A potential link between transgene silencing and poly(A) tails

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REPORT

A potential link between transgene silencing and poly(A) tails

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ABSTRACT

Argonaute proteins function in gene silencing induced by double-stranded RNA (dsRNA) in various organisms. In *Drosophila*, the Argonaute proteins AGO1 and AGO2 have been implicated in post-transcriptional gene-silencing (PTGS)/RNA interference (RNAi). In this study, we found that AGO1 and AGO2 depletion caused the accumulation of multicopied enhanced green fluorescence protein (EGFP) transgene transcripts in *Drosophila* S2 cells. Depletion of AGO1, the essential factor for miRNA biogenesis, led to an increased transcriptional rate of the transgenes. In contrast, depletion of AGO2, the essential factor for siRNA-directed RNAi, resulted in EGFP mRNA stabilization with concomitant shortening of the EGFP mRNA poly(A) tail. Our findings suggest that AGO1 and AGO2 mediate multicopied transgene silencing by different mechanisms. Intriguingly, Dicer2 depletion phenocopies AGO2 depletion, with an increase in EGFP protein levels and shortening of the EGFP mRNA poly(A) tail. The possibility that AGO2 and Dicer2 involve, at least in part, poly(A) length maintenance of transgene mRNA suggests a potentially important link between transgene silencing and poly(A) tails.

Keywords: argonautes; TGS; PTGS; poly(A); *Drosophila*

INTRODUCTION

Double-stranded (ds) RNA induces sequence-specific post-transcriptional gene silencing (PTGS) of cognate genes in a wide range of organisms (Meister and Tuschl 2004; Mello and Conte 2004; Sontheimer 2005). The ribonuclease III enzyme Dicer converts long dsRNA to short interfering RNAs (siRNAs) of 21–23 nucleotides (nt) in length (Bernstein et al. 2001; Ketting et al. 2001; Knight and Bass 2001), which are, in turn, unwound and loaded onto an RNA-induced silencing complex (RISC), an enzymatic complex that mediates cleavage of the mRNA targets depending on the sequence of the siRNA molecule within the complex. This process is known as RNA interference (RNAi) (Fire et al. 1998). RNAi is not the only PTGS triggered by dsRNAs in vivo. MicroRNAs (miRNAs) are a class of endogenously encoded small noncoding regulatory RNAs that are processed from hairpin-type precursor RNA transcripts by Dicer (Ambros 2004; Bartel 2004). miRNAs negatively control translation of mRNA targets containing sequences that are imperfectly complementary to them (Ambros 2004; Bartel 2004). Many factors that function in small RNA-mediated PTGS have been identified in various organisms. Among these, members of the Argonaute family of proteins are the most extensively studied (Carmell et al. 2002). In *Caenorhabditis elegans*, genetic analyses suggest that RDE-1, a member of the Argonaute family, is required for the initiation of RNAi, whereas Alg-1 and Alg-2, other members of the family, are required for the accumulation of stable mature miRNAs, but not for RNAi driven by dsRNA (Grishok et al. 2001). In cultured *Drosophila* S2 cells, AGO2 was identified as a core component of biochemically purified RISC (Hammond et al. 2001). Further, it has been shown that embryos lacking AGO2 are siRNA-directed RNAi defective, but are still capable of miRNA-directed target RNA cleavage (Okamura et al. 2004). In contrast, AGO1 is dispensable for siRNA-directed RNA cleavage, but is necessary for the accumulation of stable mature miRNAs, thus impacting on miRNA-directed target RNA cleavage. Recently, human AGO2 was shown to be the small RNA-guided endonuclease that cleaves the mRNA targets in RNAi (Liu et al. 2004; Song et al. 2004). In contrast, human AGO1,

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AGO3, and AGO4 did not show such target cleavage activity (Liu et al. 2004; Meister et al. 2004). These findings indicate that the Argonautes are not functionally redundant.

It has been also shown that some of the Argonaute family of proteins direct transcriptional gene silencing (TGS) induced by dsRNAs complementary to DNA sequences in the nucleus. For instance, Ago1, the only member of the Argonaute in fission yeast, is required for silencing transcription at centromeres (Volpe et al. 2002) and initiation of silent heterochromatin at the mating-type locus (Hall et al. 2002), which suggests that the RNAi mechanism is closely related to that of TGS. Very recently, Ago1 was shown to be necessary not only for TGS, but also for the RNAi pathway in fission yeast (Sigova et al. 2004). Genetic studies in plants have likewise demonstrated that the RNAi mechanism is closely related to that of TGS (Finnegan and Matzke 2003; Baulcombe 2004). In *Arabidopsis thaliana*, Ago1 is involved in RNAi and miRNA-directed gene silencing pathways (Vaucheret et al. 2004), whereas Ago4 is implicated in guiding the chromatin silencing pathway (Zilberman et al. 2003; Chan et al. 2004).

Although for many years transgene silencing was thought to be a phenomenon unique to plants, recent genetic studies have shown that it also occurs in *Drosophila* (Birchler et al. 2003). *piwi*, a member of the Argonaute family, is necessary for post-transcriptional transgene silencing and some aspects of transcriptional transgene silencing (Pal-Bhadra et al. 2002, 2004). *aubergine* (a.k.a. *sting*), another member of the family, is responsible for acting post-transcriptionally to maintain silencing of the X-linked repetitive Stellate locus that is necessary for male fertility (Livak et al. 1990, Schmidt et al. 1999; Aravin et al. 2001, 2004) and is also necessary for some aspects of post-transcriptional transgene silencing (Pal-Bhadra et al. 2002). However, it is also known that transgene silencing in *Drosophila* differs from many cases in plants, in that the degree of silencing is not as strong and seldom reaches complete silencing (Birchler et al. 2003). To investigate the possible involvement of AGO1 and AGO2 in transgene silencing in *Drosophila*-cultured S2 cells, we have sought to determine the effects of these Argonaute proteins on the expression of multicopied enhanced green fluorescence protein (EGFP) transgenes in S2 cells.

RESULTS AND DISCUSSION

Depletion of AGO1 or AGO2 by RNAi results in the accumulation of multicopied EGFP transgene transcripts in S2 cells

We produced an S2 cell line stably expressing EGFP (S2-EGFP) and verified by Southern blot analysis that EGFP transgenes are present in ~20 copies per genome in this cell line (data not shown). Although little is known about transgene silencing in cultured cells, the presence of multiple copies of EGFP was expected to trigger PTGS of the EGFP transgenes. Therefore, we asked whether AGO1 or AGO2, or both, was involved in silencing of the EGFP transgenes.

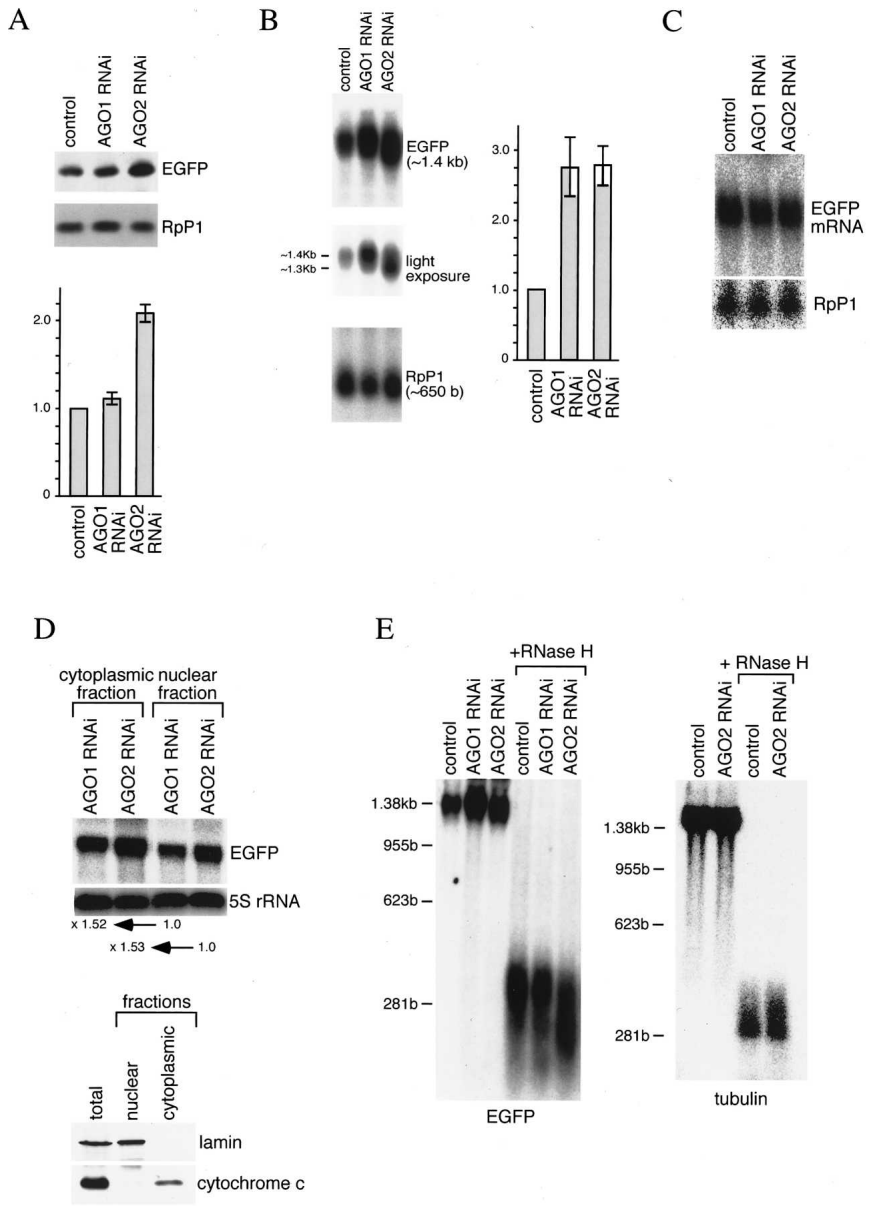

FIGURE 1. (Legend on next page)

RNAi-based reverse-genetic methods have been widely applied to study gene function in S2 cell cultures (Boutros et al. 2004). AGO2 depletion from S2-EGFP cells by RNAi caused an approximately twofold accumulation of EGFP protein compared with control (Fig. 1A). In contrast, AGO1 depletion did not cause significant accumulation of EGFP protein. These experiments were repeated four times and the quantitative data are shown in Figure 1A. We then determined whether the levels of EGFP mRNA were affected upon depletion of AGO1 or AGO2 in S2 cells. We carried out Northern blot analysis on total RNAs from S2-EGFP cells and found that the steady-state concentration of EGFP mRNA was increased approximately threefold upon depletion of AGO1 or AGO2 (Fig. 1B). In the case of EGFP mRNA transiently expressed by transfection, depletion of AGO1 or AGO2 did not cause any significant change in EGFP transcript levels (Fig. 1C). These findings suggest that AGO1 and AGO2 function in the silencing of multicopied EGFP transgenes.

AGO1 and AGO2 depletion both caused the accumulation of EGFP mRNA (Fig. 1B). On the other hand, EGFP protein accumulation was only observed in AGO2-depleted cells (Fig. 1A). From these results, it could be speculated that the depletion of AGO1 in some way affects the nuclear and cytoplasmic distribution of EGFP mRNA. Thus, we performed Northern blot analysis on total RNAs isolated separately from the nucleus and the cytoplasm of AGO1- and AGO2-depleted cells. The relative amounts of the EGFP transcripts in the cytoplasmic fraction to those in the nuclear fraction were calculated; they were approximately one and a half fold in both cases (Fig. 1D), indicating that EGFP mRNA distribution in the cellular compartments is the same in both cases. Why increased EGFP mRNA does not result in increased production of EGFP protein in AGO1-depleted cells is unclear.

AGO2 depletion by RNAi leads to poly(A) shortening of the EGFP transcripts

Intriguingly, we noticed that when AGO2 was depleted, EGFP transcripts were shorter by ~100 nucleotides (nt) (Fig. 1B). In contrast, AGO1 depletion did not affect the size of mRNA. In the case of an endogenous gene, ribosomal protein P1 (RpP1), the size of the transcripts was not altered upon depletion of AGO1 or AGO2 (Fig. 1B). The size of EGFP mRNA transiently expressed in S2 cells was also not affected by either AGO1 or AGO2 depletion (Fig. 1C), suggesting that mRNA shortening is specific for transgenes. We hypothesized that poly(A) tails of EGFP transcripts might be shortened in the AGO2-depleted cells. To test this, we isolated total RNAs from cells depleted of AGO1 or AGO2 and examined the poly(A) length of EGFP transcripts by RNaseH cleavage assay (Fig. 1E). When AGO2 was depleted, the poly(A) tails of EGFP transcripts were indeed shorter by ~100 bases than those present in control and AGO1-depleted cells. In contrast, the poly(A) length of tubulin mRNA were not altered in AGO2-depleted cells (Fig. 1E, right). In most of the cases, the length of poly(A) tails determines the translational status of a regulated transcript (for review, see Parker and Song 2004), such that translational silencing correlates with poly(A) shortening. In the case of EGFP transgene transcripts in AGO2-depleted cells, however, the poly(A) shortening did not down-regulate the translation; rather, EGFP protein expression level was observed to be higher than that in controls (Fig. 1A). This is, admittedly, a rare example of poly(A) shortening, leading to increased protein expression, although there are precedents; for example, in mice, the activation of translation of some mRNA during spermiogenesis has been shown to be accompanied by poly(A) shortening (Kleene 1989).

FIGURE 1. Depletion of members of the Argonaute family of proteins by RNAi causes an increase in expression of multicopied EGFP transgenes. (*A*) S2-EGFP cells were transfected with double-stranded RNAs (dsRNAs) corresponding to the indicated cDNAs (AGO1 and AGO2). Four days later, cells were harvested and the expression of EGFP protein analyzed. Values shown represent the expression of EGFP protein in AGO1 or AGO2 dsRNA-transfected cells relative to EGFP protein expression from cells transfected with control dsRNA. Four independent experiments were performed. Expression levels of ribosomal protein P1 (RpP1) were also checked as a control. (*B*) Northern blots show that depletion of AGO1 or AGO2 causes the accumulation of EGFP transcripts. Values shown represent the expression of EGFP mRNA in AGO1 or AGO2 dsRNA-transfected cells relative to EGFP mRNA expression from cells transfected with control dsRNA. Three independent experiments were performed. EGFP mRNAs are ~100 nt shorter, specifically when AGO2 is depleted. The levels of the RpP1 transcripts are also shown as a control, indicating that both amount and length of RpP1 mRNA are not altered upon depletion of AGO1 or AGO2. (*C*) Northern blots show that the levels of transiently expressed EGFP mRNA do not significantly change upon depletion of AGO1 or AGO2. The levels of RpP1 mRNA are shown as a control. (*D*) Northern blots show the nuclear and cytoplasmic distribution of EGFP mRNA. EGFP mRNA levels in the cytoplasmic fractions relative to those in the nuclear fractions are 1.52- and 1.53-fold in AGO1- and AGO2-depleted cells, respectively. Total RNAs (5 μg) prepared either from the nuclear or the cytoplasmic fraction were applied per lane. 5S rRNA is shown as an internal control. Western blots (*bottom*) show that lamin is only observed in the cytoplasmic fraction and cytochrome c only in the nuclear fraction, indicating that both fractions were well separated from each other. (*E*) AGO2 depletion leads to poly(A) shortening of the EGFP transcripts. The length of the EGFP mRNA poly(A) was compared under conditions with or without AGO2 expression. Northern blots after treatment of total RNAs with RNaseH and a specific oligo DNA hybridizing to EGFP 3′ UTR sequence show that the poly(A) tails are shorter by an ~100 nt, but only in AGO2-depleted cells (*left*). The length of tubulin mRNA poly(A) tail was not altered upon AGO2 depletion (*right*).

AGO1 and AGO2 mediate transgene silencing by different mechanisms

There are at least two possibilities explaining the observed accumulation of EGFP mRNA upon depletion of AGO1 or AGO2, namely, an increase in the transcriptional rate or an increase in mRNA stability. To distinguish these, we first examined the levels of EGFP mRNA by quantitative Northern blotting in AGO1- or AGO2-depleted cells after treatment with Actinomycin D (to inhibit synthesis of nascent transcripts). Only

AGO2 depletion led to the stabilization of EGFP mRNA (Fig. 2A). Tubulin mRNA stability was, on the other hand, not affected by AGO2 depletion (Fig. 2A). These results indicate that AGO2 is involved in destabilizing EGFP transgene transcripts in the stable cell line. The stabilization of EGFP mRNA upon AGO2 depletion might explain the mild increase in EGFP protein observed in AGO2-depleted cells. We also measured the nuclear transcriptional rate of EGFP transgenes by nuclear run-on experiments in cells where AGO1 or AGO2 was depleted. AGO1 depletion, but not that of AGO2, led to an increase in EGFP transgene transcription levels (Fig. 2B), suggesting that AGO1 is involved in TGS of the EGFP transgenes in S2 cells.

It has been shown that siRNAs guide heterochromatin formation or DNA methylation of cognate DNA sequences, result-ing in transcriptional silencing in plants, fission yeast, and human (Baulcombe 2004; Lippman and Martienssen 2004). In plants, heterochromatin formation has been shown to be strongly related to DNA methylation at the promoter regions (Soppe et al. 2000; Matzke et al. 2003; Chan et al. 2004). The clear difference between plants and S2 cells in transgene silencing is the degree of the silencing; in plants, the expression is completely silenced, whereas in S2-EGFP cells, the silencing degree is so low that the EGFP expression is still partly apparent. This could be due to the low amount of EGFP antisense transcripts. In agreement with this, we could not detect EGFP antisense transcripts by Northern blot analysis in S2-EGFP cells (data not shown). However, we were able to detect the presence of EGFP antisense transcripts in S2-EGFP cells by RT–PCR (Fig. 2C), which suggests that duplex forms of EGFP transcripts could exist in S2-EGFP cells. We found that methylation of the EGFP promoters in S2-EGFP cells was quite scarce by bisulfite DNA sequencing analyses (data not shown). Therefore, unlike in plants, DNA methylation does not appear to participate in TGS of the EGFP transgenes in S2 cells. It is known that heterochromatin negatively effects gene expression (Richards and Elgin 2002), and that the formation and maintenance of heterochromatin is characterized by addition of specific modifying groups of histones, which are consequently bound by heterochromatin protein 1 (HP1) (Maison and Almouzni 2004). We found that EGFP transgenes are heterochromatinized at the promoter areas as judged by heterochromatin immunoprecipitation assay using anti-HP1 antibody (Fig. 2D). However, we were unable to detect any significant change in heterochromatin for-

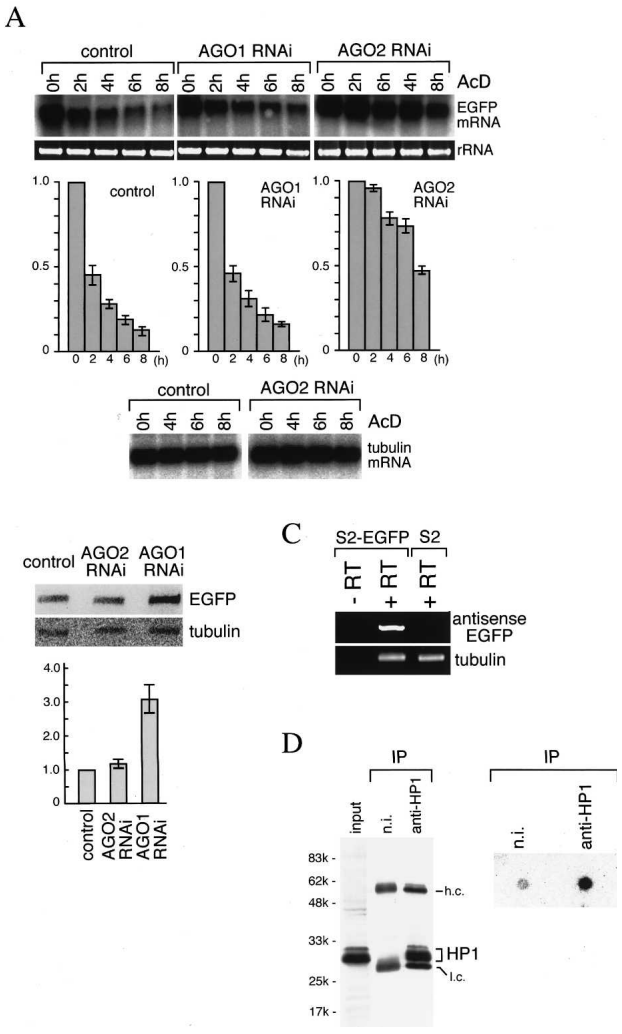

FIGURE 2. AGO1 and AGO2 are involved in silencing of EGFP transgenes in two ways. (*A*) AGO2 functions in PTGS of the transgenes. S2-EGFP cells were first treated with dsRNAs corresponding to AGO1 or AGO2 cDNAs. Four days later, transfected cells were treated with ActinomycinD. At the times indicated, total RNAs in each case were isolated and EGFP transcripts analyzed by Northern blot analyses. Ribosomal RNAs (rRNA) stained with ethidium bromide are shown as a loading control. A rapid decrease of the EGFP mRNA was observed in AGO1-depleted cells, as in the control. In contrast, the rate of decrease was much slower in AGO2-depleted cells, indicating that the EGFP transcripts are stabilized upon AGO2 depletion. Quantitation of the RNA bands by a PhosphorImager from three independent experiments is shown below. Tubulin mRNA levels were not altered upon AGO2 depletion in contrast to EGFP mRNA levels. (*B*) Nuclear run-on shows that an increase in transcription of the EGFP transgenes was observed only upon AGO1 depletion. Four days after dsRNA treatment, cells were harvested and the nuclei isolated. Transcription was then performed in nuclei in the presence of [^{32}P]UTP. The data shown below are the average ± standard deviation for three trials. No increase in transcription of tubulin was observed when AGO1 was silenced by RNAi. Tubulin mRNA levels are shown as an internal control. (*C*) Detection of EGFP antisense transcripts in S2-EGFP cells. EGFP antisense transcripts were not detected in S2-EGFP cells by Northern blotting (data not shown). RT–PCR analysis, however, reveals that EGFP antisense transcripts are indeed present in S2-EGFP cells, suggesting that EGFP dsRNAs may exist in S2-EGFP cells. RT–PCR was also performed for tubulin as an internal control. (*D*) Heterochromatin formation at the EGFP promoter region. Dot-blot hybridization was performed on DNA fragments isolated from the immunoprecipitated complexes with anti-HP1 antibody (*right*). The probe used contains the sequence corresponding to the EGFP promoter. (n.i.) nonimmune IgG shows the background level of this experiment. Western blot using anti-HP1 (*left*) shows that anti-HP1, but not n.i., specifically immunoprecipitates HP1 protein. (h.c. and l.c.) Protein bands corresponding to IgG heavy and light chains, respectively.

mation at the EGFP promoter region upon AGO1 depletion (data not shown).

Dicer2 depletion phenocopies AGO2 depletion

Drosophila Dicer2 and AGO2 act in concert in the RNAi pathway, while Dicer1, together with AGO1, is primarily involved in miRNA processing, and consequently, in miRNA function (Liu et al. 2003; Lee et al. 2004; Okamura et al. 2004). We sought to determine whether depletion of Dicer1 or Dicer2 by RNAi could also affect expression of the EGFP transgenes. Depletion of Dicer2, but not Dicer1, resulted in accumulation of EGFP protein (Fig. 3A), as was the case for depletion of AGO2 (Fig. 1A). Both Dicer1 and Dicer2 depletion caused the accumulation of EGFP mRNA (Fig. 3B), but poly(A) tail shortening was observed only in Dicer2-depleted cells (Fig. 3B).

The CCR4–NOT complex is known to be the major enzyme catalyzing mRNA deadenylation in *Saccharomyces cerevisiae* (Denis and Chen 2003). The fly homologs of the catalytic subunits of the complex, CCR4 and CAF1, were shown to be associated with a cytoplasmic poly(A)-specific 3′ exonuclease activity, and RNAi knockdown of each of these genes led to a lengthening of bulk mRNA poly(A) tails (Temme et al. 2004). Thus, we performed double depletion of AGO2 and CAF1, or Dicer2 and CAF1 in S2-EGFP cells, and examined the poly(A) length of EGFP transcripts. Depletion of AGO2 and CAF1, or Dicer2 and CAF1, led to the restoration of the length of the EGFP transcript poly(A) tails (Fig. 3C). These findings imply that AGO2 and Dicer2 protect poly(A) tails of EGFP mRNA from being shortened by deadenylase activity in vivo.

Conclusion

Transgene silencing has been observed in a wide variety of organisms, including plants, fungi, nematodes, and *Drosophila melanogaster* (Birchler et al. 2003). Our findings clearly show that the presence of multiple copies of EGFP triggers PTGS and probably TGS of the EGFP transgenes in cultured S2 cells. Both AGO1 and AGO2 were observed to function in the silencing of the EGFP transgenes, but their mechanisms of action clearly differ from each other. A general model has been proposed for heterochromatin formation and epigenetic gene silencing in different species (Lippman and Martienssen 2004). We found that EGFP transgenes are heterochromatinized at the promoter areas, indicating that the model also fits well to S2 cultured cells. Nuclear run-on assays show an increase in EGFP mRNA only in AGO1-depleted cells, but no differences in EGFP mRNA turnover. Our failure to detect any significant change of heterochromatin formation at the EGFP promoter areas upon AGO1 depletion could reflect a loss of heterochromatinization at only a few of the multicopied EGFP promoters.

EGFP mRNA stabilization upon depletion of AGO2 is accompanied by shortening of the EGFP poly(A) tail (~100 nt shorter). Increased EGFP mRNA does not appear to result in increased production of EGFP protein in AGO1-depleted S2-EGFP cells. However, in AGO2-depleted cells, EGFP protein levels were increased as EGFP mRNA. The nuclear and cytoplasmic distributions of EGFP mRNA in both cases are the same. It is speculated that the length of the poly(A) tail may directly correlate with the expression levels of EGFP protein. Interestingly, Dicer2 depletion phe-

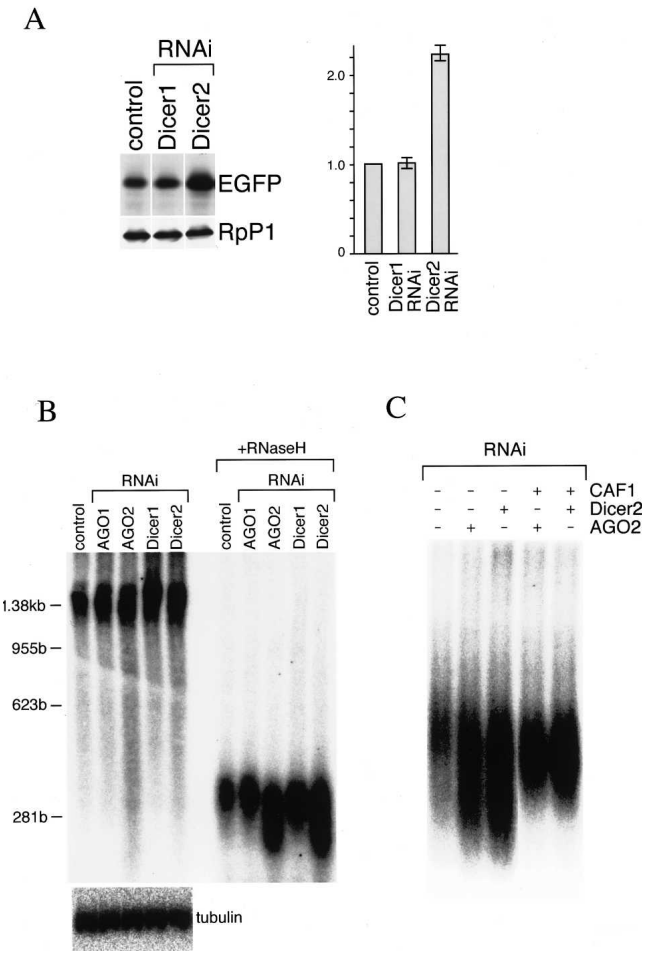

FIGURE 3. Dicer2 depletion phenocopies AGO2 depletion. (*A*) Western blots show that depletion of Dicer2, but not Dicer1, causes the accumulation of EGFP protein. S2-EGFP cells were transfected with dsRNAs corresponding to the indicated cDNAs. Three days later, cells were harvested and the expression of EGFP protein analyzed by Western blotting. Values shown represent the expression of EGFP protein in Dicer1 or Dicer2 dsRNA-transfected cells relative to EGFP protein expression from cells transfected with control dsRNA. Three independent experiments were performed. (*B*) Dicer2 depletion phenocopies AGO2 depletion, with an increase in EGFP mRNA levels and poly(A) shortening. Of note, is that an increase in EGFP mRNA levels was observed in both Dicer1-depleted and Dicer2-depleted cells. Tubulin mRNA levels are also shown as an internal control. (*C*) Depletion of CAF1, a component of the mRNA deadenylation complex, together with depletion of AGO2 or Dicer2 caused the restoration of the poly(A) length of the EGFP transcripts.

nocopies AGO2 depletion, with an increase in EGFP protein levels and shortening of EGFP mRNA poly(A) tails. In flies, Dicer2 and AGO2 are part of a holo-RISC and act in concert in the processing and function of siRNAs derived from dsRNA (Liu et al. 2003; Lee et al. 2004; Okamura et al. 2004). Thus, our findings raise the possibility that in addition to their role in RNAi, AGO2 and Dicer2 also function in PTGS of multicopied transgenes and in protection of the poly(A) tails from being shortened. The precise mechanisms involved in the shortening of the poly(A) tails of EGFP transcripts upon depletion of AGO2 or Dicer2 are unclear. Whatever the mechanisms might be, our findings provide a functional connection between RNAi complexes and enzymes that mediate poly(A) tail modifications. Recent findings in fission yeast and *C. elegans* also appear to point to a potentially important link between an RNAi-mediated process and poly(A) tails. RNAi-mediated heterochromatin assembly in fission yeast requires the RNA-induced transcriptional silencing (RITS) complex that interacts with a complex termed RNA-directed RNA polymerase complex (RDRC) containing Cid12, a member of the poly(A) polymerase family, in a manner that requires Dicer (Motamedi et al. 2004). The *C. elegans* gene *rde-3*, which is required for siRNA accumulation and for efficient RNAi in all tissues, also encodes a member of the poly(A) polymerase family (Chen et al. 2005).

MATERIALS AND METHODS

Antibodies and Western blot analyses

Anti-EGFP antibody was described previously (Okamura et al. 2004). Anti-RpP1 antibody was a kind gift from T. Uchiumi (Niigata University). Anti-HP1 antibody, C1A9, was purchased from the Developmental Studies Hybridoma Bank of the University of Iowa. Antibody against cytochrome c was purchased from BD Biosciences Pharmingen. Ant-lamin (lamin C) was a kind gift from K. Furukawa (Niigata University). Western blot analyses were performed as described in Ishizuka et al. (2002).

Northern blot analyses and RT–PCR

Northern blotting was performed as described in Ishizuka et al. (2002). For EGFP, the full-length open reading flame (ORF) was used as a probe. In Figure 1B, dsRNA of AGO1 or AGO2 was introduced into S2-EGFP cells by soaking for depletion, and Northern blotting was performed using 1.5% agarose gels. Total RNAs used was 5 μg/lane in each case. In Figure 2A, RNAi for AGO1 or AGO2 was performed for 4 d, and the cells were treated with ActinomycinD at 5 μg/mL. At each of the indicated times, total RNAs were isolated and Northern blotting performed. Total RNAs used were 5 μg/lane in each case. RT–PCR for detecting EGFP antisense and tubulin was performed using 3 μg of total RNAs isolated from S2-EGFP or parental S2 cells and treated with DNase. The RT–PCR kit (ProSTAR Ultra-HF RT-PCR system) was purchased from Stratagene. The primer used for RT for detecting EGFP antisense was 5′-GCTGTTCACCGGGGTGGTGC CC-3′. For tubulin, oligo d(T) was utilized for RT reaction. For fractionating the nuclear and the cytoplasmic fractions, S2-EGFP cells were suspended into Lysis buffer (30 mM HEPES at pH 7.3, 2 mM MgOAc, 5 mM DTT, 2 μg/mL Leupeptin, 2 μg/mL Pepstatin, and 0.5% Aprotinin) and lysed by passing through a 30G needle. After centrifugation, the supernatant (cytoplasmic fraction) and the precipitate were separated. The pellet was washed twice with Lysis buffer and lysed by sonication in Lysis buffer containing 100 mM KOAc and 20% glycerol. After centrifugation, the supernatant was collected and used as the nuclear fraction. RNAs were isolated from both fractions using ISOGEN LS (Nippon Gene).

HP1 immunoprecipitation and dot-blot hybridization

For cross-linking, S2-EGFP cells were first incubated for 10 min in medium containing 1% formaldehyde. The cells were then washed twice with PBS, resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, 2 μg/mL leupeptin, 2 μg/mL pepstatin, and 0.5% aprotinin, and then sonicated on ice. After centrifugation at 4°C, the supernatant was subjected to immunoprecipitation with anti-HP1 or nonimmune IgG on Gamma Bind beads (Amersham Bioscience). Prior to immunoprecipitation, the supernatant was diluted with a buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 170 mM NaCl, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 0.5% aprotinin and DNA carriers. After extensive washing, the beads were treated with a buffer containing 0.1 M NaHCO3 and 1% SDS. The eluates were then treated with NaCl to reverse the cross-link. All of the contained protein was digested with proteinase K; DNAs were then precipitated after being extracted with phenol/CHCl3. A portion of the DNA solution obtained was immobilized on Hybond-N+ nylon membranes and probed with a specific DNA fragment recognizing the EGFP promoter. Western blotting analysis was performed using anti-HP1 antibody to show that anti-HP1, but not nonimmune IgG precipitated HP1 protein as expected.

Nuclear run-on assays

Nuclear run-on assay was performed as described (Hirayoshi and Lis 1999). Briefly, 4 d after dsRNA treatment, the cells were harvested and the nuclei isolated. Transcription was then performed in the nuclei in the presence of [^{32}P]UTP. After reaction, all transcripts were isolated from nuclei and probed with both EGFP and tubulin cDNA fragments (both being full-length ORF) immobilized on Hybond-N+ nylon membranes (Amersham Bioscience).

Poly(A) length determinations

Four days after dsRNA treatment, total RNAs were isolated from the cells and 15 μg of each sample were incubated with an oligo DNA hybridizing to EGFP 3′UTR (~130 nt upstream of polyadenylation signal). After incubation, samples were treated with RNaseH enzyme for 30 min at 37°C. The resultant RNAs were separated on 2% agarose gels and subjected to Northern blotting using a specific probe recognizing just downstream of the cleaved site by RNaseH. Total RNAs used in the lanes without RNaseH

treatment were 5 μg/lane in each case. To determine the poly(A) length of tubulin mRNA, an oligo DNA hybridizing to the transcript just upstream of the stop codon was used.

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