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RNAi-Dependent and -Independent RNA Turnover Mechanisms Contribute to Heterochromatic Gene Silencing

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SUMMARY

In fission yeast, the RNAi pathway is required for heterochromatin-dependent silencing of transgene insertions at centromeric repeats and acts together with other pathways to silence transgenes at the silent mating-type locus. Here, we show that transgene transcripts at centromeric repeats are processed into siRNAs and are therefore direct targets of RNAi. Furthermore, we show that Cid14, a member of the Trf4/5 family of poly(A) polymerases, has poly(A) polymerase activity that is required for heterochromatic gene silencing. Surprisingly, while siRNA levels in *cid14Δ* cells are dramatically reduced, the structural integrity of heterochromatin appears to be preserved. Cid14 resides in a complex similar to the TRAMP complex found in budding yeast, which is part of a nuclear surveillance mechanism that degrades aberrant transcripts. Our findings indicate that polyadenylation by a TRAMP-like complex contributes to robust silencing of heterochromatic genes in fission yeast via the recruitment of the exosome and/or the RNAi machinery.

INTRODUCTION

Heterochromatic DNA domains are required for stable chromosome transmission and regulation of gene expression in a variety of organisms ranging from yeast to human. In the fission yeast *Schizosaccharomyces pombe*, heterochromatin is associated with telomeres, the silent mating-type loci, and repetitive DNA elements surrounding centromeres. The assembly of heterochromatin at these loci involves a series of steps that ultimately lead to the association of specific histone modifications and structural proteins with extended DNA domains. One of the key steps is the methylation of histone H3 lysine 9 (H3K9) by the Clr4 methyltransferase, which creates a binding site for the Swi6, Chp1, and Chp2 chromodomain proteins (Bjerling et al., 2002; Nakayama et al., 2001; Partridge et al., 2000; Sadaie et al., 2004). Histone H3K9 methylation is a conserved hallmark of heterochromatin and has been proposed to spread along the chromatin fiber through sequential cycles of methylation that are coupled to oligomerization of Swi6, a homolog of the *Drosophila* and mammalian HP1 proteins (Grewal and Moazed, 2003; Richards and Elgin, 2002).

Heterochromatin assembly at fission yeast centromeres also requires components of the RNA interference (RNAi) pathway. RNAi is a conserved silencing mechanism that is triggered by double-stranded RNA (dsRNA) (Bartel, 2004; Hannon, 2002). RNAi-dependent posttranscriptional gene silencing (PTGS) involves the generation of small RNA molecules of ~22 nucleotides from the longer dsRNAs by an RNase III-like enzyme called Dicer (Bernstein et al., 2001). These small interfering RNAs (siRNAs) then load onto an effector complex called RISC (RNA-induced silencing complex). The RISC complex contains Argonaute, which is a member of the conserved Argonaute/PIWI family of proteins that are required for RNAi in a variety of systems (Caudy et al., 2002; Hammond et al., 2001; Hutvagner and Zamore, 2002; Mourelatos et al., 2002; Gregory et al., 2005). siRNA-programmed RISC acts in *trans* to target cognate mRNAs for degradation by an endonucleolytic cleavage event, also referred to as slicing, which is executed by Argonaute (Liu et al., 2004; Song et al., 2004).

S. pombe contains only a single gene for each of the main RNAi enzymes, Dicer, Argonaute, and RNA-directed RNA polymerase, called *dcr1*$^+$, *ago1*$^+$, and *rdp1*$^+$, respectively, all of which are required for heterochromatin formation (Volpe et al., 2002). Ago1, together with Chp1, Tas3, and siRNAs, forms a complex called RNA-induced transcriptional silencing (RITS) (Verdel et al., 2004), whereas Rdp1, together with the helicase Hrr1 and the putative poly(A) polymerase Cid12, forms a complex called RNA-directed RNA polymerase complex (RDRC) (Motamedi et al., 2004). Deletion of any of these genes results in defects in the spreading of H3K9 methylation and Swi6 localization (Volpe et al., 2002; Motamedi et al., 2004; Verdel et al., 2004; Jia et al., 2004). Recent results have led to a model in which the association of the RITS

complex with chromatin and the initiation and spreading of chromatin modifications are proposed to involve base-pairing between siRNAs and the nascent RNA polymerase II (RNApII) transcripts. Subsequently, RITS would recruit RDRC and histone-modifying enzymes to the targeted locus, leading to the generation of additional dsRNA, dsRNA processing into siRNA, and spreading of heterochromatin (Motamedi et al., 2004; Buhler et al., 2006; Kato et al., 2005; Djupedal et al., 2005; Noma et al., 2004).

In *S. pombe*, the insertion of reporter genes within or adjacent to heterochromatic regions results in clonally heritable gene silencing that exhibits the characteristic properties of classical position effect variegation (PEV) observed in multicellular eukaryotes (Allshire et al., 1994; Thon and Klar, 1992; Grewal and Klar, 1996). Unexpectedly, in wild-type cells and in cells defective in heterochromatin formation, similar levels of RNApII are associated with *ura4*$^+$ genes inserted within centromeric heterochromatin. In addition, tethering of the RITS complex to *ura4*$^+$ transcripts, which also results in the formation of heterochromatin, neither excludes RNApII from the *ura4*$^+$ gene nor reduces the rate of transcription (Buhler et al., 2006). Furthermore, heterochromatin has little or no effect on transcription of the reverse strand of centromeric repeats, even though these transcripts are less abundant in wild-type cells compared to RNAi mutant cells (Volpe et al., 2002). Finally, point mutations in subunits of RNApII that do not affect growth rate result in defects in RNAi-mediated heterochromatic gene silencing (Kato et al., 2005; Djupedal et al., 2005). These findings have challenged the paradigm that heterochromatin is transcriptionally inert. Instead, it seems that some promoters can be transcribed within heterochromatic domains, but the resulting RNA is rapidly degraded by the RNAi pathway. However, the mechanism of this degradation is distinct from RNAi-mediated PTGS. Unlike classical PTGS, RNAi-mediated degradation of heterochromatic transcripts is a chromatin-dependent process that requires the histone H3K9 methyltransferase Clr4 (Motamedi et al., 2004; Noma et al., 2004). The fact that RNAi-mediated degradation of transcripts in wild-type *S. pombe* is *cis*-restricted is consistent with the conclusion that degradation is chromosome-associated and further distinguishes this process from classical PTGS (Buhler et al., 2006). To explain these observations, we proposed a cotranscriptional gene silencing (CTGS) model, in which the targeting of nascent transcripts by the RITS complex not only mediates chromatin modifications (Motamedi et al., 2004) but also promotes the degradation of nascent transcripts (Buhler et al., 2006).

In this study we show that insertion of a *ura4*$^+$ gene into centromeric heterochromatin leads to the generation of siRNAs, which appear to have escaped previous detection due to their low abundance. Such transgene siRNAs could potentially mediate silencing through direct slicing of the *ura4*$^+$ transcript by Ago1. Furthermore, we show that a second RNA-processing pathway, involving the Cid14 poly(A) polymerase, is required for efficient silencing within centromeric DNA repeats. Surprisingly, in *cid14Δ* cells, siRNA levels are dramatically reduced, but the structural integrity of heterochromatin appears to be preserved. This observation indicates that Cid14-dependent siRNAs are largely dispensable for heterochromatin assembly and may be degradation products. Cid14 homologs in budding yeast are components of the TRAMP polyadenylation complex, which promotes the degradation of aberrant transcripts by the exosome (Wyers et al., 2005; Lacava et al., 2005; Vanacova et al., 2005). We show that point mutations in the catalytic core of Cid14 abolish its in vitro polyadenylation activity and disrupt its silencing function in vivo. Unlike other proteins required for RNAi, Cid14 is also required for silencing of a *ura4*$^+$ gene inserted at the silent mating-type locus, revealing the existence of an RNA-processing pathway that has a general role in heterochromatic gene silencing. Together, our results establish a role for the poly(A) polymerase subunit of the fission yeast TRAMP complex in heterochromatic gene silencing and suggest that this RNA processing pathway acts downstream of H3K9 methylation and Swi6 recruitment to promote robust silencing. We propose a model for CTGS in which assembly of DNA into heterochromatin interferes with normal RNA processing events, thus targeting transcripts for recognition by Cid14 and degradation by either the exosome or the RNAi pathway.

RESULTS

Detection of siRNAs from Reporter Genes Inserted into Centromeric Heterochromatin

The observation that similar levels of RNApII are associated with centromeric *ura4*$^+$ transgenes whether they are active or silenced suggests that silencing occurs primarily at the level of RNA degradation (Buhler et al., 2006). In order to determine whether RNAi is responsible for the degradation of heterochromatic transcripts, we set out to test whether the silencing of a *ura4*$^+$ gene inserted at different heterochromatic loci is accompanied by *ura4*$^+$ siRNA production (Figure 1A). In the strains we used for our analysis, the *ura4*$^+$ gene has been inserted at the innermost centromeric repeat region (*imr1R::ura4*$^+$), the outermost centromeric repeat region (*otr1R::ura4*$^+$), or the silent mating-type locus *mat3M* (*mat3M::ura4*$^+$). We and others were unable to detect siRNAs originating from various *ura4*$^+$ insertions in wild-type cells (Iida et al., 2006; Irvine et al., 2006). We suspected that this may be due to low levels of transgene siRNAs and therefore employed two different strategies to increase the sensitivity of our northern blot siRNA detection assay. First, we size selected the total RNA preparation (<200 nt) from cells that produce higher levels of siRNAs, such as cells lacking the exoribonuclease Eri1 (Iida et al., 2006) or cells that overexpress the RNA-dependent RNA polymerase Rdp1 (Figure 1B). Despite this, we were unable to detect *ura4*$^+$ siRNAs in either *eri1Δ* or Rdp1 overproducing cells (Figure 1B, middle panel).

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