Conjugation-specific small RNAs in *Tetrahymena* have predicted properties of scan (scn) RNAs involved in genome rearrangement

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We proposed a scan-RNA model for genome rearrangement based on finding small RNAs that hybridized preferentially to micronuclear-specific sequences and on the properties of Twi1p, a PPD protein required for both sequence elimination and small RNA accumulation in *Tetrahymena*. Here we show that Twi1p interacts with the small RNAs in both the old and the developing macronucleus, and is required for their stability. We show that the specificity of the small RNAs for micronuclear-limited sequences increases during conjugation. These results indicate that the small RNAs observed in conjugating cells have the properties predicted for scan RNAs.

Received May 10, 2004; revised version accepted June 22, 2004.

Like most ciliated protozoans, *Tetrahymena thermophila* (referred to as *Tetrahymena* below) have two structurally and functionally different nuclei in a single cell (see Karre 2000). The dipleid, germ-line macronucleus and the polyploid, somatic macronucleus are derived from the same zygotic nucleus formed by fertilization of two micronucleus-deriven, haploid, meiotic nuclei during the sexual process of conjugation. Concomitant with formation of a new macronucleus during conjugation, the old macronucleus is destroyed. Most, if not all, transcription required for vegetative cell growth occurs in the macronucleus.

*Tetrahymena* undergoes extensive programmed genome rearrangement during conjugation (see Yao et al. 2002) resulting in elimination of ~15% of the genome. About 6000 internal eliminated sequences [IESs], varying from 0.5 to ~20 kb in length, are eliminated, and flanking, macronucleus-destined sequences are ligated during macronuclear development in *Tetrahymena*. The precise ends of IESs can occur reproducibly at a specific site or at a limited number of alternative sites. Because no obvious consensus sequence had been found in and around IESSs, it was not clear how IESSs were precisely recognized until recently. We and others showed that an RNAi-related mechanism was involved in the IES elimination in *Tetrahymena* (Mochizuki et al. 2002; Yao et al. 2003). A PPD (PAZ-Piwi Domain) protein, Twi1p, was required for IES elimination, and for accumulation of the small RNA-like small RNAs homologous to micronuclear-specific (largely IES) sequences. Twi1p was detected only during conjugation and accumulated first in the cytoplasm, then in parental [old] macronuclei and then relocated to developing [new] macronuclei.

Based on the results above, on the presence of micronuclear transcripts from both strands of the IESSs in conjugating cells (Chalker and Yao 2001), and on the demonstration that sequences in the old macronucleus could epigenetically affect IES elimination (Chalker and Yao 1996), we proposed a model to explain how IESSs, lacking any consensus sequences, are recognized during macronuclear development (Mochizuki et al. 2002; Mochizuki and Gorovsky 2004). First, the micronuclear genome is transcribed bidirectionally to make double-stranded [ds] RNAs that are processed to small RNAs by a Dicer-related RNase. We named these hypothetical small RNAs scan [scn] RNAs. We proposed that the scnRNAs are localized first to the old macronucleus, along with Twi1p, and that the scnRNAs homologous to any DNA found there are degraded. We hypothesized that the remaining [i.e., micronucleus-specific] scnRNAs, still complexed with Twi1p, are then transferred to the new macronucleus, where they promote elimination of the IESS sequences to which they are homologous.

Here we test three critical predictions of the scnRNA hypothesis: (1) that the small RNAs are associated with Twi1p; (2) that the small RNAs are transferred from old to new macronuclei; and (3) that macronuclear-specific sequences are eliminated from the small RNA population as conjugation proceeds. We show that a significant fraction of the ~28-nt RNA can be communoprecipitated with Twi1p when this PPD protein is localized in either the old or the new macronucleus. Thus, much of the small RNAs is associated with Twi1p and probably is transferred with it from the old to the new macronucleus. We also show that small RNAs present early in conjugation are much less specific for micronuclear DNA than for macronuclear DNA, suggesting that the enrichment for IESS sequences predicted by the scnRNA hypothesis actually occurs. These results argue strongly that the small, ~28-nt RNAs specifically expressed during conjugation have the predicted functions of the hypothetical scnRNAs.

**Results and Discussion**

Expression of small RNA is greatly reduced but not eliminated in TWI1 knockout cells

Previously we reported that small RNAs specifically expressed during conjugation in wild-type cells could not be detected in *TWI1* (somatic) knockout cells in which all somatic [macronuclear] copies of *TWI1* were replaced with a drug resistance marker (Mochizuki et al. 2002). However, low and asynchronous mating of these *TWI1* somatic knockout cells prevented us from determining whether the small RNAs were absent or just greatly reduced in the absence of *TWI1*. Although the cause of this low and asynchronous mating was not clear, we suspected that senescence [Nanney 1974] or cellular damage...
occurred during the long period (40 d = 300–400 generations) of culture and treatment with high concentration of the drug (up to 50 mg/mL of paromomycin sulfate) required for phenotypic assortment to produce somatic knockouts. To avoid these problems, we used a different strategy to make TWI1 knockout cells. One of the two copies of the TWI1 genes in the diploid micronucleus was replaced by the drug resistance marker and homozygous micronuclear knockout strains were made by uniparental pronuclear transfer during conjugation [Hai et al. 2000]. Cells created by this process are homozygous TWI1 germ-line knockout heterokaryons, homozygous for disrupted TWI1 genes in their micronucleus and containing wild-type TWI1 genes in their macronucleus [see Fig. 1A]. Two homozygous heterokaryon strains were crossed and homozygous TWI1 knockout homokaryon progeny cells, referred to as ΔTWI1 cells, were obtained. These cells contained disrupted TWI1 genes in both macro- and micronuclei and have not been subjected to long periods of drug selection.

Complete replacement of TWI1 loci with the drug resistance markers was confirmed by PCR analyses [Fig. 1A]. In contrast to the original somatic knockout strains, two ΔTWI1 strains mated almost normally and without delay [data not shown] and expressed the conjugation-specific PDD1 gene normally [see Fig. 1C]. Thus, the low and asynchronous matings of TWI1 somatic knockout strains were not due to the absence of TWI1. As seen for somatic TWI1 knockouts [Mochizuki et al. 2002], when two ΔTWI1 strains were mated, no progeny grew upon refeeding of >10 million progeny tested [see Materials and methods]. Again, like TWI1 somatic knockout cells, elimination of the M-region IES [Austerberry and Yao 1987] in the progeny of the ΔTWI1 strains also failed to occur [Fig. 1B], confirming that TWI1 is required for genome rearrangement.

Next, total RNA was extracted from mating wild-type or ΔTWI1 cells at different stages of conjugation and analyzed by acrylamide-urea gel electrophoresis [Fig. 1C]. The small RNAs could be detected in ΔTWI1 cells although the amount was greatly reduced compared with wild-type cells. In early conjugation (4 h), the amount of the small RNA in TWI1 knockout cells was about one-fourth of that of wild-type cells but these small RNAs are associated with each other in early to late stages of conjugation [see Fig. 2]. These results indicate that formation of the small RNAs in the micronucleus precedes and is independent of its association with Twi1p. Because IES elimination was not observed in the progeny of TWI1 germ-line knockout cells [Fig. 1B], it is likely that
munoprecipitation was performed with (+) or without (−) DNase either the amount of the small RNAs detected in high-molecular-weight (genomic) DNA.

To address this, we constructed germ-line knockout heterokaryons, producing viable progeny when mated with ΔTWI1 cells. By contrast, as described above, two ΔTWI1 strains could not produce viable progeny (Table 1). Thus, Flag-Twi1p expressed from pD5H8-Flag-TWI1 can replace the essential function of Twi1p during conjugation, arguing that it retains normal physical interactions with other molecules required for the function of Twi1p.

Next, a Flag-TWI1 strain was crossed with a wild-type strain and a whole cell lysate was prepared from the mating cells at 5 h (when Flag-Twi1p is localized mainly in the old macronucleus) and 9 h (when it is mainly in the new macronucleus) postmixing. Anti-Flag antibody was used to immunoprecipitate Flag-Twi1p and molecules associated with it and the immunoprecipitated components were eluted. RNA was extracted from the eluted products and analyzed by acrylamide-urea gel electrophoresis followed by ethidium bromide staining. As a control, two wild-type strains were crossed and processed in parallel.

Table 1. Viability of the progeny

| Types of mating cells | Pairs tested | Progeny |
|-----------------------|--------------|---------|
| heΔTWI1/C4-4-3 × heΔTWI1/C4-4-3 | 288 | 30 |
| ΔTWI1/C4-4-3 × ΔTWI1/C4-4-3 | 288 | 26 |
| ΔTWI1-5-1 × ΔTWI1/C4-4-3 | 287 | 0 |
| ΔTWI1-5-1 × ΔTWI1/C4-4-3 | 288 | 35 |

At ~10 h postmixing, single mating pairs were placed into drops of SPP medium to allow postconjugation growth. Completion of conjugation was confirmed by testing for expression of a drug resistance marker specific either for parental macronuclei or newly developed macronuclei (see Materials and Methods).
expected result. First, because Flag-Twi1p was overexpressed from the rDNA based pDSH8 vector, the majority of Twi1p in mating cells should be Flag-tagged. Alternatively, Twi1p–small RNA complexes could contain multiple Twi1p molecules. We repeated this experiment, using mating of 5TW11 and Flag-TW11 strains. In this case, all Twi1p molecules should be tagged. We also ran quantitative standards to ensure that the measured signals for small RNAs and for Twi1p were in the linear range of quantification. In this experiment, ∼27% (5 h) and 51% (9 h) of the small RNA and 41% (5 h) and 52% (9 h) of Flag-Twi1p in the original lysate was communoprecipitated (data not shown). Again, if immunoprecipitated and unimmunoprecipitated Flag-Twi1p are similarly complexed with the small RNA, it is estimated that ∼66% (5 h) and 97% (9 h) of the small RNAs were in the Twi1p complex.

Because Twi1p and the small RNAs were proposed to function in genome rearrangements controlled by DNA–RNA interactions [Mochizuki et al. 2002], it was possible that the association of Twi1p and the small RNAs was bridged by DNA. To test this, immunoprecipitation was performed with or without DNase (Fig. 2C) treatment. Similar amounts of small RNAs were precipitated in the presence or absence of DNase, suggesting that the interaction of Twi1p and the small RNAs was not mediated by DNA.

As noted, at 5 and 9 h postmixing, the most Twi1p is present in old and new macronuclei, respectively [Mochizuki et al. 2002]. Thus, a large fraction of the small RNAs is likely to be associated with Twi1p, and travel with it from old to new macronuclei, as predicted.

In the experiments described here, we did not detect any proteins that interacted with Flag-Twi1p by silver staining although Flag-Twi1p was clearly detected [data not shown]. In addition the PAZ domains of five different PPD proteins in fly and human have been shown to interact directly with RNA in vitro [Lingel et al. 2003; Song et al. 2003; Yan et al. 2003]. Thus, Twi1p probably interacts with the small RNA directly.

**Small RNAs homologous to micronucleus-specific sequences are gradually enriched as conjugation proceeds**

The scnRNA model hypothesizes that the whole micronuclear genome is transcribed bidirectionally and processed to the small RNAs, and that the small RNAs then localize to the old macronucleus where those homologous to the macronuclear DNA are degraded [Mochizuki et al. 2002]. If this is correct, small RNAs homologous to micronuclear-specific sequences should be enriched as conjugation proceeds. To test this prediction, purified macro- and micronuclear DNAs were probed with radioactively labeled small RNAs purified from conjugating cells at different time points. When small RNAs isolated at 2 h postmixing were used as probe, hybridization to micronuclear DNA was approximately three times greater than to macronuclear DNA [Fig. 3]. This ratio gradually increased over 20 times at 8 h postmixing, when macronuclear development starts [Fig. 3]. These results argue that small RNAs homologous to micronucleus-specific DNA sequences are enriched from early to mid-conjugation. The observation that small RNAs isolated even at early stages (2 h postmixing) of conjugation already hybridize approximately three times more to micronuclear DNA than to macronuclear DNA has four possible explanations. First, micronuclear IESs might be preferentially transcribed in early conjugation. Second, the whole micronuclear genome could be transcribed but the micronuclear sequences, many of which are repeated, might preferentially form the dsRNA substrate required for production of scnRNAs. Third, “selection” might occur so rapidly that some of small RNAs corresponding to the macronuclear sequences were already eliminated by 2 h. Fourth, it is possible that the repeated nature of many IESs causes their transcripts to be enriched and their concentration on the blot to be high, favoring their detection. Regardless of the explanation, it is clear that the specificity of the small RNAs for micronucleus sequences increases dramatically from early to mid-conjugation. At later stages (10 and 12 h postmixing), the ratio decreased [Fig. 3]. We speculate that small RNAs are continuously produced until late stages of conjugation (either by transcription or by amplification by RNA-dependent RNA polymerase [RdRP]) but, after new macronuclear development and old macronuclear degra-
dation are initiated, the small RNAs cannot enter the "selection pathway" that eliminates small RNAs homologous to macronuclear sequences. At present, the detailed mechanism of "selective enrichment" of IES-derived sequences is not clear. We previously proposed that a RNaseH-like activity, able to degrade RNA in RNA–DNA hybrid, may be involved in degradation of small RNAs homologous to macronucleus-derived sequences. Alternatively, because the absence of Twi1p prevents accumulation of the small RNAs [Fig. 1C], it is possible that DNA–RNA pairing displaces Twi1p from the small RNA–Twi1p complex, allowing the small RNA to be degraded by a nonspecific RNase. Selective amplification, but not degradation, of small RNAs homologous to the micronuclear-specific DNA by RdRP could occur.

The small RNAs act as scnRNAs

In the present study, we show that Twi1p is associated with 28-nt small RNA in both old and new macronuclei [Fig. 2] and is probably required for its stability [Fig. 1C]. Thus, Twi1p might function as a molecular "chaperone" of the small RNAs. Because, Twi1p relocates from old to new macronuclei [Mochizuki et al. 2002] and a large fraction of the small RNAs is associated with Twi1p, the movement of the small RNAs from old to new macronuclei also is likely to occur. The increase in specificity of small RNAs for micronuclear sequences during conjugation [Fig. 3], also argues for the existence of a post-transcriptional mechanism that selects the small RNAs homologous to the micronuclear-specific sequences. These results argue that the small RNAs observed in conjugating Tetrahymena cells have functions like those hypothesized for scnRNAs [Mochizuki et al. 2002; Mochizuki and Gorovsky 2004]. Thus, we propose to name the 28-nt small RNAs specifically expressed during conjugation in Tetrahymena scnRNAs.

Materials and methods

Strains and culture conditions

Wild-type B2086 and CU428 strains of Tetrahymena thermophila (provided by Dr. P.L. Bruns, Cornell University, Ithaca, NY) were grown in SPP medium (Gorovsky et al. 1975) at 30°C. For conjugation, logarithmically growing cells of different mating types were washed (16–24 h at 30°C), and mixed in 10 mM Tris [pH 7.5].

Construction of Flag-TWI1 strain

Flag-encoding sequence was added immediately after the initiation codon of the TWI1 gene by overlapping PCR. The primers used for the PCR were 5′-TWI1-NotI, 5′-CAGCGGCCGCCGATCCTTTTCTCTAT GTGTCAC-3′; Flag-TWI1-RV, 5′-CTTATCCTGTCATCTTGGTAA TTCAGGTATTTAATATTCACTGCC (Yao et al. 2003). The remaining neo3 cassettes were eliminated by uniparental micronuclear transfer [Hai et al. 2000]. Then two homozygous germ-line knockout heterokaryon strains he∆TWI1-C4-4-3 and he∆TWI1-F3-1 were mated and pFlag-TWI1 was introduced at 10 h postmixing as above. Cells were incubated in 10 mM Tris [pH 7.5] for 24 h at 30°C and then refed by adding an equal amount of 2× SPP. One hundred micromers per milliliter of pm (paromomycin sulfate, Sigma) was added 3 h after refeding and cells were aliquoted to the microtiter plates. The cells were incubated until resistant clones were grown up. Although the progeny of he∆TWI1-C4-4-3 and he∆TWI1-F3-1 had pm resistance genes in the disrupted TWI1 loci, these were driven by the cadmium inducible MTT1 promoter and thus were not expressed in the absence of Cd2+ in the medium. To select cells with increasing numbers of the pFlag-TWI1-derived rDNA, the transformants were subjected to step-wise selection in increasing concentrations of pm, starting from 0.1 to 10 µg/mL to a final of 3–4 mg/mL above which the cells failed to grow. One of these strains ΔTWI1-Flag-TWI1-18 was used for further experiments.

Viability test

To test the viability of the progeny of ΔTWI1 cells, started ΔTWI1-5-1 and ΔTWI1-WG7-1 cells (1 × 10^7 cells each) were mixed to initiate mating and refed at 24 h postmixing by adding an equal amount of 2× SPP. CdCl2 was added (0.5 µg/mL final) at 3 h after refeding and cells were cultured for 1 h. Then CdCl2 [to 1 µg/mL total] and pm (120 µg/mL final) were added and the culture was aliquoted to microtiter plates. Microscopic analyses indicated that ~65% of the cells were exconjugants at 24 h postmixing. No pm resistant progeny were obtained. Thus, the viability was estimated as less than one out of 1.3 × 10^7.

To determine the functionality of Flag-Twi1p, ΔTWI1-Flag-TWI1-18 and ΔTWI1-5-1 were crossed, individual pairs were placed into SPP drops at 30°C and cultured at 30°C. At 48–60 h after cloning, drops were examined for growth of cells. Then, the phenotypes of growing cells were tested to determine whether cells had completed or aborted conjugation. These cells were incubated with 120 µg/mL pm with or without 1 µg/mL CdCl2. Clones that showed pm resistance with CdCl2, but were sensitive in its absence were scored as progeny. This assay depends on the fact that ΔTWI1-Flag-TWI1-18 had pDS5 derived rDNA gene that conferred pm resistance in Tetrahymena without CdCl2 in the macronucleus while both ΔTWI1-Flag-TWI1-18 and ΔTWI1-5-1 had neo3 that conferred pm resistance in Tetrahymena with CdCl2 in the micronucleus. As controls, conjugations of ΔTWI1-5-1 × he∆TWI1-F3-1 or ΔTWI1-5-1 × ΔTWI1-WG7-1 were also tested. To determine the completion of conjugation in these control experiment, cells were cultured in 1× SPP including 120 µg/mL pm and 1 µg/mL CdCl2 and scored for cells resistant to pm.

Small RNA and IES elimination analysis

Total RNA extraction and analysis of the small RNAs were performed as described [Mochizuki et al. 2002]. IES elimination was analyzed as described [Mochizuki et al. 2002] except cells were analyzed at 48 h postmixing.

Coimmunoprecipitation

CU428 or ΔTWI1-WG7-1 and ΔTWI1-Flag-TWI1-18 (2 × 10^6 cells/mL of each) were mated. At 5 and 9 h postmixing, 2 × 10^6 cells were collected and homog-
enized in 1 mL lysis solution [50 mM Tris at pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1% Tween 20, 1x Complete Proteinase Inhibitor [Roche], 2 mM phenyl methyl sulfonylfluoride [Roche], 0.2 mg/mL Ribonucleoside Vanadyl Complex (New England Biolabs)]. Insoluble materials were sedimented at 10,000 × g for 15 min and the supernatant was used for immunoprecipitation. Anti-Flag M2 agarose affinity gel [Sigma] was mixed with the supernatant to precipitate the Flag-Twi1p complex. After overnight incubation at 4°C, the gel was rinsed in wash buffer [50 mM Tris at pH 7.5, 150 mM NaCl, 2 mM MgCl2] and the complex was eluted in 150 µg/mL 3× Flag peptide [Sigma] in wash buffer. Nucleic acids were extracted from the eluted fraction with phenol/chloroform and then ethanol precipitated. The small RNAs were analyzed as described [Mochizuki et al. 2002]. The intensities of bands were measured using NIH Image version 1.59. For DNase treatment, 0.1 mg/mL of DNase I (RNase free, Sigma) was added to the initial lysate. After immunoprecipitation, nucleic acid complex was eluted in 150 µg/mL 3× Flag peptide (Sigma) in wash buffer.

Acknowledgments

We thank Josephine Bowen for critical reading of the manuscript. This work was supported by grant GM21793 from the National Institutes of Health.

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*Genes Dev.* 2004, 18:
Access the most recent version at doi:10.1101/gad.1219904

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