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N^6-methyladenine DNA modification in the unicellular eukaryotic organism *Tetrahymena thermophila*

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Abstract

N^6-methyladenine DNA (6mA) modification exists in both prokaryotes and eukaryotes in which it plays a crucial role in regulating numerous biological processes. In prokaryotes, 6mA is a marker to distinguish self from foreign DNA. Its role in eukaryotes, however, remains elusive. Ciliates were among the first eukaryotes reported to contain 6mA. In the model organism *T. thermophila*, cellular localization and features of 6mA have been extensively studied, especially in ribosomal DNA (rDNA) molecules. Here, we summarize the features and potential functions of 6mA in *Tetrahymena thermophila* and other ciliates, and the major findings and contributions of the *Tetrahymena* model in studies of 6mA methylation. We also discuss other questions in order to improve understanding of the function and evolution of 6mA in eukaryotes.

Keywords: Ciliate; Methylation; N^6-methyladenine; *Tetrahymena thermophila*

Introduction

DNA methylation is an epigenetic process involving the addition of a methyl group to DNA. It plays an essential role in a number of key reactions in eukaryote cells including maintenance of DNA structure (Zacharias 1993), DNA replication (Reisenauer et al. 1999), virus latency (Bednarik 1993), and regulation of gene expression (Bird 1992). The most widely characterized DNA methylation processes are the covalent additions of the methyl group at the exocyclic amino group of adenine (Ade) (N^6), 5-carbon of the cytosine ring (Cyt) (C^5), and the exocyclic amino group of cytosine (Cyt) (N^4) resulting in N^6-methyladenine (6mA), 5-methylcytosine (5mC) and N^4-methylcytosine (4mC) respectively (Fig. 1A–C) (Cheng 1995; Ratel et al. 2006; Wion and Casadesús 2006). These processes are catalyzed by adenine- or cytosine-specific DNA methyltransferases (MTases) that transfer a methyl group from the donor *S*-adenosyl-L-methionine (AdoMet) to the substrate and generate methylated DNA and *S*-adenosyl-L-homocysteine (AdoHcy) (Hattman 2005). 6mA and 5mC have been observed both in prokaryotes and eukaryotes (Hattman et al. 1978), whereas 4mC is only found

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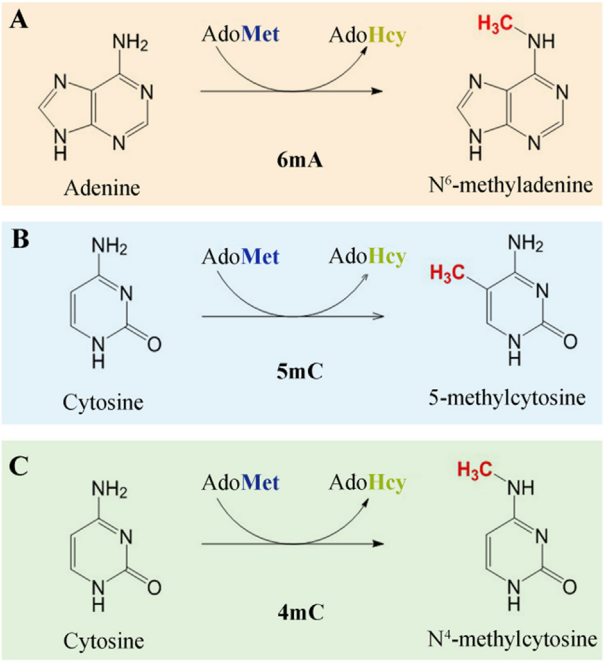

Fig. 1. (A–C) Chemical reaction equations of three methylations. (A) The reaction of adenine N^6 methylation. N^6-methyladenine (6mA) is catalyzed by adenine-specific DNA methytransferases that transfer a methyl group from donor *S*-adenosyl-L-methionine (AdoMet) to the exocyclic amino group of adenine (Ade) (N^6) to generate 6mA and *S*-adenosyl-L-homocysteine (AdoHcy). (B) The reaction of cytosine C^5 ring-carbon methylation. 5-methylcytosine (5mC) is catalyzed by cytosine-specific DNA methytransferases that transfer a methyl group from donor AdoMet to the C^5 ring-carbon of cytosine to generate 5mC and AdoHcy. (C) The reaction of cytosine N^4 methylation. N^4-methylcytosine (4mC) is catalyzed by cytosine specific DNA methytransferases that transfer a methyl group from donor AdoMet to the exocyclic amino group of cytosine (Cyt) (N^4) to generate 4mC and AdoHcy.

in prokaryotes (Cheng 1995). Historically, 5mC has been considered the sole DNA methylated modification in higher eukaryotes (Hattman et al. 1978), the main function of which is to regulate developmental processes and transcriptional silencing of transposons (Bestor 2000; Tajima and Suetake 1998). As knowledge of DNA methylation advances, 6mA has been reported in many eukaryote groups, including vertebrates (e.g. mouse, pig, zebrafish and *Xenopus*), plants (e.g. *Chlamydomonas*), insects (e.g. *Drosophila*), nematodes (e.g. *Caenorhabditis elegans*), and ciliates (Fu et al. 2015; Greer et al. 2015; Hattman et al. 1978; Koziol et al. 2016; Liu et al. 2016; Ratel et al. 2006; Zhang et al. 2015).

In prokaryotes, 6mA is often present in high levels and serves a number of roles including that as a marker to distinguish self from foreign DNA (Harrison and Karrer 1985), regulation of DNA mismatch repair (Messer and Noyer-Weidner 1988), chromosome replication (Lu et al. 1994), and cell-cycle regulation (Collier et al. 2007). By contrast, 6mA is present at extremely low levels in the genomic DNA of most multicellular eukaryotes and might occur in a tissue-specific or a development-dependent pattern. Therefore, it has been undetectable in many organisms due to the limitation of previously used technologies. Recent studies, however, have greatly advanced our knowledge of 6mA function in eukaryotes. In *Chlamydomonas*, 6mA mainly resides in ApT dinucleotides where it flanks transcription start sites (TSS) with a bimodal distribution and appears to mark active genes (Fu et al. 2015). In *Drosophila*, 6mA levels are dynamic and mainly located in transposon regions, which correlate with regulating development (Zhang et al. 2015). In the nematode *C. elegans*, 6mA was proposed to function reciprocally with histone methylation as a potential carrier of non-genetic information across generations (Greer et al. 2015). In mouse embryonic stem cells, an unregulated level of 6mA results in transcriptional silencing (Wu et al. 2016). The above evidence indicates that 6mA plays roles in both epigenetic silencing and activation, depending on the cellular environment. Multiple groups have reported 6mA accumulation in more vertebrates, including zebrafish, pig, *Xenopus*, and mammals (Koziol et al. 2016; Liu et al. 2016). These discoveries corroborate the biological relevance of 6mA modification in eukaryotes.

Ciliates were among the first eukaryotes reported to contain 6mA (Cummings et al. 1974; Gorovsky et al. 1973; Rae and Spear 1978). As in most other ciliates, the model organism *Tetrahymena thermophila* (Ciliophora, Oligohymenophorea), possesses within the same cell compartment one diploid micronucleus (MIC) and one polyploid macronucleus (MAC) (Fig. 2A). During vegetative growing, the MAC divides amitotically while the MIC splits by regular mitosis (Fig. 2C); during the sexual stage- conjugation, the new MAC and MIC are reconstructed from the parental MIC and further differentiate from each other (Fig. 2B) (Orias et al. 2011).

In this article, we review the features and potential functions of 6mA DNA modification in *Tetrahymena thermophila* and other ciliates.

6mA in *Tetrahymena thermophila*

The main features of 6mA

6mA is the only detectable methylated nucleotide base in *Tetrahymena thermophila* so far discovered. It is present in the MAC but absent in the MIC (Gorovsky et al. 1973). As the MIC is transcriptionally inert during vegetative growth, it was speculated that 6mA is required for transcriptional activation. However, the level of 6mA remains constant (~0.8%) in the MAC of vegetative and starved cells (Gorovsky et al. 1973; Pratt and Hattman 1981). Given that transcription levels are dramatically reduced in starved cells (Bannon et al. 1983), the correlation of 6mA with transcription may be more complicated than expected.

A level of 0.8% methyladenine amounts to approximately one methylated adenine per 165 bp of DNA, given that there is 75% A–T in the genome of *T. thermophila* (Karrer and

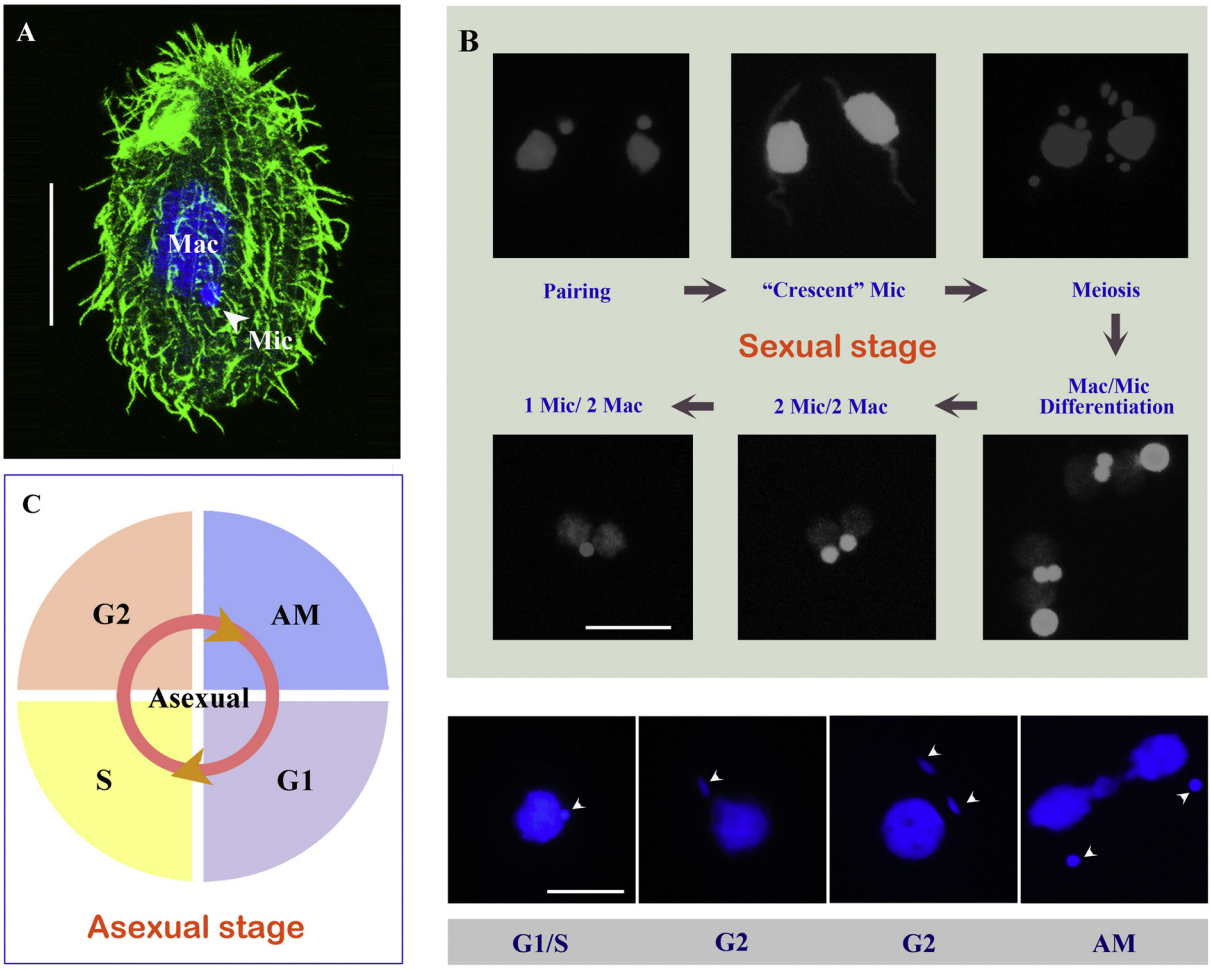

Fig. 2. (A–C) Nuclear apparatus of *Tetrahymena thermophila* in vegetative cells (A), during sexual reproduction (B) and during asexual reproduction (C). (A) Immunofluorescence staining showing the macronucleus (MAC), micronucleus (MIC) and tubulin in a vegetative cell; MAC and MIC (arrow heads) were stained by DAPI (4′,6-diamidino-2-phenylindole) in blue, and tubulin was stained by an anti-tubulin antibody in red. (B) Sexual stage reproduction: after starvation, two different mating types fuse to begin conjugation; MICs start to elongate into the linear structure called "crescent"; MICs undergo meiosis and the migratory gametic nucleus crosses the conjugation bridge; two of the products differentiate into the anlagen, whereas the other two remain as MICs; the parental MAC is degraded; one new MIC is retained. (C) Asexual stage reproduction: MAC divides by amitosis whereas the MIC divides by mitosis. Cell synthesizes various RNA and proteins rapidly and then initiates the synthesis of DNA (S phase); MIC elongates (G2 phase) and separates into two parts; this process results in two cells. White arrowheads depict the MIC. Bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

VanNuland 2002). 6mA is found specifically in the sequence of 5′-N(AT)-3′ (Bromberg et al. 1982), but no more extensive consensus sequence was revealed. The most extensively studied 5′-GATC-3′ sites, which include both uniformly and partially methylated sites of all copies of the GATC sequence, represent approximately 3% of total methylated adenines (Capowski et al. 1988).

A majority of 6mA is preferentially located in the linker DNA (Harrison et al. 1986), which indicates that 6mA distribution is not only determined by sequence per se but also by the chromatin environment (Karrer and VanNuland 2002). It should be noted that 6mA seems to be inversely correlated with 5mC that locates in nucleosomal core particles (Razin and Cedar 1977; Solage and Cedar 1978). 6mA and 5mC are both likely to be involved with the remodeling of chromatin structures in the MAC (Harrison et al. 1986; Razin and Cedar 1977; Solage and Cedar 1978).

6mA of rDNA

The ribosomal RNA gene (rDNA) of *Tetrahymena thermophila*, whose molecules have a mass of 12.6×10^6 Da and code for one precursor rRNA molecule, exists as free, extrachromosomal molecules in the MAC (Truett and Gall 1977). It has the typical palindrome structure that carries two rRNA gene copies with a length of 21 kb and amplifies to a copy number of about 10^4 from just a single copy of the riboso-

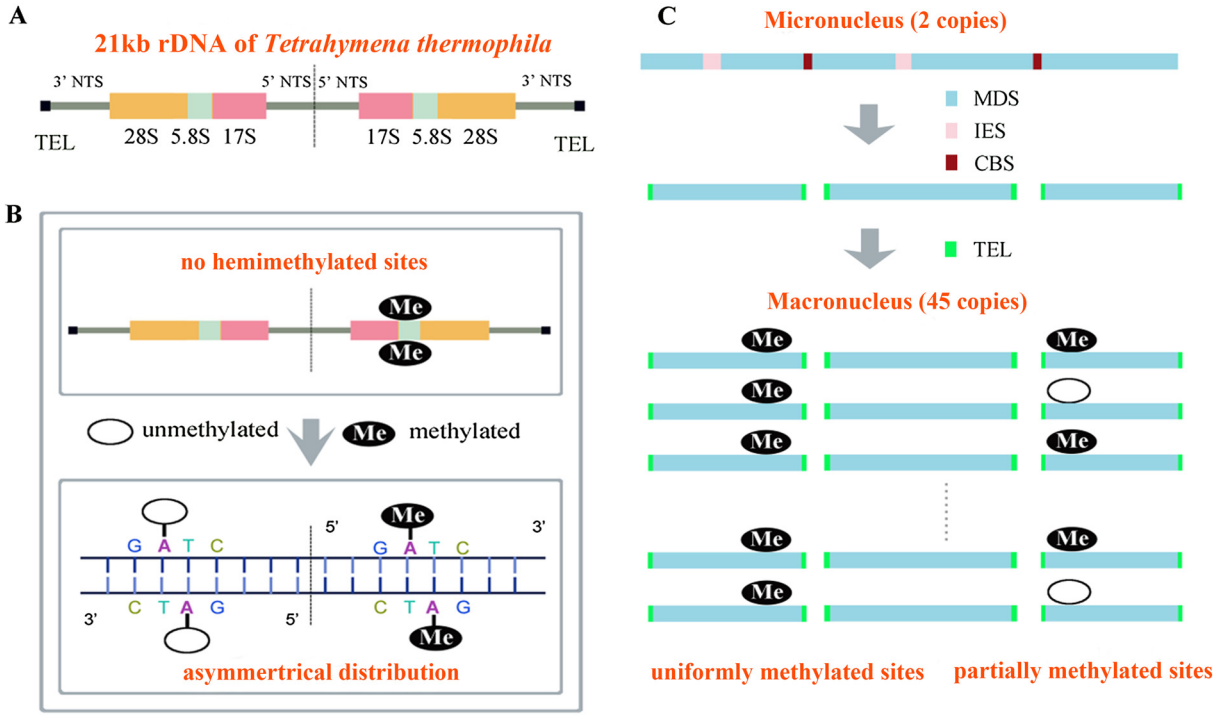

Fig. 3. (A–C) The methylated model of different DNA sites in *Tetrahymena thermophila*. (A) Structural feature of the macronuclear palindromic rDNA. Thick bar, rDNA transcription unit; thin pen bars, 5′ non-transcribed spacer (5′ NTS) and 3′ non-transcribed spacer (3′ NTS); vertical black lines, telomeric DNA repeat region (TEL). (B) The distribution pattern of rDNA methylation: no hemimethylated sites, methylated sites contain methylations in both strands of rDNA molecules; asymmetrical distribution, 6mA distribution is asymmetrical in palindromic rDNA. Me, DNA methylated by 6mA. (C) Two types of methylation sites in macronuclei: uniformly methylated sites, all 45 copies are methylated; partially methylated sites, only a portion of the molecule copies (10%–50%) are methylated. MDS, macronuclear destined sequences; IES, internal eliminated sequences; CBS, chromosome breakage sequence; TEL, telomeric DNA repeat region.

mal RNA gene in the MIC (Fig. 3A) (Yaeger et al. 1989). The presence of multiple copies of the rRNA gene in the MAC greatly facilitates the study of 6mA as does the high rate of rRNA synthesis in exponentially-growing cells compared to that in starved cells (Sutton et al. 1979). Previous research has shown that approximately 0.4% of the adenine residues of palindromic rDNA are N^6-methyladenine (Rae and Steele 1978). Due to technical limitations and convenience, the majority of previous studies on rDNA focused on the restriction site GATC.

According to Blackburn et al. (1983), 6mA of rDNA possesses the following features: (1) only about 10% of rDNA molecules are methylated with 6mA, which amounts to less than one methylated GATC site per molecule; (2) hemimethylated sites are not found in rDNA; in other words, all methylated sites contain methylations in both strands of the rDNA molecule; (3) 6mA distribution is asymmetrical in the palindromic structure of rDNA (Fig. 3B). It was proposed that either the two sections of the 21 kb palindromic rDNAs are formed from two independently methylated single rRNA gene copies or some yet unknown mechanism(s) could distinguish the two arms of the rDNA molecule. It should be noted that 11 kb rDNA molecules are detectable in the early stages of cell growth after conjugation (Pan et al. 1982). It was speculated that 6mA might serve to differentiate those rDNA molecules to be reserved from those to be degraded in the MAC, as methylation only occurs on 21 kb, but not 11 kb, rDNA molecules (Blackburn et al. 1983).

6mA of conjugation

Conjugation, the sexual process of *T. thermophila*, can be induced when at least three conditions are satisfied: mature cells, two different mating types, and environmental stress such as starvation (Elliott and Hayes 1953). It is an orderly developmental process during which cell pairing, meiosis, zygotic division, genetic exchange and formation of new somatic and germinal nuclei occur (Fig. 4A–H) (Martindale et al. 1982). The old MAC is destroyed while the zygotic MIC divides forming a new MIC and a new MAC. The developing MAC (anlagen) undergoes great changes on the way to maturity, such as the increase in DNA content from two copies to 45 copies (Gorovsky 1980), the elimination of Internal Eliminated Sequences (IES), rearrangement of DNA sequences and de novo methylation (Blackburn et al. 1983; Iwamura et al. 1982; Yao and Gorovsky 1974).

De novo methylation occurs between 13.5 and 15 h after the onset of conjugation (Fig. 4G) (Harrison et al. 1986). Once initiated, methylation will be completed within 90 min and the total level of methylation is constant thereafter (Harrison

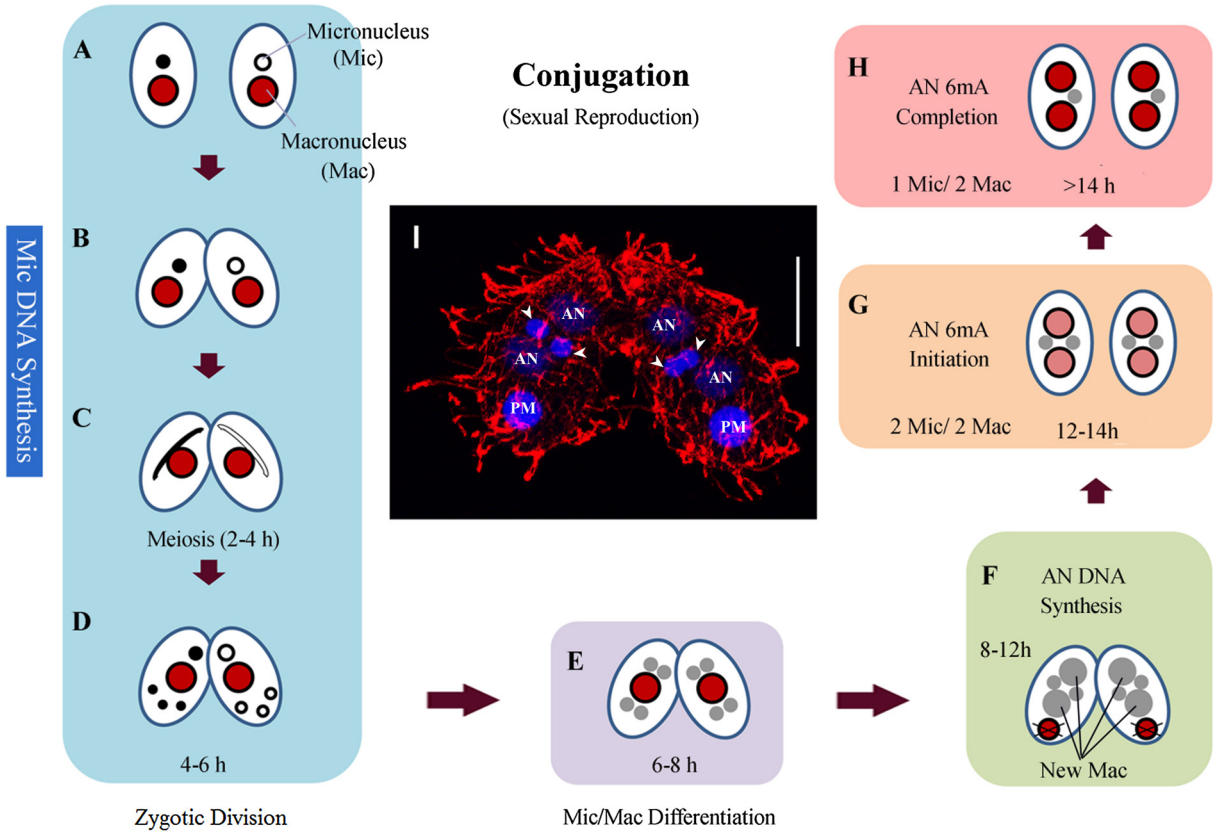

Fig. 4. (A–I) Schematic diagrams of key events and 6mA distribution during conjugation in *Tetrahymena*. (A) Cells of different mating types meet. The content of 6mA is almost equal and complete in the macronucleus (MAC). (B) Conjugation is initiated and the content of macronuclear 6mA is uniform. (C) Micronucleus (MIC) is elongated and meiosis commences 2–4 h after the onset of conjugation; the content of macronuclear 6mA is unchanged. (D) Zygotes start to divide 4–6 h after the onset of conjugation; the content of macronuclear 6mA is constant. (E) MIC develops precursors of new MIC and new MACs 6–8 h after the onset of conjugation, the content of macronuclear 6mA is constant. (F) Early anlagen has been formed during 8–12 h, the old MAC gradually decays at the same time. 6mA has not yet appeared in anlagen, but it still persists in the old MAC. (G) 6mA begins to occur in the anlagen but the content of 6mA is not complete 12–14 h after the onset of conjugation. (H) The content of 6mA is complete in the anlagen 14 h after the onset of conjugation. (I) Immunofluorescence staining shows the old MAC, anlagen, MIC and tubulin; Old MAC (PM), anlagen (AN), and MIC (arrow heads) were stained by DAPI (4′,6-diamidino-2-phenylindole) in blue, and tubulin was stained by an anti-tubulin antibody in red. Red color, the complete 6mA; light red color, the partial (incomplete) 6mA. Bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and Karrer 1985). It should be noted that methylation initiates after the formation of macronuclear anlagen (at about 8 h after the onset of conjugation). This lag phenomenon indicates that 6mA in *Tetrahymena* is unlikely to be involved in mismatch repair at least in the first rounds of DNA synthesis as is the case in prokaryotes since replication has already been underway in the macronuclear anlagen (Harrison and Karrer 1985). The onset of transcription also precedes the occurrence of 6mA (Chicoine and Allis 1986) although their correlation remains to be determined.

Degradation of the parental MAC (PM) is initiated during anlagen development. We observed that 6mA signals gradually disappear in PM while building up in new MAC in our experiment (data not shown). This raises the possibility that 6mA in *Tetrahymena* acts as a defense mechanism, marking the old MAC in order to ensure its degradation, which might be functionally similar to the recognition and degradation of foreign DNA in prokaryotes (Harrison et al. 1986).

Maintenance of 6mA patterns

Patterns of cytosine methylation are stably maintained in clonal lines, but vary with cell types and developmental stages of tissues (Razin and Szyf 1984). After replication, methylated sites of parental cells become hemimethylated. DNA methyltransferases that predominantly recognize hemimethylated sites will modify newly replicated daughter strands so that methylation patterns are maintained (Bird 1978).

Unlikely cytosine methylation, the 6mA pattern in *Tetrahymena* can be divided into uniformly methylated sites, which represent almost all 45 copies (Fig. 3C) (Harrison et al. 1986),

Table 1. Content of methylated adenine in the DNA of different ciliates.

Ciliate	6 mA[a] (%)	5mC [a](%)	Reference
Oxytricha fallax	0.6–0.7	–	Rae and Spear (1978)
Stylonychia mytilus	+[b]	–	Ammermann et al. (1981)
Paramecium aurelia	2.5	–	Cummings et al. (1974)
Tetrahymena pyriformis	0.65–0.80	–	Gorovsky et al. (1973)
Tetrahymena thermophila	0.8	–	Pratt and Hattman (1981)
Blepharisma japonicum	1.5	0.5	Salvini et al. (1986)
Colpoda inflata	–	+[b]	Palacios et al. (1994)

[a] 6mA (%)/5mC (%) were calculated on the basis of total adenine/cytosine residues.
[b] "+" Exist but no specific data.

and partially methylated sites, which represent only a fraction of the 45 copies (Fig. 3C) (Blackburn et al. 1983; White et al. 1986). During vegetative cell division, macronuclear DNA of *T. thermophila* is randomly distributed to the daughter cells (Allen and Nanney 1958; Orias and Flacks 1975), although the methylation pattern is stable (Capowski et al. 1989; Karrer and Stein-Gavens 1990; Karrer and Yao 1988). This semi-conservative copying model may be sufficient for the maintenance of uniformly methylated sites, but not for partially methylated sites. There must exist a mechanism to compensate for macronuclear assortment, with some de novo methylation activity in daughter cells.

During sexual reproduction, macronuclear anlagen are reconstructed from the unmethylated parental MIC and will be methylated ab initio (Capowski et al. 1989; Gorovsky et al. 1973). The methylase, therefore, must possess de novo activity and the capability to catalyze uniform and partial methylation.

The pattern of methylation must rely on sequence patterns to some degree, such as 3′ of thymine in methylated adenines. However, it has been clearly demonstrated that sequence target sites are not sufficient for methylation (VanNuland et al. 1995). For example, DNA fragments containing uniformly methylated sites are not methylated after cloning into the extrachromosomal rDNA (Karrer and VanNuland 1998); meanwhile, 6mA is preferentially distributed in the linker DNA (Pratt and Hattman 1981) and the methylation level is positively correlated with proximity to linker DNA (Karrer and VanNuland 2002). The preferred 6mA localization in linker DNA could be explained by two scenarios (Karrer and VanNuland 2002). First, linker DNA is more accessible to the MTase; the phenomenon of partial methylation would therefore reflect variability of methylation state in a single MAC rather than individual cells. Second, linker DNA is more AT-rich than nucleosomal DNA as AT repeats can cause steric hindrance for nucleosome formation (Chen et al. 2016; Field et al. 2008; Nelson et al. 1987; Segal and Widom 2009; Suter et al. 2000; Xiong et al. 2016). In line with this, partial methylation might be due to limited accessibility to MTase for the specific chromatin structure (Karrer and VanNuland 1998).

6mA in other ciliates

Ciliates are highly diverse and its members differ dramatically from one another in numerous morphological and genetic features, including genome structure and organization. Epigenetic regulation mechanisms of ciliates are also diversified. One of the most extensively studied topics is the complicated process of genome rearrangement (reviewed in Chalker et al. 2013), during which small RNAs are employed in distinct ways in different ciliates (Fang et al. 2012; Lepere et al. 2009; Mochizuki et al. 2002; Zhao et al. 2016). In terms of 6mA, ciliates exhibit a wide range of variation, some species having only 6mA, some having only 5mC, and some having both (Table 1).

The amount of 6mA differs slightly in two species of *Tetrahymena* accounting for approximately 0.65%–0.80% of adenine residues in the MAC of *T. pyriformis* and *T. thermophila* (Gorovsky et al., 1973; Pratt and Hattman, 1981).

The hypotrichous ciliate *Oxytricha fallax* evolved far from *Tetrahymena*. During the new macronucleus development, its germline micronucleus undergoes extensive deletion, permutation and unscrambling, resulting a much simplified macronucleus genome (Chen et al. 2014). *Oxytricha* has macronuclear DNA molecules are about 3.2 kb in average length, with variation from 0.5 to 22 kb (Rae and Spear 1978). The macronuclear genome exists as a collection of relatively short segments (20,000 molecules) with up to several hundred copies, most of which are single-gene "nanochromosomes" (Dawson and Herrick 1982; Dawson and Herrick 1984; Swart et al. 2013). The only detectable modified nucleotide in vegetative cells of *Oxytricha* is 6mA, which accounts for approximately 0.6%–0.7% adenine of its genome, but the functions of 6mA in this ciliate are not fully understood (Rae and Spear 1978). A recent study has detected 5mC methylation during the nuclear development and genome rearrangement process in *O. trifallax* but not in vegetative cells (Bracht et al. 2012). It was speculated that 5mC might be involved in: (1) DNA degradation as it accumulates in the parental MAC; (2) MIC-limited repetitive sequences; (3) aberrantly spliced gene rearrangement products (Bracht et al. 2012). However, since no DNA methylase could be recog-

nized in the *Oxytricha* genome, it remains unknown whether 5mC is evolutionarily equivalent to that in higher eukaryotes.

In the stichotrich ciliate *Stylonychia*, more than 98% of micronuclear DNA is eliminated with the development of polyploid MAC (Ammermann et al. 1974). 6mA is the only detectable methylation in *S. mytilus* (Ammermann et al., 1981). However, 5mC was found in its congener *S. lemnae* (Juranek et al. 2003), in which de novo methylation occurs in a significant percentage of cytosines during macronuclear differentiation. A low level of cytosine methylation was also founded in the micronuclear DNA of *Stylonychia lemnae* at the sequence of CCWGG. It was proposed that methylation of cytosine could play an essential role in the silencing of genes and the formation of heterochromatin in *Stylonychia lemnae*.

Paramecium is a close relative to *Tetrahymena*, both of which belonging to the class Oligohymenophorea. However, these two ciliates differ from each other in many aspects, the most dramatic one of which is whole-genome duplication (WGD) in *Paramecium* species that is absent in *Tetrahymena* (Aury et al. 2006; Eisen et al. 2006). The MAC of *P. aurelia* has approximately 800 copies per haploid genome (Gibson and Martin 1971). The percentage of 6mA is from 2.1% to 2.5% in the different syngens (Cummings et al. 1974). 6mA could participate in the silencing of partial sequences in the macronucleus of *Paramecium* (Kwok and Ng, 1989), but this needs further validation. It was proposed that 5mC might regulate the program of genomic function during development of the somatic macronucleus of *Paramecium tetraurelia*, even though the existence of cytosine methylation remained uncertainty (Kwok and Ng 1989).

The nuclear apparatus of the heterotrich ciliate *Blepharisma japonicum* consists of one MAC and a variable number of MIC (6–30) (Salvini et al. 1983). It has both 6mA (1.5%) and 5mC (0.5%) in the vegetative MAC (Salvini et al. 1986). It was found that gene activation is closely related to adenine demethylation in *Blepharisma japonicum* (Salvini et al., 1986). By contrast, in the colpodid ciliate *Colpoda inflata*, 5mC is the only detected methylation form in the MAC. Demethylation of 5mC, by restriction enzyme digestion or 5-azacytidine, was proposed to induce encystment, indicating a role of 5mC in gene expression regulation (Palacios et al. 1994).

Perspectives and Concluding Remarks

As described above, 6mA in *Tetrahymena thermophila* has the following characteristics. (1) Cellular localization: 6mA is present in the transcriptionally active MAC, but not in the transcriptionally inactive MIC; de novo 6mA methylation occurring 12–14 h after the initiation of conjugation in the newly developed MAC. (2) Content: approximately 0.8% of adenines in macronuclear DNA are methylated, in either uniformly or partially methylated sites. (3) Motif: 6mA is preferentially located in the linker DNA, in the sequence of 5′-NAT-3′.

Despite numerous studies, the role of 6mA methylation in eukaryotes has remained elusive. According to recent reports, the functions of 6mA are rather divergent. For example, 6mA is linked to gene activation in *Chlamydomonas reinhardtii* (Fu et al. 2015), whereas it is associated with epigenetic silencing in mouse embryonic stem cells (Wu et al. 2016). The original function and evolution of 6mA in eukaryotes remain unknown.

Ciliates such as *Tetrahymena thermophila* have been widely used as model organisms. Significant progress has been made toward understanding the function of 6mA in ciliates, yet several major features remain unknown including: (1) patterns of whole-genome distribution; (2) the indentity of methylase and demethylase; (3) the reason for the sporadic expansion of 6mA in various ciliates. The findings presented here will help to decipher the sequence preference and chromatin environment, characterize the enzymatic complex and the regulation mechanisms, and deduce the original function of 6mA in the last universal common ancestor of the eukaryotes.

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