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Methylation of ribosomal RNA genes in the macronucleus of *Tetrahymena thermophila*

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ABSTRACT

We have investigated the occurrence of methylated adenine residues in the macronuclear ribosomal RNA genes of Tetrahymena thermophila. It has been shown previously that macronuclear DNA, including the palindromic ribosomal RNA genes (rDNA), of Tetrahymena thermophila contains the modified base N-6-methyladenine, but no 5-methylcytosine. Purified rDNA was digested with restriction enzymes Sau 3AI, MboI and DpnI to map the positions and levels of N-6-methyladenine in the sequence 5' GATC 3'. A specific pattern of doubly methylated GATC sequences was found; hemimethylated sites were not detected. The patterns and levels of methylation of these sites did not change significantly in different physiological states. A molecular form of the rDNA found in the newly developing macronucleus and for several generations following the sexual process, conjugation, contained no detectably methylated GATC sites. However, both the bulk macronuclear DNA and palindromic rDNA from the same macronuclei were methylated. Possible roles for N-6-methyladenine in macronuclear DNA are discussed in light of these findings.

INTRODUCTION

Methylated bases constitute a widespread feature of the DNAs of both prokaryotes and eukaryotes. In eukaryotes, the modified base 5-methylcytosine is commonly found as a minor fraction of the cytosine residues (1). The level of methylation of cytosine residues in and around gene coding regions has been correlated in an inverse manner with transcriptional activity for several different genes in a number of vertebrates (1,2). However, much less is known concerning the role of the modified base N-6-methyladenine, which is not detectable in the DNA of higher eukaryotes, but which does occur in the nuclear DNAs of several groups of lower eukaryotes. These include the unicellular green alga Chlamydomonas (3), several dinoflagellates (4), and the ciliated protozoa. In the DNAs of the ciliated protozoa so far examined, N-6-methyladenine is the only modified base detectable. It has been found in the DNAs of Paramecium (5), Oxytricha (6), Tetrahymena (7), and the tetrahymenid Glaucoma (S. Hattman and E. H. Blackburn, unpublished results).

Ciliated protozoa are characterized by nuclear dimorphism. In the single cell that comprises each organism, two different types of nuclei are found: a diploid, transcriptionally inert micronucleus, which functions as a germinal nucleus to maintain genetic continuity between generations, and a transcriptionally active polyploid macronucleus. The somatic macronucleus is destroyed in the course of the sexual cyle, conjugation, and is replaced by a division product of the newly formed zygotic nucleus produced by meiosis and cross-fertilization of parental micronuclei.

A role for N-6-methyladenine in the structural and functional differentiation of these two nuclei was suggested by the finding that in Tetrahymena, N-6-methyladenine comprises 0.65 to 0.8% of the adenine residues in the macronuclear DNA of vegetatively growing cells, but is undetectable in micronuclear DNA (7). In Tetrahymena, starvation or conjugation produce a generalized shutdown of transcription of macronuclear DNA, yet the level of N-6-methyladenine was found to be the same in macronuclei of starved and rapidly-growing Tetrahymena (7). Hence, no correlation of the total level of N-6-methyladenine in macronuclear DNA with its overall transcriptional activity was observed. This contrasts with the situation in vertebrates with respect to levels of 5-methylcytosine in specific gene sequences.

Since previous studies had been carried out on overall levels of methylation in Tetrahymena nuclei, we decided to examine the distribution and levels of N-6-methyladenine in a well characterized gene, the ribosomal RNA gene (rDNA). rDNA is amplified in the macronucleus, and is found as free, linear DNA molecules, 21 kb in length, each carrying two rRNA gene copies, arranged as a palindrome (8,9). The rRNA transcription unit has been mapped (10), and is 6 kb in length. Earlier analysis has shown that N-6-methyladenine comprises about 0.4% of the adenine residues of palindromic rDNA (11; P. M. M. Rae and E. H. Blackburn, unpublished results). Bromberg et al. (12) have shown that in Tetrahymena DNA, N-6-methyladenine (MeA) is found exclusively in the sequence 5'MeA-T 3'. We therefore used restriction endonucleases whose ability to cut DNA at the tetranucleotide sequence 5' GATC 3' depends on the state of methylation of adenine residues in this sequence. Sau 3AI cuts DNA at this sequence regardless of the methylation of the adenine residues (13), and can cut hemimethylated DNA. MboI cuts double stranded DNA at this sequence only if the adenine residues on both strands are unmodified (14), and DpnI cuts at this sequence only if its adenine residues on both strands are methylated (15). The inability of DpnI to cut unmethylated or hemimethylated GATC sequences makes it a sensitive assay for

doubly methylated GATC.

We decided to use the rRNA genes to investigate possible roles for N-6-methyladenine for two reasons. First, the rate of transcription of the rRNA genes is strongly dependent on the physiological state of the cell: exponentially-growing cells have a high rate of rRNA synthesis, which is markedly decreased during starvation (16). Accordingly, we analyzed levels of methylation of rDNA in exponentially-growing, starved and conjugating cells. Second, the amplified rDNA is produced from a single, chromosomally-integrated rRNA gene present in one copy per haploid genome in the micronucleus (17). Amplification takes place during the development of the newly differentiating macronucleus following conjugation (18). During amplification, single, free rRNA genes, consisting of linear DNA molecules 11 kb in length, are synthesized from the micronuclear rRNA gene copies (19,20). These molecules are lost as the macronucleus matures, becoming undetectable after several generations following conjugation. However, whether the 11 kb rDNA molecules are direct precursors of palindromic rDNA is not clear. Thus, in the developing macronucleus, the rDNA is found in two molecular forms, one of which is destined to be lost in the maturing macronucleus. We decided to examine methylation of both these molecular forms in the developing macronucleus, to determine whether there is any relationship between their levels of methylation and their respective fates in macronuclear development.

MATERIALS AND METHODS

Tetrahymena thermophila inbred strains B-1868 (IV) and C_3-368 (V), where the number in parentheses refers to the mating type, were provided by D. Nanney. Maintenance of stocks and growth of cells were carried out as described previously (19). Cells were starved, conjugated, and labeled with [^{3}H]-thymidine also as previously described (19).

rDNA was purified as described by Wild and Gall (21). ^{32}P-labeled DNA probes for hybridization were prepared by nick-translation of either purified rDNA, or of segments of the rDNA molecule cloned in recombinant DNA plasmids as described in Pan and Blackburn (19). The cloned rDNA segments were generously provided by P. Challoner.

Restriction enzyme digestions, gel electrophoresis, fluorography and Southern (22) hybridizations were performed as described previously (19). Restriction enzymes MboI and Sau 3A1 were purchased from New England Biolabs; DpnI from Bethesda Research Laboratories. Digestions were carried out according to the manufacturer. In all restriction enzyme digestions, a known

amount (usually 0.4 µg) of the plasmid pBR322 DNA was included in the reaction mixture as a control. The extent of its digestion was determined, after electrophoresis in an agarose gel, by examination of the fragments produced by staining with ethidium bromide and illumination with 366 nm uv light on a transilluminator box. The rDNA fragments produced by restriction enzyme digestions were analyzed by Southern blotting and hybridization to ^{32}P-labeled, cloned segments of rDNA. Alternatively, the Smith and Birnstiel method (23) for mapping restriction sites was used. In some experiments, rDNA was digested first with Eco R1, which cuts once in each half of the palindromic rDNA molecules to produce a central 15.6 kb fragment and two identical 2.6 kb terminal fragments. End-labeling of the central fragment produced, because the fragment is palindromic, a unique set of end-labeled partial digestion products upon its subsequent partial digestion with Sau 3A1 or MboI. The Eco R1 fragments were end-labelled essentially according to Maxam and Gilbert (24), either by the polynucleotide kinase procedure, or by filling in single stranded termini of the fragment using the Klenow fragment of DNA polymerase I. Labelling of the terminal 2.6 kb fragment by DNA polymerase Klenow fragment was carried out under conditions in which only the Eco R1-cut end was labelled, using α^{32}PdATP and dTTP. The termini of the rDNA molecules were inaccessible to such a labelling protocol (25).

RESULTS

Digestion of rDNA from exponentially growing cells with restriction enzyme Sau 3A1

rDNAs from inbred strains B and C3 of *T. thermophila* were digested with Sau 3A1, and their Sau 3A1 restriction maps were determined as described in the Materials and Methods section. These restriction maps are shown in Fig. 1a. Two strain specific differences were seen. First, there is a Sau 3A1 site in strain C_3, 1.5 kb from the rDNA ends, which is not found in strain B. This corresponds to an extra Bam H1 site (which should also be a Sau 3A1 site) found only in strain C_3 (19,20). Second, there is a Sau 3A1 site, 0.9 kb from the rDNA ends, present in strain B but not strain C_3 rDNA. Both these polymorphisms are in the non-transcribed spacer region.

Digestion of rDNA from exponentially growing cells with restriction enzymes MboI and DpnI

Having identified the positions of 5' GATC 3' sequences within the palindromic rDNA using Sau 3A1, we used MboI and DpnI to determine which of these sites were methylated based on the following expectations.

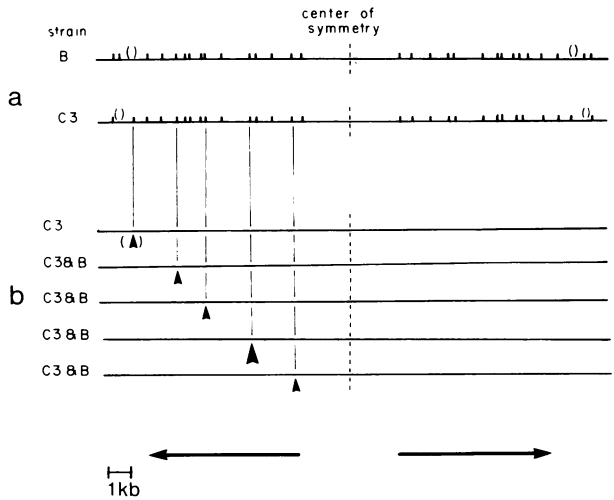

Figure 1. Restriction map of GATC sequences in palindromic rDNAs from strains B and C_3. a. Sau 3A1 and MboI cutting sites are shown (|). Sites absent in one strain but not in the other are indicated as a pair of parentheses for the strain lacking the site. b. DpnI cutting sites (▲) are shown. On average, there is less than one DpnI cutting site per palindromic rDNA molecule in the population of molecules. A preferred cutting site is indicated by a larger arrowhead and results in the strong band indicated by the arrow in Fig. 2. The pre-rRNA transcription units are indicated at the bottom of the figure.

Hemimethylated sites will be cut by Sau 3A1, but not by MboI or DpnI. Unmethylated sites will be cut by both MboI and Sau 3A1, but not by DpnI. Doubly methylated sites will be cut by DpnI and Sau 3A1, but not by MboI. However, if there is heterogeneity in the population of rDNA molecules with respect to methylation of a given 5' GATC 3' site, then the fraction of this same site susceptible to DpnI or resistant to MboI is a direct measure of its methylation level in the rDNA population.

rDNAs from both strains B and C_3 were readily digestible overall with MboI, indicating the majority of GATC sequences were unmethylated (Fig. 2, lanes 1 and 4). In Fig. 2, the strong band (1.3 kb) present in lane 1 and absent in lane 4 is a result of the differences in GATC restriction sites between strains B and C_3 referred to above. However, in addition to the fragments resulting from complete digestion with MboI, which were the same as those produced by Sau 3A1 digestion, additional fragments in submolar yields were also seen, even after prolonged digestion with excess MboI (arrowheads at Lane 4, Fig. 2). As described below, these fragments were produced as a result of methylation of both strands of a specific subset of GATC sequences

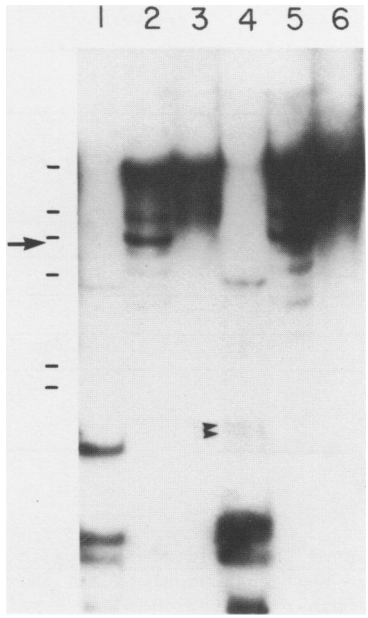

Figure 2. MboI and DpnI digests of palindromic rDNA. Electrophoresis of palindromic rDNA purified from either strain B or strain C_3 T. thermophila was carried out in a 1% agarose slab gel. The DNA was transferred to nitrocellulose and hybridized with ^{32}P-labeled nick-translated rDNA probe. Lanes 1-3: strain B rDNA, digested with MboI (lane 1), DpnI (lane 2) or undigested (lane 3). Lanes 4-6: strain C_3 rDNA, digested with MboI (lane 4), DpnI (lane 5), or undigested (lane 6). The undigested controls were incubated under the same conditions as the DpnI digests, but with no enzyme added. The strong band indicated with an arrow results from a single preferred site of methylation of a GATC sequence (see Fig. 1). Arrowheads indicate submolar MboI fragments. Horizontal bars indicate positions of 23.7, 9.6, 6.7, 4.4, 2.3 and 2.1 kb fragment markers.

in a fraction (≤10%) of the rDNA molecules. The pBR322 plasmid DNA included in the digestions as carrier and control was not detectably digested with MboI. This was the expected result, because it was prepared from dam$^+$ E. coli cells in which the sequence GATC is fully methylated.

The restriction enzyme DpnI was used to digest rDNA purified from either strain B or strain C_3 T. thermophila cells. One set of analyses was carried out by digesting to completion the purified, unlabeled rDNA with DpnI, electrophoresis of the resulting fragments in an agarose gel, and Southern hybridization to whole ^{32}P-labeled rDNA (Fig. 2, lanes 2 and 5), or cloned rDNA fragments (not shown). In another set of analyses, rDNA was first digested with EcoRI, the EcoRI fragments were end-labeled as described above,

and then digested with DpnI. The resulting fragments were electrophoresed in an agarose gel, and the dried gel autoradiographed. For both kinds of analysis, pBR322 DNA grown in dam$^+$ F. coli was included in the digestion reactions as a control.

The majority (≈90%) of the rDNA molecules purified from vegetatively growing cells were resistant to digestion with DpnI, under conditions in which the pBR322 DNA included in the same reaction mix as a control was quantitatively digested to limit digest fragments. However, a low percentage (≈10%) of the rDNA molecules were cut by DpnI. When the positions of these cutting sites were determined and related to the Sau 3A1 and MboI restriction maps, the fragments generated were found to be the result of cutting at a specific subset of the GATC sites mapped as described above (Fig. 1b). Furthermore, the submolar fragments seen after MboI digestion (Fig. 2, lane 4) could all be accounted for by the positions of the DpnI cutting sites. The positions of these DpnI cutting sites (that is, doubly methylated GATC sequences) were the same for four sites in both strains C_3 and B rDNAs. These sites were 3.6, 4.8, 6.4 and 8.4 kb from one end of the rDNA molecule. The only difference observed was a cutting site corresponding the extra BamH1 site 1.5 kb from the ends of strain C3 rDNA molecules (see Fig. 1).
These results showed that, of the 15 GATC sites we have mapped in each half of the palindromic rDNAs of both strains, only a small and specific subset of sites was doubly methylated. For any given site, the percentage of molecules methylated was characteristic for that site, although the most highly methylated site still was found in only ≈10% of the molecules. Furthermore, all the fragments seen in Sau 3A1 digests were present in MboI digests. We were not able to detect any Sau 3A1 cutting sites which were partially resistant to MboI, but which were not cut by DpnI. Therefore, we find no evidence for sites in the rDNA which are fully or partially hemimethylated, but not doubly methylated. The highly specific pattern of doubly methylated GATC sequences strongly suggests that the methylation sites are conserved over many generations of vegetative growth of cells. A likely mechanism for conservation of this pattern, once laid down, is that methylation of a newly replicated DNA strand is directed by the position of methyl groups on the AT sequences of the parental DNA strand.

Methylation of rDNA in different physiological states

In order to determine whether the methylation pattern of palindromic macronuclear rDNA was altered in different physiological states, rDNA was purified from exponentially-growing cells, and from cells that had been

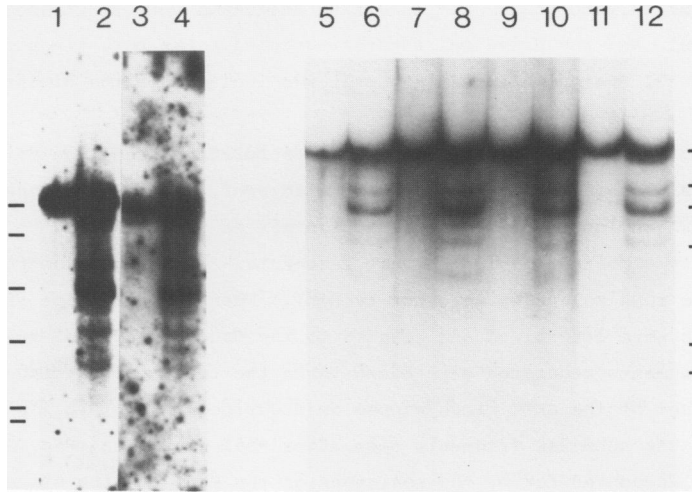

Figure 3. DpnI digests of palindromic rDNA from cells in different physiological states. Palindromic rDNA was purified from exponentially growing cells (lanes 1,2,11 and 12), starved cells (lanes 3 and 4), starved and mock-conjugated cells (lanes 5 and 6), cells conjugated with A* strain cells (lanes 7 and 8), and conjugated cells (lanes 9 and 10). For the conjugation experiments, cells were starved for 20-22 hr prior to mixing to initiate conjugation, then refed after 8 hr, and DNA was prepared 12 hr after refeeding. rDNA was digested to completion with DpnI (lanes 2,4,6,8,10 and 12), or incubated under the same conditions in the absence of added enzyme (lanes 1,3,5,7,9 and 11). Electrophoresis was in 0.8% (lanes 1-4) or 1.2% (lanes 5-12) agarose slab gels. Horizontal bars indicate positions of 23.7, 9.6, 6.7 4.4, 2.3 and 2.1 kb fragment markers.

starved for 24 hr. In addition, the effect of conjugation on palindromic rDNA methylation was examined by starving cells in preparation for conjugation, and initiating conjugation by mixing starved cells of two different mating types. Cultures were refed 8 hr after mixing, and DNA was prepared 12 hr after refeeding. In control experiments, cells were "mock-conjugated" by mixing starved cells of the same mating type, which are unable to conjugate, or mixing with a culture of strain A* strain cells, in which meiosis takes place, but new macronuclei do not develop (26). The palindromic rDNA from each of these preparations was digested with DpnI (Fig. 3). The patterns and relative intensities of the fragments produced were not significantly different in each preparation. Since starved cells have a greatly reduced level of rRNA synthesis compared with rapidly-growing cells, these results indicate that the level of N-6-methyladenine in rDNA does not detectably vary with the transcriptional activity of the rRNA genes.

Methylated GATC sites are not symmetrically located on individual palindromic rDNA molecules

The low percentage of methylated GATC sites in the rDNA population as a whole was consistent with the presence of no more than one methylated GATC sequence, on average, per individual rDNA molecule. This was supported by our finding that, for each end-labeled rDNA molecule or R1 restriction fragment digested with DpnI, we could detect two end labeled fragments produced, the sum of whose sizes was the original uncut size. However, since the palindromic, amplified rDNA molecules are produced during macronuclear development from a single, chromosomally integrated rRNA gene copy, it was important to test by additional means the possibility that each half of an individual palindromic rDNA molecule carries the same pattern of methyl groups. This was determined by carrying out digestion of rDNA "snapbacks" with DpnI. Because the rDNA is a palindrome, upon denaturation and intramolecular reassociation, each original molecule forms two double stranded "snapback" molecules, half the length of the original native palindromic rDNA (8). We found that the MboI cutting patterns of these rDNA snapback molecules were similar to those of native palindromic rDNAs. In contrast, the rDNA snapback molecules were found to be completely resistant to digestion by DpnI. If the same site on each half of the molecule had been fully methylated, the snapbacks would have regenerated this fully methylated site. Therefore, it was concluded that the majority of the rDNA molecules containing fully methylated GATC sites had any given site methylated on only one half of each molecule; that is, the palindromic rDNA is asymmetric with respect to its methylation pattern. This finding suggested that the pattern of methylation of GATC sites in the rDNA that is maintained during vegetative growth is not derived from a pattern laid down very early in the macronuclear rDNA amplification process; that is, before single rRNA gene copies were replicated into palindromes. Alternatively, this lack of symmetry of methylation on palindromic rDNAs suggests that each molecule was formed from two independently methylated single rRNA gene copies.

Methylation of rDNA in developing macronuclei

Single, free rRNA gene copies are synthesized during rDNA amplification in the developing macronuclei of conjugating cells (19). These 11 kb rDNAs are linear molecules, derived from the micronuclear rRNA gene copies in both chromosomal homologues (20). Using DNA prepared from a cell population 32 hr after mixing cells to initiate conjugation (about two generations after conjugation), 11 kb and 21 kb palindromic rDNAs were each purified as

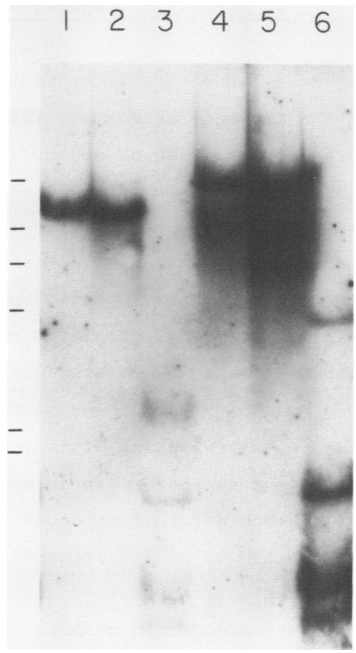

Figure 4. Digestion of 11 kb rDNA with DpnI and MboI. 11 kb and 21 kb rDNAs were purified from cells two generations after conjugation. Digestions with DpnI and MboI were carried out, the fragments were fractionated on a 1.0% agarose gel by electrophoresis, and blotted and hybridized to ^{32}P-labeled nick-translated rDNA probe. Lanes 1-3: 11 kb rDNA undigested (lane 1), digested as far as possible with DpnI (lane 2), and digested to completion with MboI (lane 3). Lanes 4-6: palindromic rDNA from the same preparation, and purified from the same gel as 11 kb rDNA, undigested (lane 4), digested as far as possible with DpnI (lane 5) and with MboI (lane 6). Even when the autoradiogram was exposed more than ten times longer than the one shown, no bands were visible below the uncut 11 kb rDNA in lane 2.

described previously (19) and digested with MboI or DpnI. The 11 kb rDNA was found to be fully digested to limit fragments by MboI, but was completely resistant to digestion by DpnI (Fig. 4). In contrast, palindromic 21 kb rDNA from the same conjugated cell DNA preparation, purified by electrophoresis on the same agarose gel as the 11 kb rDNA, was cut by DpnI to a similar extent, and produced the same fragments, as palindromic rDNA prepared from long term vegetatively growing cells. It was concluded that 11 kb rDNA molecules are considerably undermethylated relative to mature palindromic rDNA.

One possible explanation for the lack of methylation of 11 kb rDNA is that macronuclear DNA in general is not methylated at the early stages of growth following conjugation. Since DNA was prepared from whole cell

populations following conjugation, it was a mixture of DNA from starved cells in the population that had not undergone conjugation, and newly replicated DNA from the cells that had successfully completed conjugation. To examine methylation of newly-replicated DNA in conjugation, we examined the digestion by DpnI and MboI of ^{3}H-thymidine-labeled DNA, pulse-labeled at different stages during conjugation. We previously found that 11 kb rDNA is first detectable 10-11 hr after conjugation is initiated and persists for about 10 generations thereafter (19). At 10-11 hr, macronuclear development has already begun, the first events of macronuclear differentiation being detectable cytologically by 6-7 hr following the initiation of conjugation (E. Orias, personal communication; 27). We therefore labeled conjugating T. thermophila cultures with ^{3}H-thymidine, using pulses ranging from 15 min to 4 hr, at different times throughout the conjugation process. DNA was extracted and purified from cells immediately following each labeling period. This purified DNA was digested with DpnI. As a control, the DNA samples were incubated under the same conditions as the enzyme digestions, but without enzyme. The digested samples were electrophoresed in an agarose gel, stained with ethidium bromide and photographed to visualize the total DNA, then fluorographed to visualize newly synthesized ^{3}H-labeled DNA.

As expected, DNA synthesized before 6 hr, corresponding to micronuclear S-phases (28), was resistant to digestion with DpnI. However, as shown by visualization of total cell DNA by ethidium bromide staining, unlabeled parental macronuclear DNA, and pBR322 DNA, in the same preparation were digested (data not shown). The bulk ^{3}H-thymidine labeled DNA in conjugating cells was not detectably digested by DpnI before about 12 hr following the initiation of conjugation, as judged by the retention of high molecular weight (>23 kb) ^{3}H-DNA at the limit mobility position after agarose gel electrophoresis and fluorography (Figure 5A and B, lanes 1 and 2). At 12 hr, early synthesis of macronuclear DNA is occurring, during the development and polyploidization of the new macronuclei. The susceptibility of pulse-labeled bulk macronuclear DNA to digestion by DpnI began by 14 hr (Fig. 5). In lanes 5, 8 and 11 of Figures 5A and B, both ethidium staining and ^{3}H label are depleted at the limit mobility position by DpnI digestion compared with the undigested controls in lanes 4, 7 and 10 respectively. This susceptibility to DpnI digestion persisted up to at least 24 hr following the initiation of conjugation, the latest stage in these experiments in which analyses were made. These results show that even though methylation of GATC sequences in bulk macronuclear DNA and the palindromic rDNA had begun early in macronuclear

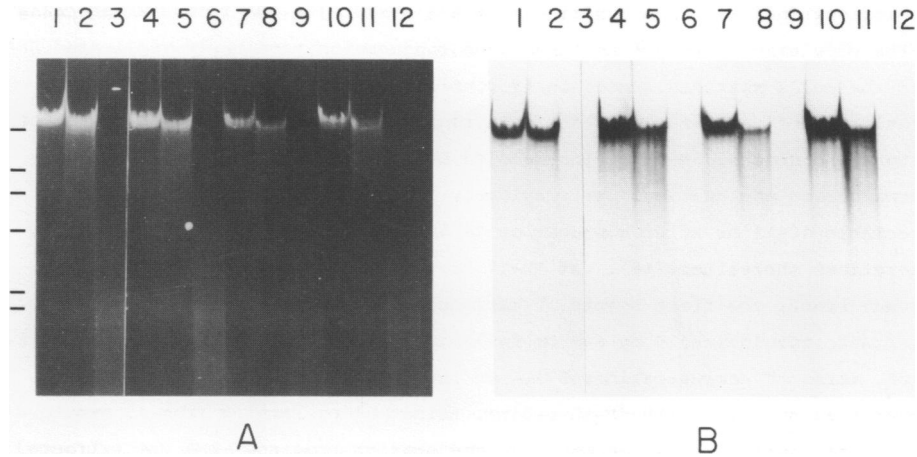

Figure 5. DpnI and MboI digests of DNA newly replicated during conjugation. Conjugating cells were pulsed with ^{3}H-thymidine at different times following mixing of cells of different mating types to initiate conjugation. DNA was purified after the pulse and digested with DpnI or MboI. After electrophoresis in a 0.7% agarose gel, the DNA was stained with ethidium bromide and photographed (A). The DNA in the gel was then fluorographed (B). Lanes 1-3: 40 min pulse beginning at 11 hr. Beginning at 14 hr, pulses of 40 min (lanes 4-6), 60 min (lanes 7-9) and 120 min (lanes 10-12) were given. Undigested control DNA samples were run in lanes 1, 4, 7 and 10, DpnI digests in lanes 2, 5, 8 and 11 and MboI digests in lanes 3, 6, 9 and 12.

development, there was no detectable methylation of GATC sequences in 11 kb rDNA molecules.

DISCUSSION

These results on the methylation of palindromic rDNA molecules in macronuclei demonstrate that, at least for the N-6-methyladenine residues in GATC sequences, a highly specific subset of sites is methylated. This pattern of methylation is very similar in the two strains of T. thermophila analyzed. Since in this analysis only GATC sequences could be examined, extension of these findings to methylation in general carries the implicit assumption that the methylation of these sequences is representative of methylation of adenine residues in 5' AT 3' sequences in general. Since the G+C content of rDNA is 37% (8), GATC sequences can be calculated to comprise, on a random basis, 3.4% of all 5' AT 3' sequences. Hence, the GATC sequences examined are expected to represent 3.4% of the total methylation sites in the rDNA. Since N-6-methyladenine comprises 0.4% of the adenine residues in rDNA, there are on average 26 N-6-methyladenines per palindrome half. Therefore, about 0.8

N-6-methyladenines per palindrome half are expected to occur in the sequence GATC. Our results show that there are only about 0.1 N-6-methyladenines in GATC per rDNA molecule, which is consistent with a non-random placement of methylated residues. GATC sequences (15 per palindrome half) were located in both the coding and non-transcribed regions of the molecule. However, although one of the specifically methylated sites was found in the non-transcribed spacer, 1•5 kb from the rDNA ends in strain C_3, the most highly methylated site (large arrowhead in Fig. 1) is located in a transcribed sequence.

Our finding only doubly methylated GATC sequences in rDNA suggests that methylation of newly-replicated DNA strands during vegetative growth is determined by the positions of N-6-methyladenine residues on the parental (template) strand. If this were not the case, the positions of methylated residues would become random over several generations, and hemimethylated sites would be seen, neither of which was observed. These results agree with the evidence of Pratt and Hattman (29), demonstrating that there is conservation over many vegetative generations of methylated residues in linker regions of DNA in chromatin.

The simplest explanation for the observed pattern of methylated GATC sequences is that the complete methylation pattern occurs on not one, but a population, of rDNA molecules. This happens during or soon after rDNA amplification in macronuclear development. To account for the observation that even though there are on average 26 N-6-methyladenines on each palindrome half, the specifically methylated GATC sequences are found in only a low percentage of molecules, it can be postulated that on each individual rDNA molecule (or palindrome half), none or only one of the possible GATC sites is methylated as one of several different possible patterns of methylation is laid down. Any methylated site is then conserved during the replication of rDNA for the duration of further vegetative cell divisions.

We have observed no differences in GATC methylation patterns or levels in the palindromic rDNAs from cells in different physiological states. These results are consistent with the previous findings made for overall levels of N-6-methyladenine in macronuclear DNA (7). However, in other eukaryotic systems, there is a strong correlation between the level of methylated cytosine residues in rRNA genes and transcriptional activity (2, 30). Our observations and those of others therefore suggest that the role of methylation of DNA with respect to transcription in the macronucleus of Tetrahymena is quite different from the postulated role of 5-methylcytosine in

vertebrates.

Our findings on the methylation of the different molecular forms of the rDNA in the developing macronucleus may therefore be of considerable relevance to the definition of a role for N-6-methyladenine in macronuclear DNA. The 11 kb rDNA molecules, destined to be lost after several generations from the mature macronucleus, are greatly undermethylated, or possibly unmethylated, at their GATC sequences, relative to both the palindromic, mature rDNA and the bulk macronuclear DNA synthesized at the same time in the same differentiating macronucleus. One possible function of N-6-methyladenine residues suggested from these results is that they serve to distinguish DNA molecular forms to be retained in the mature macronucleus from those to be rearranged or lost by degradation.

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