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Molecular Analysis of $N^6$-Methyladenine Patterns in Tetrahymena thermophila Nuclear DNA

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We have cloned two DNA fragments containing 5’-GATC-3’ sites at which the adenine is methylated in the macronucleus of the ciliate Tetrahymena thermophila. Using these cloned fragments as molecular probes, we analyzed the maintenance of methylation patterns at two partially and two uniformly methylated sites. Our results suggest that a semiconservative copying model for maintenance of methylation is not sufficient to account for the methylation patterns we found during somatic growth of Tetrahymena. Although we detected hemimethylated molecules in macronuclear DNA, they were present in both replicating and nonreplicating DNA. In addition, we observed that a complex methylation pattern including partially methylated sites was maintained during vegetative growth. This required the activity of a methylase capable of recognizing and modifying sites specified by something other than hemimethylation. We suggest that a eucaryotic maintenance methylase may be capable of discriminating between potential methylation sites to ensure the inheritance of methylation patterns.

Postreplicative addition of methyl groups to DNA bases is a common modification of the DNAs of most organisms. Most higher eucaryotes contain exclusively 5-methylcytosine, whereas protozoans, some plants, and procaryotes contain $N^6$-methyladenine either exclusively or in addition to 5-methylcytosine. It is not known whether cytosine and adenine methylations of DNA serve similar or different functions.

Methylated bases are not distributed at random in the DNA but are found in stable, tissue- or cell-type specific patterns. In the case of the cytosine methylases, it is thought that methylation patterns are established with the differentiation of cell type and maintained by a methylase with limited de novo activity (for review, see reference 18). Because of the palindromic nature of many methylation sites in eucaryotes, a model based on the semiconservative nature of DNA replication has been proposed for the mechanism of activity of cytosine methylases (3, 14, 19). According to this model, DNA molecules of the parent cell, which are modified on both strands, yield hemimethylated sites after DNA replication. These sites become the target for a nondiscriminating maintenance methylase with a strong preference for a hemimethylated substrate. This enzyme methylates the base on the newly replicated daughter strand, thus faithfully maintaining the pattern through cell division.

Support for this model has come from several lines of evidence. DNA reassocation experiments and subsequent restriction endonuclease analysis suggest that most Cpg dinucleotides are symmetrically methylated (3). Purification of methylases from somatic nuclei and in vitro assays of their activities showed that in general, their preferred substrate was hemimethylated DNA (9, 30). In addition, DNA-mediated gene transfer experiments showed that clonal inheritance of methylation patterns in vivo was restricted to the palindromic dinucleotide Cpg (27). Finally, kinetic studies have shown that the bulk of DNA methylation in eucaryotic cells occurs at the replication fork (10).

We have been using the ciliated protozoan Tetrahymena thermophila as a model system to study the maintenance of methylation patterns. T. thermophila is a single-celled organism that contains two nuclei, a germ line micronucleus and a somatic macronucleus. The micronuclear DNA is unmethylated, whereas the macronuclear DNA contains 0.8 mol% $N^6$-methyladenine at the palindromic dinucleotide sequence ApT (5, 8). The macronuclear methylation pattern is established de novo as a new macronucleus develops from a mitotic product of the zygotic micronucleus during conjugation (4, 12). The pattern established during macronuclear development appears to be stable through changes in the physiological state of the organism (11; K. Karrer, unpublished data). The function of methylation in T. thermophila is unknown.

Little is known about what specifies a methylation site in the developing macronucleus, but molecular analysis of mature macronuclei has shed some light on the distribution of modified residues. These analyses take advantage of a family of methyladenine-sensitive restriction endonucleases. By using these enzymes, it has been shown that there are two types of methylation sites in macronuclear partially methylated sites at which a fraction of the copies of the sequence in the polygenomic macronucleus are methylated whereas the rest of the copies are unmethylated (4, 31) and uniformly methylated sites at which greater than 90% of the copies in the macronucleus are modified on both strands (11). We have cloned examples of both types of sites from macronuclear DNA and used them to study the maintenance of methylation patterns during vegetative growth of the organism. Our primary concern was to determine whether the semiconservative model for maintenance of methylation patterns applies for methylation of adenine in T. thermophila. According to this model, methylation occurs on the daughter strand of replicating DNA, and hemimethylated
DNA would be the presumed target of a maintenance methylase. It was previously demonstrated that most but not all methylation occurs on the newly synthesized DNA strand in vegetatively growing Tetrahymena cells (13), as expected for a methylase functioning semiconservatively. We show that hemimethylated DNA is detectable in macronuclear DNA as a small fraction of the methylated molecules at both uniformly and partially methylated sites but is not exclusively associated with replicating DNA. In addition, we demonstrate the faithful inheritance of a complex methylation pattern that requires the activity of a methylase with a more discriminating mechanism than that provided for by a semiconservative copying model.

MATERIALS AND METHODS

Cell culture. T. thermophila 1868 BVII, CU399, and CU427 were kindly provided by P. Bruns. CU427 is the product of a round 1 genomic excision mating between CU399 and A* and contains the same macronucleus as does CU399. Strain C2-468-3 was a gift of S. Allen. G9-3 and G9-4 are clonal isolates from the whole-genome homozygous strain G9, made by B. Allitto (1). Stocks were maintained in an axenic medium containing 1% Proteose Peptone (Difco Laboratories, Detroit, Mich.)-0.1% yeast extract-0.003% Sequestrene and were grown in 2% Proteose Peptone medium to cell densities of 2 \times 10^5 to 3 \times 10^7/ml. All manipulations were at 29°C. To starve cultures, logarithmic-phase cells were transferred to 10 mM Tris (pH 7.4) as described previously (11) for 48 h. To maximize DNA replication, logarithmic-phase cultures were starved for 24 h in 10 mM Tris (pH 7.4) to arrest the majority of the cells in G1 (23). Upon refeeding, these partially synchronized cells incorporate [3H]thymidine, with maximal incorporation at 6 h after refeeding (E. E. Capowski and K. M. Karrer, unpublished observation).

Isolation of DNA. Macronuclear and micronuclear DNAs were isolated as described previously (15) from cultures of logarithmically growing, starved, or partially synchronized dividing cells. Whole-cell DNA was isolated from clonal lines by the method of Austerberry and Yao (2).

DNA digestion and Southern hybridization. DNA was digested by restriction endonucleases (Boehringer Mannheim Biochemicals, Indianapolis, Ind., and International Biotechnologies, Inc., New Haven, Conn.) as described by Karrer and Yao (16) or according to the specifications of the manufacturers. Sau3A, DpnI, and MboI digests were done in the following manner: 7 or more units of enzyme was added every hour for 3 h to a reaction mix containing 0.8 μg of macronuclear DNA, and incubation was continued for at least 2 h more. This should result in a theoretical 20-fold or more overdigestion of the DNA. Restriction fragments were separated on 0.6 or 2% agarose gels and blotted to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) by the method of Allitto and Karrer (1). Isolated fragment was labeled by the random-primer labeling method of Feinberg and Vogelstein (7) and used to probe the filters, which were washed as described previously (11) except that the wash temperature was 60°C and the final wash was in 0.1× SSC (SSC is 0.15 NaCl plus 0.015 sodium citrate) for 30 min.

Probes. pTIIIC7BX, and 4.4-kilobase (kb) BglII-XbaI fragment, was cloned from a partial library of sized restriction fragments. Macronuclear DNA from strain 1868 BVII was digested with the restriction endonucleases BglII and XbaI and fractionated on a 0.8% agarose gel for 400 v-h. A well was cut in front of the predicted position of the sized fragment, and fractions were collected into 25% glycerol in running buffer. The correct fraction was identified by Southern blot analysis and ligated to a pUC18 vector. which was used to transform the JM83 strain of Escherichia coli.

pTIIIC7BX.8 was subcloned by isolating a 0.8-kb HindIII-XbaI fragment from pTIIIC7BX and ligating it to pUC18. pTIIIC7BX.8A and pTIIIC7BX.8B are subclones of pTIIIC7BX.8 resulting from a Sau3A digest of isolated fragment, yielding a 0.25-kb HindIII-Sau3A fragment and a 0.3-kb Sau3A fragment, respectively, each ligated to pUC18. pTIIIC7.1a has been described previously (28).

pTtcydl was selected from a library of micronuclear DNA from strain CU399 (21) on the basis of its hybridization pattern to macronuclear DNA cut with restriction enzymes. When used to probe BglII-cut macronuclear DNA, for example, it hybridized to a single fragment that was cut by DpnI into two smaller fragments whose sizes added up to the size of the original fragment. This indicates that the clone contains a DpnI site, which was subcloned as pTtcydl.D. A 0.5-kb HindIII-PstI fragment.

Phenotypic assortment mating. Strain C2-468-3 (III), which carries a restriction-fragment-length polymorphism at the IIC7 locus in its micronucleus, was crossed with CU427. Chx/Chx (cy-s, VI) as described previously (11). Cells were referred to 0.1% Proteose Peptone medium at 12 h after mixing: at 20 h, concentrated medium plus cycloheximide was added to a final concentration of 20 μg of drug per ml in 1% Proteose Peptone to kill nonmatters. Cells were grown for 48 h with shaking in selective medium and cloned into drops of 1% Proteose Peptone-20 μg of cyclohexamide per ml. Whole-cell DNA was isolated from clonal lines approximately 25 generations after mating, and six lines containing both versions of the IIC7 locus were maintained in a logarithmic growth by serial transfer into nonselective media for more than 200 generations. Single cells from the aged cultures were isolated into drops of 1% Proteose Peptone medium, and whole-cell DNA was isolated from individual clonal populations.

RESULTS

Hemimethylation. To facilitate our study of methylation patterns, we have cloned two loci, cydLD and IIC7, which contain 5'-GATC-3' sites that are methylated in Tetrahymena macronuclear DNA. A small percentage of 5'-GATC-3' sites are methylated in T. thermophila, representing approximately 3% of the total methylation in the macronucleus (11). The level of modification at these sites could be tested by Southern blot analysis, using the restriction endonuclease isoschizomer DpnI, which cuts this sequence only in the presence of N'-methyladenine on both DNA strands, or MboI, which cuts only unmethylated 5'-GATC-3' sites (Table 1). The restriction endonuclease Sau3A cuts this sequence regardless of the methylation state of the adenine residues, allowing for mapping of the sites. A genomic map of the macronuclear locus homologous to pTtcydl.D is
Tetrahymena DNAs from starved and dividing cells were digested with the appropriate restriction endonucleases, and Southern blots were probed with the two subclones shown in Fig. 1A and 2A. DNA molecules that were hemimethylated at site D in Fig. 1A were resistant to \( MboI \) and \( DpnI \) and were identified as a 0.7-kb fragment in genomic DNA digested with both enzymes (Fig. 1B). Densitometric analysis showed that approximately 10% of the molecules existed in this form. This species was not found in unmethylated micronuclear DNA (Fig. 1B, lane 4).

Hemimethylated molecules at the \( IIC7 \) sites marked M/D in Fig. 2A were detected as resistant fragments of 2.1 and 0.5 kb (Fig. 2B, lanes 5 and 6) in macronuclear DNA digested with \( MboI \) and \( DpnI \) and probed with the clone shown in Fig. 2A. Resistant molecules of the size predicted for hemimethylation at the third \( IIC7 \) methylation site (marked D in Fig. 2A) have also been identified (data not shown). In all cases, the resistant species were found in macronuclear DNA from dividing and starved cells but not in micronuclear DNA. Very faint hybridization in the micronuclear lane (Fig. 2B, lane 2) can be accounted for by contamination of the micronuclear DNA preparation with macronuclear DNA.) Thus, hemimethylated DNA molecules were detected in \( Tetrahymena \) macronuclei, but these species were not exclusively associated with replicating DNA.

The \( IIC7 \) locus. The \( IIC7 \) locus is a cloned fragment of macronuclear DNA containing three methylation sites that showed a complex pattern of methylation upon molecular analysis. A restriction map of the cloned region, including the two subclones used to probe Southern blots, is shown in Fig. 3A.

The complex pattern was revealed by analysis of \( DpnI \) digestion patterns of large restriction fragments containing the locus. A probe that did not cover the methylation sites (Fig. 3A) hybridized to a 9.0-kb fragment in macronuclear DNA digested with \( HpaI \) (Fig. 3B, lane 1). In macronuclear DNA double digested with \( HpaI \) and \( DpnI \), this fragment was cut into three smaller fragments (Fig. 3B, lane 2). However, the sizes of the three \( HpaI-DpnI \) fragments did not add up to the size of the original \( HpaI \) fragment. This suggested that the two methylation sites closest to the probe were partial methylation sites. Thus, whereas all three fragments shared a left end at the \( HpaI \) site, the smallest fragment consisted of molecules methylated at the proximal site labeled M/D in Fig. 3A. The middle fragment contained molecules unmethylated or hemimethylated at this site but methylated at the distal site M/D. Finally, the largest fragment represented molecules that were unmethylated or hemimethylated at both M/D sites but methylated at a third site (D) in Fig. 3A.

To determine whether sites resistant to \( DpnI \) were unmethylated, we used subclone pTIIC7B7X, which covered two of the three methylation sites, to probe \( MboI \)-cut macronuclear DNA (Fig. 3C, lane 2). This resulted in the complex pattern of six bands corresponding to the sizes shown in Fig. 3A. The methylation sites marked M/D were targets of \( MboI \) restriction. Therefore, some of the molecules that were not digested with \( DpnI \) in the previous blot were unmethylated, as shown by \( MboI \) digestion. \( MboI \) fragments of the sizes predicted for cutting at the site designated D were not detected, which showed that none of the molecules were unmethylated at this site. Taken together, the data in Fig. 3B and C demonstrated that the sites marked M/D were partially methylated and that site D was uniformly methylated.

Clonal inheritance of methylation pattern. At least two models could explain partial methylation. Because of exten-

![FIG. 1. Hemimethylation at the cydl locus. (A) Restriction map of the cydl macronuclear locus. Symbols and abbreviations: @, pTicyd1.D; *, N\textsuperscript{6}-methyladenine-containing site; S, Sau3A; M, MboI; D, DpnI. The hemimethylated molecule detected by the probe is shown below. (B) Southern blot of genomic DNA from strain CU427 digested with Sau3A (lanes 1 to 3) or MboI plus DpnI (lanes 4 to 6) and hybridized with the probe shown in panel A. Lanes: 1 and 4, micronuclear DNA; 2 and 5, macronuclear DNA from starved cultures; 3 and 6, macronuclear DNA from partially synchronized dividing cells.](image_url)
of the other member of the pair. We tested round 2 offspring of a genomic exclusion mating between strains CU399 and A* for the presence of partially methylated sites at the IIC7 locus. Macronuclear DNA from the progeny, clonal cell lines G9-3 and G9-4, showed MboI patterns identical to that found in the parental line, CU399 (Fig. 4A, lanes 6 and 7). Thus, the data in Fig. 4A demonstrate that the complex methylation pattern at the IIC7 locus is clonal and that partial methylation cannot be ascribed to genetic differences between the molecules.

Densitometric analysis (data not shown) of the Southern blot shown in Fig. 4 indicated that the partially methylated sites (M/D) were doubly methylated in approximately 30% of the molecules and unmethylated in the other 70%; the third site (D) was uniformly methylated. All possible combinations of methylation states at the three sites were present at roughly equal levels within the macronucleus (Fig. 4A). Therefore, to generate the complex MboI pattern found, the DNA molecules in a clonal cell line (and presumably in each individual macronucleus) must be present in all four molecular configurations outlined in Fig. 4B.

**Phenotypic assortment.** During vegetative growth, *Tetrahymena* macronuclei divide amitotically. Because macronuclei contain no functional centromeres, alleles are randomly distributed to daughter cells during fission. Therefore, the progeny of a heterozygous cell contain primarily one allele or the other after about 100 cell fissions. All molecular and genetic loci studied thus far (more than 90) have been shown to undergo this phenotypic assortment (for review, see reference 6). However, the clonal analysis presented above indicates that the four molecular species found at the IIC7 locus are maintained in a single macronucleus during vegetative growth. There are two possible explanations for this result: either the IIC7 locus is not subject to phenotypic assortment, or the methylation pattern is actively maintained against assortment. In the following experiment, we show that the IIC7 locus does assort.

Macronuclear DNA from strain C2-468-3 contains a restriction-fragment-length polymorphism at the IIC7 locus (Fig. 5A). This polymorphism is heterozygous in the macronucleus (data not shown); therefore, half of the progeny would be expected to be heterozygous for this allele. Strain C2-468-3 was mated to strain CU427, offspring were isolated as single cells, and clones were grown for 25 generations to obtain enough DNA for analysis. Of 14 lines tested, 6 were heterozygous for the IIC7 polymorphism. These six strains were grown for more than 200 generations to allow phenotypic assortment to occur. Clonal lines were isolated from these aged cultures and grown for an additional 25 fissions. Whole-cell DNA from these lines was restricted with Sau3A and probed with the subclone shown in Fig. 5A to assay for the polymorphism (Fig. 5B). All of 15 clonal lines that were isolated from originally heterozygous cultures were enriched for either one or the other of the two variants. Thus, the IIC7 locus assorted during vegetative growth.

These assorted clones carrying either one or the other variant of the locus were also assayed for the IIC7 methylation pattern. Because of the restriction enzyme polymorphism, the 1.8-, 2.1-, and 3.0-kb MboI fragments in CU427 are 4.0, 4.3, and 5.2 kb, respectively, in strain C2-468-3. As expected, the MboI pattern was characteristic of the allele retained in the macronucleus (Fig. 5C). Thus, although the two alleles assorted, the pattern persisted. If methylation were maintained by a semiconservative methylase activity, unmethylated sites would be expected to assort to purity. Therefore, our data imply that a methylase must recognize
and replace sites lost by assortment and suggest that the enzyme that maintains patterns of adenine methylation in vegetatively growing *Tetrahymena* cells may have de novo methylation activity.

**DISCUSSION**

The transient existence of hemimethylated DNA molecules is an expected consequence of semiconservative DNA replication (10), and hemimethylated 5′-GATC-3′ sites have been found in plasmid DNA propagated in bacterial cells (22). We have shown that hemimethylated DNA is detectable on a Southern blot of *Tetrahymena* macronuclear DNA from dividing cells. One concern in an experiment that assays hemimethylation on the basis of resistance to enzymatic digestion is the possibility of incomplete digestion. Care was taken to ensure that the DNA shown in Fig. 2 was digested to completion (see Materials and Methods). Additional controls included (i) the demonstration that the pattern of bands in macronuclear DNA double digested with *Mbol* and *DpnI* did not change over a range of enzyme concentrations twofold lower and higher than the conditions used for these experiments and (ii) reprobing of the enzyme titration blot of *Mbol-DpnI*-double-digested DNA with a clone of the ribosomal DNA, which is unmethylated at most 5′-GATC-3′ sites. A more compelling observation is the fact that bands indicative of hemimethylation are not present in the unmethylated macronuclear DNAs in lane 4 of Fig. 1B and lane 2 of Fig. 2B. The consistent appearance of these fragments in macronuclear but not micronuclear DNA in these and other blots suggests that the fragments resulted from hemimethylation and not partial digestion.

Unexpectedly, macronuclear DNA from starved cells, which are not engaged in DNA replication, show similar levels of the hemimethylated molecules. Several factors...
A more interesting hypothesis is that hemimethylation serves some function in *T. thermophila*. The levels at which this species is found in macronuclei suggest that hemimethylated molecules persist for a significant fraction of the cell cycle. As many as 10% of the molecules at the uniformly methylated *cycl* locus were hemimethylated, and detectable levels of hemimethylation were observed at partially methylated sites modified on only a fraction of the molecules at the *HIC7* locus. In addition, we have detected hemimethylation in macronuclear DNA from unsynchronized cells at levels similar to those found in macronuclear DNA from synchronized cultures (E. Capowski, unpublished observation). In procaryotic cells, hemimethylation of 5'-GATC-3' sites appears to play a role in the regulation of DNA replication (17, 22) and of IS10 transposition (20).

The *HIC7* locus consists of one uniformly methylated and two partially methylated 5'-GATC-3' sites within a span of 500 base pairs in the macronuclear genome. The partial sites are methylated on 30% of the macronuclear molecules and unmethylated on the rest of the molecules. It is not clear whether this 30:70 ratio, which is conserved across strains, is a function of the relative affinities of the sites for the methylase or whether some structural feature of the chromatin at the two sites limits the access of the methylase.

Partial methylation of particular sites cannot readily be reconciled with a semiconservative model for maintenance of methylation patterns in *T. thermophila*. Since macronuclear DNA fragments contain no functional centromeres, there is a random partitioning of molecules to daughter cells during vegetative growth. This somatic assortment results in homozygous subclones from lines that began as heterozygotes for a given allele. The loss of alleles in the somatic nucleus does not affect the germ line macronucleus, which divides mitotically. We show that the *HIC7* molecular locus undergoes assortment over time during vegetative growth. However, the various fragments resulting from the complex methylation pattern do not assort, which indicates that the mechanism responsible for maintaining this pattern can compensate for assortment of macronuclear DNA molecules. It is not possible for a methylase functioning solely according to a semiconservative copying model to fulfill this role.

One implication of the retention of the *HIC7* methylation pattern in assorting macronuclei is that the methylase responsible for maintaining this pattern in the mature macronucleus must be capable of recognizing and modifying sites specified by something other than hemimethylation. This result supports the conclusions of microinjection experiments in which it was shown that the *Tetrahymena* methylase requires more than a hemimethylated site to signal maintenance of in vitro-established methylation patterns (16).

During sexual reproduction in *T. thermophila*, the methylation pattern of the developing somatic nucleus is established de novo on the unmethylated DNA of the progenitor nucleus. This pattern may be maintained in part through a semiconservative mechanism of methylation, since the majority of the methylation occurs on the daughter strand in replicating *Tetrahymena* DNA (13). However, the data described here indicate that a semiconservative activity is not sufficient to maintain partially methylated sites. We propose that the methylase may be capable of de novo activity in the vegetative as well as in the developing macronucleus.

De novo methylation of eucaryotic DNA during somatic proliferation has been described in systems in which cytosine is the methylated base. In *Neurospora crassa*, a
methylase active in vegetative cells is capable of recognizing and de novo methylating unmethylated DNA introduced by transformation to give the pattern characteristic of the endogenous DNA (25). This methylation may be part of a system that scans the genome for sequence duplications and mutates them (24). The basidiomycete *Corpinus cinereus* also appears capable of de novo methylating its DNA during mitotic growth (32). In mammalian cells, methylation patterns are set down in the germ line, and the pattern depends on whether the chromosome is inherited from the maternal or the paternal parent (26, 29). *T. thermophila* provides a highly accessible system in which to study the contributions such factors as DNA sequence and localized chromatin structure make to the design of methylation patterns in eucaryotes.

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**LITERATURE CITED**


**FIG. 5.** (A) Restriction maps of the *HIC7* macronucleus loci of strains C2-468-3 and CU427. Abbreviation and symbols: S, *Sau3A*; *, *N* methyladenine-containing site; ■, probe pTIIC7BX.8A. (B) Phenotypic assortment at the *HIC7* locus. Shown is a Southern blot of *Sau3A*-digested whole-cell DNA hybridized with the probe shown in Fig. 5A. DNA came from two parental strains of a mating, CU427 (lane 1) and C2-468-3 (lane 2), from an offspring (3B6) 25 generations after the mating (lane 3), and from two clonal lines isolated from the aged culture of 3B6 200 generations after the mating, 3B6A1 (lane 4) and 3B6A10 (lane 5). (C) Southern blot of *Mbol*-digested whole-cell DNA hybridized with probe pTIIC7BX.8, showing that the methylation pattern at the *HIC7* locus assorts as a unit during vegetative growth. DNA is from strains CU427 (lane 1) and C2-468-3 (lane 2) and from two clonal lines, isolated from the aged culture of 3B6. 3B6A1 (lane 3) and 3B6A10 (lane 4). The high-molecular-weight smear in lane 1 represents nonspecific sticking to protein and carbohydrate contamination of the whole-cell DNA preparations.