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N⁶-methyl-adenine: an epigenetic signal for DNA-protein interactions

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Summary

N⁶-methyl-adenine is found in the genomes of bacteria, archaea, protists, and fungi. Most bacterial DNA adenine methyltransferases are part of restriction-modification systems. In addition, certain groups of Proteobacteria harbor solitary DNA adenine methyltransferases that provide signals for DNA-protein interactions. In γ -Proteobacteria, Dam methylation regulates chromosome replication, nucleoid segregation, DNA repair, transposition of insertion elements, and transcription of specific genes. In *Salmonella*, *Haemophilus*, *Yersinia*, *Vibrio*, and pathogenic *E. coli*, Dam methylation is required for virulence. In α -Proteobacteria, CcrM methylation regulates the cell cycle in *Caulobacter*, *Rhizobium*, and *Agrobacterium*, and plays a role in *Brucella abortus* infection.

Introduction

In microbial genomes, the most common DNA modification is postreplicative base methylation. C⁵-methyl-cytosine and N⁶-methyl-adenine are found in the genomes of many fungi, bacteria, and protists, while N⁴-methyl-cytosine is only found in bacteria¹. N⁶-methyl-adenine is also present in archaeal DNA². Base modification in bacterial genomes is performed by two classes of DNA methyltransferases: (i) those associated with restriction-modification systems³, and (ii) solitary methyltransferases that do not have a restriction enzyme counterpart. Examples of the latter are the N⁶-adenine methylases Dam and CcrM, and the C⁵-cytosine methylase Dcm⁴⁻⁷. In eukaryotes, C⁵-methyl-cytosine (m5C) plays roles in gene expression, chromatin organization, genome maintenance and parental imprinting, and has attracted ample interest because of its involvement in human disease⁸. In contrast, the functions of the prokaryotic Dcm enzyme remain unknown⁴.

Diverse groups of Proteobacteria use N⁶-methyl-adenine (m6A) as a signal for genome defense, DNA replication and repair, nucleoid segregation, regulation of gene expression, control of transposition, and host-pathogen interactions⁴⁻⁷ (Figure 1). Methylation of the

amino group of adenine lowers the thermodynamic stability of DNA⁹ and alters DNA curvature¹⁰. Such structural effects can influence DNA-protein interactions, especially for proteins that recognize their cognate DNA binding sites by both primary sequence and structure¹¹. All known functions of m6A in Proteobacteria rely on regulating the interaction between DNA-binding proteins and their cognate DNA sequences. Typically, m6A is used as a signal to indicate *when* and *where* a given DNA-protein interaction must occur¹².

DNA adenine methylation and restriction-modification systems

The discovery of restriction-modification systems (R-M) was the consequence of observations made in the early 1950's on host-controlled variation of bacterial viruses^{13,14}. The molecular explanation of this phenomenon came several years later, when it was shown that growth of phages in bacteria was restricted by endonucleases which attack unmethylated viral DNA whereas host DNA is protected by a specific methylation pattern¹⁵. R-M systems are found in both Eubacteria and Archaea, and are believed to have evolved to protect bacteria against viruses. m6A is with m5C and m4C, one of the three methylated bases ensuring the protection of DNA against endonuclease digestion. Hence, R-M systems contain both a restriction enzyme and a cognate DNA adenine or cytosine methyltransferase³.

R-M systems have been classified in three types on the basis of structural features, position of DNA cleavage, and cofactor requirements. In types I and III, the DNA adenine or cytosine methyltransferase is part of a multisubunit enzyme catalyzing both restriction and modification³. In contrast, type II R-M systems have two separate enzymes, a restriction endonuclease and a DNA-adenine (or cytosine) methyltransferase which recognize the same target³. In addition to these three types of R-M systems in which adenine or cytosine methylation protects against endonuclease cleavage, restriction systems specific for methylated bases have also been described¹⁶. Thus, the pattern of adenine methylation extends the coding capacity of DNA to a new function, the distinction between “self” and “non-self”¹⁷. This classical viewpoint considering R-M systems as a barrier against foreign DNA invasion has progressively evolved to include new biological functions or concepts such as

the involvement of R-M systems in genetic exchange¹⁸ and maintenance of species identity¹⁹. Other evidence suggests that, in type II R-M systems, the couple made by the methyltransferase and the cognate restriction endonuclease behaves as a “selfish” element in a host parasite-type interaction with bacteria²⁰. This view originates from the observation that type II R-M genes are often linked with mobile elements and that their loss may cause cell death through restriction cleavage of the genome. This cell death occurs because R-M methyltransferases are distributive and not processive. Therefore, the residual methyltransferase activity is unable to protect all the restriction sites against cleavage by the remaining endonuclease molecules.

Dam: a solitary methyltransferase of γ -Proteobacteria

The first solitary DNA adenine methyltransferase found to provide signals with physiological significance was the Dam enzyme of *E. coli*^{4,6,7}. Dam transfers a methyl group from S-adenosyl-methionine to the amino group of the adenine moiety embedded in 5'-GATC-3' sites⁴. Methylation occurs shortly (but not immediately) after DNA replication; hence, passage of the replication fork leaves GATC sites transiently hemimethylated. The *E. coli* Dam enzyme methylates both hemimethylated and unmethylated GATC sites with similar efficiency⁴. Based on the organization of amino acid domains, Dam is classified in the α group of DNA amino methyltransferases, and shares significant identity with *DpnIIA*, *MboIA* and other DNA methylases which are part of restriction-modification systems^{6,7}. This relatedness suggests that Dam has evolved from an ancestral restriction-modification system. A difference, however, is that the Dam methylase is highly processive while R-M modification methylases are not²¹.

Homologs of the *E. coli* Dam methylase are found in the orders Enterobacteriales, Vibrionales, Aeromonadales, Pasteurellales and Alteromonadales⁶. In *E. coli* and *Salmonella*, *dam* mutations cause pleiotropic defects but do not impair viability^{4,22}. In *Vibrio cholerae*, a *dam* mutation is lethal²³, and may prevent replication initiation in both chromosomes²⁴. Dam methylation is also essential in certain strains of *Yersinia*²³.

Chromosome replication, nucleoid segregation and Dam methylation

The replication origin (*oriC*) of the *E. coli* chromosome contains 11 GATC sites in 254 bp, a density 10-fold higher than expected from a random distribution⁴. Chromosome replication starts when the initiator protein DnaA binds at the *oriC* and separates the two strands of the double helix²⁵. DnaA binding at the *oriC* region is only possible if the *oriC* GATCs are methylated; a hemimethylated origin is inactive^{4,25}.

Upon DNA replication, the GATC sites of *oriC* are not immediately methylated, and remain hemimethylated for up to one-third of the cell cycle²⁶. The cause of this delay is that the hemimethylated origin is sequestered by a protein called SeqA; as a consequence, in the daughter *oriC*'s, methylation of the newly synthesized strand by the Dam methylase is prevented^{27,28}. As long as SeqA-mediated hindrance of GATC methylation persists, the *oriC* remains hemimethylated, and the start of a new replication cycle is delayed²⁹.

Dam methylation also has a role in the regulation of DnaA synthesis. One of the promoters of the *dnaA* gene (*dnaA2*) is only active if its 3 GATC sites are methylated^{30,31}. Methylation of the *dnaA2* promoter is regulated by a sequestration mechanism analogous to that operating at *oriC*^{25,26}. Because *oriC* and *dnaA* are separated by only 50 kb, both regions may be sequestered almost simultaneously by SeqA^{26,32}. Besides SeqA, a protein of unknown function that binds to hemimethylated DNA has been shown to contribute to *dnaA* repression³³.

Hemimethylation of *oriC* provides a signal for nucleoid segregation. The hemimethylated origins of the two daughter chromosomes bind to segregation-driving proteins located in the outer membrane^{34,35}. SeqA also has a role in the process, as indicated by the observation that SeqA overproduction inhibits sister chromosome segregation and cell division³⁶. SeqA binds also to hemimethylated GATC sites behind replication forks, and may play a role in nucleoid organization³⁷⁻³⁹.

Dam-directed mismatch repair

Mismatched base pairs inevitably arise during DNA replication, and their repair requires a discrimination between the template strand and the newly synthesized, error-prone strand. This information is provided by transient lack of adenine methylation in the newly synthesized DNA strand⁴⁰. After recognition of a mismatched base pair by the MutS protein, a complex involving MutS, MutL and MutH is formed⁴⁰ (Figure 2). When part of the ternary complex assembled at a DNA mismatch, MutH is a GATC-specific endonuclease that cleaves the phosphodiester bond 5' to the G nucleotide in the non-methylated, newly synthesized DNA strand. Transient GATC hemimethylation in the newly synthesized strand thus provides the signal for strand discrimination by MutH⁴⁰. The resulting 3' end is a substrate for the UvrD helicase, and for exonucleases which degrade the daughter strand past the erroneously incorporated nucleotide^{40,41}. Resynthesis of the gap by DNA polymerase III and phosphodiester bond formation by ligase then follow⁴⁰.

MutH endonuclease is also active on unmethylated GATC duplexes. Hence, in Dam⁻ strains, assembly of the MutHLS complex at a mismatch can result in MutH-mediated cleavage of GATC sites in both DNA strands⁴². This explains the sensitivity of Dam⁻ mutants to agents that cause mismatches and other DNA lesions recognized by MutS⁴³⁻⁴⁶, as well as the dependence of Dam⁻ mutants on recombination and other DNA repair functions^{4,22}. Another relevant phenotype of Dam⁻ mutants is an elevated frequency of transition mutations, consistent with the failure of MutHLS to deal properly with mismatches^{4,22}. Interestingly, overproduction of Dam methylase results in higher mutation rates than its absence⁴. An explanation is that excess Dam methylase shortens the transient hemimethylation of newly replicated DNA molecules, thereby preventing MutH-mediated incisions at hemimethylated GATC's⁴. It must be noted that the level of Dam methylase is tightly adjusted to growth rate⁴⁷, probably to permit sufficient (but not excessive) delay in the methylation of newly replicated DNA.

Regulation of transposition by Dam methylation

In the bacterial transposons *IS10* and *IS50*, Dam methylation represses transposition by two independent mechanisms: (i) In the *IS10* transposase promoter, methylation of a GATC site overlapping the -10 module hinders binding of RNA polymerase and inhibits transcription of the transposase gene. Passage of the replication fork renders the GATC transiently hemimethylated, and permits transcription⁴⁸. In an analogous fashion, transcription of the *IS50* transposase gene from the P₁ promoter is repressed by methylation of two GATC sites located in the -10 module⁴⁹. (ii) In both *IS10* and *IS50*, methylation of GATCs at the ends of the transposon inhibit transposase activity at these ends⁴⁸⁻⁵⁰. In *IS10*, only one of the two hemimethylated species is active for transposition⁴⁸, an asymmetry that further reduces the frequency of *IS10* transposition and contributes to lower the mutation rate. An additional advantage of using Dam hemimethylation as a signal is that transposition occurs during DNA replication: a host cell with two chromosomes will more likely survive transposition events affecting essential loci. As in every host-pathogen interaction, increased host survival can be viewed as a favorable factor for transposon endurance.

Regulation of phase variation by Dam methylation

Phase variation, the reversible generation of variants of surface antigens, is frequent among pathogenic bacteria. Pap (pyelonephritis-associated pili) of uropathogenic *E. coli* mediate adhesion to the mucosa in the urinary tract⁵¹. Synthesis of Pap pili is turned ON or OFF by a mechanism that gives rise to two populations, one with pili, the other without. The switch rate from ON to OFF is 100-fold higher than from OFF to ON⁵¹. Because pili are highly immunogenic, phase variation may contribute to stealth invasion of the urinary tract. Furthermore, reduction of energetically expensive pili synthesis may have selective value for the population⁵².

Switching of Pap phase variation is controlled at the transcriptional level by a mechanism

involving Dam methylation and the leucine-responsive regulatory protein, Lrp⁵². The upstream-regulatory sequence of the *papBA* operon contains six binding sites for Lrp (Figure 3). Two of these sites contain GATC motifs (GATC^{dist}, located in site 5, and GATC^{prox}, located in site 2). In the OFF state, Lrp binds cooperatively and with high affinity to sites 1, 2, and 3, and prevents RNA polymerase binding and transcription of the *pap* operon. Lrp binding at sites 1-3 reduces the affinity of Lrp for sites 4, 5, and 6. Owing to Lrp binding at sites 1-3, methylation of nascent DNA molecules after passage of the replication fork is prevented, and GATC^{prox} becomes unmethylated after two rounds of replication. In contrast, the GATC^{dist} located in the unbound site 5 can undergo a normal cycle of hemimethylation / methylation. The high affinity of Lrp for the unmethylated GATC^{prox} and its inability to bind a methylated GATC^{dist} will create a feedback loop that propagates the OFF state. Hence, methylation of GATC^{dist} and unmethylation of GATC^{prox} are hallmarks the OFF state (Figure 3).

Switching to the ON state requires that Lrp is translocated to the cognate binding sites 4-6. One of them (number 5) contains GATC^{dist}. Translocation requires the ancillary protein PapI, which increases the affinity of Lrp for both sites 4-6 and 1-3, likely by binding to the GATCs present within these regions^{52,53}. However, the affinity of PapI/Lrp for sites 4-6 is much higher than for sites 1-3, tending to move PapI-Lrp to sites 4-6 at low PapI levels. Methylation of GATC^{prox} inhibits binding of PapI/Lrp, thus facilitating movement of PapI/Lrp to sites 4-6⁵³. Hence, unmethylation of GATC^{dist} and methylation of GATC^{prox} define the pattern of the ON state⁵². One of the *pap* products, PapB, activates *papI* transcription, thereby creating a positive feedback loop that will propagate the ON state⁵² (Figure 3).

David Low's model for *pap* regulation envisages that switching in both directions requires replication, and that a critical factor for switching is the PapI level⁵³. The latter is regulated by hitherto unknown mechanisms, and an appealing hypothesis is that PapI synthesis may be indirectly controlled by the ribosome by regulating PapB expression (D. A. Low, pers. comm.). Additional regulators of the *pap* operon include the nucleoid protein H-NS⁵² and the RimJ N-acetyltransferase⁵⁴ which repress Pap pili synthesis at low temperature, the cyclic AMP-dependent regulator CRP⁵², and the envelope stress system CpxAR⁵⁵.

In *E. coli*, synthesis of Prf and S pili, related to urinary tract infections, and Afa, K88, and CS31a pili, related to diarrheal infections, is also regulated by Dam and Lrp⁶. In *S. enterica*, synthesis of Pef fimbriae is regulated by a phase variation mechanism involving Dam, Lrp, the nucleoid protein H-NS, and the sigma factor RpoS⁵⁶. Hence, regulation by Dam methylation and Lrp seems to be frequent among fimbrial operons.

In the *E. coli agn43* gene, which encodes a non-fimbrial adhesin involved in autoaggregation and biofilm formation, Dam methylation prevents binding of the redox-sensitive regulator OxyR, a repressor of *agn43* transcription, to an operator located downstream of the transcription start site^{57,58}. Hence, Dam methylation is an activator of *agn43* transcription. As a consequence, Dam⁻ mutants are locked in the OFF state, and OxyR⁻ mutants are locked in the ON state^{58,59}. The *agn43* operator contains 3 GATC sites, and methylation of any two sites can block OxyR binding⁵⁷. In turn, OxyR binding to the *agn43* operator competes with Dam methylase, and prevents methylation of the operator⁵⁸. Switching from the ON to the OFF state requires OxyR binding to a hemimethylated operator after DNA replication, competing with the hemimethylated GATC-binding protein SeqA⁶⁰. Switching from the OFF to the ON state simply requires a decrease in active (oxidized) OxyR, which will lead to operator release; after DNA replication, the *agn43* operator will be methylated (Figure 4). Besides hindrance of repressor binding, Dam methylation plays a second role in the *agn43* promoter: methylation of the upstream GATC site increases transcription initiation, which occurs precisely at the G nucleotide of the GATC⁶¹.

Inheritance of Dam methylation patterns

Early studies envisaged that GATC sites remained hemimethylated for a short time lapse, and the occurrence of unmethylated DNA was viewed as a non-physiological condition^{4,6}. The discovery of SeqA introduced the notion that the duration of hemimethylation at certain GATCs could be extended by sequestration and concomitant hindrance of Dam methylase activity. However, SeqA-mediated sequestration is temporary, and the sequestered GATC

sites are fully methylated before cell division. In contrast, methylation-blocking proteins like Lrp and OxyR permit the formation of GATC sites that are stably undermethylated, and inherited as such⁶.

Heritable methylation patterns like those found in *pap* and *agn43* may not be rare exceptions. The *E. coli* chromosome contains at least 50 sites that are stably hemimethylated or unmethylated (see Box 1), and a subset of these are located in putative regulatory regions⁶. Interestingly, the distribution of undermethylated GATC sites in the *E. coli* genome varies depending on growth conditions, raising the possibility that transduction of environmental or physiological signals can change the methylation state of certain GATCs^{62,63}. DNA demethylases have not been described in bacteria; hence, active demethylation of GATC sites is unlikely to occur. Current evidence indicates that GATC undermethylation is caused by binding of proteins involved in gene regulation or nucleoid organization⁶. Local differences in the processivity of Dam methylase, which is influenced by the sequence context of GATC sites, may also contribute to GATC undermethylation⁶⁴.

Regulation of bacterial conjugation by Dam methylation

Conjugal transfer of the *Salmonella* virulence plasmid (pSLT) and the F sex factor is repressed by Dam methylation^{65,66}. In pSLT, repression of mating by Dam methylation involves two concerted actions: (i) transcriptional activation of the *finP* gene, the product of which is a small RNA that inhibits conjugation⁶⁷; and (ii) transcriptional repression of the *traJ* gene, which encodes a transcriptional activator of the transfer operon⁶⁸. At the *finP* promoter, Dam methylation prevents transcriptional repression by the nucleoid protein H-NS⁶⁷. At the *traJ* promoter, Dam methylation prevents binding of Lrp, an activator of *traJ* transcription⁶⁸. The upstream activating sequence (UAS) of *traJ* contains two Lrp-binding sites (Figure 5), both necessary for transcriptional activation. One site contains a GATC, and its methylation state affects Lrp binding: GATC methylation reduces Lrp binding, and residual Lrp protein bound to the UAS forms a complex that does not permit promoter activation. In contrast, GATC unmethylation and GATC hemimethylation permit Lrp binding, and formation of an

activating complex near the -35 module⁶⁸. This mechanism may regulate conjugal transfer in response to the occurrence of plasmid replication and the presence of sufficient Lrp⁶⁸.

A remarkable trait of *traJ* regulation by Lrp and Dam methylation is that hemimethylated DNA molecules exhibit different properties depending on the location of m6A, in a fashion reminiscent of *IS10* control⁴⁸. When the *traJ* non-coding strand is methylated, Lrp-mediated activation is permitted; when m6A lies on the *traJ* coding strand, activation by Lrp is hindered⁶⁸. As a consequence, newly replicated plasmid molecules will be in two different epigenetic states: one will permit Lrp binding and subsequent *traJ* synthesis, while the other will not. This epigenetic switch may moderate the activation of conjugal transfer, thereby limiting the metabolic and energetic burden placed on the host by the plasmid conjugation apparatus.

Roles of Dam methylation in bacterial virulence

Initial evidence for a relationship between Dam methylation and bacterial virulence was provided by the regulation of adhesin-encoding genes^{6,52}. However, the effect of *dam* mutations on the infection of model animals was first reported in *Salmonella enterica*⁶⁹⁻⁷¹. *Dam*⁻ mutants of *Salmonella* are attenuated in the mouse model⁶⁹⁻⁷¹, and present pleiotropic virulence-related defects: (i) Envelope instability with leakage of proteins and release of membrane vesicles⁷²; (ii) Ectopic expression of the Dam-repressed fimbrial operon *stdABC*, resulting in export of highly immunogenic fimbrial proteins (R. Balbontín *et al.*, in preparation); (iii) Reduced secretion of the SipC protein translocase encoded on pathogenicity island I⁷⁰; and (iv) sensitivity to bile^{46,72,73}. The relative contribution of these factors to the avirulence of *Salmonella* *Dam*⁻ mutants remains to be established, and additional components may be found. Envelope instability is not found in *E. coli* *Dam*⁻ mutants⁷², and this difference might partly explain why Dam methylation is not a major virulence factor in *Shigella flexneri*, a close relative of *E. coli*⁷⁴.

Salmonella *Dam*⁻ mutants persist in the liver, the spleen, and the lymph nodes of infected mice^{69,70}. This persistence, combined with loss of cytotoxicity to M cells⁷⁰, favors a strong

immune response in the infected animal^{71,75}. Use of *dam* mutations, alone or combined with others, may offer unique advantages for the design of *Salmonella*-based live vaccines⁷⁵. An undesirable trait, however, is the increased mutation rate typical of Dam⁻ mutants²².

Inactivation of Dam methylase in *Haemophilus influenzae* causes defects similar to those described in *Salmonella*, including avirulence in an animal model and reduced invasion of epithelial cells⁷⁶. Dam⁻ mutants are also attenuated in *Yersinia pseudotuberculosis*⁷⁷.

In bacterial species in which Dam⁻ mutants are not viable (e. g., *Vibrio cholerae* and certain strains of *Yersinia*) overproduction of Dam methylase is tolerated, and causes virulence attenuation²³. Dam-overproducing strains of *Yersinia pseudotuberculosis* and *Y. enterocolitica* show increased secretion of Yops (Yersinia outer proteins), a group of virulence proteins involved in inhibition of phagocytosis and of pro-inflammatory cytokine release^{78,79}. Overproduction of *E. coli* Dam methylase has also been shown to attenuate virulence in *Pasteurella multocida*⁸⁰.

Transcriptome analysis and proteomic studies have identified *E. coli* and *S. enterica* genes with impaired expression in Dam⁺ and Dam⁻ hosts^{38,81}. In *E. coli*, evidence exists that Dam methylation regulates metabolic pathways, respiration and motility in response to environmental cues⁸¹. In *S. enterica*, Dam methylation regulates genes encoding flagellar subunits, Braun lipoprotein and certain fimbriae, as well as genes required for invasion of epithelial cells (R. Balbontín *et al.*, in preparation). These data fulfill the prediction that Dam methylation regulates the expression of virulence genes in *Salmonella*⁶⁹, and raises the possibility that Dam-regulated genes are also found in other pathogens.

Dam methylation and bacteriophage infection

The genomes of certain phages of enteric bacteria have few GATC sites, while other phage genomes show a GATC content similar to that found in the bacterial host genome^{82,83}.

Scarcity of GATC sites in the genomes of virulent phages may protect against cutting by host

MutH⁸³. On the other hand, certain phages have *dam* genes which may contribute to protection against MutH by ensuring that GATC sites are methylated during the lytic cycle⁸³.

Dam methylation regulates transcription of the *cre* gene of phage P1⁸⁴ and the *mom* gene of Mu⁸⁵. The *mom* gene encodes a DNA modification function that generates N⁶-carboxy-methyl-adenine⁸⁶. This unusual, modified base protects Mu DNA from a variety of host-controlled restriction systems. Transcription of *mom* is regulated by Dam methylation in a fashion reminiscent of *agn43*: methylation of three closely spaced GATC sites prevents binding of the repressor protein OxyR. In the *mom* promoter, however, the OxyR binding region is located upstream the -35 module⁸⁵. OxyR is able to bind a hemimethylated *mom* promoter, and may limit *mom* expression during lytic phage development⁸⁵. This action may explain why Mom-mediated modification of Mu DNA is more efficient after induction of a lysogen than during an exogenous lytic cycle⁸⁵. Note that DNA methylation of phage DNA is usually incomplete during the lytic cycle, probably because the speed of rolling-circle replication exceeds the capacity of the Dam methylase⁸⁵.

Packaging of P1 DNA into capsids proceeds by a processive **headful mechanism** that uses concatemeric phage DNA molecules produced by rolling circle replication. Packaging is initiated at the *pac* site, a 162 bp sequence that contains seven GATC sites. Protein 9, the P1 packaging enzyme, can only cut *pac* if most of its GATC sites are methylated⁸⁷. P1 produces its own Dam methylase, and the importance of m6A in P1 infection is indicated by the observation that a P1 Dam⁻ mutant produces only 5% of the normal phage titer upon infection of a Dam⁻ host. A tentative explanation is that protein 9, which is the product of an early phage gene, binds hemimethylated *pac* sites produced by **theta replication**, and protects them from the host Dam methylase⁸⁸. P1 circular molecules with hemimethylated and unmethylated *pac* sites are thus produced. In the second stage of replication (**rolling circle**), P1 Dam methylase, the product of a late gene, is allowed to methylate only one *pac* site per concatemer; the other *pac* sites are protected (but not cut) by protein 9. This mechanism permits headful packaging, and avoids cutting of *pac* sites inside a concatemer⁸⁸.

The solitary CcrM methylase of α -Proteobacteria

The CcrM methylase was discovered in *Caulobacter crescentus*^{89,90}. Unlike Dam, CcrM belongs to the β group of DNA amino methyltransferases, and shares homology with the HinfI methylase of *H. influenzae*⁵. The target for CcrM is 5'-GANTC-3'. Like Dam, CcrM is highly processive; however, unlike Dam, CcrM is more active on hemimethylated DNA than on unmethylated DNA⁵. CcrM is an essential cell function, and participates in the regulation of the *Caulobacter* cell cycle^{5,89,90}.

CcrM homologs have been found in *Agrobacterium tumefaciens*⁵, *Rhizobium meliloti*⁵, and *Brucella abortus*⁹¹. In both *Agrobacterium* and *Rhizobium*, CcrM is essential and participates in cell cycle regulation, suggesting that the roles of CcrM methylation may be conserved among α -Proteobacteria⁵. CcrM is also essential in *Brucella*, and aberrant CcrM expression impairs the ability of this pathogen to grow inside macrophages⁹¹.

Regulation of the *Caulobacter* cell cycle by CcrM methylation

Caulobacter crescentus is a dimorphic bacterium with two different cell types: the replicating stalked cell and the non-replicating swarmer cell⁹⁰. These cells differ in morphology and behaviour: the swarmer cell harbors a flagellum and is motile, while the stalked cell is non-motile. Cell division yields one swarmer cell and one stalked cell⁹⁰. CcrM methylase is only produced during the late stage of chromosome replication, which occurs only in the stalked cell^{5,90}; the *Caulobacter* chromosome of stalked cells therefore remains hemimethylated until CcrM is produced. Activation of the *Caulobacter* replication origin (*Cori*) requires full methylation; hence, synthesis of CcrM methylase is a hallmark for completion of one cell cycle, and CcrM-mediated methylation of *Cori* provides a signal for the initiation of the next replication round⁵ (Figure 6).

Shortly after cell division, CcrM is degraded in both daughter cells^{5,90}. In the non-dividing

swarmer cell, chromosome replication is prevented by CtrA, a global regulator that plays a major role in cell cycle regulation. CtrA binds to the replication origin, which is methylated, and prevents replication initiation⁹⁰. During swarmer-to-stalked cell differentiation, CtrA is degraded, and remains undetectable until chromosome replication has started. Absence of CcrM prevents methylation after DNA replication, and leaves the origin (and most of the chromosome) hemimethylated until the late stages of replication, when a burst in CcrM synthesis occurs. Transcription of the *ccrM* gene is activated by CtrA, which progressively accumulates in the stalked cell as chromosome replication progresses^{5,90}. However, activation of *ccrM* transcription by CtrA is inhibited by methylation of two GANTC sites located in the leader of the *ccrM* coding sequence⁵. This inhibition may contribute to delay *ccrM* transcription until the replication fork reaches *ccrM*, and may serve to prevent earlier activation by CtrA^{5,90}.

Synthesis of the key cell cycle regulator CtrA is also regulated by GANTC methylation. One of the two *ctrA* promoters (P₁) contains a GANTC site near its -35 module⁹². Transcription from P₁ is repressed when the GANTC is methylated. Passage of the replication fork renders the promoter hemimethylated, and activates transcription. This mechanism may serve to boost *ctrA* gene transcription in response to replication progression. In turn, CtrA accumulation will turn on synthesis of the CcrM methylase as soon as the replication fork reaches the *ccrM* gene⁹². This model is supported by elegant genetic evidence: if the *ctrA* gene is moved to an ectopic position near the replication terminus, *ctrA* transcription from the methylation-sensitive P1 promoter remains repressed, and CtrA accumulates more slowly⁹².

m6A in eukaryotic genomes

The numerous functions performed by m6A in prokaryotic DNA contrast with the paucity of data regarding the role or even the presence of m6A in eukaryotes. Indeed, the first attempts to determine the base composition of eukaryotic DNA found m5C as the only methylated base in these genomes⁹³. However, there is now accumulating evidence demonstrating the presence of m6A in several lower eukaryotes⁹⁴. Thus, m6A has been

detected in the DNA of *Penicillium chrysogenum*⁹⁵, the green alga *Chlamydomonas reinhardtii*⁹⁶, and several ciliates such as *Oxytricha fallax*, *Paramecium aurelia*, *Stylonichia mytilius*, and *Tetrahymena pyriformis*⁹⁴. A distinct feature of ciliate protozoa is the existence of a nuclear dimorphism leading to two kinds of nuclei in the same cell: a germ line nucleus (micronucleus) and a polyploid somatic nucleus (macronucleus)⁹⁷. The finding that adenine methylation is restricted to macronuclear DNA has led to the suggestion that m6A could be involved in the control of deletions and chromosome fragmentation which occur during macronucleus formation⁹⁴. Unfortunately, proteins interacting with m6A in lower eukaryotes have not yet been characterized. Also, adenine DNA methyltransferase genes have been reported in *Chlorella* viruses⁹⁸. In some cases, these adenine DNA methyltransferases are associated with cognate restriction enzymes⁹⁹. The existence of viral adenine DNA methyltransferases suggests a potential for horizontal gene transfer among unicellular eukaryotes¹⁰⁰. A systematic study of the phylogenetic distribution of m6A in eukaryotes could determine whether vertical transmission or lateral gene transfer may explain the presence of m6A in some protists.

Perspectives and concluding remarks

Since the discovery of methylated bases in DNA, investigations on DNA methylation have mainly concerned m5C and mammals. Consequently, considerable progress has been made in understanding how DNA methylation affects transcriptional regulation, chromatin structure, and genome stability in higher eukaryotes. Quantitatively, less work has been performed on the role of adenine methylation in bacteria. However, an emerging idea is that adenine methylation in bacteria could be the counterpart of cytosine methylation in mammals. This offers an opportunity for the cross-fertilization of ideas between bacterial and mammalian epigenetic research.

The widespread functions of GATC and GANTC methylation in bacteria illustrate both the efficiency and the versatility of m6A as an epigenetic signal for the control of DNA-protein interactions. Methylation patterns at the *pap* operon^{51,52} and the *agn43* gene⁵⁸ illustrate how

E. coli lineages can inherit loci in different epigenetic states, a phenomenon reminiscent of **genomic imprinting**. In turn, differential regulation of *IS10* and *traJ* in sister DNA molecules^{48,68} shows that bacterial DNA molecules in different epigenetic states can co-exist in the same cell, as in **X-chromosome inactivation**. These examples show that epigenetic phenomena are not restricted to the kingdom Eukarya, and outline the utility of m6A as an epigenetic signal. Furthermore, the discovery of CcrM suggests that other solitary adenine methyltransferases may exist in bacteria. They can be expected to be identified when new whole genome sequences become available, and their study is likely to provide new insights into the biological roles of m6A.

Use of m6A to control DNA-protein interactions avoids the problem created by the high mutability of m5C, which undergoes relatively high frequencies of deamination, giving rise to thymine¹⁰¹. This might also explain why, in bacterial species that use m6A as an epigenetic signal, solitary m5C methyltransferases do not seem to have been selected to control crucial DNA-protein interactions. For example, no biological function has been yet found for Dcm in *E. coli*⁴. In higher eukaryotes, unrepaired T:G mismatches give rise to CG → TA transitions, which are known to be involved in many hereditary diseases and cancer^{8,102}. Hence, use of m5C instead of m6A by eukaryotes to control gene expression may be a less fortunate evolutionary event¹⁰³.

The finding that m6A is essential for growth or virulence in certain bacteria^{5,6} raises the possibility of using Dam or CcrM inhibitors as antimicrobial agents⁶⁴. However, the use of such alternative antibiotics requires the absence of m6A in the human genome. The current opinion that mammalian DNA is devoid of m6A is based upon analyses which have a detection limit of about 0.01%; hence the presence of thousands of m6A in the human genome cannot be excluded. Moreover, the recent discovery of a N⁶-adenine DNA methyltransferase in wheat coleoptiles¹⁰⁴ may stimulate a re-examination of the presence and the potential roles of m6A in higher eukaryotes.

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Legends to figures

Figure 1. Overview of the roles of m6A in enteric bacteria: (i) defense against phages and transposons; (ii) regulation of chromosome replication, chromosome segregation, and reorganization of the nucleoid after DNA replication; (iii) DNA strand discrimination for mismatch repair; (iv) regulation of conjugal transfer of plasmids; (v) packaging of phage DNA into capsids; and (vi) transcriptional regulation of fimbrial operons and other virulence genes. When known, the methylation-sensitive DNA-binding proteins involved in each process are also indicated.

Figure 2. Dam-directed mismatch repair. Assembly of the MutHLS complex at a mismatch is followed by MutH-mediated cleavage of the newly synthesized strand (shown in yellow) at the nearest GATC. Transient GATC hemimethylation in the newly synthesized strand provides the signal for strand discrimination. Depending on the distance, cleavage may require DNA looping (not drawn for simplicity).

Figure 3. Regulation of the *pap* operon of uropathogenic *E. coli* (reproduced from ref. 52). Top panel: DNA adenine methylation states of the *pap* regulatory region. In the ON state, binding of Lrp to sites 4, 5, and 6 prevents methylation of GATC^{dist}, while the unbound GATC^{prox} is methylated. In the OFF state, Lrp binding to sites 1, 2, and 3 prevents methylation of GATC^{prox}, while the unbound GATC^{dist} is methylated. Bottom panel: Model for switching from phase OFF to phase ON. (A) In the OFF state, Lrp binds to sites 1-3, and prevents methylation of GATC^{prox}. Non-methylated GATCs are shown as open circles, and methylated GATCs are shown as closed circles. Binding of Lrp to sites 1-3 reduces the affinity of Lrp for sites 4-6. (B) Every replication round offers an opportunity for switching. (C) In the presence of PapI, Lrp will translocate from sites 1-3 to sites 4-6. Lrp binding to GATC^{dist} will prevent methylation of the nascent DNA strand, while the unbound GATC^{prox} will undergo methylation. (D, E). If the intracellular concentration of cAMP is high, CRP will activate *papI* transcription. (F) PapB will stimulate *papI* transcription, creating a positive feedback loop that will perpetuate the ON state.

Figure 4. (Adapted from ref. 60). Model for switching in the *agn43* gene. Methylation of GATC sites in the *agn43* operator prevents binding of the OxyR repressor, and defines the ON state. After DNA replication, SeqA and OxyR compete for binding to the hemimethylated operator. SeqA binding is only transient and permits GATC methylation, thus propagating the ON state. In contrast, OxyR binding hinders GATC methylation, and *agn43* is switched OFF in the following replication round. Switching to the ON state occurs when OxyR leaves the operator, permitting GATC methylation.

Figure 5. Model for regulation of *traJ* transcription in the *Salmonella* virulence plasmid. The transcriptional activator Lrp binds to two cognate sites in the *traJ* UAS. Lrp binding to the downstream site is inhibited by GATC methylation; as a consequence, *traJ* is not transcribed in a non-replicating plasmid. Passage of the replication fork leaves the *traJ* UAS hemimethylated. However, activation of transcription occurs only in one daughter plasmid DNA molecule, because methylation of the coding strand does not permit formation of the Lrp-DNA activating complex.

Figure 6. (Adapted from ref. 5). In *Caulobacter crescentus*, asymmetric cell division produces a non-replicating swarmer cell and a replicating stalked cell. In the swarmer cell, CtrA binding to the chromosomal origin of replication (*Cori*) inhibits DNA replication initiation, whereas CtrA proteolysis allows replication to occur in the stalked cell. Hence, DNA replication is only initiated on the methylated *Cori* of the stalked cell. Because CcrM methylase is degraded after cell division, passage of the replication fork is not followed by methylation of the daughter DNA strands. The extent of hemimethylation indicates the distance covered by the replication machinery, and can be viewed as a measure of time. The existence of DNA-binding proteins (e. g., transcription factors) that recognize hemimethylated sites could thus permit an ordered sequence of events occurring during DNA synthesis.