2'-O-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in *Tetrahymena*

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ABSTRACT

Small RNAs ~20–30 nucleotides (nt) in length regulate gene expression at the transcriptional and post-transcriptional levels. In the plant *Arabidopsis*, all small RNAs are 3'-terminal 2'-O-methylated by HEN1, whereas only a subset of small RNAs carry this modification in metazoans. This methylation is known to stabilize small RNAs, but its biological significance remains unclear. In the ciliated protozoan *Tetrahymena thermophila*, two classes of small RNAs have been identified: RNAs ~28–29 nt long (scnRNAs) that are expressed only during sexual reproduction, and constitutively expressed ~23–24 nt siRNAs. In this study, we demonstrate that scnRNAs, but not siRNAs, are 2'-O-methylated at their 3' ends. The *Tetrahymena* HEN1 homolog Hen1p is responsible for scnRNA 2'-O-methylation. Loss of Hen1p causes a gradual reduction in the level and length of scnRNAs, defects in programmed DNA elimination, and inefficient production of sexual progeny. Therefore, Hen1p-mediated 2'-O-methylation stabilizes scnRNA and ensures DNA elimination in *Tetrahymena*. This study clearly shows that 3'-terminal 2'-O-methylation on a selected class of small RNAs regulates the function of a specific RNAi pathway.

Keywords: HEN1; 2'-O-methylation; Piwi; DNA elimination; *Tetrahymena*

INTRODUCTION

Small noncoding RNAs produced by RNAi-related mechanisms regulate gene expression. They form functional effector complexes with conserved Argonaute proteins, which can be divided into the Ago and Piwi subfamilies. Most eukaryotes possess a number of different Argonaute–RNA complexes, which are engaged in a wide variety of functions, including mRNA degradation, translational repression, and chromatin remodeling (for review, see Chu and Rana 2007).

Expression of small RNA is tightly regulated, mostly at the level of precursor RNA transcription (for example, see Johnson et al. 2003). In some cases, however, small RNA expression is post-transcriptionally regulated through control of precursor RNA processing (Obernosterer et al. 2006; Thomson et al. 2006; Yang et al. 2006a; Viswanathan et al. 2008). In addition, RNA degradation is likely to regulate the accumulation of small RNAs. One potential mechanism

is the protection of small RNAs from degradation by 2'-O-methylation at their 3' terminus (Li et al. 2005; Horwich et al. 2007).

In the plant *Arabidopsis*, all small RNAs (both si- and miRNAs) that have been studied are 2'-O-methylated at their 3' ends. In contrast, in metazoans, only a subset of small RNAs carries 2'-O-methylation at their 3' ends. In vertebrates, this modification has yet to be found on any miRNAs associated with Ago proteins that have been studied to date (Houwing et al. 2007; Kirino and Mourelatos 2007a; Ohara et al. 2007). In *Drosophila*, Ago2-associated small RNAs (mainly siRNAs) are modified at their 3' ends, most likely by 2'-O-methylation, whereas Ago1-associated small RNAs (mainly miRNAs) are largely unmodified (Horwich et al. 2007; Kawamura et al. 2008). Interestingly, most if not all Piwi-associated small RNAs (piRNAs) are 2'-O-methylated at their 3' ends in all metazoans that have been studied so far (Vagin et al. 2006; Horwich et al. 2007; Houwing et al. 2007; Kirino and Mourelatos 2007a; Ohara et al. 2007; Saito et al. 2007). Therefore, 3'-terminal 2'-O-methylation occurs on selected small RNAs, and it may regulate specific RNAi pathways in metazoans.

The RNA methyltransferase HEN1 is responsible for 3'-terminal 2'-O-methylation of small RNAs in *Arabidopsis*

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(Yu et al. 2005) and *Drosophila* (Horwich et al. 2007; Saito et al. 2007). Mouse HEN1 is also able to add 2'-O-methyl groups to small RNAs in vitro (Kirino and Mourelatos 2007b), but its role in vivo has yet to be studied. *Arabidopsis* HEN1 methylates double-stranded small RNAs (Yu et al. 2005; Yang et al. 2006b), probably before they are loaded into Argonaute proteins. This is consistent with the fact that all small RNAs are 2'-O-methylated, regardless of the Argonaute proteins to which they bind. In contrast, the HEN1 enzymes from *Drosophila* and mouse add 2'-O-methyl groups only to single-stranded small RNAs (Horwich et al. 2007; Saito et al. 2007). Thus, in these animals, 2'-O-methylation most likely occurs on small RNAs that have already complexed with Argonaute proteins. *Drosophila* HEN1 interacts with Piwi proteins (Piwi, Aubergine, and Ago3), whose binding partners (piRNAs) are 2'-O-methylated, but not with the Ago protein Ago1, whose small RNA partners are not modified (Saito et al. 2007). Therefore, the substrate specificity of HEN1 homologs in metazoans probably reflects the fact that HEN1 proteins interact with a specific class of Argonaute proteins.

The role of 3'-terminal 2'-O-methylation of small RNAs is well studied in *Arabidopsis*. *Arabidopsis* HEN1 mutants phenocopy the loss of activity of the general RNAi machinery, including Dicers and Argonautes (Chen et al. 2002; Boutet et al. 2003). This is because 2'-O-methylation protects small RNAs from degradation by exonucleases (Ramachandran and Chen 2008). The loss of this modification causes a general reduction in the levels of small RNAs in plants (Boutet et al. 2003; Li et al. 2005). Therefore, generally all small RNAs in *Arabidopsis* depend on 2'-O-methylation for their stability. Thus, this modification does not seem to control a specific RNAi pathway in plants.

In *Drosophila* mutant strains that lack the activity of the HEN1 homolog DmHEN1 (Pimet), the length and abundance of piRNAs, which are normally 2'-O-methylated at the 3' end, are lower than in wild-type flies. In contrast, the levels of miRNAs, which are unmodified also in wild-type flies, are the same in both mutant and wild-type *Drosophila* (Horwich et al. 2007). Therefore, selective 2'-O-methylation of small RNAs regulates the turnover of a specific class of small RNAs, at least in flies. Horwich et al. (2007) reported that the HeT-A transposon mRNA was increased in the absence of DmHEN1. Nevertheless, because the *Drosophila* mutant strain lacking DmHEN1 is viable and fertile (Saito et al. 2007), the significance of this regulation remains unclear. Moreover, the functions of 2'-O-methylation of small RNAs in other eukaryotes have not been reported. Thus, despite the attractiveness of the idea that 3'-terminal 2'-O-methylation of a selected class of small RNAs regulates a specific RNAi pathway, this regulation has yet to be proven necessary for any biological process.

The ciliated protozoan *Tetrahymena thermophila* has 12 Piwi proteins, but no Ago proteins have been identified in

the sequenced *Tetrahymena* genome. *Tetrahymena* expresses at least two classes of small RNAs generated by RNAi-related mechanisms. The first class comprises small RNAs of ~28–29 nucleotides (nt) (scnRNAs) that are expressed exclusively during sexual reproduction (conjugation) and are probably synthesized from many different genome loci (Chalker and Yao 2001; Mochizuki et al. 2002). scnRNAs bind specifically to the Piwi protein Twilp (Mochizuki and Gorovsky 2004), and the scnRNA–Twilp complex plays a pivotal role in developmentally programmed DNA elimination (Mochizuki et al. 2002). The other class of small RNAs consists of constitutively expressed siRNAs of ~23–24 nt that map to a small number of genomic loci (Lee and Collins 2006, 2007). The function of these siRNAs has not yet been identified.

In this study, we demonstrate that scnRNAs, but not siRNAs of ~23–24 nt, are 2'-O-methylated by the *Tetrahymena* HEN1 homolog Hen1p. Hen1p-mediated 2'-O-methylation specifically stabilizes scnRNAs and ensures DNA elimination and the consequent survival of *Tetrahymena* progeny. Our findings lend support to the view that 3'-terminal 2'-O-methylation of a selected class of small RNAs regulates a specific RNAi pathway in eukaryotes.

RESULTS

scnRNA, but not ~23–24 nt siRNA, is 2'-O-methylated

T. thermophila expresses two classes of small RNAs made by RNAi-related mechanisms: RNAs of ~28–29 nt (scnRNAs) that are expressed only during sexual reproduction (Mochizuki et al. 2002) and constitutively expressed siRNAs of ~23–24 nt (Lee and Collins 2006).

In order to analyze whether scnRNAs are modified at their 3' ends, total RNA from conjugating *Tetrahymena* cells enriched for scnRNAs was subjected to periodate oxidation/ β -elimination (Akbergenov et al. 2006). After these reactions, RNAs containing both 2'- and 3'-hydroxyl groups at their 3' ends are shorter by one nucleotide and they have an extra negative charge from the phosphate group left after the nucleotide removal. Modifications in 2'- and/or 3'-hydroxyl groups prevent the periodate oxidation reaction and keep the RNA intact. As a result, unmodified RNA migrates as if it was ~1.5 nt shorter than the modified RNA in a denaturing polyacrylamide gel after the treatment. The mobility of the scnRNAs isolated at different stages of conjugation did not increase after periodate oxidation/ β -elimination, while unmodified control synthetic RNAs in the same reactions did (Fig. 1A). This result indicates that most, if not all, scnRNAs are modified at their 3' ends throughout the process of conjugation.

To test whether the siRNAs of ~23–24 nt are modified at their 3' ends, total RNA enriched for small RNAs was

FIGURE 1. *Tetrahymena* scnRNAs are 2'-O-methylated at their 3' ends. (A) scnRNAs are modified at their 3' ends throughout conjugation. Small RNAs isolated at different time points of conjugation (2.5, 4.5, 6.5, and 8.5 h post-mixing) were combined with a synthetic, 21-nt RNA and subjected to periodate oxidation/β-elimination. The reactions were subjected to denaturing gel electrophoresis and visualized using a nucleic acid-specific fluorescent dye. Untreated RNAs isolated from cells at 4.5 h post-mixing were analyzed in the same gel. The positions of the RNA markers are shown on the left. At all the stages tested, the mobility of the scnRNAs did not increase after the reaction, whereas that of the unmodified, synthetic 21-nt RNA did, indicating that scnRNAs were modified at their terminal nucleotides. (B) The constitutively expressed small RNAs in *Tetrahymena* are not methylated. Small RNAs from vegetative (lane 2) and mating (lane 4) cells were subjected to periodate oxidation/β-elimination. The reactions were separated in a denaturing gel, and the RNA was visualized using a nucleic acid staining dye. Untreated small RNAs (lane 1, vegetative cells; lane 3, mating cells) were analyzed on the same gel. The constitutively expressed siRNAs of ~23–24 nt (arrowheads) displayed a shift after the reaction. (C) scnRNAs are 2'-O-methylated. Gel-purified scnRNAs were radiolabeled by pCp ligation and treated with P1 nuclease. The reactants and the synthetic unlabeled dinucleotide standards (pCmpC, pGmpC, pAmpC, and pUmpC) were resolved by 2D-TLC on the same plate and visualized by autoradiography (left) and UV shadowing (middle). The four spots detected by UV shadowing are circled (middle), and their identities were determined by comparing the relative positions of these spots using a dinucleotide mobility map described previously (Keith 1995). Four radiolabeled spots comigrated with the dinucleotide standards (right), suggesting that scnRNAs were 2'-O-methylated. Asterisks in the left panel indicate unidentified reaction products.

isolated from vegetatively growing and mating *Tetrahymena* and subjected to periodate oxidation/β-elimination. As shown in Figure 1B, the mobility of endogenously expressed siRNAs of ~23–24 nt increased after this

treatment, indicating that most, if not all, siRNAs of ~23–24 nt are not modified at their 3' ends in vivo.

scnRNA is 2'-O-methylated

Because the presence of a 3'-hydroxyl terminus on scnRNAs has been demonstrated previously (Mochizuki et al. 2002), the above result indicated a modification of the 2'-hydroxyl termini of scnRNAs. We next tested whether this modification was 2'-O-methylation. PAGE-purified scnRNAs were ligated to [5'-³²P]cytidine 5',3' bisphosphate (pCp) at the 3'-hydroxyl terminus, and the reaction products were digested with nuclease P1. Because the presence of a bulky modification at the 2'-position reduces the rate at which nuclease P1 hydrolyzes the neighboring phosphodiester bond (Grosjean et al. 2007), RNA lacking such a modification produces cytidine 5'-monophosphates (pC) as a [³²P]-labeled product; in contrast, modified RNA produces labeled dinucleotides as well as pC. The digested scnRNA was resolved by two-dimensional thin-layer chromatography (2D-TLC) and analyzed by autoradiography (Fig. 1C, left). Synthetic 2'-O-methylated dinucleotides (pAmpC, pCmpC, pGmpC, and pUmpC) were separated on the same plate and visualized by UV shadowing (Fig. 1C, middle). The migration distance of the four spots detected by autoradiography matched those of the synthetic 2'-O-methylated dinucleotide standards (Fig. 1C, right), indicating that the 3' termini of the scnRNAs were indeed modified by 2'-O-methylation.

Hen1p has scnRNA methyltransferase activity in vitro

In *Arabidopsis*, the RNA methyltransferase HEN1 modifies the terminal 2' hydroxyl group of siRNAs and miRNAs (Yu et al. 2005). In the fly and mouse, HEN1 homologs methylate terminal 2'

hydroxyl groups of piRNAs (Horwich et al. 2007; Houwing et al. 2007; Kirino and Mourelatos 2007b; Ohara et al. 2007; Saito et al. 2007). The *Tetrahymena* genome possesses a single gene (TTHERM_00433810) that encodes a protein

with strong similarity to these HEN1 homologs (Supplemental Fig. S1A,B). We named this gene *HEN1*. Hen1p, the protein predicted from the *HEN1* mRNA sequence (DDBJ/EMBL/GenBank FM199973), has a conserved methyltransferase domain (Supplemental Fig. S1B). Although *Arabidopsis* HEN1 has a double-stranded RNA-binding domain, *Tetrahymena* Hen1p lacks this domain, as do metazoan HEN1 proteins (Supplemental Fig. S1A).

To test whether Hen1p has scnRNA methyltransferase activity, recombinant Hen1p (rHen1p) was expressed in *Escherichia coli*. Purified rHen1p was incubated with synthetic RNAs of 29 nt whose 3' ends carried either a 2'-hydroxyl or a 2'-O-methyl and S-adenosyl-L[methyl-¹⁴C] methionine, which served as a methyl group donor. rHen1p methylated the RNA with the 2'-hydroxyl terminus but did not methylate the RNA with a 2'-O-methyl terminus (Fig. 2A). These results indicate that Hen1p can methylate the terminal 2'-hydroxyl group of scnRNAs.

rHen1p does not methylate double-stranded synthetic scnRNAs that possess blunt ends or 3'-overhangs of 2 nt, which mimic Dicer-processed RNA (Fig. 2A). This suggests that rHen1p methylates only single-stranded scnRNAs. Consistent with this, the gel mobility of scnRNAs in a *Twil1* mutant strain, which is incapable of dissociating

double-stranded scnRNAs (T. Noto and K. Mochizuki, unpubl.), increased after periodate oxidation/ β -elimination treatment (Supplemental Fig. S3). We conclude that Hen1p 2'-O-methylates only single-stranded scnRNAs both in vitro and in vivo. This also indicates that 2'-O-methylation of scnRNAs occurs after they are loaded into Twil1p and the "passenger" strand of double-stranded scnRNA is removed.

rHen1p also transferred methyl groups to synthetic RNAs of 16, 20, 24, 28, and 32 nt (Fig. 2B). Therefore, at least in vitro, Hen1p can methylate not only scnRNAs of $\sim$ 28–29 nt but also small RNAs that are the size of the siRNAs of $\sim$ 23–24 nt constitutively expressed in *Tetrahymena*.

Hen1p and Twil1p colocalize and interact

An anti-Hen1p antibody was produced and used to study the expression and localization of Hen1p. Hen1p was not detected by Western blotting in either vegetatively growing or starved cells (Fig. 3A), but *HEN1* mRNA was detected in growing cells by RT-PCR (Supplemental Fig. S4). This suggests that vegetative cells may express a small amount of Hen1p. Hen1p was detected at a very early stage of conjugation at 2 h post-mixing, but the protein quickly disappeared thereafter (Fig. 3A). A similar result was obtained using immunofluorescent staining. Hen1p was not detected in starved cells (Fig. 3B) but was first detected in the macronucleus when the micronucleus was in meiotic prophase at $\sim$ 2 h post-mixing (Fig. 3C,D). Hen1p subsequently became undetectable in post-meiotic stages at $\sim$ 4 h post-mixing (Fig. 3E). In conclusion, Hen1p accumulation is developmentally regulated and specifically occurs in the macronucleus.

Both Hen1p and the Piwi protein Twil1p, which associates with scnRNA (Mochizuki and Gorovsky 2004), localize to the macronucleus during micronuclear meiotic prophase (Fig. 3C,F). We therefore examined whether Twil1p and Hen1p interact. Recombinant GST-Twil1p was mixed with recombinant Hen1p, and a pull-down assay was performed using glutathione beads. As a negative control, recombinant GST protein was used instead of GST-Twil1p. Hen1p copurified with GST-Twil1p, but not with GST (Fig. 3G), indicating that Hen1p and Twil1p directly interact.

Hen1p is essential for scnRNA methylation in *Tetrahymena*

To address whether Hen1p is required for scnRNA methylation, *HEN1* knockout (KO) *Tetrahymena* strains were constructed. The entire Hen1p coding sequence of all polyploid macronuclear copies was replaced with a drug resistance marker in all *HEN1* loci (Supplemental Fig. S5A,B). scnRNAs from *HEN1* KO strains showed increased

FIGURE 2. *Tetrahymena* Hen1p methylates scnRNAs. (A) Recombinant *Tetrahymena* Hen1p methylates single-stranded scnRNAs in vitro. Recombinant *Tetrahymena* Hen1p (lanes 2–5) and control GST protein (lane 1) were incubated with S-adenosyl-L[methyl-¹⁴C] methionine ([¹⁴C]SAM) as a methyl donor and synthetic, single-stranded RNA of 29 nt (lanes 1,2), single-stranded RNA of 29 nt with a 2'-O-methyl group at the terminal nucleotide (lane 3), and double-stranded RNAs of 29 bp with blunt ends (lane 4) or 3' 2-nt overhangs (lane 5). The reactions were analyzed by denaturing gel electrophoresis followed by autoradiography. Hen1p transferred methyl groups only to single-stranded unmodified RNA (lane 2). (B) The activity of Hen1p is not size-specific. Recombinant *Tetrahymena* Hen1p was incubated with [¹⁴C]SAM and synthetic RNAs of 16, 20, 24, 28, and 32 nt. The reaction was analyzed by denaturing gel electrophoresis followed by autoradiography. Hen1p transferred methyl groups to RNA substrates of all sizes tested.

FIGURE 3. Hen1p colocalizes and interacts with Twi1p. (A) Hen1p is expressed early in conjugation. Cell lysates were prepared at the vegetative growing stage (log) and at different developmental stages of sexual reproduction (0 h post-mixing, before mating; 2 h, Mic pre-meiosis; 4 h, Mic meiosis; 6 h, post-zygotic mitosis-Mac anlagen; 8 h, new macronuclear development). For detailed developmental stages, see Cole et al. (1997). The lysate was analyzed by Western blotting using antibodies against Hen1p (top) and α -tubulin (bottom). Hen1p was detected only during the early conjugation stage (2–4 h post-mixing). (B–F) Hen1p and Twi1p localize in the parental macronucleus early in conjugation. Starved (B) or mating (premeiosis, C,D,F; post-second meiosis, E) wild-type cells were processed for immunofluorescence using an anti-Hen1p antibody (leftmost columns in B–E), preimmune serum (third column from the left of B–E), or an anti-Twi1p antibody (F, left). Cells were counterstained with DAPI (second and fourth columns from the left) to visualize the nuclei. Arrowheads indicate micronuclei; P, parental macronuclei. (G) Association between Hen1p and Twi1p. Recombinant GST (left) or GST-Twi1p (right) was immobilized on glutathione beads and incubated with recombinant Hen1p. After washing, proteins on the beads were eluted and analyzed by Western blotting using an anti-Hen1p antibody. Hen1p specifically bound to GST-Twi1p but not to GST alone.

gel mobility after periodate oxidation/ β -elimination treatment (Fig. 4A), indicating that scnRNAs are not modified at their 3' termini in the absence of *HEN1*. Therefore, *HEN1* is essential for 2'-O-methylation of scnRNAs in *Tetrahymena*.

Hen1p is essential for stable accumulation of scnRNAs

Total RNA was extracted from wild-type and *HEN1* KO cells, and scnRNA accumulation was analyzed at several different time points during conjugation. Although comparable levels of scnRNAs were detected in the early stages of conjugation (4 h post-mixing) in wild-type and *HEN1* KO cells, the amount of scnRNAs from *HEN1* KO cells in late stages of conjugation (6–10 h post-mixing) was substantially lower than in wild-type cells. In addition, the scnRNAs were gradually shortened by a few bases in the absence of *HEN1* (Fig. 4B). In contrast, the accumulation of constitutively expressed siRNAs of $\sim$ 23–24 nt was unaffected by the absence of *HEN1* (Fig. 4B). Therefore, the turnover of scnRNAs and siRNAs of $\sim$ 23–24 nt is independently regulated, and only scnRNAs are destabilized in the absence of Hen1p. Since scnRNAs are synthesized only during the early stages of conjugation, these results suggest that Hen1p protects scnRNA from degradation by 2'-O-methylating their 3' ends.

Viability of progeny is greatly reduced in *HEN1* KO strains

The *HEN1* KO strains showed no obvious defects during vegetative growth (data not shown). They mated normally and produced exconjugants (progeny) containing new micro- and macronuclei (data not shown). Thus, at the cytological level, nuclear differentiation appeared to occur normally without *HEN1*.

To test the viability of progeny from the *HEN1* KO strains, single mating pairs were placed into nutrient medium and their growth was analyzed. In wild-type cells, $\sim$ 40% of isolated pairs produced viable sexual progeny, whereas only $\sim$ 3% of *HEN1* KO pairs gave rise to viable sexual progeny (Supplemental Fig. S5C). These data indicate that Hen1p is not essential for the production of functional sexual progeny, but it does play an important role in the efficiency of this process.

DNA elimination is inhibited in *HEN1* KO strains

In the sexual reproduction of *Tetrahymena*, the micronucleus produces both new micro- and macronuclei. During macronuclear development, $\sim$ 6000 internal eliminated sequences (IESs) are removed, and the remaining macronuclear-destined sequences are religated (for review, see Yao and Chao 2005). scnRNAs play essential roles in DNA elimination

FIGURE 4. *HEN1*-mediated 2'-*O*-methylation stabilizes scnRNAs in *Tetrahymena*. (A) scnRNAs in *HEN1* knockout ($\Delta HEN1$) cells are not modified. Small RNAs from wild-type (wt) and $\Delta HEN1$ (Δ) strains extracted at 4 h post-mixing were combined with synthetic, unmodified RNA of 21 nt and were treated with (+) or without (-) periodate oxidation/ β -elimination. The reactions were then mixed with synthetic RNA of 32 nt and analyzed by denaturing gel electrophoresis followed by staining with a nucleic acid-specific fluorescent dye (left). Densitometric analysis of the fluorescent signal is shown on the right. The gel mobility of scnRNAs from $\Delta HEN1$ strains increased after the treatment, indicating that they have free 3' hydroxyl groups. (B) scnRNAs are destabilized in $\Delta HEN1$ strains. Total RNA from wild-type (wt) and $\Delta HEN1$ (Δ) strains was extracted at the indicated time points of conjugation, run on a sequencing gel, and stained with a nucleic acid-specific fluorescent dye. As conjugation proceeded, scnRNAs in $\Delta HEN1$ cells became shorter and less abundant than wt scnRNAs at the same time points.

(for review, see Meyer and Chalker 2006). Therefore, we examined whether 2'-*O*-methylation of scnRNA plays a role in eliminating DNA in the new macronucleus.

We analyzed the elimination of four different IESs (M, R, Cal, and Tlr1 elements) (Austerberry et al. 1989; Katoh et al. 1993; Wells et al. 1994) in single sexual progeny (Fig. 5A–C). In 18 of 56 progeny of the *HEN1* KO strains, one or more of the four examined IES loci were not eliminated (Fig. 5C). In contrast, all of these loci were eliminated in the progeny of the control strain (Fig. 5C). Thus, we conclude that DNA elimination is affected in the absence of Hen1p. Although 38 of 56 progeny of the *HEN1* KO strains showed complete or only reduced elimination of the four IESs, the removal of many of the other ~6000 IESs was likely to be affected in these progeny. Since DNA elimination is believed to be essential for producing viable sexual progeny (Coyne et al. 1999; Nikiforov et al. 1999;

Mochizuki et al. 2002; Liu et al. 2007; Aronica et al. 2008), the reduced production of viable offspring in the *HEN1* KO strains (Supplemental Fig. S5C) is likely due to this partial DNA elimination defect.

DISCUSSION

Function of 2'-*O*-methylation with respect to scnRNA

In this study, we demonstrated that *Tetrahymena* scnRNAs are 2'-*O*-methylated by the RNA methyltransferase Hen1p.

FIGURE 5. (Legend on next page)

Loss of Hen1p renders scnRNAs unstable, partially disables DNA elimination, and makes the production of sexual progeny inefficient. Since the defect in DNA elimination and production of sexual progeny can be explained by the instability of scnRNAs, we believe that the main function of 2'-O-methylation is scnRNA stabilization.

The manner by which 2'-O-methylation stabilizes scnRNAs remains unknown. It may directly protect scnRNA from exonuclease attack. Alternatively, scnRNAs lacking a 2'-O-methyl group may associate less tightly with Twi1p, and dissociated scnRNAs may be more susceptible to ribonuclease degradation. In light of this suggestion, it is worth noting that the 3' terminus of siRNA provides a binding site for the PAZ domains of human Ago1 and Ago2 (Ma et al. 2004; Lingel et al. 2004) and that scnRNAs are unstable in the absence of Twi1p (Mochizuki and Gorovsky 2004).

Similar to the effects reported here for scnRNAs in *Tetrahymena*, HEN1-mediated 2'-O-methylation was reported to stabilize small RNAs in *Arabidopsis* and *Drosophila* (Chen et al. 2002; Li et al. 2005; Horwich et al. 2007). Plants can convey silencing information, which is probably mediated by siRNAs, over long distances (Hamilton et al. 2002; Klahre et al. 2002; Yoo et al. 2004). 2'-O-Methylation of siRNAs in plants may enable them to travel long distances, thus enhancing the defense against viruses (Boutet et al. 2003). In all metazoans studied to date, piRNAs are 2'-O-methylated (Vagin et al. 2006; Horwich et al. 2007; Houwing et al. 2007; Kirino and Mourelatos 2007a; Ohara et al. 2007; Saito et al. 2007) and Piwi proteins function primarily in germline cells (Cox et al. 1998; Deng and Lin 2002; Das et al. 2008). Since germline cells are transcriptionally inactive at certain developmental stages in many animals (Zalokar 1976; Newport and Kirschner 1982; Seydoux et al. 1996; Leatherman et al. 2002), piRNAs may

have to be 2'-O-methylated to increase their turnover time and ensure transposon silencing, even in the absence of their de novo production. Also, scnRNAs in *Tetrahymena* must be stable because they are produced at early stages of conjugation (Malone et al. 2005; Mochizuki and Gorovsky 2005) but they play a role in DNA elimination during late stages of conjugation. Thus, 2'-O-methylation appears to have evolved as a way to protect small RNAs from degradation in pathways that require their stable accumulation.

A specific class of small RNAs is 2'-O-methylated in *Tetrahymena*

We show in this study that scnRNAs, but not siRNAs of ~23–24 nt, are 2'-O-methylated at their 3' ends. Therefore, only this specific class of small RNAs is 2'-O-methylated in the ciliate *Tetrahymena*. Since all 12 Argonaute proteins identified in *Tetrahymena* belong to the Piwi subfamily, the siRNAs of ~23–24 nt are most likely associated with some of those Piwi proteins. If this is the case, this is the first report describing Piwi-associated small RNAs that are not modified by 2'-O-methylation. This suggests that 2'-O-methylation is not a universal feature of Piwi-associated small RNAs.

Tetrahymena Hen1p can methylate various sizes of small, single-stranded RNAs in vitro (Fig. 2B), whereas only scnRNAs seem to be methylated in vivo (Figs. 1A, 2C). This specific methylation of scnRNAs is likely ensured by (1) the restricted accumulation of Hen1p early in conjugation (Fig. 3A), when scnRNAs begin to accumulate (Mochizuki et al. 2002); (2) the localization of Hen1p to the parental macronucleus (Fig. 3B), where Twi1p is also localized (see also Fig. 3F; Mochizuki et al. 2002); and (3) the interaction of Hen1p with Twi1p (Fig. 3G), which specifically binds to scnRNA (Mochizuki and Gorovsky 2004). The selective interaction between HEN1 protein and Piwi proteins has also been reported in *Drosophila* ovaries (Saito et al. 2007). In addition to this HEN1–Argonaute interaction, the specificity of small RNA 2'-O-methylation in metazoans may be controlled by the spatial and temporal regulation of HEN1 homolog expression, as in *Tetrahymena*.

Evolution of piRNA biogenesis

Although all detectable scnRNAs are methylated at their 3' ends throughout conjugation (Fig. 1A), Hen1p, which is responsible for methylating the 3' end (Fig. 4A), only transiently appears during early conjugation (Fig. 3A–E). Therefore, scnRNAs that are present in the middle and later stages must have been methylated by Hen1p during the early stages of conjugation. This argues that most, if not all, scnRNAs are produced during the early stages of conjugation, when the Dicer homolog Dcl1p processes micronuclear noncoding RNA to produce scnRNAs (Malone et al. 2005; Mochizuki and Gorovsky 2005). This also indicates that little or no secondary Dicer-independent scnRNA synthesis

FIGURE 5. DNA elimination is affected in Δ HEN1 progeny. (A) Schematic diagram of the IES elimination assay. Four primers per locus (M and R loci, *top*) or six primers per locus (Cal and Tlr1 loci, *bottom*) were used for nested PCR. Primers marked 1 were used first, followed by those marked 2. Shorter Mac-form products (a) indicate that the locus was rearranged, whereas longer Mic-form products (i) indicate a defect in DNA elimination. (B) An example of the results of DNA elimination assays in Δ HEN1 progeny. Single cells were picked at 30 h post-mixing and used in nested PCR reactions as shown in A. M, R, Cal, and Tlr1 loci were tested in each progeny cell for DNA elimination. Representative PCR results of 19 progenies of Δ HEN1 cells are shown. The positions of PCR products from unrearranged (i) and rearranged (a) loci are indicated. The progeny that showed only an unrearranged PCR product (highlighted with asterisks) in at least one of the four loci were counted as cells with defective DNA elimination. (C) Summary of the results obtained from 56 progeny cells of the Δ HEN1 and control cells. M, R, Cal, and Tlr1 loci were tested in each progeny cell (1–56) for DNA elimination. Open boxes indicate rearranged (a) DNA; filled boxes, unrearranged (i) DNA. Eighteen of 56 progeny cells from Δ HEN1 had DNA elimination defects in at least one of the four loci examined, whereas none of the progeny from control cells showed such defects.

occurs during the later stages of conjugation. In addition, siRNAs of ~23–24 nt in *Tetrahymena*, which likely associate with Piwi proteins, are produced through a Dicer-dependent mechanism (Lee and Collins 2007). These processes differ markedly from the Dicer-independent production of piRNA in metazoans (Vagin et al. 2006; Houwing et al. 2007; Das et al. 2008; Wang and Reinke 2008). In *Drosophila*, it has been postulated that piRNAs are produced by sequential actions of the endoribonuclease (slicer) activities of Piwi proteins (Brennecke et al. 2007; Gunawardane et al. 2007). At this time, we cannot conclude whether the Dicer- or Piwi-dependent form of piRNA production is the ancestral form. It would be interesting to know how piRNAs are produced and whether all of these are 2'-O-methylated in *Dictyostelium*, which is an amoeba more closely related to metazoans than to ciliates.

MATERIALS AND METHODS

Cell lines and culture conditions

The wild-type CU428 and B2086 strains of *T. thermophila* were provided by Dr. P. J. Bruns (Cornell University). These strains are also available at the *Tetrahymena* Stock Center (Cornell University, <http://tetrahymena.vet.cornell.edu>). The cells were grown at 30°C in SPP medium (Gorovsky et al. 1975) containing 2% proteose peptone. Before mating, the cells were washed and resuspended (5×10^5 cells/mL) in 10 mM Tris buffer (pH 7.5). After 12–18 h of incubation at 30°C, equal numbers of cells were mixed and mated. In the experiments shown in Figures 3A and 4B, the cultures were refed at 2 and 4 h post-mixing, respectively, by adding one-third volume of 4× SPP medium to limit the initiation of mating after that period.

Analysis of small RNAs

For the experiments shown in Figures 1A and 4A and Supplemental Figure S3, total RNA enriched in small RNAs was extracted using the mirVana Kit (Ambion). For the experiment shown in Figure 1B, total RNA was first extracted with Trizol (Invitrogen), and the small RNA fraction was enriched according to the method described by Lee and Collins (2006). For the experiment shown in Figure 4B, total RNA was extracted with Trizol. Oligo ribonucleotides were synthesized by Dharmacon. The periodate oxidation/ β -elimination reactions were performed as described (Akbergenov et al. 2006). RNA was separated in 12%–20% polyacrylamide-urea sequencing gels and was visualized using Gel Red (Biotium).

Two-dimensional thin-layer chromatography

Total RNA was isolated with Trizol (Invitrogen) at 4 h post-mixing and separated in a preparative sequencing gel; the scnRNAs (~26–31 nt) were purified from the gel. The purified scnRNAs were ligated to [5'-³²P]pCp using T4 RNA ligase (Amersham). The ligated scnRNAs-pCp were gel purified and digested for 20 min at 37°C using P1 nuclease (400 mU/mL final) (Sigma). The digest was mixed with synthetic 2'-O-methylated dinucleotide standards (pAmpC, pCmpC, pGmpC, and pUmpC), spotted onto HPTLC

cellulose glass plates (10 × 10 cm; Merck), and resolved with isobutyric acid/25% ammonium hydroxide/water (66:1:33 by volume) in the first dimension and then with isopropanol/HCl/water (70:15:15 by volume) in the second dimension. The labeled dinucleotides were visualized by autoradiography, while the synthetic dinucleotide standards were visualized by UV shadowing.

Expression and purification of Hen1p protein from *E. coli*

Because of the differential codon usages in *Tetrahymena*, the entire Hen1p coding sequence was synthesized (GenScript) for expression in *E. coli*. The sequence of the synthetic *HEN1* gene is available upon request. The synthetic gene was subcloned into a modified pGEX-4T-1 vector (pGEX4T1-TEV), which has a TEV protease recognition sequence at the C terminus of the GST (gift from T. Clausen, IMP, Vienna) to allow production of a GST-TEV-Hen1p fusion protein. The construct was expressed in the *E. coli* BL21 (DE3) strain overnight at 25°C in the presence of 50 μ M IPTG. The proteins were purified using glutathione Sepharose 4B resin (GE Healthcare), and the recombinant Hen1p was eluted from the beads by cleaving the TEV sequence with AcTEV protease (Invitrogen).

Methyltransferase assay

In vitro methyltransferase assays were performed as described previously (Yu et al. 2005) with slight modifications. Hen1p (~35 μ g) was incubated for 5 h at 37°C with 0.6 nmol of synthetic oligoribonucleotides (Dharmacon) (for sequences, see Supplemental Fig. S2) and 1.5 μ Ci S-adenosyl-L[methyl-¹⁴C] methionine (Amersham). After proteinase K treatment, followed by phenol/chloroform extraction and ethanol precipitation, half of the sample was separated in a thin (0.5-mm) 12% polyacrylamide-urea sequencing gel. The gel was dried, wrapped in Saran wrap, and exposed overnight to a storage phosphor screen (GE Healthcare). Images were captured using a Typhoon Imager (GE Healthcare) and analyzed using ImageQuant software (GE Healthcare).

Indirect immunofluorescent staining

Cells were fixed in 3.7% formaldehyde and 0.5% Triton-X 100 for 30 min at room temperature (RT), resuspended in 3.7% formaldehyde and 3.4% sucrose, and dried on poly-L-lysine (Sigma)-coated cover slips. The samples were blocked for 1 h with 3% BSA (Sigma), 10% normal goat serum (Invitrogen), and 0.1% Tween 20 in PBS followed by overnight incubation at 4°C in blocking solution containing a 1:500 dilution of anti-Hen1p or a 1:200 dilution of anti-Twi1p (Aronica et al. 2008). The anti-Hen1p antibody was obtained by immunizing a rabbit with recombinant Hen1p. After washes with PBT, samples were incubated with a 1:1000 dilution of anti-rabbit antibody conjugated with Alexa 488 (Invitrogen) for 1 h at RT. The samples were washed, incubated with 10 ng/mL DAPI (Sigma) in PBS, mounted with ProLong Gold (Invitrogen), and observed by fluorescent microscopy.

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) from the wild-type strains B2086 and CU428, and residual genomic DNA was eliminated using the Turbo DNase Kit (Ambion). cDNA was

synthesized from 5 µg of total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) and a random hexamer as a primer. cDNA from 12.5 ng of total RNA was amplified by PCR (30 sec at 94°C, 30 sec at 51°C, 40 sec at 72°C; 32 cycles) with primers 5'-AAAATTAATTCGAAGATGGTTCATAC-3' and 5'-TAGGAGAATAAGTTATCTGCAGTGG-3'.

GST pull-down assay

GST, GST-Twilp, and Hen1p were expressed in *E. coli* and purified as described above. GST or GST-Twilp (0.1 µg) were incubated for 30 min at 4°C with glutathione Sepharose 4B resin (GE Healthcare) in GST pull-down buffer (GPB) (20 mM Tris at pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, 10% glycerol, 0.1 mM EDTA, 1 mM DTT). The beads were washed once with 1% BSA in GPB and once with 0.1% BSA in GPB. Hen1p (0.1 µg) was added, and the samples were incubated for 2 h at 4°C. The beads were washed five times with 0.1% BSA in GPB and boiled in SDS loading buffer. The samples were loaded onto an SDS-PAGE gel, and the presence of Hen1p was monitored by Western blotting using the anti-Hen1p antibody.

Construction of *HEN1* KO strains

The *HEN1* KO construct (see Supplemental Fig. S5A) was generated by overlapping PCR. First, the 5'UTR and 3'UTR were amplified using the following primers (sequences overlapping with a *neo4* cassette are underlined):

5_UTR_fw, 5'-GGACTCGAGTGATAAAAAATGAGTTGTTTGCTTATT-3';
 5_UTR_rv, 5'-GTCTATCGAATTCCTGCAGCCCAACCGGCTAGTTTTACTTAG-3';
 3_UTR_fw, 5'-CTGGAAAAATGCAGCCCTTGAAGCATTACAAATAAATGG-3'; and
 3_UTR_rv, 5'-GGAATTCTCAACAACTAAATTCAAAC-3'.

The products were combined with the *neo4* cassette (Mochizuki 2008) using 5_UTR_fw and 3_UTR_rv and the PCR extender system (5PRIME). The B2086 and CU428 wild-type strains were transformed with the PCR product as described (Cassidy-Hanley et al. 1997), and the transformants were selected with 100 µg/mL paromomycin in the presence of 1 µg/mL cadmium chloride. The endogenous Mac *HEN1* loci were replaced by phenotypic assortment and selection using increasing concentrations of paromomycin. Complete replacement was confirmed by Southern hybridization (Supplemental Fig. S5B).

DNA elimination assay

Elimination of M, R, Cal, and Tlr1 IES elements (Austerberry et al. 1989; Katoh et al. 1993; Wells et al. 1994) was performed as described previously (see also Fig. 5A; Aronica et al. 2008) with a slight modification. In this assay, the control cells were wild-type cells possessing a *neo4* marker at the 3' flanking region of the *HHT2*-mCherry fusion gene in the macronuclei (K. Kataoka and K. Mochizuki, unpubl.). Single pairs of control and *HEN1* KO strains were placed into drops of SPP medium at ~8 h post-mixing and were allowed to complete conjugation. The separated exconjugants were analyzed at 30 h post-mixing by nested PCR. In

the first PCR, all five sets of primers—the outer primers for the four IES regions and the outer *neo4* primers—were used in a single reaction. In the second PCR, only one set of inner primers was used per reaction. In this way, DNA elimination of all four IES loci in a single exconjugant could be analyzed. The control and *HEN1* KO strains contain *neo4* genes in their parental Macs, but their progeny lack *neo4* genes in their micronuclei and new macronuclei. Samples that provided *neo4* amplification products, indicating that the parental cells aborted conjugation, were excluded from the study.

Progeny viability test

Viability of progeny was analyzed as described previously (Mochizuki et al. 2002) with a slight modification: The mating pairs were isolated at 6–8 h post-mixing, and the growth of the cells was examined ~60 h after cloning.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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