SURVEY AND SUMMARY

Beta class amino methyltransferases from bacteria to humans: evolution and structural consequences

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ABSTRACT

S-adenosyl-L-methionine dependent methyltransferases catalyze methyl transfers onto a wide variety of target molecules, including DNA and RNA. We discuss a family of methyltransferases, those that act on the amino groups of adenine or cytosine in DNA, have conserved motifs in a particular order in their amino acid sequence, and are referred to as class beta MTases. Members of this class include M.EcoGII and M.EcoP15I from Escherichia coli, Caulobacter crescentus cell cycle–regulated DNA methyltransferase (CcrM), the MTA1-MTA9 complex from the ciliate Oxytricha, and the mammalian MettL3-MettL14 complex. These methyltransferases all generate N6-methyladenine in DNA, with some members having activity on single-stranded DNA as well as RNA. The beta class of methyltransferases has a unique multimeric feature, forming either homo- or hetero-dimers, allowing the enzyme to use division of labor between two subunits in terms of substrate recognition and methylation. We suggest that M.EcoGII may represent an ancestral form of these enzymes, as its activity is independent of the nucleic acid type (RNA or DNA), its strandedness (single or double), and its sequence (aside from the target adenine).

INTRODUCTION

MTase families defined by amino acid sequences

Early analysis, of the amino acid sequences of 13 bacterial DNA methyltransferases (MTases) generating 5-methylcytosine (5mC), revealed a set of ten conserved blocks of amino acid residues (1). These conserved motifs, numbered I to X from amino to carboxyl end, were found to have a constant linear order, simplifying their identification in protein sequences (particularly for the shorter or less-conserved motifs), though one alternative permutation of 5mC MTase motif order was later found (2,3). This aided in the discovery of mammalian 5mC-generating DNA methyltransferases Dnmt1 (4) and Dnmt3 (5). The structural analysis of HhaI MTase, from the bacterium Haemophilus haemolyticus, allowed the functions of conserved sequence motifs to be inferred, particularly those responsible for SAM binding (motif I consensus: FxGxG) and for the chemistry of methyltransfer reaction on an inert carbon (motif IV consensus: PC), along with a varied target recognition domain (TRD) for specific binding of the substrate DNA sequence (6,7).

Not surprisingly, the same set of sequence motifs also occur in mammalian Dnmt2 (8–10), a tRNA 5mCMTase (11). The conservation in Dnmt sequence and structure reflects the conserved nature of SAM binding, which occurs so as to optimize the catalysis of methyl transfer onto cytosine-C5 in nucleic acids. Before and after the discovery that Dnmt2 homologs are actually tRNA MTases, a number of studies on the activity of Dnmt2 on DNA yielded kinetic rate constants varying between zero and very low, though the human enzyme can methylate cytidine in a short segment of single-stranded DNA ligated into a tRNA molecule (12).

In addition to cytosine-C5, methylated bases on DNA include N4-methylcytosine (N4mC) and N6-methyladenine (N6mA), both of which are modified on their exocyclic primary amines. Bacteriophages, particularly lytic ones, modify their DNA in many additional ways, including other methylations (13), but our focus here is on methylations that are consistent with normal cell physiology. Recognition
of the base to be methylated, and access to it by catalytic residues, occurs after the base is swung out of the DNA duplex into a typical concave binding pocket, in a process known as ‘base flipping’ (14–16). Interestingly, the DNA MTases that generate N4mC and N6mA do not group separately from one another on the basis of sequence (17). This highlights the constraints of the chemistry of amino methylation as being evolutionarily dominant, instead of recognition of cytosine versus adenine in the active-site binding pocket. In fact, there is evidence of independent derivations of N4mC MTases from N6mA MTases (18), and at least one MTase that generates N4mC can generate N6mA on a DNA substrate in which a C-to-A substitution is made at the target base (19).

A multiple sequence alignment of 42 known DNA amino MTases revealed the existence of (at least) three classes of amino MTases, differing from one another by circular permutation in their order of motifs important for three essential functions: binding the methyl donor SAM, binding substrate DNA and catalyzing the chemical reaction between the donor and substrate (20) (Figure 1). Briefly, class α is arranged in the order (N-to-C termini): motif I (SAM-binding), TRD (substrate recognition and binding), and motif IV (methylation catalysis). Class β is arranged in the order: motif IV-TRD-motif I. Class γ is arranged in the order: motif I-motif IV-TRD. The class γ amino MTases, exemplified by M.TaqI (21,22), have the motif order comparable to that of the 5mC MTases (23). The residues of motif IV vary with the target atom—the PC (proline-cysteine) motif in 5mC MTases is responsible for ring carbon-C5 methylation of cytosine, with the Cys sulfhydryl acting as a nucleophile to attack the cytosine ring and initiate the reaction (24–27). In contrast, motif IV for MTases catalyzing exocyclic amino methylation to generate N6mA or N4mC have the four-residue consensus (D/N/S)-P-P-(Y/F/W) [(Asp/Asn/Ser)-Pro-Pro-(Tyr/Phes/Tpr)], where the last bulky hydrophobic side chain stacks against the target base, while the first one abstracts a proton from the target amino group (22,28).

As was illustrated by Dnmt2, the apparent sequence and structural similarities do not reveal with certainty whether an enzyme acts on a particular methylation substrate (DNA, RNA or protein). Other examples include *Escherichia coli* HemK and human HemK2, which were thought to be DNA N6mA MTases (29,30), yet were found to be protein MTases active on glutamine (31–36). The human HemK2 is also active as a histone lysine MTase (37). The common feature of the potential substrates is the amino group (NH2) of protein glutamine or lysine (in the deproto- nated state) or DNA adenine, at which three methylation reactions are catalyzed by a NPPY motif (a subset of the motif IV possibilities).

While the MTase families differ in motif order, their structures are remarkably similar, comprising a seven-stranded β sheet (1–to-7) with a central topological switchpoint between strands β1 and β4, and a characteristic reversed β hairpin (β6 and β7) at one end of the sheet next to strand β5 (Figure 2A and B). This topology allows for circular permutation, where the same structure simply has the break between its amino beginning and carboxyl end at different points (38) and, in fact, it is possible to circularly permute a MTase in the laboratory with retention of function (3). The two most significant motifs – motif I (FxGxG) for binding SAM and motif IV (DPPY) for binding substrate Ade – juxtapose the target N6 atom of Ade in line with the methyl group and sulfur atom of SAM for catalysis, and are positioned at the carboxyl ends of the two parallel neighboring strands β1 and β4 (Figure 2C). Based on the chemical similarity of the SAM-adenosyl and DNA-adenosyl moieties, it makes sense that the two structural elements responsible for the binding (β1–β2 and β4–β5) do, in fact, have striking similarity (20). It was suggested that the original MTases arose after tandem gene duplication (39) converted a SAM-binding domain into a protein that bound two molecules of SAM (20), and there is some evidence to support that hypothesis (40).

Based on the proposal that the ancestral tandem duplication had two copies of motif I (represented as I–I), one evolutionary model is that the two halves diverged with one SAM-binding motif I changing to an adenine-binding motif IV (Figure 1A). If this change involved the amino-proximal motif I, it would yield the ancestor to Class β MTases (IV–I), while conversion of the carboxyl-proximal SAM binding site would yield the ancestor to Class α and γ MTases (I–IV). An alternative would be that just one of the two changed, with the other motif order occurring subsequently via a true circular permutation event (38,41) (not shown). Presumably, the ancestral MTase(s) as described here would methylate free adenine or a free adenine nucleotide, perhaps contributing to pre-protein metabolism (42,43).

This ancestral nucleotide MTase could then have gained the ability to modify DNA or RNA adenines following an additional fusion of the target (nucleic acid substrate) recognition domain (TRD, Figure 1B). The three classes are roughly the same size in number of known members (Figure 1C). While the majority of 5mC and N4mC DNA MTases each fall into single classes defined by motif order, the N6mA MTases are fairly evenly distributed among the three classes (α, β and γ) (20). This could also be explained by the tandem duplication model in which the ancestral nucleic acid MTase(s) generated N6mA. In addition to considering MTase evolution, we also note here that the β arrangement of motifs has intrinsic structural consequences that, we suggest, impose a unique requirement for this family of MTases to dimerize in order to function. We begin by comparing two MTases that play similar biochemical and physiological roles, but one of them is a β MTase and the other is not.

### *Escherichia coli* Dam versus *Caulobacter crescentus* CcrM (monomer vs. dimer)

The great majority of bacterial and archaeal DNA MTases are associated with restriction–modification (RM) systems, where the MTase protects a cell’s own DNA from digestion by the paired (cognate) restriction endonuclease (44,45). RM systems are important for defense against bacteriophage predation (46,47), although they play other roles as well (48,49). Bacterial ‘orphan’ MTases are so named as, unlike most bacterial DNA MTases, they are
not paired with a restriction endonuclease as part of a RM system (50). Orphan MTases are sometimes involved in chromosome replication, DNA repair, and epigenetic gene regulation (51). Examples of such regulatory orphan MTases include the DNA adenine MTase (Dam) in *Escherichia coli* and cell cycle-regulated DNA MTase (CcrM) in *Caulobacter crescentus* (Alphaproteobacteria) which are, respectively, responsible for maintenance of daughter strand adenine methylation at GATC or GAnTC sequences (n = any nucleotide), immediately after their replication (52,53). We will first compare these two ‘orphan’ enzymes, and then consider β MTases associated with RM systems, and finally examine the mammalian β MTases.

Although the substrate sequences of Dam (GATC) and CcrM (GAnTC) are both palindromic, both enzymes act on hemi-methylated DNA substrates (Figure 2G-H) – that is, sequences that already contain N6mA in one strand. Both enzymes can act on unmethylated sites, but most often encounter hemimethylated sites resulting from replication of fully-methylated sites, and both enzymes show a preference for hemimethylated over unmethylated sites (54,55).

Besides the aforementioned difference in the order of motifs (Dam is a member of class α and CcrM is a class β MTase), there are three major differences between the two enzymes. First, though both enzymes use an arginine to interact with a 5′ guanine (Figure 2G and H) and both Arg-Gua interactions are sequence-discriminatory contacts, these contacts are made to different strands relative to the substrate Ade. In Dam, the recognized Gua is on the opposite strand, and 2-base pairs away, from the target Ade; whereas in CcrM, the recognized Gua is on the same strand as, and adjacent to, the target Ade. Second, except for the flipped-out target adenine, the Dam-bound DNA conformation has intact intra-helical paired bases, whereas CcrM pulls the two DNA strands apart, creating a bubble comprising four enzyme-recognized, unpaired bases (Figure 2I). These two features of CcrM, strand separation and base recognition on the same strand that contains the target Ade, allow CcrM (but not Dam) to be active on both double-stranded (ds) and single-stranded (ss) DNA, as well as on mismatches within or immediately outside of the recognition sequence (56). Neither enzyme is active on ssRNA. Lastly, Dam is a monomer under most conditions (57,58), while CcrM is a homodimer (Figure 3A) (59), reflecting a broader phenomenon that we will discuss next.

**Are class-β MTases required to form homodimers for activity?**

The homodimeric subunit structure of CcrM has been observed previously in other MTases of structurally-characterized class β MTases. M.PvuII of *Proteus vulgaris* is (at this writing) the only structurally-characterized N4mC MTase (60), and yielded the first structure for a MTase in the β family. It methylates the central cytosine in its symmetric recognition sequence 5′-CAGCTG-3′. The M.PvuII structure, consistent with the circular permutation model, shares a common fold with MTases of other families, while having the major functional regions (particularly motifs I and IV) permuted into distinct linear order. The major unexpected finding was that M.PvuII forms a homodimer (Figure 3B). This dimeric feature was subsequently observed by others in other β MTase structures. M.RsI, from *Rhodobacter sphaeroides* (61,62) (Figure 3C), methylates the internal adenine of the palindromic DNA sequence GAATTC, and its dimeric structure was consistent with earlier biochemical evidence for this MTase (63,64). M1.MboII, from *Moraxella bovis* (Figure 3D), methylates the 3′ adenine of an asymmetric sequence 5′-GAAGA-3′, and the homodimeric structure again supported by biochemical results (65). Dimer formation was also observed in...
Figure 2. Comparison of Dam (class α) and CcrM (class β). (A, B) The catalytic domain of the seven-stranded β structure in Dam (panel A) and CcrM (panel B). (C) Interactions with the SAM–adenosyl and DNA–adenosyl moieties in the active-site of CcrM. The phenylalanine of motif I provides an edge-to-face interaction to the face of SAM-adenosyl ring. The DPPY motif interacts with DNA adenine. (D–F) The locations of motif I and motif IV are reversed in the amino acid sequences of Dam and CcrM. (G) Dam interacts with guanine G4 of the non-target strand. (H) CcrM interacts with guanine G1 of the target strand. The underlined letter A in red is the methylation target. (I) Two strand separation in the CcrM-bound DNA.

structures for THA0409 from *Thermus thermophilus* HB8 (Figure 3E) (66) and M1.HpyAVI from Helicobacter pylori (Figure 3F) (67). Furthermore, even where structures do not yet exist, there is biochemical evidence for dimerization in several other class β MTases (M.BamHI (68), M.LiaCI (69), M.KpnI (70,71), M.HpyAXVII (72)). However, none of these class β MTases were structurally characterized in complex with DNA, and their postulated TRDs were disordered in the absence of bound DNA, so the significance of the dimeric character was not grasped until the structure determination of M.EcoP15I.

‘Division of labor’ between two dimeric subunits of M.EcoP15I homodimers

M.EcoP15I is an archetype of the Type III RM family, and consists of two methylation (M) and 0–2 restriction (R) subunits (73), resulting in M₂, M₂R₁ or M₂R₂ complexes. The M.EcoP15I M-subunit functions as a dimer (74,75) but recognizes an asymmetric sequence, 5‘-CAGCAG-3‘ and methylates the 3‘ internal adenine of the A-containing strand of the dsDNA (the complementary strand is 5‘-CTGCTG-3‘). Other Type III MTases have also been shown biochemically to form dimers in solution (76,77).

The M.EcoP15I structure is the first one for a class-β amino MTase bound to its substrate DNA, and it suggests a division of labor between two M subunits in terms of DNA recognition (one M subunit) and catalysis of methyltransfer (the other M subunit) (78) (Figure 3G). The DNA-recognition subunit of the homodimer provides side chains, such as Arg305 and Arg350, for recognition of guanines on the non-target (non-A-containing) strand (Figure 3H). These Arg-Gua interactions with the non-target strand are reminiscent of *Escherichia coli* Dam (Figure 2G), and (like Dam) limit M.EcoP15I to methylating dsDNA and not ssDNA. In contrast, while differing roles for each subunit are also seen for CcrM, each protomer contacts primarily a different DNA strand. Specifically, one CcrM subunit binds the target strand, recognizes the target sequence and catalyzes methyl transfer, while the second molecule simply binds the non-target strand (in the case of a dsDNA substrate) (Figure 3A) (59). This division of labor allows CcrM to methylate both ds and ssDNA.

Looking at these dimeric class β MTase structures together, whether in the presence or absence of DNA, they share a striking feature—the catalytic domain of one subunit and the DNA binding domain of the second subunit are arranged so as to face each other, forming one integral binding surface appropriate for one DNA duplex (dashed circles in Figure 3A, C and G). In contrast, the catalytic site and DNA binding of the same subunit face away from one another, such that they cannot cooperate to accomplish the DNA recognition and methylation in one binding event. This is distinct from the association of MTases with other proteins in order to modulate their activity (see Discussion); and also implies that—while there may be kinetic or other reasons for which dimerization may be advantageous to other MTases (examples include mammalian Dnm3L-3a-3a-3L tetramer (79–81))—for the β MTases dimerization is essential for activity.
A sequence non-specific class $\beta$ MTase: M.EcoGII

M.EcoGII is encoded in the genome of the pathogenic strain $E. coli$ O104:H4 C227–11 (82), which was responsible for a severe outbreak of hemorrhagic uremia in Europe (83). The gene appears to reside within an integrated prophage genome, but is not expressed during normal bacterial growth. When the gene encoding M.EcoGII is expressed in vivo - using a high copy plasmid vector and a methylation-deficient $E. coli$ host—extensive in vivo adenine methylation activity is revealed. It appears that M.EcoGII methylates adenine residues in any sequence context, in DNA and RNA, double-stranded and single stranded, and even adenine bases in either strand of a DNA/RNA-hybrid oligonucleotide duplex (84). There is not yet a M.EcoGII-DNA complex structure, though modeling based on a homolog (using threading with the M.EcoP15I-DNA structure) predicts a dimeric M.EcoGII complexed with one duplex (Figure 4A). Strikingly, from Figure 4A it appears to be impossible for M.EcoGII to function as a monomer. If it does function as a dimer, which seems likely, it is surprising that there appears to be no quadratic rate dependence of activity on the enzyme concentration [see figure 4 in (84)]. In contrast, such rate dependence is clear for two other $\beta$ MTases, M.KpnI and M.RsrI [see figure 4 in (64) and (70)]. One possible explanation of this apparent paradox is that M.EcoGII has a particularly high dimerization constant. In fact, the known homodimer CcrM also shows a linear rate dependence on the enzyme concentration [see figure 7 in (85)].

Even if we assume that M.EcoGII functions as a dimer, however, it is unclear how the 'division of labor' between the two M.EcoGII subunits is accomplished, given its lack of sequence specificity, and the fact that recognition and methylation occur to the same nucleotide. Considering the aforementioned gene duplication model of a tandemly-duplicated SAM-binding protein having one SAM pocket diverge to bind adenine, M.EcoGII might be an example of such a primordial adenine MTase, lacking a well-defined and functional TRD.

However, while it is difficult to identify TRDs from sequence analysis alone (86–88), there is no evidence that the region in which the TRD is expected to occur in M.EcoGII and its orthologs is unusual (e.g., smaller than in other MTases; not shown). Thus it is also possible that M.EcoGII began as a standard N6mA MTase and lost specificity, under selection for its role in the physiology of the phage that carries it. In this regard, it is noteworthy that some class-$\beta$ RM MTases tested under certain solvent conditions can in fact methylate the DNA strand of a DNA/RNA hybrid duplex (89).

A heterodimeric class $\beta$ MTase: MTA1-MTA9 in the ciliate Oxytricha

In contrast to metazoas, DNA N6mA is abundant in various unicellular eukaryotes, including ciliates, the green alga Chlamydomonas, and early-diverging fungi (90–92). A recent study identified four ciliate proteins—two class-$\beta$-like MTases (MTA1 and MTA9) and two homeobox-like DNA binding proteins—as being necessary for deposition of N6mA in the Oxytricha genome (93). Instead of
Figure 4. Homology models of class β MTases. (A) A model of the M.EcoGII–DNA complex based on the actual M.EcoP15I–DNA complex structure. (B) Sequence alignment of motif IV and motif I of class β MTases analyzed in this study. (C) A model of ciliate MTA1 and MTA9 heterodimer based on MettL3-MettL14. (D) A structure of human MettL3-MettL14 heterodimer complex (Structural Genomics Consortium). (E) A dimer model of human MettL4 based on MettL3–MettL14.

a gene-targeting approach, the authors used classic biochemistry by identifying candidate proteins, in nuclear extracts, that co-purified with DNA MTase activity. A conserved catalytic motif IV (DPPW) is preserved in MTA1, but not MTA9 (which has an eroded motif NALW) (Figure 4B). The four-subunit ciliate MTase complex preferentially methylates ApT dinucleotides in dsDNA – in agreement with earlier observations that in the unicellular eukaryotes Saccharomyces cerevisiae, Chlamydomonas reinhardtii and Tetrahymena thermophila, N6mA is enriched in ApT dinucleotides (92) within nucleosome linker regions near promoters (94). This ApT sequence is symmetrical, like the CpG methylation target in mammals, suggesting a similar mechanism for maintaining methylation following DNA replication. Indeed, the MTase activity was even higher on hemimethylated than on unmethylated dsDNA, though it showed no activity on ssDNA or RNA (93). Homology modeling suggests a heterodimeric association of MTA1 and MTA9 (Figure 4C), in analogy to mammalian MettL3-MettL14, which we will discuss next.

Heterodimeric complex of mammalian MettL3–MettL14

Detection of N6mA in mammalian DNA was reported only recently, but the role of MettL3 and MettL14 in generating N6mA in RNA has been known for some time. In mammalian (HeLa) cells, the mRNA N6mA MTase activity on the degenerate consensus sequence RRACH (R = purine, H is not a G) (95) requires at least two separate subunits, MT-A and MT-B (96). MT-A is itself a multimeric protein, that contains a 70-kDa MT-A70 subunit (now known as MettL3, containing 580-residues and with a predicted molecular weight of 65 kDa). The amino acid sequence of MT-A70/MettL3 was noted to include conserved motifs (e.g. motif IV: DPPW) of the bacterial DNA MTases but not of known RNA MTases (97). MettL3 and MettL14 form a heterodimeric complex (98), and have been studied widely for their role in generating N6mA in RNA (99,100) and references therein). Like the ciliate MTA1 and MTA9 (preceding section), mammalian MettL3 is the catalytically active subunit, while MettL14 has an eroded motif IV (EPPL) (Figure 4B). An earlier study suggested that MettL14 catalyzed RNA methylation (98). Unlike the ciliate complex, which requires two additional DNA binding subunits, MettL3 itself contains two tandem CCCH-type zinc fingers within its polypeptide, located N-terminal to its MTase domain, that are necessary for RNA binding and thus enzymatic activity (101). Four research groups have independently determined the structures of the heterodimeric complex of the MTase domains of MettL3 and MettL14 ((102–104) and Structural Genomics Consortium) (Figure 4D). However, these partial complexes, lacking the CCCH-
zinc fingers, possess no enzymatic activity and there is no RNA substrate-bound complex structure currently available for MettL3–MettL14.

Low levels of N6mA have been reported in DNA from mouse (105,106), human (30,107), and human malignant brain tumor glioblastoma (108), though other studies have failed to detect N6mA in mammalian genomes (109–111). Two reports present evidence that N6mA in mammalian DNA can result from incorporation of RNA-derived ribo-N6mA that was converted to deoxy-N6mA via the nucleotide salvage pathway (112,113). Interestingly, the three class β MTases MettL3, MettL4, and MettL14, from mouse and human, were recently assessed for the ability to generate N6mA on DNA (114,115). These three proteins were previously considered to be analogs of the MT-A70 subunit of the human mRNA N6mA MTase (116).

Prompted by the observations noted above, revealing that characterized class β MTases are active for DNA N6mA methylation, with some members having activity on ssDNA (CcrM) as well as ssRNA (M.EcoGII), we investigated whether the MettL3/MettL14 heterodimer also possesses methyl transfer activity onto DNA adenine. With synthetic substrate oligonucleotides, MettL3-MettL14 shows >10-fold stronger catalytic efficiency of methylation on ssDNA than the corresponding ssRNA under the same conditions (114). Furthermore, MettL3-MettL14 is active on an unpaired region in overall context of dsDNA. This appears to be consistent with the requirement of MettL3 methylation activity for DNA repair (117).

Mammalian MettL4

The third member of MT-A70 class β MTase in human and mouse is MettL4 (116). The C. elegans homolog was the first of the MettL4 family members to be considered as a candidate DNA N6mA MTase, but there was no enzymatic evidence demonstrating that C. elegans MettL4 has intrinsic DNA N6mA activity (118). Murine MettL4 was reported to be responsible for N6mA deposition in genic elements associated with transcriptional silencing (106). Curiously, recombinant human MettL4 expressed in HEK293T (human embryonic kidney) cells has in vitro enzymatic activity on mitochondrial DNA (115), whereas recombinant human MettL4 purified from E. coli has RNA MTase activity (119). The former study showed that MettL4 localizes within mitochondria in tested tissues (115), and examination of 23 high-confidence N6mA-enriched sites of mitochondrial DNA revealed an ApT containing consensus sequence—CTTATC (in the main text) or CT(C/A)ATC (in the figure S2E of (115))—agrees at least partially with an earlier study that N6mA sites across the mitochondrial genome were generally at an ApT dinucleotide (107).

In contrast, the latter study found mainly nuclear localization of MettL4 expressed from a lentiviral vector, and failed to identify appreciable levels of N6mA in mitochondrial DNA, but did find N6mA in a small nuclear RNA (the spliceosome-associated U2 snRNA) (119). The differences among these various studies illustrates the complex nature of DNA versus RNA adenine methylation in mammalian genomes (genomic vs. mitochondrial). The accumulation of N6mA in DNA and/or RNA might also reflect diverse cellular and mitochondrial stress responses in different cell lines under different laboratory conditions. Homology modeling suggested a dimeric MettL4, but was indeterminate as to whether it is a homodimer or heterodimer (the latter of which would require a yet-to-be-identified binding partner) (Figure 4E). A proper biochemical approach, such as the one used to identify the ciliate complex (93), will be needed to identify necessary components of the catalytically active MettL4 MTase complex.

DISCUSSION

Here, we build on earlier suggestions that an ancestral protein that bound a single molecule of SAM evolved into an adenosine-binding protein; undergoing first tandem gene duplication, and then divergence of the two SAM-binding pockets (Figure 1A). M.EcoGII might reflect properties of the ancestral enzymes, as it acts on any form of nucleic acid (ss or ds, RNA or DNA), in any sequence context, though many nucleic acid-modifying enzymes are still able to modify both DNA and RNA ((120) and references therein). To become a sequence-specific DNA/RNA adenine MTase, an additional fusion (or selection acting on an exposed surface loop) would have brought in a target recognition domain (TRD) (Figure 1B). Distinguishing between gain of specificity from a promiscuous ancestral MTase, and loss of specificity from a more recent sequence-specific MTase, is not straightforward. This question has been addressed in some cases, for example among metabolic enzymes (121), where increased specificity is seen in essential or higher-flux enzymes, or via reconstruction of ancestral enzymes from...
phyllogenetic analyses, e.g. (122–126). It might be revealing to carry out such analysis with M.EcoGII, or even to determine if it can methylate various adenine derivatives.

The unique dimeric feature of class β MTases, either in homo- or hetero-form, allows the enzyme to use ‘division of labor’ between two subunits in terms of DNA recognition and methylation. This division could well have resulted from the development or importation of a TRD in an orientation opposite to that of the methylation domain of the same polypeptide, thus requiring the two domains from each subunit to face each other to form one integral functional surface. In contrast, the monomeric enzymes in class α (E. coli Dam), class γ (M.TaqI) and 5mC class (M.HhaI) have TRDs in the same orientation as the methylation domain. Examining the evolved to be non-nucleic acid enzymes (127). We wish MTases discussed here—have the seven-stranded catalytic core of each subunit to face each other to form one integral function.

The opposing TRD orientation in the class β MTases may help explain why they are primarily nucleic acid enzymes. Examining ~30 human methyltransferase-like proteins (MettL1 to 27), all—except the three class β MTases discussed here—have the seven-stranded catalytic domain arising from motif order of I-to-IV, and many of them evolved to be non-nucleic acid enzymes (127). We wish to clarify a distinction between the ‘division of labor’ in homodimeric MTases, and non-β MTases that participate in protein–protein interactions for other purposes. For example, Trm112, named for its role in tRNA methylation, is a relatively small protein conserved in all three domains of life (128). In S. cerevisiae, Trm112 interacts with and activates other dimers, while other MTase classes may or may not form dimers, and that this is an intrinsic property distinct from other protein-protein interactions in which MTases may engage.

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**CONFLICT OF INTEREST STATEMENT**

None declared.

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