[^{6}N]METHYL ADENINE IN THE NUCLEAR DNA OF A EUCARYOTE, *TETRAHYMENA PYRIFORMIS*

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ABSTRACT

DNA isolated from macronuclei of the ciliate, *Tetrahymena pyriformis*, has been found to contain [^{6}N]methyl adenine (MeAde); this represents the first clear demonstration of significant amounts of MeAde in the DNA of a eucaryote. The amounts of macronuclear MeAde differed slightly between different strains of *Tetrahymena*, with approximately 0.65–0.80% of the adenine bases being methylated. The MeAde content of macronuclear DNA did not seem to vary in different physiological states. The level of MeAde in DNA isolated from micronuclei, on the other hand, was quite low (at least tenfold lower than in macronuclear DNA).

INTRODUCTION

The existence of methylated bases in the DNAs of both procaryotes and eucaryotes has been known for some time (1, 2). Only recently, however, has the biological importance of these methylated bases been demonstrated in bacteria and their viruses, where it appears that both [^{6}N]methyl adenine (MeAde)[1] and 5-methyl cytosine (MeCyst) play important roles in host restriction and modification systems (3–11). On the other hand, eucaryotes are reported to contain only MeCyt (1, 2, 12–25), but its biological function remains unknown. In an attempt to elucidate the function of methylated bases in the DNA of eucaryotes, we have initiated studies on the DNA of macro- and micronuclei of the ciliate, *Tetrahymena pyriformis*. In *Tetrahymena*, as in most ciliates, these two nuclei are derived from a common zygotic nucleus during conjugation and, therefore, contain the same or related DNA sequences. However, in vegetatively growing cells, the macro- and micronuclei are remarkably different in several respects: ploidy level (26–28), ultrastructure (29–31), ability to synthesize RNA (32-34), time in the cell cycle at which they synthesize DNA (27, 35, 36), and in the time and manner in which they divide (30, 37). Finally, during conjugation the macronucleus breaks down, and the micronucleus undergoes meiosis and fertilization, giving rise to a new line of both macro- and micronuclei.

In this report we demonstrate that, unlike that in other eucaryotes, DNA isolated from the macronuclei of *Tetrahymena* contains significant amounts of MeAde. DNA isolated from micronuclei, by contrast, contains at least tenfold less MeAde than macronuclear DNA. The MeAde content of macronuclear DNA varies slightly in different strains but is not influenced by different physiological states of the cells.

MATERIALS AND METHODS

Strains

Strain WH-14 (mating type I, syngen I) was obtained from Dr. George Holz, Syracuse University. Strain HSM was obtained from Dr. Ivan Cameron, University of Texas, San Antonio. Strain B-7 (mating

[1] *Abbreviations used in this paper*: MeAde, [^{6}N]methyl adenine; MeCyt, 5-methyl cytosine; SSC, 0.15 M NaCl + 0.015 M Na citrate.

type VII, syngen I, inbred family B) was obtained from Dr. Peter Bruns, Cornell University. All three strains have micronuclei. An amicronucleate strain, GL, was obtained from Dr. Jytte Nilsson, Carlsberg Laboratories.

Culture Methods

Cells were grown axenically in an enriched proteose-peptone broth as described previously (33). Cell densities were measured by growing cells in 50 ml of medium in 300-ml nephelo culture flasks (Bellco Glass Co., Inc., Vineland, N. J.), and measuring the absorbance in a Bausch and Lomb Spectronic 20 colorimeter (Bausch and Lomb Incorporated, Rochester, N. Y.) at 550 nm. Under these conditions one absorbance unit equals approximately 1×10^6 cells/ml. Starvation was performed in Dryl's salt solution (37) in nephelo flasks.

Isotopic Labeling

Cells were grown for appropriate times in medium containing 1–2 μCi/ml of tritiated adenine ([2-^{3}H]-adenine, 22 Ci/mmol obtained from Schwarz/Mann, Orangeburg, N. Y.). 0.5 ml of antibiotic-antimycotic mixture (Grand Island Biological Co., Grand Island, N. Y.) was added to the 50-ml cultures at the time of labeling.

Isolation of Macro- and Micronuclei

Macronuclei were isolated as previously described (31). A micronuclear fraction was isolated from postmacronuclear supernatants by centrifugation at 16,000 *g* for 10 min (M. A. Gorovsky, in preparation). In one experiment, this micronuclear fraction was further purified by sedimentation at unit gravity (38).

Isolation of DNA

Cells or nuclei were pelleted by centrifugation, washed once in distilled-deionized water (cells) or in 0.15 M NaCl + 0.015 M Na citrate (SSC) (nuclei), and resuspended in a solution of 0.5% Sarkosyl NL 97 (Geigy Chemical Corp., Ardsley, N. Y.), 0.1 M EDTA, 0.05 M Tris-HCl, pH 8.4, and 100 μg/ml selfdigested pronase. The cell or nuclear suspension was incubated 2–4 h at 37°C and then extracted twice with 2 vol of water-saturated phenol. After centrifugation, the aqueous phase was precipitated with 2 vol of 95% ethanol overnight at −20°C. The nucleic acid precipitate was washed three times with 70% ethanol, resuspended in 0.1 × SSC, and digested with boiled pancreatic RNase (100 μg/ml) and T_1 RNase (1,000 units/ml) and with 250 μg/ml of α-amylase for 2 h at 37°C. 100 μg/ml pronase was added, and the digestion was continued for another 2 h. The DNA was precipitated with 2 vol of 95% ethanol at −20°C.

Determination of MeAde Content

DNA samples were dissolved in 0.5 N KOH and digested at 37°C for 4–12 h to hydrolyze any RNA remaining in the DNA preparations. The samples were neutralized with 1 N HCl, and the DNA was precipitated by addition of TCA to a final concentration of 5%. The precipitate was collected by centrifugation, washed in 5% TCA, 70% ethanol, and resuspended in 1 N HCl. Hydrolysis and paper chromatography were carried out as described previously (5, 6).

RESULTS

Presence of MeAde in Tetrahymena DNA

Various strains of *Tetrahymena* were grown in the presence of [2-^{3}H]adenine and the DNA isolated from whole cells. Due to the known presence of methylated adenine derivatives in RNA, extensive RNase and alkali treatments were included in the preparation of the DNA (see Materials and Methods). Under these conditions, all DNA preparations examined contained ^{3}H label which cochromatographed with marker MeAde in 86% *n*-butanol (NH_3 atmosphere). To establish that the radioactivity was in MeAde, this material was eluted from the paper and rechromatographed in 2-propanol, 1% $(NH_4)_2SO_4$ (2:1, vol/vol) or 2-propanol, HCl, H_2O (68:17:14.4, vol/vol/vol). The ^{3}H label and UV absorbance again were observed to coincide; the R_f's in the latter two solvent systems were 0.78 and 0.37, respectively, in good agreement with values from the literature. We conclude that the ^{3}H label is in MeAde.

As indicated above, the preparation of total cell DNA routinely involved steps to ensure removal of any contaminating RNA. If the MeAde is in DNA then treatment with DNase should release the methylated bases as acid-soluble nucleotides. This was tested as follows: ^{3}H-labeled DNA was treated with electrophoretically pure DNase (1 μg/ml) for 2 h at 37°C in 0.1 M sodium acetate, pH 5.0, + 5 mM $MgSO_4$ (under these conditions, no acid solubilization of [^{3}H]uridine-labeled phage f_2 RNA was observed). The digest was made 5% in cold perchloric acid and sedimented. The acid-soluble supernatant was collected, neutralized with KOH, and the insoluble $KClO_4$ was removed by centrifugation. The bases were then liberated by hydrolysis in 1 N HCl for

60 min at 100°C and analyzed by paper chromatography. The proportion of MeAde observed (0.63%) was the same as that obtained when the same DNA preparation was treated in the standard fashion (0.65%). These results demonstrate that the MeAde is present in DNA.

MeAde-Containing DNA is Located in the Macronucleus

The high level of MeAde observed in the DNA isolated from the amicronucleate strain GL (Table I) ruled out the possibility that micronuclei are the major source of MeAde-containing DNA. To rule out the possibility that MeAde is present exclusively in the DNA of cytoplasmic organelles, macronuclei were isolated from [2-^{3}H]adenine-labeled cells. Macronuclei isolated by this technique have been shown to be essentially free of cytoplasmic organelles and to be contaminated only by a small number of micronuclei (31). Due to the large difference in DNA amounts in macro- and micronuclei, at least 95% of the DNA isolated from the "macronuclear fraction" is actually derived from macronuclei. As can be seen in Table I(A), the MeAde content of DNA extracted from isolated macronuclei was consistently found to be similar to that of total cell DNA. While it is still possible that "cytoplasmic" and micronuclear DNAs may also contain MeAde, these results clearly demonstrate that the bulk of the MeAde-containing DNA is present in the macronucleus.

Micronuclear DNA Contains Little MeAde

To determine whether micronuclear DNA contained MeAde, micronuclei were isolated from late log phase cells labeled with [2-^{3}H]adenine. DNA extracted from micronuclear fractions consistently had MeAde contents far below those of macronuclei isolated from the same cells (Table II). Those preparations judged (by light microscopy) to be least contaminated with macronuclear fragments had MeAde contents of less than 0.06%. Therefore, micronuclear DNA has, at most, about one-tenth the MeAde content of macronuclear DNA (see Discussion).

MeAde Content May be Strain Specific

To see if MeAde is generally present in the DNA of *Tetrahymena*, four different strains were examined, one of which (GL) is amicronucleate. As shown in Table I, similar levels of MeAde were observed. However, in one series of experiments where the DNAs of strains GL and HSM were analyzed (three independent preparations were made in parallel) a reproducible difference in MeAde level was noted (Table I [B]). Thus, while the DNAs of all the strains tested exhibited similar levels of MeAde, the precise amount may be strain specific.

Effect of Physiological Conditions on MeAde Content of DNA

Experiments were performed to determine the effects of changing physiological conditions on the MeAde content of DNA. Cells of strain WH-14 were grown in the presence of [2-^{3}H]adenine into stationary phase (no exhaustion of exogenous adenine was observed). At various stages of growth, samples were removed and the DNA was isolated

TABLE I

MeAde Content in Total Cell DNA of T. pyriformis

Experiment	Strain	Source of DNA	Mol % MeAde*
A	1. WH-14	Whole cells	0.67‡
	2. WH-14	Macronuclei	0.77‡
B	1. GL	Whole cells	0.68 (0.68, 0.64, 0.71)§
	2. HSM	Whole cells	0.80 (0.79, 0.76, 0.86)
C	1. B-VII	Whole cells (late log)	0.73
	2. B-VII	Whole cells (starved)	0.74

* Mol % MeAde was calculated on the basis of the radioactivity found in the MeAde and Ade regions of the paper chromatograms.

‡ These figures represent the mean values obtained from at least four independent DNA preparations (some of which were analyzed in duplicate). The range of values was ±8%.

§ Values in parenthesis can be paired with values immediately below.

TABLE II

MeAde Content in the DNA of Macro- and Micronuclei of Strain WH-14 of T. pyriformis

Source of DNA	Mol % MeAde		
	Exp. A	Exp. B	Exp. C*
Micronucleus	0.12	0.12	0.06
Macronucleus	0.76	0.74	0.80

* Micronuclei isolated by differential centrifugation followed by sedimentation at unit gravity (see Materials and Methods).

and analyzed for MeAde level. Little or no difference was noted when the cells were taken at log, late log, or stationary growth phase; the values obtained were 0.65, 0.66, and 0.72 mol % MeAde, respectively.

In another experiment, cells labeled with [2-^{3}H]adenine (grown to late log phase) were taken and split into two aliquots. One sample was analyzed directly for MeAde content, and the second was starved for 24 h by incubation at 30°C in Dryl's salt solution. After this period, the DNA was isolated and analyzed. The MeAde content for both cultures was the same (Table I [C]), indicating that the starvation period had no detectable effect on the MeAde of prelabeled DNA. It should be noted that the starvation period is a necessary condition for *Tetrahymena* to become competent for mating; thus, it does not appear that there is a significant change in the methylation of preexisting DNA before the mating process.

DISCUSSION

The data presented here represent the first clear demonstration that MeAde occurs in the nuclear DNA of a eucaryote. DNA from isolated macronuclei contained levels of MeAde similar to (slightly higher than) the amounts found for total cellular DNA, while DNA from isolated micronuclear fractions contained a considerably lower proportion of MeAde. Although it is difficult to obtain a quantitative estimate of the degree of purity of the micronuclear fraction (even our best micronuclear preparations are slightly contaminated with macronuclear fragments), it is clear that micronuclear DNA contains little (or no) MeAde. However, it will be necessary to obtain completely pure micronuclei to determine whether they contain MeAde at all. Further work on this question is in progress.

Our results also indicate that the level of MeAde in *Tetrahymena* does not vary in different stages of culture growth or during starvation of cells in preparation for conjugation. It should be pointed out that in the physiological experiments described here the MeAde contents measured were mainly those of macronuclear DNA, since the DNA isolated from whole cells contains approximately 10–40 times more macronuclear than micronuclear DNA (27). In short, significant changes in the MeAde content of micronuclei would have remained undetected. Experiments are currently in progress to repeat these physiological experiments on purified preparations of labeled macro- and micronuclei.

Other eucaryotic cells contain significant amounts of MeCyt. Preliminary experiments indicate that total cell DNA isolated from *Tetrahymena* also contains MeCyt in addition to MeAde (M. A. Gorovsky and S. Hattman, unpublished observations). To date, the function of methylated bases in eucaryotes is unknown. By analogy to procaryotes, it is tempting to speculate that these methylated bases might play a role in protection against (host) nucleases which degrade foreign (viral or bacterial pathogens, phagocytosed food organisms, etc.) DNAs. However, it is difficult to explain the marked differences between the MeAde content of macro- and micronuclear DNA on this basis. Our finding that the same or related DNA sequences in two different nuclei in the same cell contain very different levels of MeAde suggests, rather, that DNA methylation in *Tetrahymena* (and perhaps in other eucaryotes as well) may play a role in the marked structural and functional differences observed between the two nuclei. To this end, a search for nuclear-specific DNA methylases in *Tetrahymena* and for cell-specific DNA methylases in metazoans might prove highly informative.

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