Active site mapping and substrate specificity of bacterial Hen1, a manganese-dependent 3' terminal RNA ribose 2'O-methyltransferase

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ABSTRACT

The RNA methyltransferase Hen1 and the RNA end-healing/sealing enzyme Pnkp comprise an RNA repair system encoded by an operon-like cassette present in bacteria from eight different phyla. *Clostridium thermocellum* Hen1 (*Cth*Hen1) is a manganese-dependent RNA ribose 2'O-methyltransferase that marks the 3' terminal nucleoside of broken RNAs and protects repair junctions from iterative damage by transesterifying endonucleases. Here we used the crystal structure of the homologous plant Hen1 to guide a mutational analysis of *Cth*Hen1, the results of which provide new insights to RNA end recognition and catalysis. We illuminated structure-activity relations at eight essential constituents of the active site implicated in binding the 3' dinucleotide of the RNA methyl acceptor (Arg273, Arg414), the manganese cofactor (Glu366, Glu369, His370, His418), and the AdoMet methyl donor (Asp291, Asp316). We investigated the effects of varying the terminal nucleobase, RNA size, RNA content, and RNA secondary structure on methyl acceptor activity. Key findings are as follows. *Cth*Hen1 displayed a fourfold preference for guanosine as the terminal nucleoside. RNA size had little impact in the range of 12–24 nucleotides, but activity declined sharply with a 9-mer. *Cth*Hen1 was adept at methylating a polynucleotide composed of 23 deoxyribonucleotides and one 3' terminal ribonucleotide, signifying that it has no strict RNA specificity beyond the 3' nucleoside. *Cth*Hen1 methylated RNA ends in the context of duplex secondary structures. These properties distinguish bacterial Hen1 from plant and metazoan homologs.

Keywords: AdoMet; transmethylation; RNA end-healing; RNA repair

INTRODUCTION

Programmed RNA breakage by site-specific nucleases is an ancient mechanism of responding to cellular stress, by activation of latent intracellular “ribotoxins,” and also for distinguishing self from nonself, by secreting ribotoxins that kill nonself-competitors (Ogawa et al. 1999; Tomita et al. 2000; Lu et al. 2005). Most known ribotoxins are transesterifying endonucleases that generate 5'-OH and 2',3' cyclic phosphate termini. Repairing this type of damage is feasible, and entails sequential enzymatic end-healing and end-sealing steps (for review, see Nandakumar et al. 2008). In the healing phase, the 2',3' cyclic phosphate end is hydrolyzed to a 3'-OH and the 5'-OH end is phosphor-

ylated by an NTP-dependent polynucleotide kinase to yield a 5'-monophosphate. The healed 3'-OH and 5'-PO₄ termini are then suitable substrates for sealing by an RNA ligase that restores the 3',5' phosphodiester backbone. The biological impact of RNA repair pathways is known for only a few cases of programmed RNA damage. For example, RNA repair can ameliorate ribotoxicity, as in phage tRNA restriction-repair (Amitsur et al. 1987), or it can generate new informational RNAs, as in the eukaryal unfolded protein response (Gonzalez et al. 1999).

There is a growing appreciation of the prevalence of RNA repair enzymes in many diverse taxa that, unfortunately, exceeds our current understanding of their physiology (in no small part because the organisms that have candidate RNA repair systems are not genetically tractable). Nonetheless, it is clear that biochemical and structural characterization of newly discovered RNA repair enzymes can provide clues to the types of substrates they might act on and propel them forward as useful tools for RNA researchers (Weitzer and Martinez 2007; Ho et al. 2004;

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Martins and Shuman 2004; Nandakumar and Shuman 2004; Englert and Beier 2005; Pfeffer et al. 2005; Nandakumar et al. 2006; Raymond and Shuman 2007; Ramirez et al. 2008; Schwer et al. 2008; Jain and Shuman 2009; Schutz et al. 2010; Englert et al. 2010).

An intriguing issue is whether and how the dynamics of RNA breakage and repair are modulated. In the event that a ribotoxin is constitutively activated, the RNA repair system is fighting an uphill battle against relentless RNA cleavage (Nandakumar et al. 2008). One way to evade the vicious cycle would be to install a “mark” on the broken RNAs that have undergone repair, which then protects the marked RNAs from recurrent damage.

Recent studies suggest a capacity for this type of “intelligent” RNA repair mechanism in the many bacterial species that encode the repair enzymes Pnkp (polynucleotide 5′-kinase/3′-phosphatase) and Hen1 in an operon-like gene cassette (Chan et al. 2009a; Jain and Shuman 2010). Bacterial Pnkp was first discovered in *Clostridium thermocellum* and the *CthPnkp* protein has been characterized extensively as the exemplary bacterial end-healing enzyme (Martins and Shuman 2005; Keppetipola and Shuman 2006a,b, 2007; Keppetipola et al. 2007). *CthPnkp* is a homodimer of an 870-aa polypeptide composed of three catalytic domains: N-terminal kinase, central phosphoesterase, and C-terminal adenylyltransferase. The kinase module catalyzes phosphoryl transfer from ATP to the 5′-OH terminus of DNA or RNA polynucleotides. The phosphoesterase domain (a binuclear metallophosphoesterase) releases P_i from 2′- PO_4 , 3′- PO_4 , or 2′,3′ cyclic phosphate ribonucleotides. The adenylyltransferase domain is homologous to RNA ligases and reacts with ATP to form a covalent enzyme-AMP adduct (Martins and Shuman 2005), just as RNA ligases do during strand joining. However, the inability to detect strand-joining activity by *CthPnkp*, using a variety of RNA and DNA substrates (Martins

and Shuman 2005), suggested that either Pnkp has a very fastidious RNA substrate requirement or its sealing function depends on a missing bacterial protein cofactor. Chan et al. (2009a) implicated bacterial Hen1, encoded by the flanking genomic ORF in all bacteria that have Pnkp, as an activator of the RNA sealing function of the Pnkp ligase-like domain.

The C-terminal half of bacterial Hen1 is homologous to the ribose 2′O-methyltransferase module of eukaryal Hen1 proteins, which modify the 3′-terminal ribonucleotide of small regulatory RNAs (Yu et al. 2005; Yang et al. 2006; Horwich et al. 2007; Kirino and Mourelatos, 2007; Saito et al. 2007). The N-terminal half is conserved among the bacterial Hen1 homologs only. Our physical and functional characterization of *Clostridium thermocellum* Hen1 (*CthHen1*) has revealed the following (Jain and Shuman 2010). (1) Purified recombinant *CthHen1* is a homodimer of a 465-aa polypeptide. (2) *CthHen1* catalyzes methyl transfer from AdoMet to the 3′ terminal nucleoside O2′ of an RNA oligonucleotide. (3) *CthHen1* methyltransferase activity requires manganese. (4) Activity is inhibited by AdoHcy and abolished by mutations D291A and D316A in the putative AdoMet-binding pocket. (5) A C-terminal polypeptide fragment, Hen1-(259–465), comprises an autonomous monomeric methyltransferase domain. (6) The N-terminal fragment of *CthHen1* is a catalytically inactive homodimer.

Raven Huang’s lab has studied *Anabaena variabilis* Hen1 methyltransferase and its connection to *Anabaena* Pnkp; they showed that the *AvaHen1*-*AvaPnkp* complex has end-healing and sealing activities on a broken tRNA substrate and that ribose 2′O-methylation of the 3′ end prior to sealing can protect the repair junction from further damage by a transesterifying ribotoxin (Chan et al. 2009a). They also solved crystal structures of the C-terminal fragments of *AvaHen1* and *CthHen1*, showing that they adopt classical methyltransferase folds (Fig. 1C; Chan et al. 2009b).

FIGURE 1. Delineating the margins of the *CthHen1* methyltransferase domain. (A) Aliquots (5 μ g) of recombinant His₆-tagged WT *CthHen1* and the truncated proteins spanning the amino acid residues as indicated were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (B) Methyltransferase reaction mixtures (10 μ L) contained 20 μ M [³H-CH₃]AdoMet, 10 μ M (100 pmol) 24-mer RNA, and *CthHen1* proteins as specified. The extents of ³H-methyl transfer to RNA are plotted as a function of input enzyme. (C) The tertiary structure of the C-terminal catalytic domain of *CthHen1* is shown as a gray cartoon trace, starting from amino acid 268. The AdoHcy in the methyl donor site, depicted as a stick model, was imported from the structure of *Anabaena variabilis* Hen1 after superimposing it on the *CthHen1* structure. The figure was prepared using coordinates from PDB entries 3JWG and 3JWH (Chan et al. 2009b).

A salient finding from our initial characterization of *CthHen1* was that its activity was strictly dependent on manganese (Jain and Shuman 2010). We proposed, by analogy to catechol O-methyltransferase (Vidgren et al. 1994), that *Hen1* uses Mn^{2+} to engage the vicinal 2' and 3' OH of the terminal ribose and to lower the pK_a of the O2' to allow its attack on the AdoMet methyl group (Jain and Shuman 2010). This prediction was immediately validated via the 3.1 Å crystal structure of plant *Hen1* in a complex with AdoHcy and a dsRNA methyl acceptor (Huang et al. 2009), which revealed a divalent cation in a coordination complex that included the 2' and 3' ribose hydroxyls (Fig. 2). The investigators surmised that the endogenous metal was Mg^{2+} , but we speculate, based on our results,

that it might be Mn^{2+} . Raven Huang and colleagues have since confirmed that human *Hen1* and *Anabaena Hen1* also require Mn^{2+} and are both inactive with Mg^{2+} (R Huang, pers. comm.). In the present study, we used the crystal structure of the plant *Hen1*–AdoHcy–RNA complex to guide a mutational analysis of *CthHen1*, the results of which provide new insights to the basis for RNA end recognition and catalysis.

In our initial study, we showed that *CthHen1* catalyzed quantitative methylation of the terminal ribose O2' of a single-stranded 24-mer RNA oligonucleotide, but was unreactive with a synonymous DNA or an RNA with a single 3'-terminal deoxyribose sugar. Here we further delineate the methyl acceptor specificity of *CthHen1*, by answering

the following: (1) How much RNA information is required? (2) Is there an RNA length requirement? (3) Is there any preference for the 3' terminal base? (4) What is the influence of RNA secondary structure? The results of this analysis elucidate the distinctive substrate recognition properties of a new bacterial clade of RNA methyltransferases.

RESULTS AND DISCUSSION

Delineation of a minimal *CthHen1* methyltransferase module

We had shown previously that the purified recombinant C-terminal domain of *CthHen1* spanning residues 259–465 is an active RNA methyltransferase (Jain and Shuman 2010). Indeed, protein titration experiments indicate that *CthHen1*–(259–465) has twofold higher specific activity in methylating our standard 24-mer RNA substrate than does full-length *CthHen1* (Fig. 1B). Crystallization of the C-terminal domain fragment, *CthHen1*–(257–465), was reported by Chan et al. (2009b). The refined 1.9 Å structure is shown in Figure 1C. The modeled *CthHen1* polypeptide starts at residue Asn268, signifying that the segment from 257 to 267 was disordered in the crystal. Conceivably, the disordered segment is simply an interdomain linker that is noncontributory to methyltransferase activity. Alternatively, the segment might be important for methyltransferase activity, e.g., by becoming ordered in the presence of the RNA methyl acceptor and/or contributing to a putative methyl acceptor binding site. To discriminate

FIGURE 2. Conserved active site of plant and bacterial *Hen1* methyltransferase domains. (A) Stereo view of the active site of *Arabidopsis thaliana* *Hen1* (Huang et al. 2009) (from PDB 3HTX). AdoHcy (SAH) and the 3' terminal dinucleotide of the RNA methyl acceptor are shown as stick models with gray carbons. The divalent cation is depicted as a magenta sphere; waters are red spheres. The amino acid residues contacting the methyl donor, the metal ion, and the RNA methyl acceptor are shown as stick models with beige carbons. The amino acids are labeled according to their *CthHen1* equivalents. Atomic contacts are indicated by dashed lines. (B) The amino acid sequences of the *Clostridium thermocellum* (Cth) and *Arabidopsis thaliana* (Ath) *Hen1* methyltransferase domains are aligned. Positions of side chain identity/similarity are indicated by a circle. The conserved AdoMet-binding motifs are shaded in yellow. Gaps in the alignment are indicated by a dashed line. A peptide segment of *AthHen1* that is disordered in the crystal structure and missing from *CthHen1* is highlighted in red font. The nine conserved amino acids that were subjected to mutational analyses in the present study are indicated by a vertical bar.

these scenarios, we produced and purified a series of incrementally truncated variants: *CthHen1*-(262–465), *CthHen1*-(265–465), and *CthHen1*-(269–465) (Fig. 1A). The specific activities of *CthHen1*-(262–465) and *CthHen1*-(265–465) were virtually the same as that of *CthHen1*-(259–465) (Fig. 1B). By contrast, the specific activity of *CthHen1*-(269–465) was an order of magnitude lower (Fig. 1B). We surmise that the interval ²⁶⁵KKLN²⁶⁸ peptide is important for RNA methylation and that residue 265 demarcates the proximal margin of a fully active methyltransferase domain. Based on the structure of the homologous catalytic domain of plant Hen1 (Huang et al. 2009), we suspect that the severe decrement in methyltransferase activity in *CthHen1*-(269–465) reflects the loss of two hydrogen bonds from protein main-chain amides to the penultimate phosphodiester of the RNA methyl acceptor (Fig. 2A). Efforts to delineate a distal margin of the catalytic domain by serial deletion of 11 or 27 amino acids from the C-terminus were not fruitful, insofar as the recombinant proteins were intractably insoluble. This is now sensible in light of the *CthHen1* structure (Fig. 1C), which clarifies that the C-terminal 11-aa peptide comprises one of the central β strands of the methyltransferase fold.

Structure-guided alanine scan of the *CthHen1* active site

A primary structure alignment of the bacterial and plant Hen1 methyltransferase domains (Fig. 2B) highlights conservation of the many side chain functional groups that comprise the binding sites for AdoMet donor, the divalent metal, and the 3' dinucleotide of the RNA methyl acceptor in the active site of plant Hen1 (Huang et al. 2009), the structure of which is depicted in Figure 2A, with residues numbered according to their *CthHen1* equivalents. We had shown by alanine scanning that AdoMet-binding residues Asp291 and Asp316 are essential for *CthHen1* methyltransferase activity (Jain and Shuman 2010). Now, we extend the alanine scan to six other conserved residues shown in Figure 2A (Arg273, Glu366, Glu369, His370, Arg414, and His418) that form the metal and RNA binding sites. We also mutated Asp417, which is conserved in plant Hen1 (Fig. 2B), but does not interact with the substrates or metal cofactor. The alanine mutations were introduced into the *CthHen1*-(259–465) catalytic domain. We purified the recombinant wild-type (WT) and mutated proteins (Fig. 3A) and assayed them for RNA methylation at equal levels of input enzyme that, in the case of WT *CthHen1*-(259–465), sufficed for quantitative modification of the 24-mer substrate RNA ends (Fig. 3B). The R273A, E366A, E369A, R414A, and H418A mutations abolished methyltransferase activity, while the H370A change reduced product formation to 1% of the WT level (Fig. 3B). By contrast, the D417A mutant methylated 83% of the input RNA ends (Fig. 3B). Chan et al. (2009b) independently reported

FIGURE 3. Effects of alanine mutations on *CthHen1* methyltransferase activity. (A) Aliquots (5 μ g) of recombinant WT *CthHen1*-(259–465) and the indicated alanine mutants were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (B) Methyltransferase reaction mixtures contained 20 μ M [³H-CH₃]AdoMet, 10 μ M (100 pmol) 24-mer RNA, and 4 μ M (40 pmol) *CthHen1* proteins as specified. The extents of RNA methylation are plotted. Each datum is an average of three separate experiments $\pm$ SEM.

drastic effects of four of these mutations (R273A, E366A, E369A, H370A) on activity relative to wild-type, albeit without quantifying the absolute level of methylated RNA product formed.

It is noteworthy that four of the six essential residues (Glu366, Glu369, His370, and His418) are constituents of the octahedral metal coordination complex. The strong selectivity of *CthHen1* for manganese as the metal cofactor, and its inability to use magnesium (Jain and Shuman 2010), can now be rationalized, insofar as the reliance on the His370 and His418 N ϵ atoms as metal ligands will favor “soft” metal interactions with manganese in contrast to the “hard” oxygen-based contacts preferred by magnesium. The other two essential residues identified presently—Arg273 and Arg414—make electrostatic contacts with the 3' terminal phosphodiester of the RNA substrate (Fig. 2A).

Structure–activity relationships at essential residues in the *CthHen1* active site

We proceeded to determine structure–activity relationships for each of the eight essential *CthHen1* residues we have identified here and previously (Arg273, Asp291, Asp316, Glu366, Glu369, His370, Arg414, and His418) by testing the effects of 18 conservative side chains substitutions. The recombinant *CthHen1*-(259–465) mutants were purified (Fig. 4A) and assayed for methyltransferase activity in parallel

FIGURE 4. Effects of conservative substitutions at essential residues. (A) Aliquots (5 μ g) of recombinant WT *CthHen1*-(259–465) and the indicated mutants were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (B) Methyltransferase reaction mixtures contained 20 μ M [3 H-CH₃]AdoMet, 10 μ M (100 pmol) 24-mer RNA, and 4 μ M (40 pmol) *CthHen1* proteins as specified. The extents of RNA methylation are plotted. Each datum is an average of three separate experiments $\pm$ SEM.

with the WT enzyme (Fig. 4B). The results are discussed below and interpreted in light of the homologous plant *Hen1* structure.

AdoMet binding site

CthHen1 has a classical AdoMet binding site that includes two conserved peptide motifs: ²⁸⁹VIDLGCG²⁹⁵ and ³¹³TGVDV³¹⁷ (Fig. 2B, highlighted in yellow). Asp316 in the distal motif engages in a bidentate hydrogen-bonding interaction with the AdoMet ribose hydroxyls (Fig. 2A). We found that the conservative asparagine change virtually abolished methyltransferase activity (1% methylation of input RNA by D316N versus 94% for WT), whereas introduction of glutamate restored activity partially, to 27% methylation. We surmise that: (1) the carboxylate moiety is critical at position 316 and (2) there is a modest steric constraint on the longer glutamate side chain versus aspartate. The inactivity of the isosteric asparagine implies that the bidentate ribose contact requires that the ribose oxygens be the proton donors in the hydrogen bonding network.

Asp291 in the proximal motif coordinates the AdoMet methionine amine via water. Here, too, the isosteric asparagine change abolished activity (0.7% methylation), while the glutamate substitution restored function to near wild-type levels (73% methylation) (Fig. 4). This result

attests to the lack of steric constraint of the main-chain to carboxylate distance. It is not obvious to us from the plant *Hen1* structure why the asparagine amide is deleterious, given it can, in principle, form a similar H-bond to the bridging water. Conceivably, the negative charge on Asp291 strengthens interaction with the positively charged amine, even though the distance separating them is 4.7 Å.

Metal coordination complex

The two metal-binding glutamates—Glu366 and Glu369—were replaced conservatively with aspartate and glutamine, either of which abolished methyltransferase activity (Fig. 4B). Thus, Glu366 and Glu369 are strictly essential. The failure of glutamine to substitute underscores the primacy of the ionic interactions of the carboxylates with the divalent metal. The inactivity with aspartate highlights a stringent requirement for the longer main-chain to carboxylate distance to place the glutamate carboxylate oxygens within the radius of the octahedral metal coordination complex.

The two metal-binding histidines—His370 and His418—were substituted conservatively by asparagine and glutamine, which can, via their amide nitrogens, mimic the positions of the histidine N δ and N ϵ atoms, respectively. The H370N, H370Q, H418N, and H418Q mutants were all catalytically inactive (Fig. 4B). We also replaced His370 with Asp and Glu (exemplary “hard” metal interactors). Whereas the H370D mutant was unreactive, H370E methylated 8% of the input substrate RNA ends (Fig. 4B). A glutamate carboxylate oxygen (but not the aspartate) can, in principle, occupy the same position as the metal-binding N ϵ atom of His370 (Fig. 2A). The relatively feeble activity sustained by glutamate compared to the native histidine fortifies our speculations that the manganese cofactor for *CthHen1* activity relies on soft interactions with the two histidine nitrogen ligands in the coordination complex.

RNA binding site

The plant *Hen1* structure provides unique insights to recognition of the RNA methyl acceptor (Huang et al. 2009), whereby the signature specificity of plant *Hen1* for double-stranded RNAs with 3' dinucleotide overhangs (Yang et al. 2006) is achieved via two double-strand RNA-binding domains (dsRBDs) that bind to opposite faces of the RNA double helix and thereby enclose it within the *Hen1*

protein, while the catalytic domain binds the 3' overhang that is the immediate methyl acceptor. Metazoan and bacterial Hen1 proteins lack the dsRBDs, and the available evidence indicates that they are adept at methylating the 3' terminal ribose of single-stranded RNA acceptors (Horwich et al. 2007; Jain and Shuman 2010). The interactions of the catalytic domain of plant Hen1 with the 3' terminal dinucleotide of the acceptor RNA provide clues to how RNA end recognition might occur, via sequence nonspecific ionic and hydrogen bonding contacts with the terminal and penultimate phosphodiester. Whereas the penultimate phosphodiester is coordinated by main-chain amide nitrogens at the proximal margin of the catalytic domain, the terminal phosphodiester is bound by two conserved arginines, corresponding to Arg273 and Arg414 in *CthHen1* (Fig. 2A). Arg414 makes a bifurcated contact with the 3' bridging oxygen and one of the nonbridging oxygens of the phosphodiester. Arg273 contacts the other nonbridging oxygen via one of its terminal guanidinium nitrogens and also makes a salt bridge to the AdoMet carboxylate via its other terminal guanidinium nitrogen (Fig. 2A). Here we replaced Arg414 and Arg273 conservatively with lysine and glutamine. The R273K and R273Q changes abolished methyltransferase activity (Fig. 4B), suggesting that both atomic contacts of Arg273—to the RNA phosphodiester and AdoMet carboxylate—are essential for *CthHen1* function. The R414K and R414Q mutants were severely defective (3% and 1% RNA methylation, respectively) (Fig. 4B), signifying that arginine is uniquely suited at this position to make the appropriate contacts with the terminal phosphodiester.

The mutational data and structure support a mechanism of substrate binding and catalysis predicated on contacts with the terminal dinucleotide phosphodiesters that dock the 3' end of the RNA in the active site in an orientation that brings the terminal ribose hydroxyls into the manganese coordination complex and orients the ribose O2' (the nucleophile) for in-line attack on the AdoMet methyl carbon. In this mechanism, manganese plays a critical role in organizing the active site, positioning the ribose hydroxyls, and, we presume, lowering the pK_a of the O2'.

Methyl acceptor specificity—preference for guanine as the 3' terminal base

We compared the methyltransferase activity of full-length *CthHen1* with a set of otherwise identical 24-mer RNA substrates with 3' terminal G, A, C, or U nucleosides. Enzyme titration experiments (Fig. 5) revealed that *CthHen1* displayed a fourfold preference for 3'-G versus the other nucleosides. The C-terminal catalytic domain *CthHen1*-(259–465) also preferentially methylated the 3'-G substrate (not shown). The crystal structure of plant Hen1 with RNA that has a 3'-G nucleoside (Fig. 2A) suggests an explanation for our findings, whereby multiple van der Waals contacts

FIGURE 5. *CthHen1* prefers guanosine at the 3'-terminal position of the RNA methyl acceptor. Methyltransferase reaction mixtures contained 20 μ M [3 H-CH $_3$]AdoMet, 10 μ M (100 pmol) 24-mer RNAs differing in their 3'-terminal nucleosides (G, U, A, or C), and *CthHen1* as specified. The extents of RNA methylation of each substrate are plotted as a function of input *CthHen1*. Each datum is an average of three separate experiments $\pm$ SEM. The primary structures of the RNAs are shown below the graph. The 3' terminal nucleobases are highlighted in gray.

between the 3' guanine-N2 atom and AdoMet methionine atoms S δ (3.5Å), C γ (3.15Å), and C β (3.75Å) appear to stabilize the RNA 3' nucleoside in the Michaelis complex. These atomic contacts would be unique to guanine.

Methyl acceptor specificity—effect of RNA length

We incrementally trimmed the RNA substrate (with a 3'-G) from its 5' end to yield a series of 9-mer, 12-mer, 18-mer, and 24-mer RNAs with the same 3' nucleotide sequence. We compared them at equimolar concentrations as methyl acceptors for *CthHen1*. (The sizes and integrity of the RNAs were first verified by 5' 32 P end-labeling equimolar aliquots with T4 polynucleotide kinase.) *CthHen1* titrations (Fig. 6) revealed modest stepwise declines in methyltransferase-specific activity as the RNA chain length was shortened from 24 to 18 ($\sim$ 10% decrement) and from 18 to 12 ($\sim$ 18% decrement). By contrast, the transition from a 12-mer to a 9-mer elicited an order of magnitude decrease in specific activity (Fig. 6). We conclude that RNA length is a significant factor when the methyl acceptor is less than 12 nucleotides (nt). (Similar effects of RNA size were seen with the isolated catalytic domain, *CthHen1*-(259–465) [not shown].) An implication of these results is that bacterial Hen1 likely makes additional contacts with the RNA upstream of those with the 3' terminal dinucleotide that are depicted in Figure 2A. A fuller understanding

FIGURE 6. Effect of RNA length on methyl acceptor activity. Methyltransferase reaction mixtures contained 20 μ M [3 H-CH $_3$]AdoMet, 10 μ M (100 pmol) 24-mer, 18-mer, 12-mer, or 9-mer RNAs, and *CthHen1* as specified. The extents of RNA methylation are plotted as a function of input *CthHen1*. Each datum is an average of three separate experiments $\pm$ SEM. The primary structures of the RNAs are shown below the graph.

of the *CthHen1*–RNA interface will depend on crystallization of the Michaelis complex (or a mimetic thereof) with methyl acceptor, manganese, and AdoMet (or AdoHcy) in the active site.

Methyl acceptor specificity—how much RNA information is required?

We had shown previously that the last nucleoside of the RNA methyl acceptor must be a ribo. Changing only the 3' nucleoside to a 2'-deoxy abolished *CthHen1* methyltransferase activity (Jain and Shuman 2010), signifying that the enzyme is unable to methylate O2' at internal nucleosides. We postulated, by analogy to catechol-O-methyltransferase, that this specificity is enforced by manganese coordination of the vicinal 2' and 3' hydroxyls of the terminal nucleoside, which could not be achieved when the ribose is at an internal position and the O3' is bonded to the internucleotide phosphate (Jain and Shuman 2010). The plant *Hen1* structure neatly reveals that this explanation is valid (Fig. 2A).

Here, we queried the extent to which there is ribo specificity elsewhere in the polynucleotide methyl acceptor. To answer this question, we tested activity with 24-mer mixed DNA–RNA chimaeras in which the 5' segment is DNA and the 3' end is RNA. In the extreme case of “no ribo specificity” outside the acceptor 3' nucleoside, we

would see that a “D23R1” polynucleotide (composed of 23 deoxys and one 3' terminal ribo) is an effective substrate. Indeed, we found by enzyme titration that D23R1 was methylated with 60% of the specific activity of R24 at equivalent substrate concentrations (Fig. 7). A D17R1 polynucleotide composed of 17 deoxys and one 3' terminal ribo was also an effective methyl acceptor (Fig. 7). A shorter D11R1 substrate composed of 11 deoxys and one terminal 3' ribo was methylated, albeit with one-fourth the specific activity of the longer DNAs with 3' ribos. The catalytic domain *CthHen1*-(259–465) was also adept at methylating the D23R1 substrate (not shown).

Methyl acceptor specificity—influence of secondary structure

Plant *Hen1* is highly specific for methylating the terminal ribose of 21–24-nt duplex RNA substrates with 3' single-strand overhangs (Yang et al. 2006). Whereas these stringent requirements are reinforced by the dsRBDs of plant *Hen1*, a recent study indicates that the isolated C-terminal methyltransferase catalytic domain per se can still discriminate in favor of annealed dsRNA substrates versus the individual component single strands (Vilkaitis et al. 2010). Here, we were interested in assessing the effects of RNA secondary structure on the activity of bacterial *Hen1*. Thus, we compared the extents of methylation by full-length *Hen1* of our standard 24-mer RNA acceptor (R24) and a series of RNA–DNA duplexes formed by pre-annealing

FIGURE 7. A single ribonucleotide at 3'-terminus of a DNA strand suffices as a methyl acceptor. Methyltransferase reaction mixtures contained 20 μ M [3 H-CH $_3$]AdoMet, 10 μ M (100 pmol) D23R1, D17R1, or D11R1 polynucleotides, and *CthHen1* as specified. The extents of polynucleotide methylation are plotted as a function of input *CthHen1*. Each datum is an average of three separate experiments $\pm$ SEM. The primary structures of the substrates are shown below the graph.

the R24 strand to complementary DNA strands D20, D24, or D28 (Fig. 8). (Efficient duplex formation was verified by 5' 32 P-labeling the single strands and annealed duplexes and analyzing the products by native PAGE.) The R24/D20 substrate consists of a 20-base pair (bp) duplex with a 4-nt 3' single-stranded RNA overhang. The R24/D24 substrate is a blunt-ended 24-bp duplex. The R24/D28 substrate is a 24-bp duplex with a 4-nt 5' single-stranded DNA overhang. We chose complementary DNA strands because the resulting RNA–DNA hybrids will adopt an A-form helical conformation similar to that of RNA duplexes, yet the DNA strands of the duplex cannot be methylated by *CthHen1* (Fig. 8), thereby restricting the observed methyl transfer to the R24 strand. Control assays were performed in which R24 was supplemented with a noncomplementary 24-mer strand (D24*). The generic DNA polynucleotide elicited a slight decrease in the extent of RNA methylation compared to a reaction containing R24 alone (Fig. 8), suggesting that DNA competes to some extent with RNA for binding to *CthHen1*. (This idea is consistent with the ability of *CthHen1* to methylate all-DNA strands with a single 3' ribo terminus.)

FIGURE 8. Effect of RNA secondary structure on methyl acceptor activity. Methyltransferase reaction mixtures contained 1 mM MnCl_2 , 20 μM [^3H -CH $_3$]AdoMet, 3 μM (30 pmol) *CthHen1*, and 10 μM (100 pmol) of 24-mer RNA strand (R24), either alone, hybridized to complementary DNA strands D20, D24, or D28, or mock-hybridized to a noncomplementary strand D24*. A control reaction mixture contained D24 in lieu of R24. The extents of polynucleotide methylation are plotted. Each datum is an average of two separate experiments; the bars denote the range. The structures of the R24 and DNA strands and the annealed duplexes are illustrated below the graph. Annealing was performed by mixing R24 with equimolar amounts of the DNA strand in 150 mM NaCl, then incubating the mixture for 10 min at 65°C, followed by 15 min at 37°C, and 15 min at 22°C. The annealed substrates were stored at -20°C and thawed immediately before use.

Our expectation, in light of the plant Hen1 structure showing projection of the single-stranded RNA 3' end into the active site, was that recessing the RNA 3' nucleotide within a 5' tailed duplex (as in R24/D28) might interfere with methyl transfer. But, to the contrary, the R24/D28 substrate was 50% more effective as a methyl acceptor than the R24 strand alone (Fig. 8). Similarly, positioning the 3' terminal ribo at a blunt duplex end in the R24/D24 substrate made it a 50% better methyl acceptor than R24 alone (Fig. 8). By contrast, the R24/D20 duplex substrate with the 3' single-stranded RNA tail was 40% less effective than the R24 strand alone (Fig. 8).

We performed additional experiments in which the D23R1 strand was annealed to complementary DNA strands D20, D24, and D28 to yield duplex DNAs (in B-form helical conformation) with a single 3' ribonucleoside methyl acceptor (Supplemental Fig. S1). These duplexes were methylated by *CthHen1* to similar extents, comparable to that of the D23R1 strand alone (Supplemental Fig. S1). Thus, *CthHen1* is largely indifferent to A-helical conformation and whether the 3'-ribonucleoside is at a blunt end, in a 4-nt single-strand tail, or recessed 4-nt from a 5' single-strand tail. (The catalytic domain *CthHen1*-(259–465) was also active in methylating the R24- and D23R1-containing duplex substrates) [not shown].

These results indicate that *CthHen1* responds to nucleic acid secondary structure in a distinctive manner compared to plant Hen1 (dsRNA specific and intolerant of ssRNA) or metazoan Hen1 (single-strand specific and intolerant of dsRNA) (Horwich et al. 2007; Vilkaitis et al. 2010). It appears that *CthHen1* has broader acceptor specificity that would enable it to mark with a methyl group a wide variety of broken RNA structures destined for repair. At the very least, we surmise that either: (1) *CthHen1* can accommodate a duplex nucleic acid end directly in the active site or (2) binding of *CthHen1* to the RNA strand 3' end melts out a short duplex segment while positioning the 3' ribose in the manganese coordination complex.

Concluding remarks

The present study provides new insights to: (1) the mechanism of manganese-dependent methyl transfer by bacterial Hen1; (2) the identification of essential active site moieties that bind the RNA substrate; and (3) the features of the RNA methyl acceptor that influence Hen1 activity. The methyl acceptor requirements for the isolated monomeric catalytic domain of *CthHen1* appear to be the same as those of the full-length *CthHen1* homodimer, consistent with the idea that the N-terminal domain of bacterial Hen1 mediates physical and functional interaction with bacterial Pnkp to form an RNA repair complex (Chan et al. 2009a; Jain and Shuman 2010), but is not required for methyltransferase activity or RNA recognition. It remains to be seen whether the intrinsic substrate preferences of bacterial Hen1

illuminated here might be altered when Hen1 is associated physically with bacterial Pnkp.

Packaging of an RNA methylase with an RNA repair enzyme is loosely reminiscent of DNA restriction-modification systems. A key difference is that the methylation mark that protects DNA from cleavage by a DNA restriction endonuclease is deposited on an intact DNA duplex before any DNA damage occurs. By contrast, because bacterial Hen1 acts exclusively at a free RNA end, its installation of a methyl mark can only occur after an RNA cleavage step (or else be restricted to the 3' end of a primary transcript). Coupling Hen1 to Pnkp can, in principle, provide an antidote to any target-specific ribotoxins that generate 2',3' cyclic phosphate/5'-OH breaks, by forming a N_{mp}N' repair junction that is immune to recleavage by the ribotoxin.

Other functions can also be envisioned for Hen1–Pnkp in bacterial RNA transactions, e.g., in the genesis and modification of regulatory RNAs. For example, CRISPRs (clustered regularly interspaced short palindromic repeats) provide bacteria with RNA-guided acquired immunity to invasive DNAs (for review, see Horvath and Barrangou 2010). CRISPRs consist of an array of short direct repeat DNA sequences (25–50 bp) separated by hypervariable spacer sequences (20–70 bp). The repeat array is flanked on one side by an AT-rich region called the leader. The CRISPR locus is transcribed into a single RNA transcript with the leader at the 5' end followed by the tandem repeats. The primary transcript is processed by Cas (CRISPR-associated) proteins to generate unit-sized CRISPR RNAs (crRNAs), each consisting of one spacer flanked by 5' and 3' sequences derived from the direct repeats. The processed crRNAs can interfere with foreign genetic elements to which they display base complementarity. Several recent studies have identified the CRISPR RNA processing enzymes as transesterifying endonucleases that leave 2',3' cyclic phosphate/5'-OH breaks at the processing sites (Carte et al. 2008; Haurwitz et al. 2010). *Clostridium thermocellum* has a CRISPR system. Thus, it is conceivable that the crRNAs could be substrates for end-healing by *Cth*Pnkp and 2'-O-methylation by *Cth*Hen1, with or without an ensuing ligation step. The fact that crRNAs target complementary nucleic acids, and that bacterial Hen1 is capable of methylating RNA ends within duplex structures, raises the prospect that Hen1–Pnkp might modify crRNAs after they identify their targets.

What would be the point of methylating the free ends of small bacterial RNAs, independent of ligation? Here again, the methyl mark could be protective. Indeed, recent studies of eukaryal small RNA metabolism converge on the theme that ribose 2'-O-methylation by Hen1 shields RNAs from 3' uridylation reactions that trigger their decay (Li et al. 2005; Kurth and Mochizuki 2009; Ameres et al. 2010; Kamminga et al. 2010). The biological ramifications of Hen1 RNA modification in bacteria are uncharted, but certainly worthy of attention.

MATERIALS AND METHODS

Recombinant *Cth*Hen1

Full-length *Cth*Hen1 and the C-terminal catalytic domain *Cth*Hen1-(259–465) were produced in *Escherichia coli* BL21(DE3) as His₆ fusions and purified from soluble bacterial lysates as described previously (Jain and Shuman 2010). Missense mutations were introduced into the *Cth*Hen1-(259–465) ORF by two-stage overlap extension PCR. Truncated ORFs encoding *Cth*Hen1-(262–465), *Cth*Hen1-(265–465), and *Cth*Hen1-(269–465) were generated by PCR amplification using sense-strand primers that introduced a start codon in lieu of the Leu258, Glu261, Glu264, or Asn268 codons, as well as an upstream BamHI site. The truncated/mutated ORFs were inserted into pETduet-1. The pET plasmid inserts were sequenced completely to verify the intended coding sequence and exclude acquisition of unwanted coding changes. The truncated/mutated Hen1 proteins were produced in *E. coli* BL21(DE3) as His₆ fusions and purified from soluble bacterial lysates by Ni²⁺-agarose and DEAE-cellulose chromatography steps (Jain and Shuman 2010). Protein concentrations were determined by using the Biorad dye reagent with bovine serum albumin as the standard. The yields of the truncated *Cth*Hen1 proteins were 12–15 mg from a 1-L bacterial culture.

Methyltransferase assay

Reaction mixtures (10 μ L) containing 25 mM Tris-HCl (pH 8.5), 0.5 mM MnCl₂, 20 μ M [³H-CH₃]AdoMet, 10 μ M (100 pmol) 24-mer synthetic RNA oligonucleotide (5'-CACUAUCGAAUAAGGGCGACACG) or other polynucleotides as specified, and *Cth*Hen1 (plus 0.2 mM EDTA 0.2 mM DTT and 10 mM NaCl contributed by the enzyme buffer) were incubated at 45°C. The reactions were quenched by adding 1 μ L of 55 mM EDTA. Aliquots (4 μ L) were spotted on a polyethyleneimine cellulose TLC plate, which was developed with 0.2 M (NH₄)₂SO₄. The ³H-AdoMet and ³H RNA-containing (origin) portions of the lanes were cut out and the radioactivity in each was determined by liquid scintillation counting.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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