Position Effect Takes Precedence Over Target Sequence in Determination of Adenine Methylation Patterns in the Nuclear Genome of a Eukaryote, *Tetrahymena thermophila*

Kathleen M. Karrer  
*Marquette University, kathleen.karrer@marquette.edu*

Teresa A. Van Nuland  
*Marquette University*

Position effect takes precedence over target sequence in determination of adenine methylation patterns in the nuclear genome of a eukaryote, *Tetrahymena thermophila*

Kathleen M. Karrer* and Teresa A. VanNuland+

Department of Biology, Marquette University, Milwaukee, WI 53201-1881, USA

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**ABSTRACT**

Approximately 0.8% of the adenine residues in the macronuclear DNA of the ciliated protozoan *Tetrahymena thermophila* are modified to $N^6$-methyladenine. DNA methylation is site specific and the pattern of methylation is constant between clonal cell lines. In *vivo*, modification of adenine residues appears to occur exclusively in the sequence 5'-NAT-3', but no consensus sequence for modified sites has been found. In this study, DNA fragments containing a site that is uniformly methylated on the 50 copies of the macronuclear chromosome were cloned into the extrachromosomal rDNA. In the novel location on the rDNA minichromosome, the site was unmethylated. The result was the same whether the sequences were introduced in a methylated or unmethylated state and regardless of the orientation of the sequence with respect to the origin of DNA replication. The data show that sequence is insufficient to account for site-specific methylation in *Tetrahymena* and argue that other factors determine the pattern of DNA methylation.

**INTRODUCTION**

The genomic DNA of most organisms is modified by methylation, which plays a role in a variety of biological processes, including regulation of gene expression (1), DNA replication (2,3), mismatch repair (4) and in defense of the host against foreign DNA (5).

In prokaryotes, cytosine and/or adenine is methylated, depending on the species. Patterns of DNA methylation in prokaryotes are determined entirely by the sequence specificity of DNA methyltransferase (MTase) (6,7).

In eukaryotes, the most common modification is methylation of cytosine residues to 5-methylcytosine. Patterns of cytosine methylation are clonally inherited, but vary with cell type and the developmental stage of the tissue. It is likely that methylation patterns in mammalian systems are established and maintained as a result of a complex series of interactions of MTase with various cis- and trans-acting factors (8).

The ciliated protozoa are unusual among the eukaryotes in that the nuclear genomes have no detectable methylcytosine, but they do contain low levels of $N^6$-methyladenine. Methylated adenine has been reported in *Tetrahymena* (9), *Paramecium* (10), *Oxytricha* (11) and *Stylonychia* (12). Of these, adenine methylation has been studied extensively only in *Tetrahymena*.

Ciliates have two different types of nuclei; germline micronuclei and transcriptionally active macronuclei. In *Tetrahymena*, micronuclear DNA is unmethylated. Approximately 0.8% of the adenines in macronuclear DNA are modified to $N^6$-methyladenine (9). Methylation occurs at the sequence 5'-NAT-3' (13) and sequencing of several methylated sites did not reveal any more extensive consensus sequence for methylation (14).

During vegetative cell division, methylation occurs predominantly on the daughter strand of the newly replicated DNA; but there is also some new methylation on the parental strand. DNA methylation is ongoing at low levels in starved cells, where there is no detectable DNA replication (15).

During sexual reproduction in *Tetrahymena* the macronucleus is degraded and new macronuclei develop in the progeny cells from the mitotic product of the zygotic micronucleus. Macronuclear development entails extensive genome reorganization (reviewed in 16), including endoreduplication of the genome to ~45 times the haploid DNA content, amplification of the rDNA and *de novo* methylation of the macronuclear genome.

Methylated sites in *Tetrahymena* DNA have been assayed by digestion with m$^6$A-T-sensitive restriction enzymes. It has been estimated that ~3% of the methylation events in *Tetrahymena* occur at the sequence GATC (17). Methylated GATC sites are readily detected by digestion with the restriction enzyme *DpnI*, which will digest the DNA only if the adenines in both strands are methylated.

Methylation in the *Tetrahymena* genome is site specific (17). Patterns of methylation are consistent in independent clonal cell lines (18) and do not vary with the physiological state of the cell or the transcriptional activity of nearby genes (17,19). Two classes of sites can be defined with respect to the level of

*To whom correspondence should be addressed. Tel: +1 414 288 1474; Fax: +1 414 288 7357; Email: karrerk@mu.edu

*Present address: Abbott Laboratories, 200 Abbott Park Road, Abbott Park, IL 60064-3537, USA*
methylation. The first class is methylated on >90% of the macronuclear DNA molecules. These sites are referred to as uniformly methylated. A second class, partially methylated sites, is methylated on a proportion of the macronuclear DNA molecules which is characteristic of the site (18, 20).

A semi-conservative model for maintenance of methylation patterns in mammalian cells has been proposed on the basis of the preference of mammalian MTase for a hemimethylated substrate (21). The mechanism whereby patterns of DNA methylation are maintained in Tetrahymena is unknown. The Tetrahymena MTase has not been purified and its substrate specificity is not well characterized. However, a simple semi-conservative mechanism is insufficient to account for the maintenance of partially methylated sites (18).

At least two models could account for methylation patterns in Tetrahymena. First, methylation is entirely dependent on sequence specificity. According to this model, partially methylated sites would be explained by a lower affinity of MTase for the sequences at those sites. Second, methylation is dependent on chromatin structure. According to this model, partial methylation would be explained by limited accessibility of these sites to MTase. The two models are not mutually exclusive and patterns of methylation must be dependent on sequence to some extent, since methylated adenines are always 5' of thymine in vivo (13).

In order to assess the relative contribution of sequence dependence and chromosomal environment to DNA methylation in Tetrahymena, we inserted a fragment of DNA containing a site which is uniformly methylated on the chromosome into the extrachromosomal rDNA. When present in the rDNA, the site is unmethylated. Thus sequence is not sufficient for methylation in Tetrahymena. The results were the same whether the DNA was methylated or unmethylated upon entry into the cell and irrespective of the orientation of the sequence with respect to the origin of DNA replication.

MATERIALS AND METHODS

Cell lines

Strains CU428, Mpr/Mpr [6-methylpurine-sensitive (6-mps), VII] and CU441, ChxA/ChxA [cycloheximide-sensitive (cys), VI] of inbreeding line B were obtained from P. Bruns (Cornell University).

Culture conditions

Vegetative growth. Tetrahymena were grown in 2% PPYS [2.0% proteose peptone (Difco), 0.1% yeast extract, 0.003% sequestrene (Ciba-Geigy)] prepared according to the method of Gorovsky et al. (22) at 29°C with constant swirling at 90 r.p.m. to a density of 1.0–5.0 × 10^5 cells/ml.

Starvation. Cells were pelleted from 2% PPYS, washed twice with sterile 10 mM Tris–HCl (pH 7.4), resuspended in 10 mM Tris–HCl (pH 7.4) at a density of 1.0 × 10^5 cells/ml and starved for 5–24 h at 29°C with constant swirling at 90 r.p.m.

Conjugation. Equal numbers of cells of complementary mating types (strains CU428 and CU441) were starved in 10 mM Tris–HCl (pH 7.4) for 5–24 h and mixed at a density of 1.0 × 10^5 cells/ml according to the methods described by Bruns and Brussard (23). Cells were mated at 29°C without shaking.

DNA isolation

Micro and macronuclei were prepared for DNA isolation from strain CU428 cells by the method of Gorovsky et al. (22). Whole cell DNA of transformed cell lines was isolated from 10 ml cultures by the method of Austerberry and Yao (24).

Southern blots

DNA was digested with the appropriate restriction enzymes (Boehringer Mannheim, American Allied Biochemical or International Biotechnologies Inc.) according to the manufacturers' specifications and size fractionated by agarose gel electrophoresis on 0.8–2.5% agarose (Seakem LE; FMC BioProducts) gels using a 1× TAE (0.04 M Tris–acetate, 0.002 M EDTA) buffer system. The sizes of hybridizing fragments were estimated based on the mobility of fragments of λ DNA digested with HindIII on one side of the gel and pBR322 DNA digested with HindIII on the other.

Probe DNA fragments were isolated from agarose gels by electrophoresis of the DNA onto a DEAE-nitrocellulose membrane (25, 26) or excised from SeaPlaque (FMC BioProducts) agarose and random primer labeled (25).

DNA constructs

The plasmid clone pTtcyd1, containing a GATC site that is methylated in the Tetrahymena macronucleus (18), was isolated from a library of partially MboI-digested micronuclear DNA fragments from Tetrahymena strain CU399 cloned in pUC18 (27). DNA fragments including the methylated site cyd1 were subcloned in pUC18 as a 3.4 kb XbaI fragment (pTtcyd1.D), and a 0.52 kb PstI-HindIII fragment (pTtcyd1.D). pTtcyd1.X DNA was digested with HindIII and the 0.95 kb HindIII fragment was cloned into the HindIII site of pBluescriptII (+) to generate pTtcyd1.XHA.

The processing vector p947H8 (Fig. 1) consists of a Tetrahymena micronuclear rDNA gene containing a polylinker region with a unique NotI site at the HindIII (8) site of the Tetrahymena rDNA, downstream of the rRNA genes (28). The micronuclear rRNA gene and flanking micronuclear-limited sequences were cloned into the bacterial vector pUC19. When microinjected into the developing macronuclei of conjugating Tetrahymena, p947H8 undergoes DNA rearrangement to produce linear extrachromosomal palindromic macronuclear rDNA (29). For insertion of fragments containing the methylated cyd1 site into the NotI site of p947H8, various fragments were first cloned into the plasmid vector pHSS6, which has NotI sites flanking its polylinker (30). The cyd1.D fragment was isolated from pTtcyd1.D as a 0.52 kb BamHI–HindIII fragment and cloned into the corresponding sites in pHSS6. The fragment was recovered from pHSS6 as a 0.58 kb NotI fragment and ligated into the unique NotI site of p947H8. Similarly, cyd1.X was released from pTtcyd1.X as a 3.4 kb XbaI fragment, cloned into pHSS6, recovered from pHSS6 as a 3.5 kb NotI fragment and cloned into the NotI site of p947H8. cyd1.D and cyd1.X were each cloned into p947H8 in both directions relative to the origin of replication on the rDNA. The constructs designated [L] or [R] represent the two alternative orientations of the cyd1 fragments within the rDNA.

Methylated plasmids were obtained by replication in DH5α or HB101, two Dam MTase-containing Escherichia coli strains. The Dam MTase of E. coli methylates the adenine residues on both strands of DNA at virtually all GATC sites (5). Unmethylated
plasmid constructs were obtained from GM2971 or GM2163, two Dam MTase-defective *E. coli* strains, a gift from E. Raleigh (New England Biolabs). Transformation of plasmids into the *dam*− strains is inefficient, due to inhibition of replication of hemimethylated plasmid DNA (2), and was achieved by transformation according to the method of Hanahan (31).

Transformation of *Tetrahymena* via microinjection

Plasmid DNA constructs were injected into the developing macronuclear anlagen of conjugating *Tetrahymena* according to methods developed in the Yao laboratory (32,33). Plasmid DNA was suspended in 1× injection buffer (114 mM KCl, 20 mM NaCl, 3 mM Na2HPO4, pH 7.4) at a concentration of 50 ng/μl. Injection needles were pulled from capillary tubing (91.2 mm OD × 0.6 mm ID/fiber; FHC). Approximately 100–200 molecules of plasmid DNA were injected directly into the developing macronuclei of one cell of a mating pair at 9.5–11.5 h after initiation of conjugation. Following microinjection, individual pairs of mating cells were cloned into drops of 2% PPYS medium and grown at 29°C for 3–4 days. Clonal cell lines were replica plated into 200 μg/ml paraomycin to select for transformants. Paraomycin resistance is conferred by a single base pair mutation in the 17S rRNA gene of the injected rDNA (40). Figure 2C shows the results of a Southern hybridization experiment designed to assay for methylation of cyd1.D in transformed cells, using the 32P-labeled cyd1.D fragment as probe. Lane 1 contained DNA isolated from untransformed strain CU428 and digested with *NotI* and *RsaI* to generate a 0.86 kb *RsaI* fragment containing the methylation site of the genomic cyd1 sequence. In lane 2, this fragment was digested with *DpnI*. *DpnI* cuts GATC sites if the adenines on both strands are methylated. The genomic cyd1 fragment was completely digested with *DpnI*, showing that the chromosomal site is uniformly methylated, as reported previously (18). The small fragments resulting from *DpnI* digestion of the *RsaI* fragment did not show detectable hybridization at this exposure.

Figure 2A and B presents restriction maps of the cyd1.D fragment situated in each orientation within the 3′-non-transcribed region of the transformant rDNA. In the [L] orientation, there is a short inverted repeat of linker sequences that was not stable in bacteria. Deletion of the inverted repeat region resulted in loss of the *NotI* restriction site at one end of the cloned fragment. For this reason the DNA was digested with *NotI* and *RsaI* to release the cyd1.D fragment from the rDNA.

Lanes 3–10 of Figure 2C were loaded with DNA isolated from transformed cell lines with cyd1.D inserted in the rDNA. In the odd numbered lanes, transformant DNA digested with *NotI* and *RsaI* generated a major band of 0.48 kb. This band was the cyd1 fragment released from the rDNA vector. The hybridization signal of cloned cyd1 sequences was more intense than the signal due to genomic cyd1 sequences in transformed lines because the cyd1.D fragment was present in the rDNA, which is amplified ~200-fold over the bulk of the macronuclear DNA (41,42). For
strains with cydl.D in the [L] orientation, several minor fragments of sizes 2.1 kb and larger were also produced. These fragments correspond to the terminal RsaI fragment of the rDNA and dimers of this fragment, which were detectable in the blot due to cydl.D sequences distal of the RsaI site.

In the even numbered lanes, transformant DNA was digested with NotI, Rsal and DpnI. DpnI digestion at a methylated cydl site in the rDNA would result in smaller fragments of 0.32 and 0.15 kb. Resistance of the 0.48 kb fragment to DpnI digestion showed that the cydl site was essentially unmethylated when the cydl.D fragment was located in the rDNA. Digestion by DpnI of the 0.86 kb fragment containing the genomic cydl site provided an internal control for DpnI digestion. The experiment showed that the 522 bp of DNA surrounding the methylated GATC site is insufficient for methylation of the site in the rDNA construct.

In Figure 2C, the chromosomal cydl.D fragment served as a positive control for digestion with DpnI. However, the small fragments did not hybridize with sufficient intensity to be detectable at this exposure. In order determine whether the GATC sites were intact on the cydl.D fragment in the transformed cell lines and to demonstrate that small fragments are detectable under the conditions of our experiments, the DNA from the same transformed cell lines was digested with an enzyme that is specific for unmethylated GATC sites.

In the experiment shown in Figure 3C, the DNA was digested with NotI and Rsal (odd numbered lanes) or NotI, Rsal and NdeII (even numbered lanes). NdeII cuts GATC sites only if the adenines are unmethylated. Lanes 1 and 2 contained DNA isolated from untransformed CU428 cells as a control. In lane 1, digestion with NotI and Rsal generated a 0.86 kb Rsal fragment containing the genomic cydl site and a second, unmethylated GATC site. NdeII digested this fragment at the unmethylated GATC site (lane 2). (The 0.75 kb fragment resulting from digestion at the unmethylated Sau3a site in the genomic DNA was faint in lane 2, but readily detectable in lanes 4, 6, 8 and 10, where slightly more DNA was loaded.)
Transformant DNAs in lanes 3–10 were digested with NotI and RsaI to generate a 0.48 kb cyd1 fragment and the larger telomeric fragments from the rDNA. In the even numbered lanes, the 0.48 kb fragment was digested with NdeII to produce smaller fragments of expected sizes 0.21, 0.15 and 0.11 kb. (An additional fragment of 0.6 kb was generated by digestion of the telomeric RsaI fragment with NdeII.) Thus both GATC sites of the cloned cyd1.D region are present and unmethylated in the transformant DNAs.

Direction of replication fork movement

Methylation of mammalian DNA is tightly linked to DNA replication (43,44) and eukaryotic MTases co-localize with the DNA replication foci during S phase (45). The rDNA transformation system in Tetrahymena provides a unique opportunity to determine whether the direction of replication across a methylation site might affect its recognition by MTase.

Replication of Tetrahymena rDNA begins at one of two origins of DNA replication near the center of the palindromic molecule (Fig. 1B) and proceeds bidirectionally towards the termini (46). The direction of DNA replication at the chromosomal cyd1 locus is not known. For this reason, cyd1.D was cloned into the plasmid vector in both orientations relative to the origin of replication in the rDNA. The constructs, arbitrarily designated cyd1.D[R] and cyd1.D[L], were microinjected into Tetrahymena and several transformed lines were obtained in each case.

Figure 2B presents a restriction map of cyd1.D[R] in the 3'-non-transcribed region of an rDNA molecule. Figure 2C (lanes 5–8) shows a Southern blot analysis of two Tetrahymena cell lines transformed with p947/H8 containing cyd1.D[R]. In lane 5, transformant DNA was digested with NotI and RsaI in order to generate a major band of 0.48 kb. This fragment contained the cloned copy of the cyd1 site. In lane 6, transformed DNA was digested with NotI, Rsal and DpnI to assay for methylation. Methylation at the cyd1 site on the cloned copy of the sequence would be expected to allow digestion of the 0.48 kb fragment by DpnI to smaller fragments of 0.32 and 0.15 kb. Resistance of the 0.48 kb fragment in lane 6 to DpnI digestion showed that the cyd1 site was largely unmethylated in the rDNA of the transformants. As expected, both GATC sites were methylated at the cyd1 site in the rDNA. Figure 4 is the expected size for the 0.48 kb fragment containing the cyd1 methylation site and an additional fragment of 0.94 or 0.61 kb, depending upon the orientation of the cyd1.D fragment in the rDNA (Fig. 4A and B). The DNA in even numbered lanes was digested with HindII and DpnI, to detect methylation of adenine at the GATC sites. The experiment showed that the cyd1.D site in the rDNA was not methylated in any of the cell lines tested. Comparison of restriction patterns in DNA of growing versus starved transformants revealed no differences in DNA methylation. The experiment showed that the cyd1.D site in the rDNA was not methylated in starved cells, where transcriptional activity was reduced and DNA replication was arrested.

The 0.73 and 0.46 kb fragments in double-digest lanes of Figure 4 are likely to be due to partial methylation of GATC sites in the rDNA (20). Partial methylation at the GATC sites of cyd1.D can be ruled out because fragments resulting from DpnI digestion would be detectable in the blot shown in Figure 2. The 0.73 kb fragment in Figure 4 is the expected size for DpnI digestion at the polymorphic BamHI site in C3-type rDNA, the type in the transformants (48,49).

Flanking sequence

In the fungus Neurospora crassa and in mammalian systems there is evidence for portable methylation signals or ‘methylation centers’ which can act as cis-acting factors to promote methylation of adjacent DNA sequences (50–52). In Tetrahymena, it is not known whether the site of binding for MTase is the same as the site of methylation. It was considered possible that the 0.5 kb cyd1.D fragment may not contain sufficient sequence information to allow for proper recognition of the site by Tetrahymena MTase.

In order to provide additional sequence information surrounding the methylation site, the transformation studies were repeated using cyd1.X, a 3.4 kb genomic fragment that contains cyd1.D plus additional flanking sequences on both sides. Figure 5A presents a restriction map of cyd1.X in the [L] orientation within the rDNA. Figure 5B shows the results of a Southern hybridization experiment designed to assay for methylation of cyd1.X.[L] in transformed lines derived from cells injected with methylated plasmid. The cyd1.D fragment was used as a molecular probe. Lanes 1 and 2 contained DNA isolated from untransformed
fragments were due to partial methylation at two different GATC sites in transformant DNA, the Southern blot shown in Figure 3 stripped and reprobed with cyd1.XHA. Figure 4 shows an experiment designed to assay for the occurrence of both the size and relative intensity of hybridization of the minor fragments, was found for 13 of 13 transformants containing cyd1.XHA. The pattern along with the results from Figure 2 allowed localization of the partial methylation to two GATC sites in cyd1.X[L] (Fig. 5A). Thus, although no methylation was detectable at the cyd1 site, other GATC sites in the cyd1.X fragment were methylated at low levels.

The transformed lines analyzed in Figure 5B and C all resulted from injections of Tetrahymena with methylated plasmid. Similarly, there was no indication of methylation of the cyd1 site in three cell lines transformed with unmethylated cyd1.X[L] plasmid (data not shown).

A single transformed line was obtained with cyd1.X in the [R] orientation. Figure 5D shows an experiment designed to assay for the methylation of the DNA of that transformant, using the cyd1.D fragment as probe. The fragment sizes seen upon methylation analysis in the transformed line were identical in size to those seen upon analysis of transformants containing cyd1.X[L]. Partial methylation occurred at the same two GATC sites in cyd1.X regardless of orientation of the subclone. This was consistent with the results shown in Figures 2 and 3 and provided further evidence...
that Tetrahymena MTase does not have a directional requirement for presentation of sites for modification.

The transformed line analyzed in Figure 5D was derived from injection of methylated plasmid. Attempts to generate a transformant containing cyd.l.X[R] using unmethylated plasmid were unsuccessful. However, the results seen in DNA of cell lines injected with either methylated or unmethylated cyd.l.X[L] suggest that the state of methylation at the time of injection had no effect on the methylation pattern in transformants.

DISCUSSION

Transformation assays have been used to investigate the role of DNA sequence as a determinant of DNA methylation in a variety of biological systems. In prokaryotes, DNA sequence is sufficient to establish specific DNA methylation patterns. The Dam MTase of E.coli modifies the adenine residue in GATC sequences without discrimination. Heterologous DNA transformed into dam+ E.coli is methylated at essentially all GATC sequences (6).

In eukaryotes, determination of cytosine methylation is considerably more complex. It has been proposed that MTase may associate with sequences known as 'methylation centers' and that methylation may spread along adjacent genomic sequences. Similarly, other sequences may block the spreading of methylation into promoter regions. Accordingly, cytosine methylation of transgenes is subject to a position effect which is dependent on chromosomal location, proximity to cis-acting elements, the presence of trans-acting factors in a particular cell type or genetic background and, in some cases, on the degree of repetition of the transgenic DNA (reviewed in 8).

The data described here demonstrate that position takes precedence over DNA sequence in determination of the adenine methylation patterns in Tetrahymena. A site that is uniformly methylated in the macronuclear chromosome is unmethylated when a DNA fragment containing the site is placed in a novel environment in the rDNA minichromosome.

One consideration in a transformation assay is the cellular compartment to which the transformed rDNA is targeted. In the experiments described here, the cyd.l.D and cyd.l.X fragments inserted into the macronuclear rDNA were presumably localized in the nucleoli of the transformants. Two lines of evidence demonstrate that the nucleoli are accessible to Tetrahymena MTase. First, the macronuclear rDNA of Tetrahymena is methylated at about half the level of the chromosomal DNA (20). Second, although there was no detectable methylation of the cyd.l site in the experiments described here, there was partial methylation of sites in cyd.l.X in the rDNA (Fig 5) and of rDNA sequences flanking the cyd.l.D insert (Fig 4).

The data presented here show that the position effect for methylation of Tetrahymena DNA cannot be ascribed to the direction in which the replication fork progresses across the methylated site. This is true both in the case of the cyd.l site, which is unmethylated in the rDNA (Fig 1) and in the case of two partially methylated sites in the cyd.l.X insert (Fig 5). To the best of our knowledge, this is the first case in which the direction of replication has been tested as a variable in the determination of methylation patterns.

The mammalian DNA MTase has two domains; a catalytic C-terminal domain and a large N-terminal regulatory domain. The regulatory domain represses de novo methylation, with the result that the enzyme acts much more efficiently on a hemimethylated substrate (53). The preference of the enzyme for a hemimethylated substrate is thought to account, at least in part, for the clonal inheritance of specific methylation patterns in differentiated cell types.

Since the Tetrahymena MTase gene has not been cloned, it is not known whether the enzyme contains a similar regulatory domain. However, several lines of evidence argue that a simple semi-conservative mechanism is not adequate to explain the maintenance of methylation patterns in Tetrahymena. First, partially methylated sites do not drift toward either the uniformly methylated or the unmethylated state as a result of amitotic division of the Tetrahymena macronucleus (18). This suggests that de novo methylation is required to maintain patterns of methylation in Tetrahymena. Second, methylation was not maintained in cells transformed with rDNA methylated in vitro at novel sites (54). Lastly, the experiments described here show that methylation was not maintained in transformants, even for a sequence that is normally methylated on the chromosome (Figs 2–4).

The position effect for methylation in Tetrahymena may depend at least in part on the chromatin structure of the transgenic DNA. Although there are seven phased nucleosomes at the center of the palindromic macronuclear rDNA (55), the chromatin structure of the bulk of the molecule is non-nucleosomal (56). Pratt and Hattman (57) showed that methylated adenine in Tetrahymena chromatin is more sensitive to micrococcal nuclease than the bulk of the adenines, suggesting that methylation occurs primarily in linker DNA. We have recently confirmed the inter-nucleosomal location of methylated adenines in Tetrahymena chromosomal DNA by indirect end labeling (K.M.Karrer and T.A.VanNuland, manuscript in preparation).

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