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REVIEW ARTICLE

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DNA methylation in ciliates: implications in differentiation processes

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Summary Much experimental evidence on the role of DNA methylation in gene expression has been reported. Here we review reports on DNA methylation in ciliated protozoa, emphasizing its implications in cell differentiation processes. Both types of methylated bases (adenine and cytosine) can be found in macronuclear DNA. The division cycle and conjugation have been studied with regard to adenine methylation, and several different functions have been assigned to the methylation changes detected in these processes. Cytosine methylation changes were analyzed during stomatogenesis of *Paramecium* and encystment of *Colpoda inflata*. A comparative analysis with other similar microbial eukaryotic differentiation processes is carried out.

Key words DNA methylation · Conjugation · Stomatogenesis · Encystment · Ciliates

Introduction

DNA methylation is involved in DNA-protein interactions [13], protection of DNA against restriction endonucleases [33], and enhancement of mutation and recombination. Besides, it affects DNA structure [49], DNA replication [39], virus latency [4] and the regulation of gene expression in eukaryotic cells [5, 10, 13, 15, 16, 38, 43, 44].

How does DNA methylation modulate gene expression? Previous studies [13, 16] have established a correlation between undermethylation and unimpeded gene expression. They have also shown that the control of the expression of certain genes and the maintenance of a cellular differentiated stage are related to increased cytosine methylation levels [13, 15, 26, 38]. 5-Methylcytosine (MeCyt) appears to be the main modified base in eukaryotic DNA. For example, it occurs predominantly in the so called CpG islands; in animal DNA, an average 70% of all CpG pairs are methylated [3]. For some genes or groups of genes, frequency clusters of the dinucleotide CpG can be found either close to or in the promoter, as well as in the leader or in the 5´ region of these genes. Site-specific methylation studies involving both viral and eukaryotic gene promoters have shown [13] that methylation of only one or a few cytosines at CpG sites can alter transcriptional activity, and that, in most cases in which expression is affected, these methylated sites are part of binding sequences for transcription factors. Therefore, a positive correlation can be established between the following conditions: (i) existence of CpG islands in gene regulation sequences; (ii) cytosine methylation generally originated in these CpG islands; and (iii) methylation in these CpG islands blocks the corresponding coding gene expression by not allowing particular transcription factors to bind to the regulatory region of the gene (a diagram of this gene expression modulation mechanism is shown in Fig. 1).

Recently, it has been suggested that DNA methylation suppresses rather than regulates gene expression, and that methylation causes permanent suppression of CpG island promoter activity. This hypothesis is supported by the genomic sequencing of inactive promoters and by observations that silenced genes rarely reactivate spontaneously, that methylation is regulated developmentally, and that in vitro methylation down-regulates the expression of reporter genes.

In this article, we present for the first time an updated study of DNA methylation in ciliated protozoa that includes a general view of the ciliate nuclear system, the methylated bases present in it with regard to other eukaryotic microorganisms, the DNA methylation pattern changes during developmental or differentiation processes, and the role of DNA methylation in the regulation of ciliate gene expression.

Ciliate nuclear system

Nuclear dualism, a main feature of the nuclear system of ciliates, consists of the presence of two different types of nuclei in the same cytoplasm. These are called, according to their sizes, micronucleus (Mi) and macronucleus (Ma). In general, ciliates

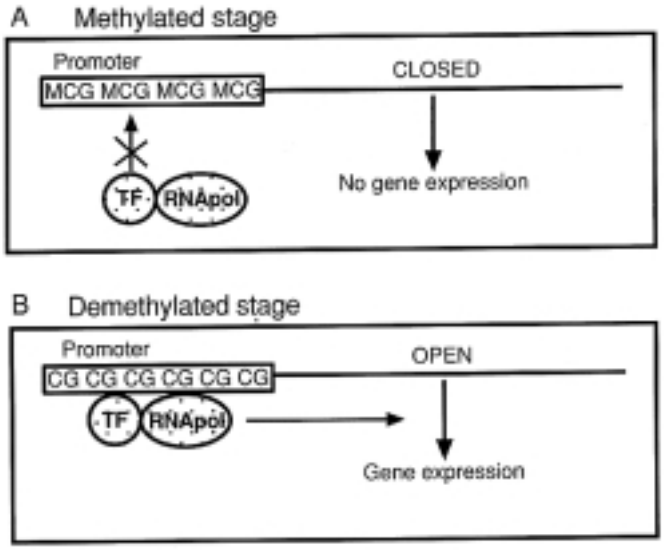

Fig. 1 Gene expression modulation mechanism to explain, in some eukaryotes, the regulation of the gene expression by MeCyt. (A) Methylated stage of the gene promoter CpG islands (MCG). This methylated stage prevents the binding of a specific transcription factor (TF) with the RNA polymerase holoenzyme (RNA pol) to the corresponding promoter. Thus, the encoding gene is maintained inactive. (B) After an active or passive demethylation process of this promoter region, the complex (TF + RNApol) can initiate transcription and the gene expression takes place

may contain one or more diploid Mi and one or more polyploid Ma in a single organism. For example, *Tetrahymena* and *Colpoda* species have one Mi and one Ma (Fig. 2), and *Oxytricha* species usually have two to four Mi and two Ma per cell. An extreme case is *Urostyla grandis* (a hypotrichous ciliate), which has 5–20 Mi and several hundred Ma [36]. Micronuclei are germ line nuclei, which carry out sexual exchange of DNA during conjugation, whereas macronuclei are somatic nuclei for vegetative transcription to support cell growth, differentiation and proliferation. For this reason, nucleoli are present exclusively in Ma. Some micronuclear genes, however, may be also expressed during vegetative growth [36]. Mi divide mitotically during vegetative growth and undergo meiosis during conjugation to form the haploid pronuclei, to be exchanged between two mating cells. Ma divide by amitotic division, which is an imprecise division mechanism, because Ma-DNA is unequally distributed to daughter macronuclei [36]. Besides, Ma degenerate during conjugation to be replaced by the newly-formed diploid Mi after an extensive polyploidization [36]. During *Tetrahymena* conjugation, the two diploid anteriorly located nuclei enlarge (they are called macronuclear anlagen), replicate six times most of their DNA content, and become the two new macronuclei. When a single ciliate cell has several Ma, they can fuse to form one only macronuclear mass during division and/or encystment processes [22, 36].

Both nuclei are typical eukaryotic nuclei. They have nucleosomes and a nuclear membrane with typical pores, but they are very different from each other with regard to their chromatin structure and DNA organization. In general, vegetative Mi-DNA occurs in uniformly, densely packed chromatin, whereas the Ma-DNA occurs in many chromatin bodies dispersed in the nucleoplasm. Macronuclei contain the usual five major histones, but the histone H1 is absent from the micronucleus of *Tetrahymena thermophila* [36]. Micronuclear DNA has a very high molecular weight and is formed by extremely long molecules. Most ciliates in the vegetative stage have these micronuclear features. In contrast, two very different types of genomic macronuclear organization can be distinguished: (i) The macronuclear genome pattern in hypotrich ciliates such as *Oxytricha*, *Stylonichia* and *Euplotes* consists of gene-sized DNA molecules. This DNA is composed of fragments ranging from about 0.4 to 20 kb, and can be detected in a standard agarose electrophoresis gel as a smear of Ma-DNA molecules (Fig. 3A). These molecules have the characteristics of mini-chromosomes: they can undergo autonomous replication and transcription, and have typical telomeric inverted repeats ($C_4 A_4 / G_4 T_4$) [36]. (ii) The second macronuclear genome pattern occurs in many different ciliates, including *Tetrahymena*, *Paramecium* and *Colpoda* [22, 36]: the chromosomes are also fragmented but not as strongly as in hypotrich ciliates, and they are named subchromosomic fragments, because they originate from micronuclear chromo-

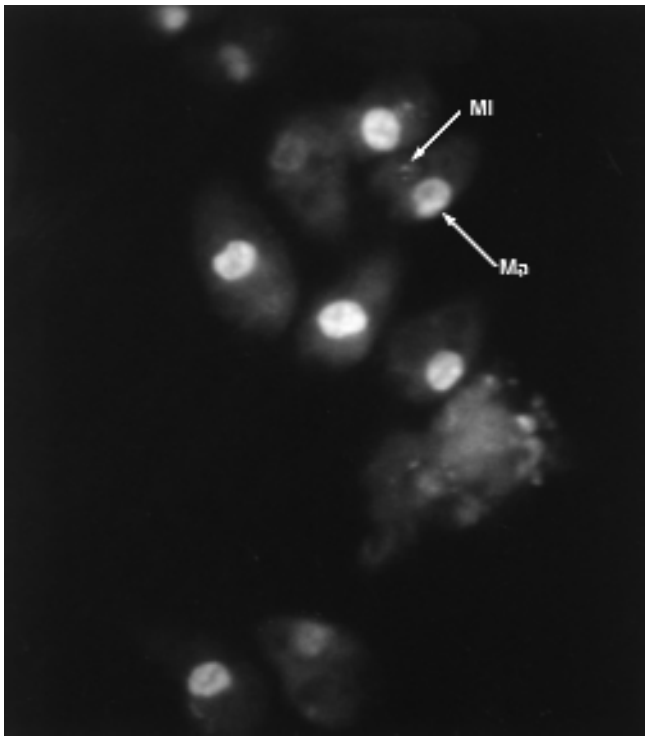

Fig. 2 Nuclear system of *Colpoda inflata* revealed after BrdU (5-bromo-2´-deoxyuridine) incorporation during DNA synthesis, and detected by immunofluorescence with a monoclonal anti-BrdU antibody labelled with fluorescein (approx. 1000×). Mi: Micronucleus; Ma: Macronucleus

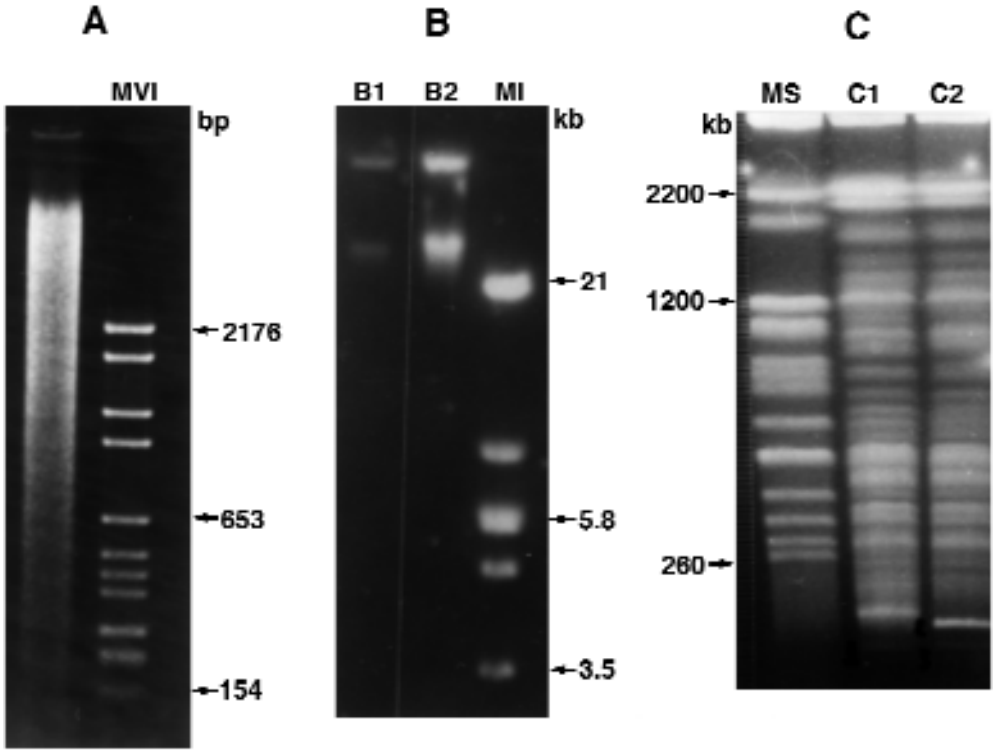

Fig. 3 The two basic macronuclear genome types of ciliates. (A) Gene-sized macronuclear DNA. Standard 0.8% agarose gel electrophoresis of total DNA from *Oxytricha nova* (smear of macronuclear DNA molecules from 0.4 to > 20 kb in size). (B) Chromosome-sized DNA macronuclear type. Standard 0.8% agarose gel electrophoresis of total DNA from *Tetrahymena thermophila* (B1) and *Colpoda inflata* (B2). (C) Pulsed-field (1%) agarose gel electrophoresis (CHEF) of two strains of *Tetrahymena thermophila* (lanes C1 and C2), one of them is the same as in B1. MS: DNA size standards (*Saccharomyces cerevisae* S13 chromosomes). MI and MVI: DNA size standards from Roche Molecular Biochemicals (Germany)

some fragmentation during conjugation to form the macronuclear genome. In standard agarose electrophoresis, these chromosomes (like most eukaryotic genomes) show only one big band at the top of the gel (Fig. 3B). The subchromosomic fragments are only separated by using pulsed-field gel electrophoresis (Fig. 3C), which separates DNA molecules up to 2000 kb.

Methylated bases in ciliates (comparison with other eukaryotic microorganisms)

Methylation of both adenine (MeAde) and cytosine (MeCyt) may occur in prokaryotic cells, though in some species only one base becomes methylated. In contrast, MeCyt is the only methylated base that has been found in multicellular eukaryotes (with the exception of salmon sperm, which has a small amount of MeAde). However, in eukaryotic microorganisms there is more variability than in multicellular eukaryotes; methylation of cytosines has been detected in many but not all fungi, and several fungi including *Saccharomyces cerevisiae* and *Aspergillus nidulands* have been reported to lack DNA methylation [43]. In many fungi, MeCyt is found mostly, if not exclusively, in CpG dinucleotides. Changes at the methylation level during development processes have been observed in the fungi *Phycomyces* [40], *Candida*, *Neurospora* [42], *Mucor* [8] and *Schizophylum* [7], and in the slime mold *Physarum* [19, 50].

In some phytoflagellates, both MeAde and MeCyt have been found, and in dinoflagellates, 5-hidroxymethyl-uracil is dominant, although some species have also MeCyt [41]. Among ciliates, both types of methylated bases have been detected in the macronuclear DNA, but they are not present together in the same ciliate. Until now, only six different ciliates have been tested for DNA methylation: two hypotrichous ciliates, *Oxytricha* and *Stylonichia* [2, 37]; two hymenostomatids, well-known ciliates, such as *Tetrahymena* [25, 35] and *Paramecium* [14]; one heterotrichous ciliate, *Blepharisma japonicum* [41]; and one colpodid ciliate, *Colpoda inflata* [34]. As shown in Table 1, MeAde has been found in macronuclear DNA of most studied ciliates, but no MeCyt has been detected , except for *Blepharisma* and *Colpoda*, which only have MeCyt. Besides, in *Paramecium tetraurelia* [28], MeCyt has been detected indirectly by using the nucleoside analogue 5-azacytidine (5-azaC), a potent demethylating agent [38]. Although it has not been biochemically detected, some authors [28, 32] consider that the limit of sensitivity of biochemical detection of 5-

methylcytosine, in several ciliates, is at best 0.01 mol % of cytosine [35]. For a genome of about 60,000 kb and a GC content of 26%—as is the case in *P. primaurelia* [18]—, the presence of fewer than 800 MeCyt bases would not be detected. This situation may also occur in other ciliates in which only one type of methylated base has been reported.

Table 1 Methylated bases in the macronuclear DNA of ciliates

Ciliate	MeAde	MeCyt	Reference
Tetrahymena pyriformis	+	–	25
Tetrahymena thermophila	+	–	35
Paramecium aurelia	+	–	14
Paramecium tetraurelia	?	+/– *	28
Stylonichia mytilus	+	–	2
Oxytricha sp.	+	–	37
Blepharisma japonicum	–	+	41
Colpoda inflata	–	+	34

* Not biochemically detected.

At present, data about methylated bases in micronuclear DNA have only been collected from *Paramecium* and *Tetrahymena*. Both types of *P. aurelia* nuclei (Ma and Mi) contained about 2.5 molepercent MeAde [14]. However, in *T. thermophila* [35] the Ma has MeAde in approximately one in every 125 adenine residues (0.8 %), but the Mi is not methylated (< 0.01 mol % MeAde) [20]. Therefore, only the transcriptionally active Ma contains methylated DNA, not the inactive Mi. The results obtained from *P. aurelia* [14] may be due to macronuclear DNA contamination during the Mi isolation, because other authors did not find Mi-DNA methylation in this ciliate. A more extensive analysis of methylation at Mi-DNA level should be carried out to elucidate this point.

DNA methylation changes during development and differentiation

DNA methylation changes involved in ciliate differentiation have been reported in *Tetrahymena thermophila* [6, 9, 24] at adenine methylation level (the only methylated base in the Ma-DNA of this ciliate) (Table 1). Those changes affect Ma-DNA methylation during cell division and conjugation processes. On the contrary, although the MeAde is also the main methylated base in the Ma-DNA of *Paramecium*, methylation changes at cytosine level (MeCyt) have been analyzed during the stomatogenic process of this ciliate [32]. Besides, MeCyt changes are involved in the encystment process of *Colpoda inflata* [34].

Methylated sequences and cell division in *Tetrahymena* Nuclease digestion and methylation have been probed in *Tetrahymena thermophila* [35]. Methylated sequences are mostly susceptible to DNase I, which preferentially digests "active" genes, as has been reported in several eukaryotes. Those methylated sequences are preferentially digested by staphylococcal nuclease, which has also been reported to discriminate between active genes and bulk DNA in chromatin. The preferential release of MeAde residues in *Tetrahymena* by these nucleases may be due to a higher number of MeAde in the exposed (linker) regulatory regions of chromatin.

The order of increasing thermal stability is MeAde : T < A: T < C : G < MeCyt : G, and easily denaturable sequences may facilitate the binding of RNA and DNA polymerases to promoters and replication origins. In multicellular eukaryotes, methylation of cytosine might inhibit local denaturation and, consequently, transcription. In contrast, methylation of adenine could decrease duplex stability and facilitate transcription. In *Tetrahymena*, with an A+T content of about 75%, and where MeAde residues are preferentially located in internucleosomal linker DNA sequences [35], adenine methylation might increase transcription by facilitating local denaturation at the promoter. Thus, there may be a relationship between denaturability (A : T richness or presence of MeAde) and accessibility to replication and transcription enzymes. However, this hypothesis has not yet been elucidated.

In growing *Tetrahymena* cells, 87% of the methylation occurs on the newly replicated daughter strand, but methylation was also detected on the parental strand [24]. Methylation of non-replicating Ma-DNA from starved *Tetrahymena* cells has also been demonstrated. In this case, like in vertebrate DNA cytosine-5-methyltransferases, two different functions can be assigned: maintenance of methylation and *de novo* methylation. Maintenance of methylation ensures clonal transmission of lineage-specific methylation patterns in somatic cells, and is stimulated by or dependent on hemimethylated DNA, which is the product of semiconservative DNA replication [29]. *De novo* methylation can establish differentiated cell-specific methylation patterns.

In contrast, of 143–278 possible methylation sites in *T. thermophila* [27], 29 of which are known to be methylated, none underwent changes in its methylation state despite changes in the transcriptional activity of nearly genes. These data suggest that the methylation pattern of the Ma-DNA remains fairly constant during growth, starvation and early conjugation, which is in contradiction with the results obtained by other authors in the same ciliate [6, 23, 24].

Conjugation and adenine methylation in *Tetrahymena* The role of adenine methylation during *Tetrahymena thermophila* conjugation has been studied by using macronuclear palindromic rDNA molecules [6]. The main reasons to use these molecules are their extrachromosomal character and abundance in the Ma-genome of *T. thermophila*, and the fact that the ratio of transcription of ribosomal genes is frequently dependent on the physiological state of the cell. For the N-6-methyladenine residues in GATC sequences in Ma-rDNA molecules, a highly specific subset of sites is methylated. The

GATC sequences examined are expected to represent 3.4% of the total methylation sites in the rDNA. Since MeAde comprises 0.4% of the adenine residues in rDNA, there are on average 26 MeAde per palindrome half. Therefore, about 0.8 MeAde per palindrome half are expected to occur in the sequence GATC. Blackburn et al. [6] reported that there are only about 0.1 M MeAde 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 coding and non-coding regions of the molecule. Whereas one of the specifically methylated sites was found in the non-transcribed spacer (1.5 kb from the rDNA ends in the strain C3 of *T. thermophila*), the most highly methylated site is located in a transcribed sequence.

No differences were detected in GATC methylation patterns or levels in the palindromic rDNAs from cells in different physiological states (exponentially growing cells, cells starved for 24 h and cells at different stages during conjugation) [6]. These results are consistent with previous findings made for overall levels of MeAde in Ma-DNA [20]. One possible function of MeAde residues is that they might serve to distinguish molecular forms of DNA to be retained in the mature Ma from those to be rearranged or lost by degradation during the Mi-DNA to Ma-DNA transformation (developing macronuclear anlagen) [6].

A second and more detailed study on possible functions of methylation during Ma development in *Tetrahymena* [23] has determinated the timing of *de novo* methylation of GATC sites relative to DNA synthesis in the anlagen and degradation of DNA in the old Ma. This study found a delay of at least several hours between anlagen DNA synthesis, beginning during Ma development II (8 hours after starting conjugation), and methylation, which began during Ma development III (11 hours after starting cojugation). Therefore, several rounds of DNA synthesis have occurred before *de novo* methylation of anlagen DNA. The authors [23] did not observe an intermediate level of methylation of newly synthesized DNA in conjugating cells, in contrast to results from Pratt and Hattman [35] in vegetative 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 [5]. It is possible that different maintenance methylases could be simultaneously active [48]. In mammalian cells, also a delayed methylation from DNA synthesis by several hours has been reported [29], and authors suggest that 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 a new message.

Genetic evidence has shown transcription of genes during Ma development in *Tetrahymena* conjugation [36]. Methylation of anlagen DNA occurs late in the Ma development [23]. Therefore, methylation might be active in transcriptional control during conjugation. Until now, however, no experimental evidence of specific gene methylation changes has been reported during ciliate conjugation.

In prokaryotic microorganisms, methylation is known to function in mismatch repair. 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. If mismatch repair occurs in Mi-DNA, newly synthesized DNA should be distinguished by something other than methylation because Mi-DNA is not methylated [23]. 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 [23]. Alternatively, methylation may function in a modification-restriction system during conjugation in *Tetrahymena*. As mentioned [6], methylation may protect DNA sequences from degradation in the developing macronuclear anlagen. Evidence supporting this notion is that a family of DNA sequences that is partially eliminated from the developing Ma includes one member which is retained in the new Ma and which is methylated at a GATC site in about 50% of the macronuclear copies [23].

Stomatogenesis and MeCyt in *Paramecium* During cell division, in a process called stomatogenesis, ciliates produce a new oral apparatus to be passed on to the posterior daughter cell. This stomatogenesis is not only dictated by the somatic nucleus (Ma) but also involves the Mi [12]. Several studies have demonstrated that the Mi plays a definitive, but replaceable, role (favouring normal oral development) in the stomatogenesis during cell division [31]. In both *Stylonichia* [1] and *Paramecium bursaria* [17], the involvement of the Ma in replacing micronuclear function has been reported. The initial working hypothesis was that, in the somatic nucleus (Ma), some cytosine bases associated with stomatogenic DNA sequences are usually methylated, and that the methylated state is maintained in the presence of the Mi. Following removal of the Mi, these sequences gradually become activated and their activation is promoted by demethylation [28]. Some experimental evidence which can corroborate this hypothesis has been obtained in amicronucleate cells of two species of *Paramecium* [28, 32]. Amicronucleate cells in the state of growth depression responded specifically to 5-cytosine-demethylating drugs by developing more normal oral membranelles. The action of these drugs was thus to promote stomatogenic recovery, which normally occurs more slowly in amicronucleates [32]. This might agree with the following points: (i) Stomatogenic recovery in amicronucleates is a function of Ma-DNA sequences. (ii) Such Ma-DNA sequences are normally repressed by methylation of cytosine in the presence of Mi. (iii) When Mi are removed from the ciliate, the Ma-DNA sequences are slowly activated by demethylation.

During this time, the oral apparatus develops abnormally. (iv) When a certain level of activation of the Ma-DNA sequences is reached, stomatogenesis proceeds normally.

A second study [28] has tested the notion that the Ma is involved in this compensation, by treating cells (with Mi) with 5-azacytidine (5-azaC), a potent demethylating agent [38], during sexual reproduction when a new somatic nucleus (Ma) develops. These cells were then propagated asexually for a number of fissions in the absence of the drug, and thereafter Mi were removed from them. The amicronucleated cells did not undergo a growth depression (marked by abnormal oral development) as severe as the controls, and they recovered much sooner. This suggests that the drug 5-azaC applied to *Paramecium* during autogamy (fusion of gametic nuclei produced by the same parental cell or self-fertilization) can alter the programming of the developing Ma. According to the authors [28], after fertilization, the normal programming of the new Ma involves repression of some of its stomatogenic DNA sequences. After removal of the Mi, such sequences are gradually activated to replace the stomatogenic function of the Mi. Treatment with 5-azaC prevents the normal repression of these sequences during development of the Ma, and the altered program is maintained during subsequent asexual division. The Ma sequences that have been activated are thus ready to take over the function of the Mi, in initiating recovery of the cell line, once the Mi are removed. How the Ma sequences are activated after the Mi is removed is still unresolved.

Changes in DNA methylation during ciliate cryptobiosis

Vegetative cells of many ciliate species can differentiate into resting cysts (the cryptobiotic stage) under certain environmental conditions [21, 22]. This process, called encystment, involves progressive morphological and physiological changes concluding with the formation of resting cysts. This microbial differentiation process depends on both RNA and protein synthesis, but not on DNA synthesis [22]. According to a generalized ciliate encystment model reported in 1990 [21], a nutritional deficiency (the exogenous inducer) triggers the "turn-off" of vegetative genes involved in the growth-division cycle and the "turn-on" of encystment genes. This activation of encystment genes involves the appearance of new transcripts, which are necessary to synthesize the cystic macromolecules (such as cyst wall proteins). There is experimental evidence that a specific gene expression takes place during ciliate encystment. The DNA methylation pattern changes during the encystment process in the ciliate *Colpoda inflata* [34], and it is the only reported study on methylation in ciliate encystment. A restriction analysis showed that MeCyt is the only methylated base in the Ma-DNA of this ciliate [44]. Ma-DNA demethylated GCGC and CCGG sequences are present in resting cyst DNA, but not in vegetative cell DNA, indicating that Ma-DNA demethylation may take place during the encystment process of this ciliate.

Experiments, also in *Colpoda inflata*, using 5-azaC have corroborated the molecular results [34]. This demethylating agent induces encystment in growth medium. The 5-azaC is incorporated into DNA during replication, which specifically inhibits methylation of cytosine residues. Increasing levels of incorporated 5-azaC inhibit the action of DNA methyltransferases, and the result after several generations is a hemimethylated or unmethylated DNA. This might "switch on" the encystment genes under adverse conditions for ciliate encystment induction. This drug does not affect the cell otherwise during encystment, and the ratio of encysted to vegetative cells is similar to that for the control. After encystment, the drug is not incorporated into DNA, because DNA replication does not take place during encystment of *C. inflata* [22].

As in other eukaryotic cell differentiation processes [38], and in the encystment process of the myxomycete *Physarum flavicomum* [50], a specific genomic DNA demethylation could be involved in the activation of ciliate encystment genes. Although demethylation does not normally involve DNA replication, the encystment demethylation should be an active mechanism. It has been shown that methyl groups can be removed in the cell cycle when DNA is not undergoing replication [38]. In contrast to the well defined biochemistry of the Dmmt1 methyltransferase, the enzymatic basis of MeCyt demethylation *in vivo* remains unknown. An "amazing demethylase" was recently described [11] and different pathways for "active" demethylation were also reported [48].

Changes in the DNA methylation pattern of specific encystment genes have not been analyzed in ciliates because no complete sequences of these genes have been reported, and only very few and still incomplete macronuclear gene sequences have been described [46]. However, we have obtained experimental evidence about the 18S rDNA methylation during encystment of *Colpoda inflata* (unpublished data). In other eukaryotic systems, there is a strong correlation between the methylation level of ribosomal genes and transcriptional activity [47]. This may explain the low or null transcriptional activity of the mature resting cysts or any cryptobiotic stage.

In *Physarum polycephalum* treated with inhibitors of DNA methylation, it has been reported that methylation of DNA must take place during the differentation process in spherules at least during the second half of the process [19]. In *P. flavicomum* [50], formation of dormant cysts was found to be accompanied by an approximately fourfold decrease in the content of MeCyt. Pronounced differences in the content of MeCyt among individual DNA preparations from spherulating cultures and spherules indicate that transient changes in DNA methylation do take place. These changes could be waves of demethylation and subsequent *de novo* methylation resulting in altered patterns of methylation of individual nucleotide sequences. The latter assumption is supported by the observation of changes in the

methylation pattern of a spherulating-specific gene named spherulin 4 [19]. As spherulin 4 mRNA is detectable only in late spherulating cultures and mature spherules, methylation of this gene apparently accompanies its transcriptional activation [19, 30]. Methylation and transcriptional activation have been correlated to explain some results obtained in multicellular systems. Not all CpG islands and methylation sites are located in known promoters, which has given rise to an interesting paradox in which methylation in the transcribed region is often correlated with expression, in contrast to the inverse correlation seen at the site of transcriptional initiation. For five other genes analyzed by these authors [19], no changes in their methylation were detected. These last results agree with those obtained by Telle and Hildebrandt [45] using the same microorganism, which did not reveal any evidence for a change in the methylation of specific genes in the course of its differentiation process.

DNA methylation changes during spore germination have been also analyzed in both *Phycomyces blakesleeamus* and *Mucor rouxii* [40]. Analysis of metallothionein gene behavior and expression during spore germination has confirmed that this gene is methylated and not transcribed in the spore, and that it becomes demethylated and transcriptionally active after germination. Also, the levels of DNA methylation in spores of both microorganisms were higher than those in mycelial cells [40]. In summary, in microbial eukaryotic cryptobiosis processes, both demethylation and/or methylation of DNA might be involved in activating and/or closing the specific gene expression in those processes.

Concluding remarks

As has been shown in this review, our knowledge of DNA methylation in ciliate differentiation processes is still quite limited and sometimes confusing. Our knowledge on the type of methylated bases present in both Mi and Ma is very limited. Also, it is unclear if MeCyt is present in ciliates such as *Paramecium*, and Mi-DNA methylation is still an enigma. MeAde is present in the Ma-DNA of most studied ciliates, but a more extensive analysis should be carried out to elucidate if it is present in other ciliate groups.

Not many studies on DNA methylation changes during ciliate differentiation have been carried out, and most of them are on *Tetrahymena thermophila* (with MeAde in its Ma-DNA). They have been carried out on cell division and conjugation, and very few specific genes involved in these processes have been analyzed with regard to methylation patterns. Several different functions have been assigned to adenine methylation changes in these processes, and some of these putative functions are confusing and sometimes contradictory. Something similar happens with the experiments on stomatogenesis, MeCyt and the presence/absence of the Mi in *Paramecium*. Regarding DNA methylation and ciliate encystment, only one study in *Colpoda inflata* has been reported, and the results are quite similar to those for other eukaryotic microorganisms. Specific encystment genes should be sought. The studies on DNA methylation changes during this differentiation process should be increased using other types of ciliates, such as *Blepharisma*, which presents both macronuclear MeCyt and encystment. Also, hypotrichous ciliates (*Oxytricha* and *Stylonichia*), which have MeAde in their Ma-DNA, might be good systems to analyze the methylation changes at adenine residues during the encystment-excystment cycle. Much new work on the Ma-DNA methylation is still necessary to establish general conclusions about the relevance of DNA methylation/ demethylation in the life of ciliated protozoa.

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