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Methylation of Replicating and Nonreplicating DNA in the Ciliate *Tetrahymena thermophila*

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Methylation of adenine in replicating and nonreplicating DNA of the ciliate *Tetrahymena thermophila* was examined. In growing cells, 87% of the methylation occurred on the newly replicated daughter strand, but methylation was also detectable on the parental strand. Methylation of nonreplicating DNA from starved cells was demonstrated.

The modified base N^6-methyladenine (N^6MeA) is present as a minor component in DNA from several unicellular eucaryotes, including members of the genera *Chlamydomonas* (9), *Chlorella* (16), *Oxytricha* (15), *Paramecium* (2), and *Tetrahymena* (9). DNA in the germ line micronucleus in *Tetrahymena thermophila* is unmethylated, while 0.8% of adenines in DNA of the somatic, polyploid macronucleus are modified to N^6MeA (3). The function of N^6MeA in *T. thermophila* is not known.

During sexual reproduction, the old macronucleus is destroyed and a new macronucleus develops from a mitotic product of the zygotic micronucleus. De novo methylation of DNA in the developing macronucleus is not random but occurs according to a specific pattern. Some GATC sites, for example, are unmethylated while others are methylated in all macronuclear DNA molecules (6). Still others are methylated in a fraction of the molecules that is characteristic of a particular site and that is consistent between cell lines (1, 17; Capowski et al., submitted for publication). Because *T. thermophila* undergoes phenotypic assortment (for a review, see reference 13), the existence of partially methylated sites implies that there is a methylase in vegetative cells which recognizes unmethylated DNA as a substrate.

Methylation of replicating DNA. If de novo methylation contributes to maintenance of the methylation pattern, then both the newly synthesized strand and, to a lesser extent, the parental strand are expected to undergo methylation in replicating DNA. Vegetatively growing cells were cultured in axenic medium containing 1% proteose peptone (4) to a density of 3×10^5 cells per ml. Bromodeoxyuridine (BUdR; 50 μg/ml) and [*methyl*-^{3}H]thymidine (55 to 80 Ci/mmol; concentration, 0.5 to 1.0 mCi/ml) were added to the medium, and the cells were grown for an additional 270 min (1.5 generations). Nuclear DNA was purified as described previously (6). The parental and daughter strands of the newly replicated DNA were separated on alkaline $CsCl_2$-Cs_2SO_4 gradients (12) with 3.9 g of $CsCl_2$ in each 4.8-ml gradient.

After BUdR was incorporated into the DNA and the DNA was labeled with [^{3}H]thymidine, approximately 40% of the DNA, which was assayed by A_{260} determination, migrated as a broad peak with a heavy density (Fig. 1a). The majority (92%) of newly replicated DNA, which was labeled with [^{3}H]thymidine, migrated in the gradient as a heavy peak. Thus, replicating DNA was efficiently labeled and there was good separation of newly replicated daughter strands from unreplicated parental strand DNA.

Since *T. thermophila* does not synthesize purines and pyrimidines (11), DNA was labeled with methionine only by postreplicative methylation of adenine (14) (see Table 2). A gradient of DNA from cells dividing in the presence of BUdR and L-[*methyl*-^{3}H]methionine (12 to 80 Ci/mmol) at 8 to 25 Ci/ml is shown in Fig. 1b. The majority of counts (87%) comigrated with newly synthesized (heavy) DNA.

In contrast to DNA labeled with [^{3}H]thymidine, [^{3}H]methionine-labeled DNA produced a small but distinct peak of

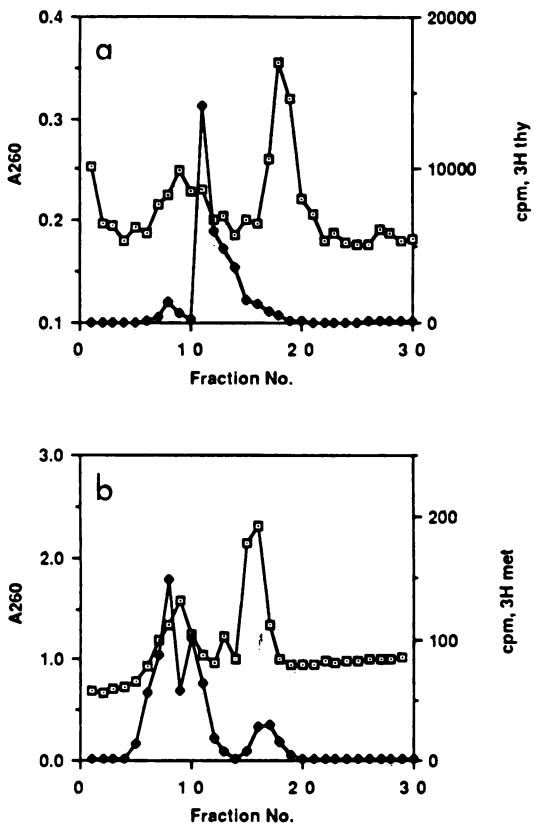

FIG. 1. Alkaline $CsCl_2$-Cs_2SO_4 gradients of [*methyl*-^{3}H]thymidine- (a) and L-[*methyl*-^{3}H]methionine- (b) labeled DNA from cells cultured vegetatively in the presence of BUdR. Density decreased as the fraction number increased. Symbols: ⊡, A_{260}; ■, counts per minute per 100 μl of sample.

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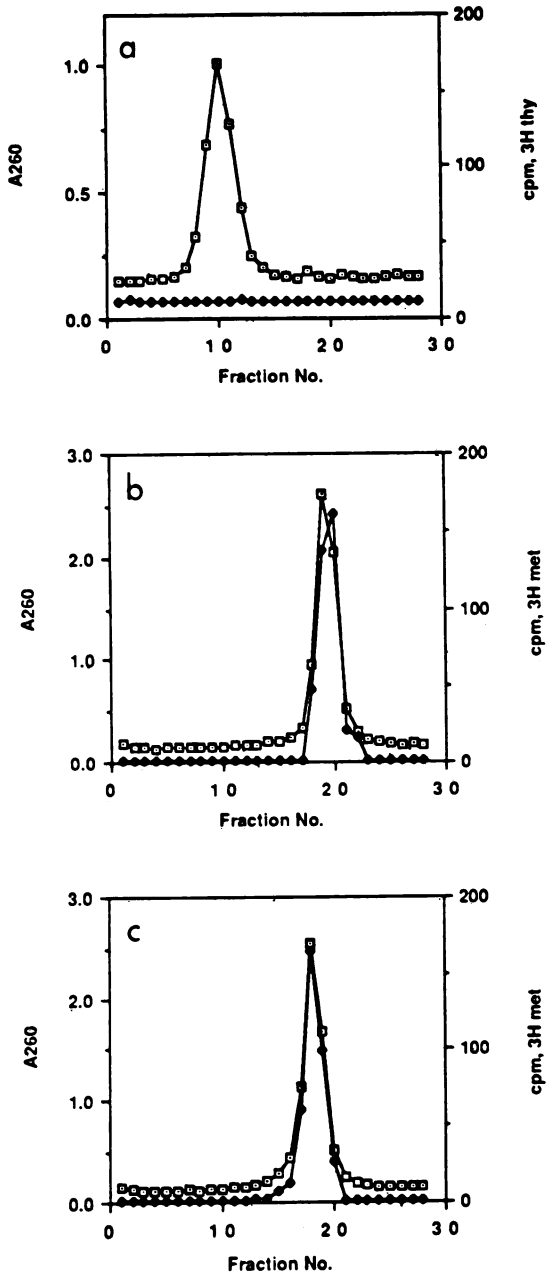

FIG. 2. Alkaline $CsCl_2$-Cs_2SO_4 gradients of [*methyl*-^{3}H]thymidine- (a) and L-[*methyl*-^{3}H]methionine- (b and c) labeled DNA from cells starved in 10 mM Tris. Cells were cultured in the presence (a and b) or absence (c) of BUdR. Gradients in panels b and c were run in a separate experiment from the gradient in panel a, which accounts for the difference in elution of the unreplicated (light) DNA. Symbols: ⊡, A_{260}; ■, counts per minute per 100 μl of sample.

radioactivity comigrating with unreplicated (light) DNA. About 13% of incorporated counts were in this peak, suggesting that a significant fraction of methylation in replicating DNA occurs on the parental strand.

Methylation of unreplicated DNA. Autoradiography of cells starved in the presence of [^{3}H]thymidine previously indicated that starved cells do not synthesize DNA (7). This was confirmed by density gradient analysis of DNA from

TABLE 1. DNase I digestion of L-[*methyl*-^{3}H]methionine- and [8-^{3}H]adenine-labeled DNA

DNA treatment	cpm (% recovery) of DNAs labeled with:	
	L-[*methyl*-^{3}H] methionine	[8-^{3}H]adenine
Minus DNase I		
Before filtration	100	192
After filtration (bound to filter)	56 (56)	151 (79)
Plus DNase I		
Before filtration	110	135
After filtration (bound to filter)	9 (8)	8 (6)

starved cells (Fig. 2a). Log-phase *T. thermophila* was transferred to 10 mM Tris and starved for 22 to 24 h, BUdR and [^{3}H]thymidine were added to the medium, and the cells were maintained for an additional 18 h before DNA extraction. When the DNA was run on an alkaline $CsCl_2$-Cs_2SO_4 gradient, there was a single peak, as measured by determining the A_{260}, and there was no evidence for the incorporation of [^{3}H]thymidine.

Although DNA synthesis was below detectable levels in starved cells (Fig. 2a), DNA methylation occurred nonetheless (Fig. 2b and c). The addition of [^{3}H]methionine to medium containing starved cells resulted in the incorporation of label into DNA both in the presence (Fig. 2b) and the absence (Fig. 2c) of BUdR. Since we demonstrated that starved cells do not synthesize DNA, incorporation of [^{3}H]methionine must have occurred in the absence of DNA replication.

We showed that radioactivity incorporated by labeling with [^{3}H]methionine was in DNA and not protein by two criteria. The first was that the counts were sensitive to digestion with DNase I. Table 1 shows the result of digesting 0.5 μg of DNA from cells which were labeled in vivo with either [^{3}H]methionine or, as a control, [8-^{3}H]adenine. Following DNase I treatment, DNA was coprecipitated from 10% trichloroacetic acid with 50 μg of calf thymus DNA. The majority of counts were released by DNase I digestion, with only 6 to 8% of the radioactivity remaining bound to the filter.

Thin-layer chromatography of bases was done to show that the DNA was labeled in methyladenine residues. Acid hydrolysis of DNA was performed as described by Hattman (8). The hydrolyzed DNA was spotted, along with standards, onto a silica thin-layer chromatographic sheet containing a UV fluorescence indicator (J. T. Baker Chemical Co., Phillipsburg, N.J.). The difference in migration between adenine and methyladenine was maximized by using an ascending, one-dimensional, two-step solvent system (ethyl acetate-methanol; [70:30] with overnight drying, followed by chloroform-methanol [40:60]). The spots were visualized with UV light, cut out, and counted. Results from one experiment are shown in Table 2. Guanine and cytosine residues ran as a smear near the origin in this system and were not measured. A total of 58 cpm (94% of the total counts per minute measured for the bases adenine, methyladenine, and thymine) was detected in the spot corresponding to N^6MeA, compared with 4 cpm (6%) for adenine and 0 cpm for thymine. The 21 cpm measured at the origin probably corresponded to partially hydrolyzed DNA. Thus, label incorporated into the DNA of starved cells was the result of adenine methylation.

TABLE 2. Thin-layer chromatography of hydrolyzed L-[*methyl*-^{3}H]methionine-labeled DNA

Base	Distance (cm) migrated from origin	R_f[a]	cpm in spot
Thymine	12.5	0.39	0
N^6MeA	11.0	0.34	58
Adenine	10.0	0.31	4
Origin	0	0	21

[a] The R_f was determined as follows: distance migrated by base/distance migrated by solvent.

We showed that the majority of methylation occurs on the daughter strand during DNA replication in *T. thermophila*. In addition, there was significant incorporation of label on the unreplicated parental strand in DNA from dividing cells and methylation of nonreplicating DNA in starved cells. One explanation for the methylation of nonreplicating DNA could be the turnover of methyl groups, with no change in steady-state levels. Another possible explanation, at least in growing cells, is delayed methylation, as has been described previously for cytosine in mammalian systems (5, 10, 12, 18). It is unlikely, however, that delayed methylation accounts for methylation of DNA in starved cells, which was synthesized 22 to 24 h before the addition of the radioisotope.

Repair synthesis cannot readily account for methylation of nonreplicating DNA in starved cells. The assay for DNA replication in growing cells by labeling with [^{3}H]thymidine is 100-fold more sensitive than labeling with [^{3}H]methionine (Fig. 1), yet no DNA synthesis was detected in starved cells. It is unlikely that DNA synthesis was masked in starved cells by a large pool of unlabeled thymidine, since replicating DNA is readily labeled by thymidine incorporation when starved cells with complementary mating types are mixed to induce conjugation (7). These results indicate that DNA methylation occurs in the absence of DNA synthesis in *T. thermophila* and are consistent with the hypothesis that unmethylated sites are a substrate for a maintenance methylase.

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