MOLECULAR AND CELLULAR BIOLOGY, July 1981, p. 600-608
0270-7306/81/070600-09$02.00/0

Vol. 1, No. 7

Deoxyribonucleic Acid Methylation and Chromatin Organization in *Tetrahymena thermophila*

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Received 26 February 1981/Accepted 30 April 1981

Deoxyribonucleic acid (DNA) of the transcriptionally active macronucleus of *Tetrahymena thermophila* is methylated at the N^6 position of adenine to produce methyladenine (MeAde); approximately 1 in every 125 adenine residues (0.8 mol%) is methylated. Transcriptionally inert micronuclear DNA is not methylated (≤0.01 mol% MeAde; M. A. Gorovsky, S. Hattman, and G. L. Pleger, J. Cell Biol. **56**:697-701, 1973). There is no detectable cytosine methylation in macronuclei in *Tetrahymena* DNA (≤0.01 mol% 5-methylcytosine). MeAde-containing DNA sequences in macronuclei are preferentially digested by both staphylococcal nuclease and pancreatic deoxyribonuclease I. In contrast, there is no preferential release of MeAde during digestion of purified DNA. These results indicate that MeAde residues are predominantly located in "linker DNA" and perhaps have a function in transcription. Pulse-chase studies showed that labeled MeAde remains preferentially in linker DNA during subsequent rounds of DNA replication; i.e., there is little, if any, movement of nucleosomes during chromatin replication. This implies that nucleosomes may be phased with respect to DNA sequence.

Deoxyribonucleic acid (DNA) methylation specifies genetic information in that specific sequences are methylated and the amount of methylation is both species and tissue specific (5, 15, 19, 42, 56, 64, 74, 78, 83, 84). In nuclear DNA of multicellular eucaryotes, the amount of 5-methylcytosine (MeCyt) varies from as little as 0.17 mol% in insects (3) to as much as 50 mol% in certain plants (72). DNA of unicellular eucaryotes generally contains either MeCyt or methyladenine (MeAde), or both (19, 31, 36, 54, 56). Recent evidence suggests that site-specific methylation of cytosine in eucaryotes may be related to the control of transcription; e.g., transcriptionally competent genes are not methylated at sites which are methylated in cells not transcribing those genes (11, 14, 16, 17, 20, 21, 24, 45, 48, 50, 61, 70, 73, 75, 78). It is important to determine the relationship between DNA methylation and transcriptional activity and the functional differences mediated by the two types of methylated residues, MeAde and MeCyt. Unlike multicellular eucaryotes, which contain only MeCyt, the unicellular ciliate *Tetrahymena* has only MeAde (36; this paper). The macronuclear DNA contains 0.8% of the adenine residues in the form of MeAde (31, 36) and occurs in specific sequences (S. Bromberg, K. Pratt, and S. Hattman, submitted for publication). Since each cell possesses a macronucleus with transcriptionally active, methylated DNA and a micronucleus with transcriptionally inert, unmethylated DNA (30, 31), we chose to use *Tetrahymena* to study the role of MeAde in genetic regulation. In this paper we show that methylated DNA sequences in *Tetrahymena* chromatin are preferentially digested by staphylococcal (staph) nuclease and to a lesser extent by pancreatic deoxyribonuclease (DNase) I, which have been shown in other organisms and in *Tetrahymena* (29) to digest DNA preferentially between nucleosomes (linker DNA) (49) and transcriptionally competent genes (active DNA), respectively. Furthermore, the differential accessibility of MeAde-containing sequences to staph nuclease persists through chromatin replications, as demonstrated by the sensitivity of methylated sequences in pulse and pulse-chase labeling experiments. Therefore, MeAde residues are preferentially located in linker regions between nucleosome cores and may also have a regulatory function in transcription.

MATERIALS AND METHODS

Growth of cells and isolation of nuclei. *Tetrahymena thermophila* strain BVII cells were grown axenically in 1% proteose peptone-0.2% dextrose-0.1% yeast extract-0.003% sequestrine (iron chelate, Geigy Chemical Corp.)-1× antibiotic-antimycotic mixture (GIBCO Laboratories), as described previously (32). Cells were continuously labeled during growth in the presence of 2 μCi of [^{3}H]adenosine (labeled at the 2, 8, or 2 and 8 positions) per ml and harvested at densities

of 2×10^5 to 6×10^5 cells per ml. Macronuclei were isolated according to the procedures described by Gorovsky et al. (32), except that all isolation media contained 0.5 mM phenylmethylsulfonyl fluoride. Washed nuclei were frozen in medium A (0.1 M sucrose, 4% gum arabic, 0.1% spermidine-HCl, 0.002 M $MgCl_2$, pH 6.75) at −20°C. Pulse-labeled nuclei were isolated from cells grown in medium without yeast extract. Pulse-chased nuclei were isolated from cells that were pulse-labeled, washed three times, and then grown in medium containing yeast extract as a source of excess unlabeled DNA precursors. Incorporation of the ^3H label was monitored during the chase and found to have been effectively blocked.

Staph nuclease digestion. Macronuclei (0.5×10^8 to 2×10^8/ml) were washed with and suspended in RSB^{++} buffer [10 mM tris (hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride). Purified macronuclear DNA (400 μg/ml) was also suspended in RSB^{++} buffer. Digestions with staph nuclease (EC 3.1.4.7; Worthington Diagnostics) at concentrations of 0 to 400 U/ml were performed at 37°C for 0 to 120 min. Digestion was stopped by addition of 4 volumes of NDS [0.5 M ethylenediamine tetraacetate, 1% sodium dodecyl sulfate, 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 9.5] and heating for 20 min at 65°C. Digests were then made 1 mg/ml in self-digested pronase and incubated for several hours at 37°C. The extent of digestion for each sample was monitored by determining the trichloroacetic acid-soluble and trichloroacetic acid-precipitable radioactivity before and after NaOH hydrolysis (to account for ^3H counts per minute in ribonucleic acid) of appropriate aliquots. The remaining DNA and DNA fragments were purified and analyzed for MeAde content as described below.

DNase I digestion. Washed macronuclei or purified DNA was suspended in RSB^{++} buffer as described for the staph nuclease digestions. Pancreatic DNase I (EC 3.1.4.5; Worthington Diagnostics) was added to final concentrations of 0 to 300 U/ml, and samples were incubated for 0 to 20 min at 37°C. Digestions were stopped and aliquots were analyzed for extent of digestion as described above.

DNA purification. After pronase digestion, nucleic acids were extracted with an equal volume of phenol-chloroform (1:1). The organic phase was reextracted with a small volume of water, and the combined aqueous phases were reextracted at least twice with an equal volume of chloroform-isoamyl alcohol (24:1). The nucleic acids in the aqueous phase were precipitated in 70% ethanol at −20°C, pelleted by centrifugation, dried, and dissolved in 0.5 ml of 1× SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Ribonucleic acid was digested at 37°C for 2 h by ribonuclease A (100 μg/ml [Sigma Chemical Co.]; previously boiled for 10 min) and ribonuclease T_1 (1,000 U/ml [Worthington Diagnostics]; previously boiled). Reactions were terminated by the addition of pronase to a final concentration of 80 μg/ml, and incubation was continued for an additional 2 h. The digests were deproteinized by extraction with phenol and chloroform-isoamyl alcohol, and the DNA was precipitated in ethanol as described above.

Analysis of MeAde content. Purified, ethanol-precipitated DNA was dissolved and incubated in 0.5 ml of 1 N NaOH at 37°C for at least 4 h to hydrolyze any residual ribonucleic acid. The samples were neutralized by the addition of 1 N HCl, and the denatured DNA fragments were precipitated in 5% trichloroacetic acid. After centrifugation, the pellets were washed in cold 10% trichloroacetic acid and then in cold 95% ethanol. The final pellets were dried in air and hydrolyzed in 1 N HCl at 100°C for 1 h or in 70% perchloric acid at 95°C for 1 h. Chromatographic analyses of the ^3H-labeled purines were as described previously (34). After neutralization of perchloric acid and removal of salt, the bases were separated with authentic markers by descending paper (Whatman no. 1) chromatography in 86% butanol in an NH_3 atmosphere for 17 to 21 h at 22°C. The chromatograph was cut into 1-cm strips, and each strip was eluted with 0.5 ml of water in vials; scintillation fluor was added, and the radioactivity was counted in a Packard 3375 scintillation counter.

HPLC. High-performance liquid chromatography analyses were carried out by D. Swinton according to the methods of Singhal (65), except that the column was 100 by 0.46 cm.

Core particle preparation. Core particles were isolated on linear 10 to 30% sucrose gradients essentially according to the methods of Giri and Gorovsky (29). Core particles analyzed by HPLC were isolated by D. Pederson.

RESULTS

Macronuclear DNA lacks MeCyt. To determine whether *Tetrahymena* DNA contains MeCyt, in addition to MeAde, DNA was purified from macronuclei isolated from cells grown in the presence of L-[*methyl*-^3H]methionine. Under these conditions only methylated bases are labeled because *Tetrahymena* does not synthesize pyrimidine or purine ring structures, which must be supplied in the medium (44). Labeled DNA was acid hydrolyzed and bases were separated by descending paper chromatography (Fig. 1). The only peak of radioactivity comigrated with authentic marker MeAde. Chromatography in two other solvent systems (65% isopropanol–18% HCl and 86% butanol–1% NH_3) revealed only labeled MeAde. Cells were also labeled with [6-^3H]uridine (a cytosine precursor) and analyzed for MeCyt content by similar methods. Three serial chromatographic separations in which the MeCyt region of the chromatograph was eluted and rechromatographed revealed no significant radioactivity in MeCyt. The absence of MeCyt in *Tetrahymena* DNA was confirmed by HPLC analyses of both macronuclear and micronuclear DNA. By all of these methods, MeCyt is less than 0.01% of cytosine. Previous studies indicated that *Tetrahymena* macronuclear DNA contains ~0.8% of the adenines as MeAde (31, 36). To illustrate the method of computing the

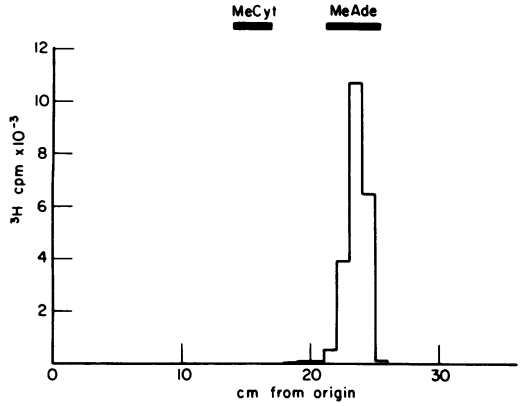

FIG. 1. *MeAde is the only modified DNA base in Tetrahymena, as determined by chromatography of [methyl-^{3}H]methionine-labeled DNA bases. Cells were grown to late log phase (~5 × 10^5 cells per ml) in 1% proteose peptone medium containing 20 μCi of [methyl-^{3}H]methionine per ml. Macronuclei were isolated and DNA was purified. Free bases were obtained by hydrolysis at 95°C for 1 h in 70% perchloric acid. Labeled bases were mixed with authentic markers, spotted on Whatman no. 1 strips, and separated by descending paper chromatography as described in the text. Marker bases were located by ultraviolet light (indicated by solid bars). Only the MeAde region of the chromatograph contained significant radioactivity.*

MeAde content (moles percent) and to confirm previous results, we grew cells in the presence of [^{3}H]adenosine and analyzed the content of MeAde. Figure 2 shows a typical chromatograph of bases obtained from [^{3}H]adenosine-labeled DNA. In a total of 23 preparations, the content was 0.79 mol% (mean) with a standard deviation of 0.11 mol% (Table 1). Variation in moles percent MeAde reflects differences among cultures (since triplicate analyses of a single hydrolysate varied by <0.03 mol%); the physiological significance of this variation is undefined.

DNA methylation lags behind DNA replication. It was observed during the course of studies on DNA methylation in *Tetrahymena* that DNA from pulse-labeled cells exhibited a lower MeAde content than cells which had been labeled for several generations. For example, in three independent experiments, cells pulse-labeled with [^{3}H]adenosine for 1 h had MeAde contents of 0.45, 0.58, and 0.59 mol% (about 70% of the steady-state value determined above). However, pulse-labeled cells which were washed and effectively chased in cold medium, for as little as one doubling time (about 2.5 h), reached the steady-state level of methylation (0.78 mol%; see Table 1). Thus, DNA methylation of adenine in *Tetrahymena* lags behind DNA replication, as does methylation of cytosine in other eucaryotes.

Methylated DNA sequences are located preferentially in linker DNA. To determine whether there is a relationship between chromatin organization and DNA methylation (i.e., whether internucleosomal DNA [linker DNA] or histone-protected DNA [core DNA] is preferentially methylated), macronuclei were digested with staph nuclease. Staph nuclease preferentially digests internucleosomal linker DNA (for a review, see reference 49). The data in Fig. 3 demonstrate that the MeAde content in DNA decreases with increasing extent of staph digestion. Thus, MeAde residues are released from chromatin by staph digestion more rapidly than adenine residues.

Furthermore, in one experiment, different size fractions of DNA were isolated (from agarose gels) at a single kinetic point (42% acid solubilization) and analyzed for MeAde content. It was

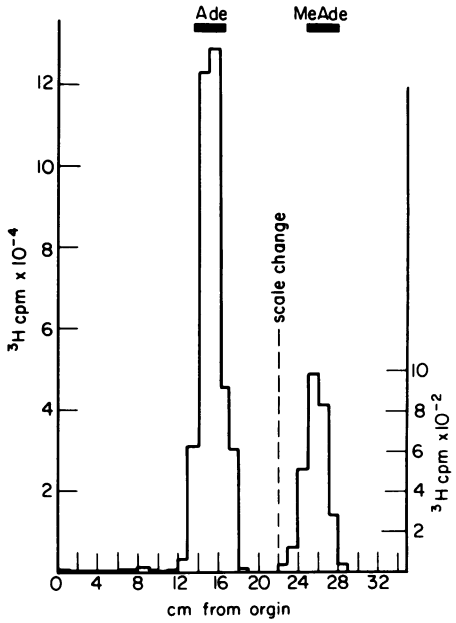

FIG. 2. *Determination of MeAde content of macronuclear DNA by chromatography of [^{3}H]adenosine-labeled bases. Cells were grown to late log phase in 1% proteose peptone medium containing 2 μCi of [^{3}H]adenosine per ml. DNA purified from isolated macronuclei was hydrolyzed in 1 N HCl for 1 h at 100°C, and the labeled purines were separated as described in the text. The positions of markers (indicated by solid bars) were located under ultraviolet light. MeAde content relative to total adenine was determined from the radioactivity in the adenine and MeAde regions: [counts per minute in MeAde/(counts per minute in adenine plus counts per minute in MeAde)] × 100 = moles percent MeAde.*

TABLE 1. *Base composition of various macronuclear DNA fractions*[a]

Fraction	Base composition (mol%)		
	MeAde		G+C (n = 1)
	Mean (SD)	n	
1. Macronuclear DNA	0.79 (0.11)	23	24
2. Newly replicated macronuclear DNA	0.53 (0.07)	3	ND
3. Chased for one generation	0.78 (0.05)	3	ND
4. Chased for two generations	0.79 (ND)	1	ND
5. Core particle DNA	0.52 (0.08)	4	26
6. Staph limit digest DNA (65% TCA soluble)	0.21 (0.02)	4	26

[a] MeAde content was determined by paper chromatography of [^{3}H]adenosine-labeled bases (described in the legend to Fig. 2) and by HPLC for fractions 1, 5, and 6. G+C was determined by HPLC. Paper chromatography and HPLC values for MeAde were in agreement. Newly replicated DNA was purified macronuclear DNA isolated from cells pulse-labeled for 1 h in medium containing [^{3}H]adenosine (doubling time, about 2.5 h). Chased cells were washed and chased in medium without isotope. The culture was incubated until the cell number had doubled (one generation chase) or quadrupled (two generation chase), and macronuclei were isolated. Core particles were isolated from nuclei incubated with staph nuclease (until 15% of the DNA was acid soluble), according to the procedure of Giri and Gorovsky (29). Limit digest DNA was prepared from nuclei incubated with excess staph nuclease until there was no further increase in trichloroacetic acid (TCA) solubility (about 65% of the DNA was rendered acid soluble). SD, Standard deviation; n, number of analyses; ND, not determined.

found that DNA fragments too small to contain linker DNA (90 to 110 base pairs) had 0.25 mol% MeAde compared with larger fragments partially depleted in linker DNA (110 to 220 base pairs), which had 0.35 mol% MeAde. Thus, the preferential linker location of methylated sites is demonstrated by both the loss of MeAde residues from overall digests and the greater decrease of MeAde in DNA fragments too short to contain linker DNA.

Preferential sensitivity of methylated sites to staph nuclease digestion is a reflection of chromatin structure since there is no decrease in the MeAde content during the course of digestion of purified DNA (Fig. 3). Thus, the enzyme exhibits no preference for MeAde per se. The possibility that staph nuclease preferentially digests adenine-rich sequences in chromatin was ruled out by HPLC analyses of core particle DNA (after staph nuclease digestion of nuclei in which 15% of the DNA was rendered acid soluble) and limit digest DNA (after staph nuclease digestion of nuclei in which 65% of the DNA was rendered acid soluble). The results in Table 1 show that these DNA fractions have guanine-plus-cytosine (G+C) contents similar to that of whole genome DNA. Therefore, any adenine-thymine (A-T) preference by staph nuclease is insignificant relative to its digestion of linker DNA, which is not enriched in A-T sequences but must be enriched in MeAde.

The linker DNA location of MeAde is stable. Because MeAde is located predominately in *Tetrahymena* linker DNA, we were able to use DNA methylation as a marker to investigate nucleosome movement during chromatin replication in vivo. For this purpose cells were pulse-labeled for less than one half of a doubling time, after which nuclei were isolated from a portion of the culture, whereas the remaining cells were

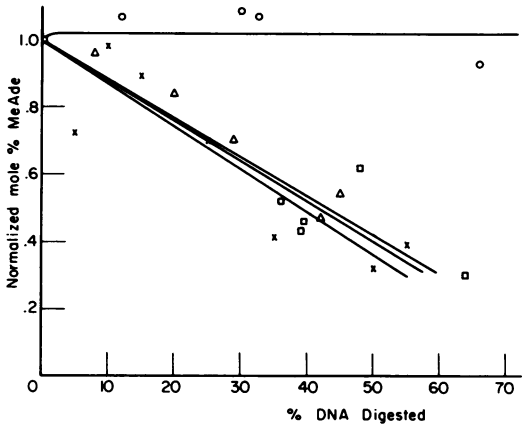

FIG. 3. *Methylated sequences are preferentially released by staph nuclease digestion of chromatin but not during digestion of purified DNA. Uniformly labeled cells* (△) *were labeled continuously overnight in the presence of [^{3}H]adenosine; pulse-labeled cells* (×) *were labeled for 1 h; pulse-chased cells* (□) *were labeled for 1 h, washed three times, and chased effectively for over four generations in medium without isotope. Isolated nuclei were washed and digested by staph nuclease in RSB buffer containing 0.1 mM CaCl and 0.5 mM phenylmethylsulfonyl fluoride at 37°C. Purified DNA* (○) *was digested under the same conditions. The slopes of the lines were calculated by a least-squares linear regression program for purposes of comparison. Slope differences for the digests of nuclei from cells grown under different labeling regimens are not statistically significant. Values for the continuously labeled cells are from five experiments; those from pulsed and pulse-chased cells are from two experiments each. The MeAde content was normalized to the values for undigested samples in each experiment. Undigested DNA was then considered as 1 mol% MeAde. (See Table 1 for actual MeAde content values.)*

washed and chased for over four generations in medium lacking isotope (before isolation of nuclei). The data shown in Fig. 3 illustrate the same kinetics of preferential staph nuclease digestion of methylated sequences in nuclei from cells that were pulse-labeled, pulse-chased, or labeled continuously for several generations. Therefore, MeAde residues are in staph nuclease-sensitive regions when pulse-labeled, and they remain at those sites for at least four rounds of chromatin replication. This stability in location of MeAde residues in linker DNA indicates that there is no randomization of nucleosomes during chromatin replication with respect to sequences methylated during the pulse-labeling period. This implies that nucleosomes are phased with respect to DNA sequence (see below).

Methylated sequences are also preferentially digested by DNase I. Since it has been shown in other eucaryotes (26, 49, 55, 67, 81) and in *Tetrahymena* (29) that DNase I preferentially digests transcriptionally active DNA in nuclei, we wished to determine whether MeAde-containing sequences in *Tetrahymena* chromatin were also preferentially digested by DNase I. For this purpose macronuclei and purified macronuclear DNA from *Tetrahymena* grown in medium containing [^{3}H]adenosine were digested to various extents with pancreatic DNase I. The precipitable DNA from each digest was purified, and the moles percent MeAde was determined. MeAde content decreased with increasing extent of DNase I digestion of DNA in nuclei but not during digestion of purified DNA (Fig. 4).

Some methylated sequences that are digested by staph nuclease are not preferentially accessible to DNase I. For example, at 60% acid solubility, the reduction in methylation level (relative to undigested nuclei) is four- to fivefold for staph nuclease, but barely twofold for DNase I (cf. Fig. 3 and 4). It may be that DNase I has some preference for linker DNA (in addition to its preference for transcriptionally active DNA) or active DNA may be slightly enriched in MeAde, or both.

DISCUSSION

When [^{3}H]adenosine-labeled *Tetrahymena* macronuclei and free macronuclear DNA are digested with staph nuclease, which preferentially digests linker DNA (reviewed in reference 49), we observe a preferential release of methylated adenine residues from chromatin, but not from free DNA (Fig. 3). The methylation content of the precipitable DNA decreases from 0.8 to 0.2 mol% as the DNA in nuclei is digested from 0 to 65% trichloroacetic acid solubility (Table 1). In addition, during staph nuclease digestion of nuclei, DNA fragments depleted of linker DNA have a lower MeAde content than longer fragments. Thus, the loss of MeAde residues is correlated with the digestion of linker DNA by both overall digestion kinetics and analysis according to fragment length. Therefore, we conclude that *Tetrahymena* linker DNA is preferentially methylated.

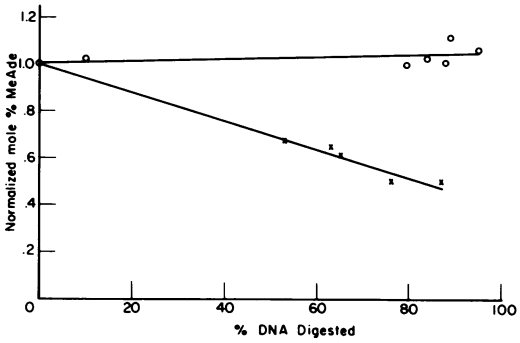

FIG. 4. *Methylated sequences are preferentially released by DNase I digestion of chromatin but not during digestion of purified DNA. Macronuclei were isolated from cells grown in 1% proteose peptone medium with [^{3}H]adenosine (2 μCi/ml). Macronuclei (10^8/ml; ×) were washed and digested with DNase I (0 to 300 U/ml) in RSB buffer containing 0.1 mM CaCl and 0.5 mM phenylmethylsulfonyl fluoride for 15 min at 37°C. Purified DNA (○) was digested under the same conditions. The DNA from each digest was analyzed for percent DNA digested as described in the text and for MeAde content as described in the legend to Fig. 2. Slopes of lines were determined with a least-squares linear regression program and MeAde content was normalized as for Fig. 3.*

We cannot attribute these results to any feature of staph nuclease sequence specificity. Although it has been demonstrated that staph nuclease prefers to cleave free DNA within A-T-rich sequences (77), there is little or no preference for A-T-rich sites during staph nuclease digestion of chromatin (39, 53; this paper). We find no difference in G+C content for undigested macronuclear DNA, DNA from core particles, or limit digest DNA, although the MeAde content decreases (Table 1). It is unlikely that staph nuclease has a preference for removing MeAde residues per se during digestion of chromatin and not during digestion of free DNA. The simplest interpretation of our data is that preferential release of MeAde residues during chromatin digestion is due to their preferential location in linker DNA.

Since MeAde content also decreases with DNase I digestion of nuclei (but less dramatically than with staph nuclease), there may be a relation between linker DNA methylation and gene activity. Our results are consistent with

several interpretations: (i) DNase I may show some preference for linker DNA; (ii) transcriptionally active regions may be enriched in MeAde by virtue of being enriched in linker DNA; or (iii) there may be two "compartments" of MeAde-containing sequences in chromatin (namely, linker DNA and transcriptionally active DNA). Perhaps staph nuclease preferentially cleaves active DNA in chromatin (6–8, 13, 39, 40, 47, 58–60, 68) because active chromatin contains more linker DNA. That DNase I is able to cleave within (undermethylated) core DNA may account for the relatively slower release of MeAde residues during DNase I digestion of nuclei, compared with staph nuclease digestion.

The mechanism by which linker DNA becomes preferentially methylated in vivo may be explained by the timing of DNA methylation relative to DNA synthesis. Deposition of histones on replicating DNA in eucaryotes is very rapid (80), and the rate of DNA methylation is slow relative to the rate of DNA synthesis in *Tetrahymena* (Table 1); lagging of methylation relative to replication has been observed in several higher eucaryote systems (1, 2, 38, 41, 43). It is likely, then, that core histones would interfere with core DNA methylation. Consistent with this view, it has been found that chemical methylation of adenine in the major groove of the DNA helix is inhibited by histones (52), and enzymatic methylation of adenine (12, 22) or cytosine (43) in isolated nuclei is confined to linker DNA. Furthermore, purified *Tetrahymena* macronuclear DNA can be methylated in vitro by the *Tetrahymena* DNA methylase, possibly in core DNA regions undermethylated in vivo (Bromberg et al., submitted for publication).

In higher eucaryotes there is disagreement as to the distribution of methylated bases (MeCyt) in chromatin. For example, MeCyt has been reported to be equally distributed in core and linker DNA (4), preferentially in core DNA (56, 57), and preferentially in linker DNA (43). In view of these contradictions, the relationship between DNA methylation and chromatin organization in MeCyt-containing organisms still needs to be elucidated.

In *Tetrahymena,* linker DNA is enriched in MeAde residues, and this preferential location is stable through chromatin replication. Since methylation is sequence specific (Bromberg et al., submitted for publication) and nucleosomes do not randomize with respect to these sequences, we may infer that nucleosomal location is sequence specific. We have investigated this phenomenon directly by DNA-DNA reassociation experiments and conclude that staph limit digest DNA is only a subset of the genome complexity and, therefore, nucleosomes are phased on the *Tetrahymena* genome (Pratt and Hattman, manuscript in preparation). We expect that the packaging of only certain sequences into nucleosomes and the preferred linker DNA location of methylated sites are functionally important.

There are several observations which suggest a possible role for MeAde in transcription in *Tetrahymena.* First, only the transcriptionally active macronucleus, and not the inactive micronucleus, has methylated DNA (30). Second, methylated sequences are preferentially susceptible to DNase I (Fig. 4), which preferentially digests "active" genes in other eucaryotes (26, 55, 70, 81) and in *Tetrahymena* (29). Third, methylated sequences are preferentially digested by staph nuclease, which has also been reported to discriminate between active genes and bulk DNA in chromatin (6–8, 13, 39, 40, 47, 58–60, 68). Fourth, it has been shown that there are exposed regions of chromatin at the 5′ ends of certain genes (82), which might function as transcription promoters, and at viral origins of replication (63, 76, 79) that are preferentially sensitive to both staph nuclease and DNase I. The preferential release of MeAde in *Tetrahymena* by these nucleases may be due to a similar phenomenon; that is, MeAde may be more concentrated in exposed (linker) regulatory regions of chromatin.

Although the specific relationship between methylated sequences and transcription in *Tetrahymena* remains to be ascertained, it appears that there is a positive correlation. However, in multicellular eucaryotes methylation appears to exert the reverse effect on transcription. For example, transcriptionally competent genes are not cytosine methylated at specific sites which are methylated when those genes are not being transcribed (11, 14, 16, 17, 20, 21, 24, 45, 48, 50, 61, 70, 73, 75, 78). When one considers the differences in base pairing stabilities, reciprocal functions for MeAde and MeCyt are not surprising. For example, the base pair MeAde·T is more easily denatured than A·T (25), but C·G is more easily denatured than MeCyt·G (23, 28, 71). The order of increasing thermal stability is MeAde·T < A·T < C·G < MeCyt·G. Easily denaturable sequences may facilitate the binding of ribonucleic acid and DNA polymerases at promoters and replication origins (37). Thus, in multicellular eucaryotes methylation of cytosine might inhibit local denaturation and, consequently, transcription. In contrast, methylation of adenine would decrease duplex stability and facilitate transcription. Consistent with this hy-

pothesis are the following observations: (i) promoter regions of at least some genes, located in the nucleus (9) or in mitochondria (18), contain A-T-rich sequences; (ii) about 15% of the adenines in the replication origin of *Escherichia coli* are in the sequence G-A-T-C (51, 69), in which the A is methylated (27, 35, 46); (iii) the C_pG dinucleotide, which is frequently methylated in many higher eucaryotes, is especially rare in transcribed sequences (10, 33, 62); and (iv) T is the only 3′ nearest neighbor of MeAde in *Tetrahymena* macronuclear DNA (Bromberg et al., submitted for publication). In *Tetrahymena*, with an A+T content of 75%, methylation of A-T might increase transcription by facilitating local denaturation at the promoter. Thus, there may be a relationship between denaturability (A-T richness or presence of MeAde) and accessibility to replication and transcription enzymes. However, it still remains to be shown unequivocally that changes in DNA methylation have a regulatory function and are not merely consequences of gene expression.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-10864 and GM-29227 to S. H. from the National Institutes of Health.

We are grateful to D. Swinton for carrying out the HPLC analyses, D. Pederson for core particle isolation, F. Calzone and D. Pederson for encouragment and suggestions during the course of this work, and R. Angerer, K. Drlica, and M. Gorovsky for critical comments and suggestions.

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