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DNA Synthesis, Methylation and Degradation During Conjugation in *Tetrahymena thermophila*

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**ABSTRACT**

We have investigated the timing of DNA synthesis, methylation and degradation during macronuclear development in the ciliate, *Tetrahymena thermophila*. DNA synthesis was first detected in the anlagen early in macronuclear development, but the majority of DNA synthesis occurred later, after pair separation. Anlagen DNA was first detectably methylated at GATC sites 3-5 hours after its synthesis. Once initiated, *de novo* methylation was rapid and complete, occurring between 13.5 and 15 hours of conjugation. The level of methylation of GATC sites was constant throughout the remainder of conjugation, and was similar to that in mock-conjugated cells. Degradation of DNA in the old macronucleus and DNA synthesis in the anlagen began at about the same time. Upon pair separation, less than 20% of old macronuclear DNA remained. A small percentage of nucleotides prelabeled prior to conjugation were recycled in the developing anlagen.

**INTRODUCTION**

The ciliated protozoan, *Tetrahymena thermophila*, contains two nuclei: a diploid micronucleus, and a polyploid macronucleus. The macronucleus is responsible for most, if not all, transcriptional activity during vegetative growth. The micronucleus is responsible for inheritance during sexual conjugation in which the old macronucleus is destroyed and the zygotic micronucleus divides to form both a new micronucleus and a new macronucleus. The developing macronucleus, called the anlage, undergoes many structural and morphological changes, including: nuclear swelling (1), DNA polyploidization to a final DNA content of 45c (2), elimination and rearrangement of germ line DNA sequences (3,4) and *de novo* methylation (5).

In vegetative cells, micronuclear DNA does not contain modified bases while, in macronuclear DNA, 0.65-0.8% of adenines are modified to N-6-methyladenine (6,7). Bromberg et al. (8) showed methylation in *Tetrahymena* occurs in the sequence 5' MeAT 3', with any of the four bases found 5'. In conjugating cells, *de novo* methylation of anlagen DNA occurs at GATC sites between 11 hours and 14 hours following initiation of conjugation, both in bulk DNA and in extrachromosomal ribosomal DNA (5).
While the presence of 5-methylcytosine has been correlated, in some cases, with transcriptional control (for review, see refs. 9 and 10), no functional role has yet been attributed to N-6-methyladenine in eukaryotes. Methylated adenine is found preferentially in linker DNA in Tetrahymena chromatin (11). Methylation levels do not change detectably in different physiological states in which transcription levels are significantly different (6). Blackburn et al. (5) have shown only 10% of extrachromosomal ribosomal DNA molecules are methylated at GATC sites. Among this population, only a subset of GATC sites are methylated, and the population displays heterogeneity as to whether a given site is methylated or not. Taken together, these results suggest methylation is not involved exclusively in transcriptional control.

We examined the timing of de novo methylation relative to DNA synthesis and degradation in the developing macronucleus. We utilized the enzyme DpnI, which cleaves at the sequence 5' GATC 3' CTAG 5' only if adenines on both strands are methylated (12). Autoradiography of fixed cells showed the cytological stage as well as the source of newly synthesized DNA.

DNA methylation is known to function in prokaryotes in a modification-restriction system (9). Foreign DNA, recognized by being unmethylated at specific sites, is selectively degraded. We examined the possible relation between de novo methylation of anlagen DNA and degradation of DNA in the old macronucleus. We show degradation of DNA in the old macronucleus began at about the time DNA synthesis was first detected in the anlagen, but several hours before methylation of anlagen DNA.

Another known function for methylation in prokaryotes is mismatch repair (13). The newly synthesized daughter strand is distinguished from the parent strand by its lack of methylation, and the mismatched nucleotide is selectively corrected in the daughter strand. Since micronuclear DNA is not methylated in Tetrahymena, daughter strands must be recognized in some way other than by methylation. In addition, since methylation of anlagen DNA occurs subsequent to several rounds of DNA synthesis, methylation is probably not involved in mismatch repair in the early anlagen. In light of these findings, we discuss possible roles for DNA methylation during macronuclear development.

MATERIALS AND METHODS

Tetrahymena thermophila strains CU399 (mating type VI) and CU401 (mating type VII) were kindly provided by P. Bruns. Stocks were maintained in 1% PPYS and cultures grown in 2% PPYS, as previously described (14).
Prior to conjugation, cells were starved in 10 mM tris for 22-26 hours. Conjugation was done as described by Martindale et al. (15). In all conjugations, greater than 90% of cells were paired at 3 hours. As a control, mock-conjugated cells were similarly starved and mixed except both cell populations were CU399. Cells were labeled with 0.5-1.0 μCi/ml of \(^3\text{H}\)-thymidine (ICN) for different pulse lengths, beginning 3 hours into conjugation. To prelabel DNA in the old macronucleus, cells were grown in PPYS with 1.0 μCi/ml of \(^3\text{H}\)-thymidine for 2-3 generations. This was followed by chase with 100-fold excess unlabeled thymidine, to a final concentration of 2.3 X 10\(^{-3}\) mM, for one generation of vegetative growth and also during starvation. The amount of thymidine added was 100-fold lower than the level reported to inhibit DNA synthesis in Tetrahymena (16).

Micronuclear and macronuclear DNA were isolated as described by Howard and Blackburn (personal communication), after Gorovsky et al. (17). 
Nuclei were isolated from conjugating and mock-conjugated cells as described by Gocke et al. (18). Nuclei were lysed in 10 mM tris, 1 mM EDTA + 1% Sarcosyl. Nucleic acid was digested with pancreatic RNase (Worthington), TI RNase (Boehringer Mannheim), and pronase (Sigma), as previously described (14). DNA was phenol extracted and ethanol precipitated twice. Purified DNA from different stages of conjugation was digested with \textit{DpnI} (International Biotechnologies, Inc.) according to manufacturer and run on 0.7-0.8% agarose gels for 390 volt hours. DNA samples were digested with at least 5 units of enzyme per μg of DNA for 4-5 hours at 37°C. In some cases, 200 ng of pBR322 was added to digestion sample to assay for complete digestion. Undigested DNA was mock-digested in \textit{DpnI} digestion buffer under the same conditions as digested DNA.

Fluorography was done using Enhance (New England Nuclear) according to manufacturer's instructions for agarose gels. Fluorographs were exposed for 4 days to a month at -70°C. Fluorographs and negatives of ethidium bromide stained gels were scanned with a Joyce-Loebel densitometer.

Cells were fixed with Schaudinn's fixative and stained with Giemsa as previously described (14), except staining was for 1/2-1 hour in 0.01M sodium phosphate, pH 6.5. Slides were coated for autoradiography with Kodak emulsion NTB-2 as described by Pardue and Gall (19), and exposed for 7 to 10 days. To determine the amount of DNA remaining in the old macronucleus at various stages, silver grains were counted in the emulsion over prelabeled cells using a 100X oil-immersion lens. To determine the proportion of recycled label, silver grains were counted in the emulsion over anlagen late in macronuclear...
development III, after complete degeneration of the old macronucleus. At least 20 cells or cell pairs were counted for every stage.

RESULTS

Cytological events occurring during anlagen stage

Upon pairing, the micronuclei of conjugating cells undergo two meiotic divisions, generating four haploid products per cell. Of these, three degenerate and the remaining haploid nucleus divides mitotically. The conjugants then exchange one nucleus and fusion of the pronuclei takes place. The resulting zygotic nuclei are diploid, and are identical in the two cells. These events have been described in detail by others (15,20).

The zygotic nucleus undergoes two mitotic divisions, generating four diploid nuclei. Two nuclei move to the anterior of the cell and form macronuclear anlagen. The remaining two move to the posterior of the cell to form micronuclei. Fig. 1 illustrates nuclear migration and degeneration occurring during anlagen stage. Macronuclear development I and macronuclear development II were defined by Martindale et al. (15). The anlagen in the anterior of the cell swell during macronuclear development I (fig. 1a). They migrate toward the center of the cell during macronuclear development II, and the micronuclei come to lie on either side of the anlagen. The old macronucleus moves to the posterior of the cell, and condenses as the DNA is degraded (fig. 1b). In our experiments, macronuclear development I began 7 hours into conjugation, while macronuclear development II began at 8 hours and lasted through 11 hours. We define macronuclear development III, beginning at 11 hours, as the stage when pair separation occurs (fig. 1c,d). Degeneration of the old macronucleus is followed by degeneration of one of the two micronuclei. In our experiments, conjugating cells were not refed in order to prevent DNA synthesis from occurring in the small percent of unpaired cells. Under these conditions, cells arrested after degeneration of one micronucleus. If refed, the remaining micronucleus would undergo a mitotic division and cell fission would occur.

DNA synthesis in the anlagen

Cytofluorimetric analysis of anlagen DNA, by Doerder and DeBault (1), has shown anlagen DNA content to be approximately 6c during macronuclear development II. The remainder of DNA synthesis occurs upon pair separation, in macronuclear development III, to a final polyploidization level of 45c. Allis and Dennison have similarly described the DNA content of the anlagen at 8c upon pair separation (21). Thus, two rounds of DNA synthesis have occurred.
Figure 1. Cytological events during anlagen stage of conjugation. 
a: Macronuclear Development I. Early anlagen are at the anterior of the 
cells and micronuclei are at the posterior. Old macronuclei are in the center 
of the cells. b: Macronuclear Development II. Old macronuclei are condensed 
and have moved to the posterior of the cell. c: early Macronuclear 
Development III. Cell pairs have separated. A remnant of the old 
macronucleus is present. d: late Macronuclear Development III. Degeneration 
of the old macronucleus is complete. Bars indicate 10 μM.

in the anlagen by the time of pair separation. While this represents almost 
half the rounds of DNA synthesis necessary to advance the anlagen to the final 
ploidy level of 45c, it is less than 25% of the total DNA synthesis, since a 
greater mass of DNA undergoes each successive round of synthesis.

We monitored DNA synthesis during anlagen stages by conjugating the cells 
in the presence of ³H-thymidine for various pulse lengths, beginning no
Figure 2. DNA synthesis during conjugation. a: Cells were labeled with \(^{3}H\)-thymidine for a 4 hour pulse during the mitotic divisions of the micronucleus. Cells are in macronuclear development I. The number of silver grains over the anlagen and the micronuclei are about equal. Seven day exposure. b-d: cells were labeled for 1 hour during macronuclear development. b: Early Macronuclear Development III. The old macronucleus is still present (arrow). c and d: labeling in the anlagen increases during macronuclear development III. Ten day exposure. Bars indicate 10 \(\mu\)M.

earlier than 3 hours into conjugation. Adding label earlier than 3 hours resulted in incorporation of labeled nucleotides into micronuclei, old macronuclei, and mitochondrial DNA of some cells (data not shown). Although most starved cells arrest at macronuclear G1 and micronuclear G2 (22,23), DNA synthesis may be necessary to advance some of the cells to this stage.

Between 3 hours and 8 hours of conjugation, DNA synthesis occurred only in the prezygotic and postzygotic micronuclei. Thus, the amount of label in early anlagen was the same as the amount of label in micronuclei of the same cell (fig. 2a). DNA synthesis was first evident in the anlagen during macronuclear development II. The intensity of labeling in the anlagen
Figure 3. De novo methylation of anlagen DNA during conjugation. a: 0.7% agarose gel of micronuclear DNA (lanes 1,2) and macronuclear DNA (lanes 3, 4). DNA was undigested (lanes 1,3) or digested with DpnI (lanes 2,4). b: 0.8% agarose gel of DNA from mock-conjugated cells (lanes 1,2) or from conjugating cells at 11 hours (3,4), 12 hours (5,6) 13.5 hours (7,8), 15 hours (9,10), 18 hours (11,12) and 21 hours (13,14). Upper panel, ethidium bromide stained gel; lower panel, fluorograph of the same gel. DNA in lanes 1,3,5,7,9,11 and 13 was undigested; DNA in lanes 2,4,6,8,10,12 and 14 was digested with DpnI. DNA in lanes 3-8 was labeled with \(^{3}H\)-thymidine during mitotic divisions of the micronucleus; DNA in lanes 9-14 was labeled during macronuclear development. In fig. 3a, the marker at the left of the gel is phage lambda DNA digested with HindIII. The bands seen in the ethidium bromide stained gels correspond to pBR322 DNA included in the digestion sample. The band seen in the fluorograph, lanes 11 and 13, is presumed to be an amplification intermediate of the rDNA.

increased during macronuclear development III (fig. 2b,c,d), suggesting that most of the anlagen DNA synthesis occurred subsequent to pair separation. We did not detect DNA synthesis in unpaired cells or in the old macronucleus of paired cells at any stage.

Methylation of anlagen DNA at GATC sites

We assessed the level of methylation by digestion with the enzyme DpnI,
which cleaves at the sequence GATC only if the adenine is methylated. Newly synthesized DNA was distinguished from old DNA by $^3$H-thymidine incorporation and fluorographic analysis. Fig. 3a shows the ethidium bromide pattern for undigested and DpnI-digested DNA from vegetative micronuclei and macronuclei (lanes 1-4). As expected, micronuclear DNA was undigested by DpnI since it does not contain methyladenine, although pBR322 DNA in the same sample was digested to completion. Macronuclear DNA in fig. 3a lane 4 was digested with DpnI, indicating the presence of methylated GATC sites. Fig. 3b shows the ethidium bromide pattern and fluorograph for DNA from mock-conjugated cells and from cells at different times of conjugation. The fragments in fig. 3a lane 4 are smaller, on the average, than in fig. 3b lane 2 due to the presence of contaminating exonuclease in the preparation of DpnI used for the experiment in fig. 3a. Lanes 3-14 show undigested and DpnI-digested DNA from 11, 12, 13.5, 15, 18 and 21 hours of conjugation. The bulk of DNA from conjugants at these stages appeared to be digested by DpnI (fig. 3b, upper panel).

Fluorographic analysis of the same agarose gel showed methylation of newly synthesized anlagen DNA occurred between 13.5 and 15 hours of conjugation (fig. 3b, lower panel). Newly synthesized DNA from 11, 12 and 13.5 hours was undigested by DpnI (lanes 4,6,8). This is confirmed in the densitometric tracings seen in fig. 4. In contrast, by 15 hours, complete methylation of GATC sites in newly synthesized DNA had occurred. As shown in fig. 3b (lower panel, lane 10) and fig. 4f, newly synthesized DNA at 15 hours appeared methylated to the same extent as DNA from the mock-conjugated control (lower panel, lanes 2,10, and fig. 4d). The same level of methylation persisted through 21 hours of conjugation (lower panel, lanes 12,14). No intermediate stage was found where the newly synthesized DNA appeared only partially methylated relative to the mock-conjugated control. The DNA from control cells was prelabeled during vegetative growth since mock-conjugated cells do not synthesize DNA (data not shown). The sharp band seen in fig. 3b, lower panel, lanes 11 and 13, is presumed to be the 11kb rDNA intermediate reported by Blackburn et al. (5).

Since newly synthesized DNA was not methylated until 15 hours of conjugation, the DNA digested by DpnI in the ethidium bromide pattern at 11, 12, and 13.5 hours must be from the old macronucleus. In the next section, we show only a small amount of DNA is still present in the old macronucleus at these stages. Thus, the proportion of unmethylated anlagen DNA is increasing. This is reflected in the ethidium bromide pattern seen in fig. 3b where bulk
Figure 4. Densitometric tracings of ethidium bromide stained gel and fluorograph. Solid lines are tracings of undigested DNA; broken lines are tracings of DNA digested with DpnI. a-c: ethidium bromide stained gel, d-f: fluorograph. Lane numbers refer to fig. 3b. a,d: lanes 1,2 (mock-conjugated cells), b,e: lanes 7,8 (13.5 hr. conjugants), c,f: lanes 9,10 (15 hr. conjugants). Tracings of the lower portion of the lanes, including peaks corresponding to pBR322 DNA, are not shown.

DNA at 11 hours and 13 hours appears less digested than bulk DNA from later times.

We have demonstrated that de novo methylation of anlagen DNA occurs several hours into macronuclear development III. Since DNA synthesis was first detected in the anlagen during macronuclear development II, there was a lag of 3-5 hours between DNA synthesis and methylation in the developing macronucleus. Once initiated, methylation at GATC sites occurred rapidly, reaching its final level in less than 90 minutes.

**Degradation of DNA in the old macronucleus**

Degradation of DNA in the old macronucleus was observed by prelabeling cells during vegetative growth, prior to conjugation, with $^{3}$H-thymidine. This was followed by chase with 100-fold excess thymidine for one generation of vegetative growth as well as during the 24 hour starvation period preceding conjugation. Autoradiograms of prelabeled cells from different stages of conjugation are shown in fig. 5. The average number of silver grains counted over the old macronucleus and over the anlagen at these stages is shown in
Figure 5. Degradation of DNA in the old macronucleus and recycling of label in the macronuclear anlagen. Cells were prelabeled with $^3$H-thymidine during vegetative growth. a: unpaired cell. b: Macronuclear Development I. Most, or all of the label in the old macronucleus is still present. c: Macronuclear development II. Label in the old macronucleus has begun to disappear. d: Macronuclear Development III, after complete degeneration of the old macronucleus. Some of the label from the old macronucleus is recycled in the anlagen. Seven day exposure. Bars indicates 10 μM.

Table 1. In two separate experiments, the number of silver grains counted over the micronucleus of unpaired cells was approximately 1/20 the number counted over the macronucleus, consistent with the known DNA content (fig. 5a and Table 1).

Conjugants through the stage macronuclear development I had the same amount of label in the old macronucleus as unpaired cells on the same slides (fig. 5a,b). This was expected, since cytological preparations showed the old macronucleus had not yet begun to degenerate. During the early part of macronuclear development II, it was not possible to count silver grains over the old macronuclei because they condensed and were stained very darkly with
### Table 1. Number of silver grains counted in emulsion over prelabelled nuclei of paired and unpaired cells.

<table>
<thead>
<tr>
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<th>old macronucleus</th>
<th>micronucleus</th>
<th>anlagen</th>
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<tr>
<td>Unpaired</td>
<td></td>
<td></td>
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<tr>
<td>Exper. 1</td>
<td>97±23</td>
<td>5±3</td>
<td>N.A.</td>
</tr>
<tr>
<td>Exper. 2</td>
<td>215±68</td>
<td>10±8</td>
<td>N.A.</td>
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<tr>
<td>Macronuclear</td>
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<td>Development II</td>
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<tr>
<td>Exper. 1</td>
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<td>N.D.</td>
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<td>Exper. 2</td>
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<td>Development III</td>
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<tr>
<td>Exper. 1</td>
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<td>2±2</td>
</tr>
<tr>
<td>Exper. 2</td>
<td></td>
<td>N.A.</td>
<td>5±3</td>
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N.A.=not applicable; N.D.=not determined
Numbers are shown with standard deviation.
At least 20 cells were counted for each stage.

Giemsa. The number of silver grains over the old macronuclei were counted late in macronuclear development II as they degenerated (fig. 5c). Table 1 shows an average of 18 silver grains counted per old macronucleus of cells late in macronuclear development II, compared to an average of 97 silver grains counted per macronucleus of unpaired cells from the same slide. The percentage of DNA remaining in the old macronucleus may be represented as:

\[
\frac{\text{av. # grains in old macronucleus of paired cell}}{\text{av. # grains in macronucleus of unpaired cell}} \times 100
\]

Therefore, before pair separation, approximately 19% of the DNA in the old macronucleus remained (=18/97). As shown in fig. 5c, degradation often appeared asynchronous in a pair.

As the old macronucleus degenerated, silver grains could be seen in the anlagen of paired cells in macronuclear development II and III (fig. 5c,d). Since the anlagen were derived from micronuclei, some of the prelabeled nucleotides in the anlagen originated from DNA synthesis in the micronuclei of prelabeled cells. The number of silver grains over anlagen increased at macronuclear development III (Table 1). This increase is unlikely to be new incorporation of \(^3\)H-thymidine since the label was chased with 100-fold excess thymidine during both vegetative growth and starvation. We suggest the increase in label in the anlagen may represent salvage of nucleotides from the old macronucleus. Since two anlagen develop in each cell, the proportion of recycled label may be estimated as:

\[
\]
2(\text{av#grains in anlage of paired cell-av#grains in micronucleus of paired cell}) \over av#grains in macronucleus of unpaired cell

Under the conditions of these experiments, 10-17% of prelabeled nucleotides were recycled in the developing anlagen.

DISCUSSION
We have determined the timing of de novo methylation of GATC sites relative to DNA synthesis in the anlagen and degradation of DNA in the old macronucleus during conjugation in Tetrahymena. Not all methylation of Tetrahymena DNA occurs at GATC sites. There are enough methylated adenines (1/125 adenines) to saturate every GATC site (approximately 1/455 bases), but it is evident from the DpnI digestion patterns this is not the case. Our results for methylation of GATC sites may or may not be representative of methylation at other sequences.

Other investigators have demonstrated that approximately two rounds of DNA synthesis have taken place in the anlagen upon pair separation. The remaining 2-3 rounds occurred subsequent to separation (1,21). Our results suggest the majority of DNA synthesis occurred upon pair separation in macronuclear development III. Since a greater mass of DNA undergoes each successive round of synthesis, our results are consistent with the last few rounds of DNA synthesis occurring subsequent to pair separation.

We found a delay of at least several hours between anlagen DNA synthesis, which began during macronuclear development II, and methylation, which began during macronuclear development III. Thus, several rounds of DNA synthesis have occurred before de novo methylation of anlagen DNA. Pratt and Hattman (11) observed an intermediate level of methylation in newly synthesized DNA of vegetative cells which reached steady state levels within one cell doubling. In contrast, we did not observe an intermediate level of methylation of newly synthesized DNA in conjugating cells. This suggests a rapid turn-on of the methylase activity.

In other eukaryotic systems, the methylation pattern is faithfully conserved by maintenance methylases. These enzymes recognize hemimethylated DNA resulting from DNA replication, and add the appropriate methyl groups on the newly synthesized strand (13). The possibility that different maintenance methylases could be simultaneously active was suggested by Woodcock et al. (24,25). In a mammalian cell line, a minor but consistent fraction of DNA showed methylation that was delayed from DNA synthesis by several hours. The investigators examined the effect of inhibitors of methylation on delayed and
non-delayed methylation, and found they were differentially effected by several inhibitors. They suggest different maintenance enzymes may be responsible for delayed or non-delayed methylation. Perhaps the methylase responsible for de novo methylation in Tetrahymena is not the same enzyme as the maintenance methylase active during vegetative growth, and its synthesis must await the transcription of new message.

What are the possible functions of methylation during macronuclear development in Tetrahymena? Genetic evidence has shown transcription of genes conferring drug-resistance begins during macronuclear development (26). In our experiments, methylation of anlagen DNA occurred late in macronuclear development in the absence of refeeding. It is therefore possible that methylation may be active in transcriptional control during conjugation.

In prokaryotes, methylation is known to function in mismatch repair. If mismatch repair occurs in micronuclear DNA, newly synthesized DNA must be distinguished by something other than methylation because micronuclear DNA is not methylated. Methylation is also unlikely to function in mismatch repair in the early anlagen, since at least two rounds of DNA synthesis have already taken place before de novo methylation is detected at GATC sites.

Alternatively, methylation may function in a modification-restriction system during conjugation in Tetrahymena. Blackburn et al. (5) suggested methylation may protect DNA sequences from degradation in the developing anlagen. They observed that the transient 11 kb rDNA molecule, which is lost several generations after conjugation, is either unmethylated or undermethylated relative to the palindromic rDNA and the bulk DNA. Further evidence supporting this notion has been reported by T. White and S. Allen (personal communication). They have identified a family of DNA sequences which is partially eliminated from the developing macronucleus. One of the members which is retained is methylated at a GATC site in approximately 50% of the macronuclear copies.

We showed significant degradation of DNA occurred in the old macronucleus before methylation of newly synthesized anlagen DNA. It is therefore unlikely that methylation protects anlagen DNA from degradation at the time DNA in the old macronucleus is being degraded. Possibly, compartmentalization of nuclease between the two nuclei may explain why the anlagen DNA is not degraded. Alternatively, enzymes degrading DNA in the old macronucleus may recognize specific methylated sites, resulting in protection of the unmethylated micronuclear and anlagen DNA.

Our data are also consistent with the hypothesis that methylation may play
a role in designating retention or elimination of germ-line sequences during macronuclear development III. Yokoyama and Yao (27) have shown germ-line sequences derived from the micronucleus are replicated for several rounds in the developing anlagen before they are actively degraded. Active degradation of sequences begins when the old macronucleus is no longer detectable, which occurs between 15-18 hours in our conjugations. Methylation of newly synthesized DNA is thus occurring at about the same time as active degradation of micronuclear sequences in the anlagen.

While the role of DNA methylation remains unclear, the macronuclear anlagen in *Tetrahymena* is an ideal system in which to investigate its possible functions. We have presented evidence that methylation of DNA at GATC sites is unlikely to be involved in mismatch repair and does not protect macronuclear anlagen DNA from degradation during degeneration of the old macronucleus. We cannot eliminate the hypothesis that methylation may serve as a signal for degradation of DNA sequences in the old macronucleus. Additionally, methylation of DNA sequences in the macronuclear anlagen occurs at a time permitting its involvement in either transcriptional activation or DNA sequence rearrangement in the developing macronucleus.

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