from human fibroblasts employing ethanol precipitation to fractionate digestion products. Unlike in the experiments described in Figures 1, 2, and 5 in which enzyme digestions were performed as described under Materials and Methods, different extents of digestion in this experiment were generated by digesting for 20 min with 0.1–2.5 units of SN/μg of DNA. Ethanol-soluble (●) and -precipitable (O) fractions of the digestion points were analyzed by HPLC, and m⁵dC content was calculated as described in Figure 1. Single HPLC determinations were performed on all soluble fractions and the precipitable fractions at 70% digestion. Two HPLC determinants were performed on precipitable fractions at 0%, 36%, and 52% digestion; the corresponding SEM's were 0.01%, 0.13%, and 0.06%, respectively. Three HPLC determinations were performed on the precipitable fraction at 19% digestion; the SEM was 0.03%.

result rules out participation of any endogenous nucleases in the digestion process.

In all the aforementioned experiments, perchloric acid precipitation was employed to separate lower molecular weight soluble products of digestion from higher molecular weight insoluble products. Cleaver & Boyer (1972) have established that, above 9 nucleotides in length, there is a progressive insolubility of oligonucleotides in PCA; 50% solubility occurs at a length of 16–18 nucleotides. This study also showed that ethanol precipitates shorter oligonucleotides than PCA. To exclude the possibility that the preferential association of m⁵dC with nuclease-resistant regions of purified DNA is based on the peculiar solubility characteristics of PCA, we digested purified DNA with SN and used ethanol precipitation to separate the digestion products into nuclease-resistant and -sensitive fractions (Figure 3). Our findings demonstrate decreased m⁵dC content in the ethanol-soluble fractions relative to the corresponding ethanol-precipitable fractions as well as increasing m⁵dC content in either fraction with increasing extents of digestion. Digestions of nuclei with ethanol precipitation show a preference of similar magnitude (data not shown).

Finally, we demonstrated the preferential association of m⁵dC with SN-resistant regions of purified DNA using a double-label procedure that eliminates the need to fractionate nucleosides by HPLC. Deoxycytidine residues were labeled with [^{14}C]dC (in the presence of 400 μM unlabeled dT) and m⁵dC residues were labeled with L-[*methyl*-^{3}H]methionine (Materials and Methods). To avoid the combined toxicity of the two labeling protocols, cells were labeled separately with each protocol and the purified DNA preparations from each were mixed to give double-labeled purified DNA. In order to obtain maximal purification of these DNA samples, particularly from ^{3}H-labeled proteins, these samples were banded twice on neutral CsCl gradients in addition to the routine purification procedure. Purity of this DNA was judged by coincidence of ^{3}H and ^{14}C radioactivity and absorbance on the final gradient (data not shown). The distribution of label in each sample was assessed by HPLC (Figure 4). In the ^{14}C-

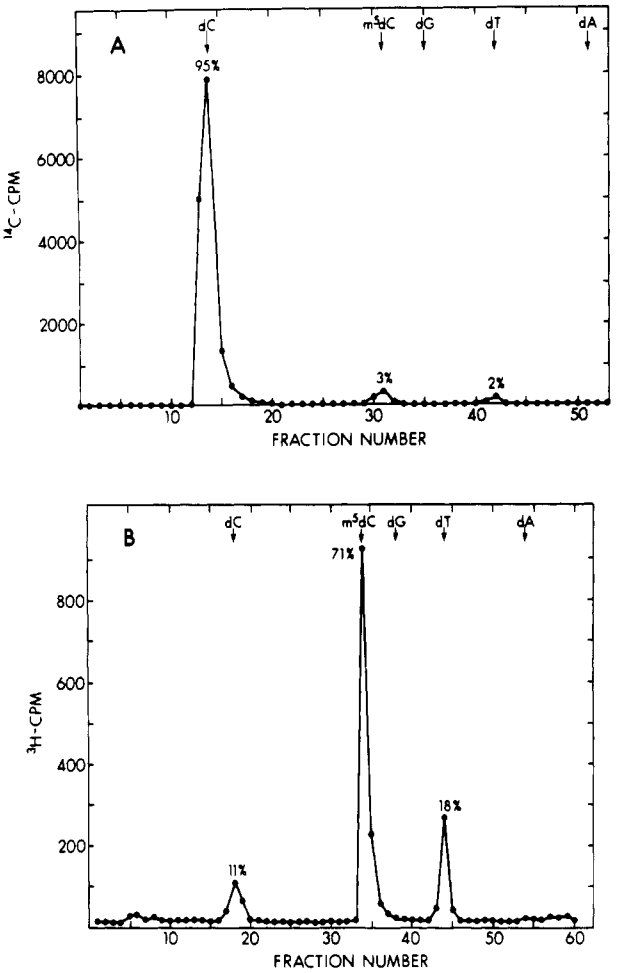

FIGURE 4: Distribution of radioactive label in DNA prepared for the double-label experiment. Cells were labeled, and the DNA was purified and prepared for HPLC as described under Materials and Methods. The positions of the nucleoside peaks from the absorbance profile of the HPLC fractionation are presented at the top of each figure. The percentage of total radioactive counts in each nucleoside peak is presented above each peak. (A) Labeled nucleoside content of DNA from cells labeled with 10 nCi/mL [^{14}C]dC in the presence of 400 μM dT. (B) Labeled nucleoside content of DNA from cells labeled with 10 μCi/mL [^{3}H]methionine in the presence of 20 mM sodium formate.

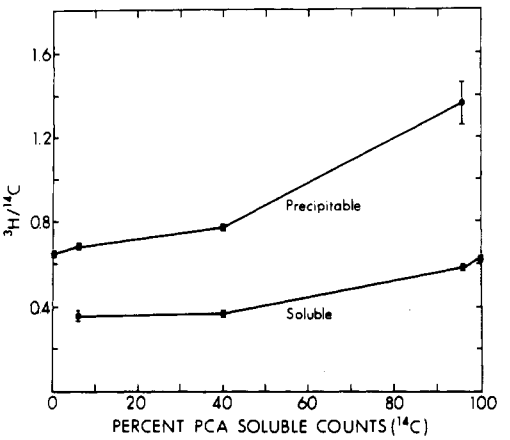

FIGURE 5: Staphylococcal nuclease digestion of a mixture of ^{14}C- and ^{3}H-labeled DNA preparations. Perchloric acid soluble and precipitable (solubilized in 0.1 N NaOH) fractions of the digestion time points were analyzed by liquid scintillation counting. To correct for background, the radioactivity in the precipitable fraction of a limit digest was subtracted from the radioactivity in precipitable fractions of the digestion points; similarly, the radioactivity in the soluble fraction of an undigested sample was subtracted from the radioactivity in the soluble fractions of the digestion points. In addition, ^{3}H cpm in each fraction were corrected for ^{14}C crossover. Each point represents the average (±SEM) of duplicate or triplicate samples.

labeled sample, greater than 95% of the counts were present in dC, and in the ^{3}H-labeled sample, greater than 70% of the counts were present in m^{5}dC.

The double-labeled DNA was digested with SN and fractionated into nuclease-sensitive and -resistant fractions by PCA precipitation. The ^{3}H/^{14}C ratio of each fraction is a measure of the level of m^{5}dC relative to dC. Comparison of this ratio for a soluble fraction with the corresponding precipitable fraction at any time point or comparison of the trends in each curve with increasing digestion (Figure 5) again indicates that m^{5}dC is preferentially associated with SN-resistant regions of purified DNA. Because dC and dT are released more rapidly than m^{5}dC by SN and because a small but significant amount of ^{3}H label occurs in dC and dT (Figure 4), the actual magnitude of the preferential association is greater than the observed result.

Discussion

Our data confirm the previously described preferential association of m^{5}dC residues with SN-resistant regions of chromatin (Razin & Cedar, 1977; Solage & Cedar, 1978). However, in purified human fibroblast DNA, a preferential association of m^{5}dC with nuclease-resistant regions of similar magnitude was observed. Furthermore, this preference in purified DNA was not altered when alternate methods were used for precipitation of nuclease-resistant DNA and for analysis of m^{5}dC content or when additional steps were employed to yield more highly purified DNA. These data indicate that highly methylated regions of DNA have an intrinsic resistance to digestion by SN that is of sufficient magnitude to explain the high degree of nuclease resistance of chromatin containing highly methylated DNA. An explanation based on the preferential association of nucleosomal or other chromosomal proteins with highly methylated DNA is not necessary.

Several hypotheses could explain the intrinsic resistance of highly methylated DNA to SN digestion. A trivial explantation that cannot be totally excluded is that a protein tightly bound to highly methylated regions of DNA could protect these regions from nuclease attack. Such a protein would have to be resistant to proteinase K digestion, SDS denaturation, organic extraction, and dissociation by cesium chloride.

A second possibility is that methylation of one or several deoxycytidine residues in a region makes that region more resistant to SN digestion. This hypothesis is consistent with the idea that the sequence preference of SN results from its preference for regions of DNA with greater single-stranded character (Wingert & Von Hippel, 1968; Nelson et al., 1979). Since the single-stranded character is, in general, inversely related to the strength of hydrogen bonding of complementary base pairs and since m^{5}dC·dG base pairing is stronger than dC·dG base pairing (Ehrlich & Wang, 1981), methylation would decrease the single strandedness of a region and hence susceptibility of that region to SN attack.

A third hypothesis to explain the intrinsic nuclease resistance of highly methylated DNA is that m^{5}dC residues are nonrandomly distributed throughout the genome in sequences of intrinsic resistance to SN. Fine structure analysis of a variety of genes has emphasized the sequence specificity of SN in chromatin and purified DNA; there are sequences with high resistance and others with high sensitivity to nuclease attack (Dingwell et al., 1981; Horz & Altenburger, 1981; Keene & Elgin, 1984). A great deal of data has accumulated that describes the nonrandom distribution of m^{5}dC in the genome;

for example, highly repetitive sequences are relatively enriched in m^{5}dC content (Solage & Cedar, 1978; Kastan et al., 1982; Ehrlich & Wang, 1981). Therefore, it is reasonable that the preferential association of m^{5}dC with nuclease-resistant regions of DNA could result from an intrinsic SN resistance of a few repetitive sequences in which a significant proportion of methylation occurs.

On a more general level, because most (perhaps greater than 90%) of m^{5}dC occurs in the sequence m^{5}dC-dG (Ehrlich & Wang, 1981), the proportion of m^{5}dC residues appearing in a dC·dG-rich region is greater than the proportion of unmethylated dC residues appearing in that region. Since dC·dG-rich regions are relatively more resistant to SN digestion, this higher proportion of m^{5}dC residues occurring in dC·dG-rich regions could explain the even higher nuclease resistance of m^{5}dC·dG-rich regions.

Registry No. SN, 9013-53-0; m^{5}dC, 838-07-3.

References

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

Cleaver, J. E., & Boyer, H. W. (1972) *Biochim. Biophys. Acta 262*, 116-124.

Davis, R. W., Thomas, M., Cameron, J., St. John, T. P., Scherer, S., & Padgett, R. A. (1980) *Methods Enzymol. 65*, 404-411.

Dingwell, C., Lomonossoff, G., & Laskey, R. (1981) *Nucleic Acids Res. 9*, 2659-2673.

Dresler, S. L., Roberts, J. D., & Lieberman, M. W. (1982) *Biochemistry 21*, 2557-2564.

Ehrlich, M., & Wang, R. (1981) *Science (Washington, D.C.) 212*, 1350-1357.

Felsenfeld, G. (1978) *Nature (London) 271*, 115-122.

Horz, W., & Altenburger, W. (1981) *Nucleic Acids Res. 9*, 2643-2658.

Kastan, M. B., Gowans, B. J., & Lieberman, M. W. (1982) *Cell (Cambridge, Mass.) 30*, 509-516.

Keene, M. A., & Elgin, S. C. R. (1984) *Cell (Cambridge, Mass.) 36*, 121-129.

Krawisz, B. R., & Lieberman, M. W. (1984) *Carcinogenesis (London) 5*, 1141-1144.

Nelson, P. P., Albright, S. C., & Garrard, W. T. (1979) *J. Biol. Chem. 254*, 9194-9199.

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

Smerdon, M. J., Kastan, M. B., & Lieberman, M. W. (1979) *Biochemistry 18*, 3732-3739.

Solage, A., & Cedar, H. (1978) *Biochemistry 17*, 2934-2938.

Von Hippel, P. H., & Felsenfeld, G. (1964) *Biochemistry 3*, 27-39.

Wingert, L., & Von Hipel, P. H. (1968) *Biochim. Biophys. Acta 157*, 114-126.

Hierarchy of Binding Sites for Chromosomal Proteins HMG 1 and 2 in Supercoiled Deoxyribonucleic Acid

Hiroshi Hamada and Michael Bustin*

Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Received August 22, 1984

ABSTRACT: The interaction of chromosomal proteins HMG 1 and 2 with various DNA structures has been examined with plasmid pPst-0.9, which contains DNA sequences that can form the Z-DNA conformation and palindromic sequences that can form cruciform structures. Direct binding and competition experiments with ^{32}P-labeled plasmid indicated that proteins HMG 1 and 2 preferentially bind to supercoiled form I DNA as compared to double-stranded linear DNA. The preferential binding to form I is due to the presence of single-stranded regions in this DNA. The binding of HMG 1 and 2 to the form I plasmid results in inhibition of S_1 nuclease digestion in a selective manner. The B-Z junction is preferentially protected as compared to the cruciform, which in turn is more protected than other minor S_1-sensitive structures present in pPst-0.9. Our results indicate that the binding of HMG 1 and 2 proteins to DNA is not random in that HMG 1 and 2 can distinguish between various S_1 nuclease sensitive sites in the plasmid. The existence of a hierarchy of DNA binding sites for these proteins suggests that they can selectively affect the structure of distinct regions in the genome.

Structural alteration in DNA may play a key role in the regulation of gene expression. Distinct DNA conformations may be facilitated and stabilized by various cellular proteins. Chromosomal proteins HMG 1 and 2 are potential candidates for affecting structural featues of DNA. These proteins are relatively abundant in both the nucleus and cytoplasm of cells and are ubiquitously distributed among various eukaryotic kingdoms, and their primary and tertiary structures are relatively conserved during evolution [see Johns (1982) for review]. They can distinguish between single-stranded and double-stranded DNA (Isackson et al., 1979), unwind the DNA double helix (Javaherian et al., 1978, 1979; Yoshida & Shimura, 1984), and influence the enzymatic digestion of DNA by various nucleases (Shasti et al., 1982). In chromatin, they seem to be bound to the linker region between nucleosomes. In spite of these findings, the cellular role of HMG 1 and 2 is not clear. It has been reported that cell division brings about quantitative changes in HMG 1 and 2 (Seyadin & Kistler, 1979). Microinjection of ant-HMG 1 and anti-HMG 2 antibodies into the nucleus of amphibian oocytes inhibits the highly active transcription on the lampbrush chromosome loops and brings about a retraction of the loops into the chromomeres (Kleinschmidt et al., 1983). In nuclei of somatic cells, however, antibody microinjection did not produce a noticeable effect on RNA synthesis mediated by RNA polymerase II (Einck & Bustin, 1983).