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Analysis of a *piwi*-Related Gene Implicates Small RNAs in Genome Rearrangement in *Tetrahymena*

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Summary

During development of the somatic macronucleus from the germline micronucleus in ciliates, chromosome rearrangements occur in which specific regions of DNA are eliminated and flanking regions are healed, either by religation or construction of telomeres. We identified a gene, *TWI1*, in *Tetrahymena thermophila* that is homologous to *piwi* and is required for DNA elimination. We also found that small RNAs were specifically expressed prior to chromosome rearrangement during conjugation. These RNAs were not observed in *TWI1* knockout cells and required *PDD1*, another gene required for rearrangement, for expression. We propose that these small RNAs function to specify sequences to be eliminated by a mechanism similar to RNA-mediated gene silencing.

Introduction

Like most ciliated protozoa, *Tetrahymena* exhibits nuclear dimorphism (see Karrer, 2000). Each cell contains a germline micronucleus and a somatic macronucleus. During the sexual process of conjugation, two cells pair and their micronuclei undergo meiosis, followed by fertilization. The zygotic nucleus divides to produce the next generation of macro- and micronuclei in each cell.

Macro- and micronuclei differ in structure and function. During vegetative growth, the micronucleus is transcriptionally inert, while the macronucleus is transcriptionally active. The micronucleus is diploid and divides mitotically; the macronucleus is polyploid and divides by amitosis. Although derived from the same zygotic nucleus, they differ in the organization of their genomes (see Yao et al., 2002). The macronucleus lacks ~15% of DNA sequences found in the zygotic nucleus and in the micronucleus. This is due to sequence elimination associated with two types of DNA rearrangements that occur in developing macronuclei during the late stages of conjugation. The first type occurs by deletion of ~6000 *i*nternal *e*liminated *s*equences (IESs), accompanied by ligation of the flanking macronucleus-destined sequences. IESs 0.5 to >20 kb in length occur in noncoding regions. Excision of IESs can occur reproducibly (within a few nucleotides) at a specific site, or (with similar fidelity) at a limited number of alternative sites. The second type of rearrangement involves chromosome breakage followed by small deletions (<50 bp) and addition of telomeres. This "chromosome healing" occurs at precise locations, producing 2–300 macronuclear chromosomes from the 5 chromosomes in the micronuclear (haploid) genome. We refer to this type of eliminated sequence as a *b*reakage *e*liminated *s*equence (BES). Little is known about the mechanisms that produce IESs or BESs. BESs contain a conserved, 15 bp chromosome breakage sequence (Cbs; Yao et al., 1987), a likely site for recognition by (unknown) proteins. The only common elements associated with IESs analyzed to date are short (<10 bp), direct repeats of varied sequences at the ends of the IESs that have no known function in IES removal. The flanking regions of some IESs have *cis*-acting sequences required for IES removal, but no sequence homology has been observed between different elements (see Yao et al., 2002). Thus, how IESs are recognized and eliminated is obscure.

DNA sequences in the parental macronucleus guide DNA rearrangements. In *Tetrahymena*, introduction of DNA containing an IES sequence into the parental macronucleus inhibits DNA elimination of that IES when the new macronucleus forms (Chalker and Yao, 1996). In another ciliate, *Paramecium*, an IES in the G surface antigen coding region that had aberrantly been retained in the macronucleus inhibited elimination of this IES when the new macronucleus formed (Duharcourt et al., 1995), and deletion of another (A type) surface antigen gene in macronuclei resulted in deletion of this locus in new macronuclei, even though the gene in the micronucleus was intact (Forney et al., 1996). These phenomena suggest that sequence-specific information is transferred from the parental to the new macronucleus.

Here, we provide evidence for involvement of small RNAs in DNA rearrangement in *Tetrahymena*, and propose that they transfer sequence information from the old to the new macronucleus. This evidence derives from analysis of a gene related to *piwi,* a member of the PPD (*P*AZ and *P*iwi *d*omains) gene family defined by conserved PAZ and Piwi domains of unknown function (Cerutti et al., 2000). Some PPD gene family members are involved in posttranscriptional (PTGS) and/or transcriptional (TGS) gene silencing evoked by introduction of transgenic DNA or RNA. For example, *AGO1* is required for PTGS in *Arabidopsis* (Morel et al., 2002), *qde-2* is required for PTGS (quelling) in *Neurospora* (Catalanotto et al., 2002), and *rde-1* is required for RNA interference (RNAi) in *C. elegans* (Tabara et al., 1999). In *Drosophila*, *AGO1* and *AGO2* are involved in RNAi (Williams and Rubin, 2002, Hammond et al., 2001), *aubergine* (*sting*) is required for RNAi (Kennerdell et al., 2002) and for silencing the Stellate locus (Schmidt et al., 1999), and *piwi* is required for silencing induced by multiple copies of *Adh* (Pal-Bhadra et al., 2002). In many of these events, small (20–26 nt) RNAs, processed by a Dicer-

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related, double-stranded RNase (see Ambros, 2001), play a pivotal role, but how PPD genes are related to small RNAs is not well understood.

We also show that a *piwi*-related gene, *TWI1*, is required for DNA rearrangement in *Tetrahymena thermophila*. In *TWI1* knockout cells, both IES elimination and chromosome breakage were affected. Small (~28 nt) RNAs specifically expressed during conjugation do not accumulate in the absence of *TWI1* or *PDD1* (another gene required for IES elimination) expression. Possible relationships between DNA rearrangement and the small RNAs are discussed.

Results

Characterization of *TWI1*

TWI1 was obtained by RT-PCR using RNA from early mating cells of *Tetrahymena thermophila*. It is a single copy gene (see Figure 2B, WT lanes). Comparison of cDNA and genomic DNA indicated that the cDNA contained six exons and encoded a putative protein, Twi1p, of 780 amino acids. Twi1p has both PAZ and Piwi domains found in all PPD proteins (Figure 1A).

Phylogenetic analyses (Figure 1B) indicate that PPD proteins form two distinct, Piwi- and AGO1-related subfamilies. Twi1p and a Piwi-related protein (PAP) from another ciliate, *Paramecium caudatum* (Obara et al., 2000), are related to the Piwi-related proteins in metazoans. Thus, *piwi*-related genes are not metazoan-specific and are conserved at least from ciliates to metazoans. *piwi*-related genes were not identified in plants or fungi, including the complete genomes of *Arabidopsis*, *S. cerevisiae* and *S. pombe*. In contrast, *AGO1*-related genes occur in diverse eukaryotes (Figure 1B). It is believed that ciliates arose earlier in evolution than fungi (Baldauf et al., 2000), suggesting that yeasts lost *piwi*-related genes during fungal evolution.

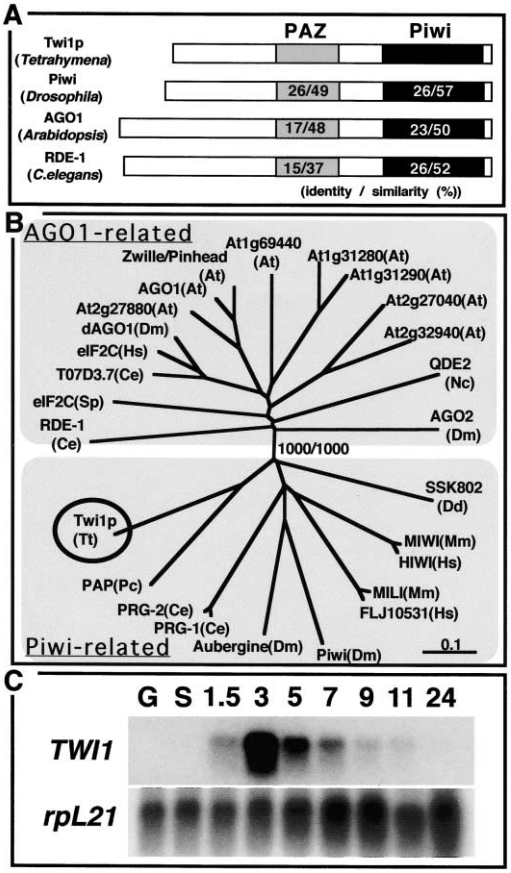


Figure 1. Characterization of *TWI1*

(A) Comparison of Twi1p and other PPD proteins.

(B) Phylogenetic relationships of PPD proteins. The sequences of Piwi domains were used to construct a phylogenetic tree using the neighbor-joining method. At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dd, *Dictiostelium discoideum*; Hs, *Homo sapiens*: Mm, *Mus musculus*; Nc, *Neurospora crassa*; Pc, *Paramecium caudatum*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tt, *Tetrahymena thermophila*.

(C) Expression of *TWI1* and ribosomal protein L21 (*rpL21*) mRNAs analyzed on Northern blot. Total RNA from log phase growing (G), starved (S), and conjugating cells (1.5, 3, 5, 7, 9, 11, and 24 hr after mixing) was used. See Figure 2D for the developmental profiles of mating cells.

TWI1 mRNA Is Expressed in Early Conjugation

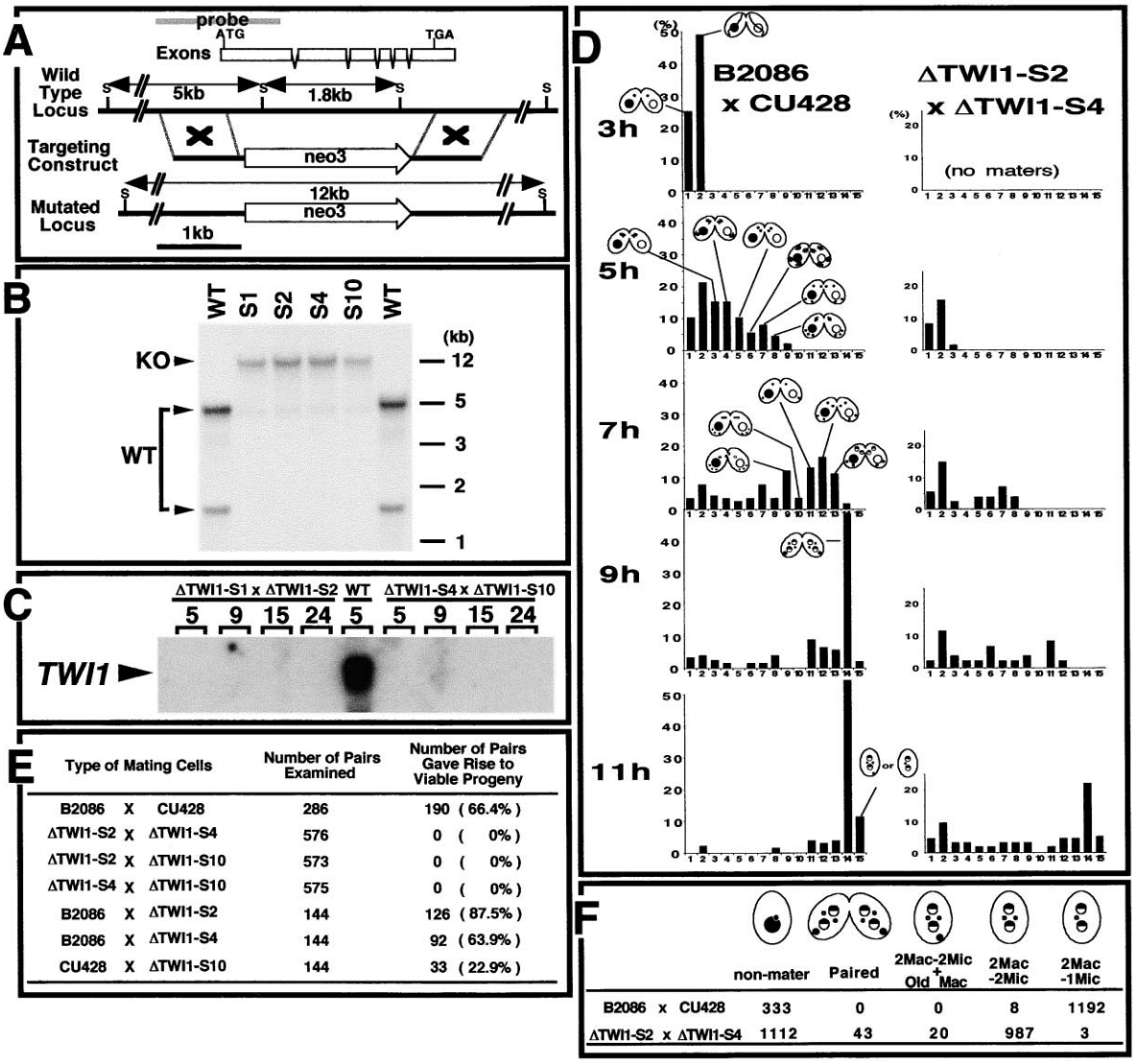
Figure 1C shows Northern blots of RNA from growing, starved, and conjugating cells. The developmental stages of the conjugating cells used for this analysis are shown in Figure 2D. No expression was detected in growing or starved cells. *TWI1* mRNA accumulated early (3 hr postmixing), just before and/or during meiosis I of conjugation, and was reduced as conjugation proceeded. It is likely that *TWI1* expression is confined to early conjugation and that the small amount of *TWI1* mRNA at late times after mixing reflects incomplete synchrony of conjugation (see Figure 2D).

TWI1 Is Not Essential in Vegetative Cells

To study *TWI1* function, the ORFs of *TWI1* genes in the polyploid macronucleus were replaced by neomycin resistance genes (Figure 2A). Replacement was analyzed by Southern and Northern blotting in four independent somatic knockout strains (S1, S2, S4, and S10). A strong band whose size is that expected from knocked out genes, and a faint band corresponding to the endogenous *TWI1* genes, were observed on Southern blots (Figure 2B). The amount of the endogenous gene was 15–20 times less than the disrupted gene, similar to the relative amounts of DNA in micro- and macronuclei in log phase cells (Woodard et al., 1972). We could not detect any *TWI1* mRNA in conjugating cells of knockout strains by either Northern hybridization (Figure 2C) or RT-PCR (data not shown). These results argue that all macronuclear genes had been disrupted and that the small amount of the endogenous gene remaining in these cells was derived from the transcriptionally inert micronucleus. The absence of *TWI1* mRNA during the mating of the *TWI1* knockout cells also argues that *TWI1* is a parentally expressed gene transcribed only from the old macronucleus. As expected from the absence of *TWI1* mRNA in growing cells (Figure 1C), the *TWI1* knockout strains showed no obvious defects during vegetative growth (data not shown).

Parentally Expressed *TWI1* Is Essential for Formation of Viable Progeny

Figure 2D compares the developmental profiles of the mating of wild-type and *TWI1* knockout cells. Although



Type of Mating Cells	Number of Pairs Examined	Number of Pairs Gave Rise to Viable Progeny
B2086 X CU428	286	190 (66.4%)
ΔTWI1-S2 X ΔTWI1-S4	576	0 (0%)
ΔTWI1-S2 X ΔTWI1-S10	573	0 (0%)
ΔTWI1-S4 X ΔTWI1-S10	575	0 (0%)
B2086 X ΔTWI1-S2	144	126 (87.5%)
B2086 X ΔTWI1-S4	144	92 (63.9%)
CU428 X ΔTWI1-S10	144	33 (22.9%)

	non-mater	Paired	2Mac-2Mic + Old Mac	2Mac -2Mic	2Mac -1Mic
B2086 x CU428	333	0	0	8	1192
ΔTWI1-S2 x ΔTWI1-S4	1112	43	20	987	3

Figure 2. Somatic Knockout of *TWI1*

(A) Diagram of the knockout construct and wild-type locus of *TWI1*. A drug resistance marker (*neo3*) was inserted into the *TWI1* gene, replacing the entire coding sequence. The position of the probe used for (B) and (C) is marked on the top. S, SacI.

(B) Southern hybridization of the *TWI1* knockout strains. Total genomic DNA isolated from knockout strains (S1, S2, S4, and S10) or wild-type cells (WT) was digested with SacI and hybridized with the *TWI1* probe.

(C) Confirmation of *TWI1* knockout by Northern hybridization. Two *TWI1* knockout strains were mated and RNA was isolated at the indicated hours after mixing and analyzed on a Northern blot probed with the *TWI1* probe.

(D) Developmental profiles of WT and *TWI1* knockout cells during conjugation. Starved wild-type cells (B2086 × CU428) and *TWI1* knockout strains (Δ*TWI1*-S2 × Δ*TWI1*-S4) were mixed and stages of conjugations were observed by DAPI staining at 3, 5, 7, 9, and 11 hr after mixing. The stages we categorized were: 1, Pair Formation; 2, Crescent; 3, Chromosome Condensation; 4, Meiosis I; 5, Meiosis I Completed; 6, Meiosis II; 7, Meiosis II Completed; 8, Prezygotic Mitosis; 9, Pronuclear Differentiation/Exchange; 10, Pronuclear Fusion; 11, 1st Postzygotic Mitosis; 12, 2nd Postzygotic Mitosis; 13, Macronuclear Anlagen; 14, Nuclear Alignment; 15, Pair Separation. For details of nuclear behavior during conjugation, see Cole et al. (1997).

(E) Viability of progeny. At 12–13 hr postmixing, single mating pairs were placed into drops of SPP medium and incubated for 48 hr at 30°C. Completion of conjugation was confirmed by testing for expression of a drug resistance marker specific either for parental macronuclei or newly developed macronuclei.

(F) *TWI1* knockout causes arrest at later stages of conjugation. At 72 hr postmixing, progeny of wild-type cells (B2086 × CU428) or *TWI1* knockout strains (Δ*TWI1*-S2 × Δ*TWI1*-S4) were fixed and nuclei were observed by DAPI staining.

the start of mating was delayed and maximum mating was low in the knockout strains, the stages of development looked normal, and exconjugants containing two new macronuclei and two new micronuclei in the center of the cell were produced (Figure 2D, column 14). Thus, at the cytological level, nuclear differentiation appeared to have occurred normally without *TWI1* gene expression.

To test for viability of progeny, single pairs were cloned and tested for growth. The progeny of the *TWI1* knockout cell did not grow upon return to nutrient and eventually died (Figure 2E). Thus, *TWI1* expressed from

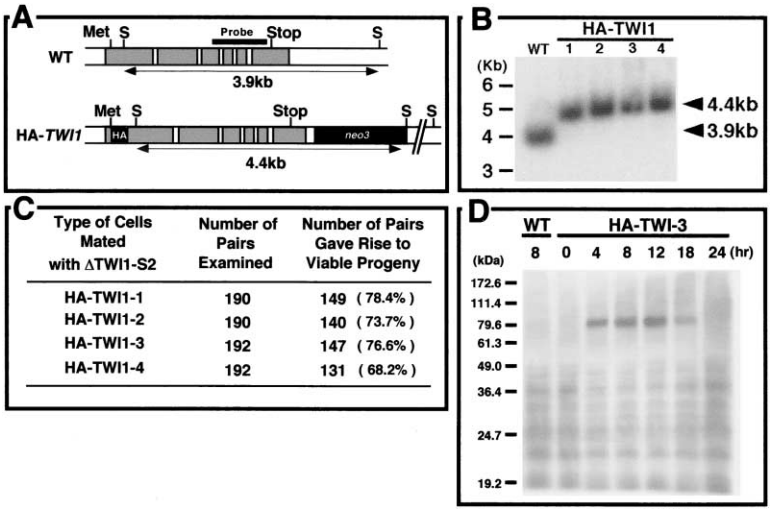


Figure 3. HA-Tagging of *TWI1*

(A) Diagram of the HA-*TWI1* construct. The HA epitope was inserted just after the initiator methionine residue of Twi1p. The *neo3* cassette was inserted into the 3′ nontranscribed sequence. The probe used for (B) is shown on top. S, ScaI.

(B) Confirmation of complete replacement of endogenous *TWI1* genes by HA-*TWI1*. Total genomic DNA isolated from HA-*TWI1* and wild-type cells was digested with ScaI and hybridized with the probe shown in (A).

(C) Viability of progeny. The method is same as in Figure 2E.

(D) HA-Twi1p expression analyzed on a Western blot. Total cell protein was prepared from the mating of HA-*TWI1* with B2086 (HA-*TWI1*) or B2086 with CU428 (WT) at 0, 4, 8, 12, 18, and 24 hr postmixing. The protein was separated by SDS-PAGE and blotted on the membrane. HA-Twi1p was localized with anti-HA mAb.

the parental macronucleus is essential for formation of viable conjugation progeny. The early conjugation defects and viability were rescued when *TWI1* knockout cells were crossed with wild-type cells (Figure 2E). This is not surprising, as Twi1p can be exchanged between members of a pair (see Figure 4).

Because the progeny of *TWI1* knockout cells did not resume growth after conjugation, we analyzed later stages of their development more carefully. During late stages of conjugation, wild-type cells undergo pair separation, degradation of old macronuclei, and resorption of one of their two micronuclei (see Figure 2F). This stage persists until cells are refed, when the micronucleus divides mitotically, followed by cytokinesis. Analysis of knockout cells (Figure 2F) indicated that they underwent pair separation and most degraded their old macronuclei, but few, if any, resorbed one of their two micronuclei. As a result, cells were arrested at the 2 Mac-2 Mic stage, even 72 hr postmixing (without nutrient). Thus, the progeny of *TWI1* knockout cells cannot enter the first cell division cycle.

HA-*TWI1* Can Replace the Function of Endogenous *TWI1*

To study expression and localization of Twi1p, a sequence encoding a HA epitope was inserted 5′ of the *TWI1* open reading frame (denoted as HA-*TWI1*, Figure 3A). This transgene was introduced into wild-type (CU428) cells, and cells were selected in which the endogenous *TWI1* genes were completely replaced, as indicated by Southern hybridization (Figure 3B).

HA-*TWI1* cells, whose endogenous *TWI1* genes were replaced by HA-*TWI1,* mated without delay (data not shown) and produced viable progeny (Figure 3C), even when mated with the *TWI1* knockout cells. Thus, HA-Twi1p can replace the essential function of Twi1p. The expression of HA-Twi1p during conjugation of wild-type and HA-*TWI1* cells was examined by Western blot using anti-HA antibody. A single band ~90 kDa was observed (Figure 3D), consistent with the predicted molecular weight of HA-Twi1p (90.6 kDa). Thus, we believe that the HA-Twi1p reflects the function and distribution of endogenous Twi1p.

HA-Twi1p Relocalizes from the Old to the New Macronucleus

Anti-HA immunofluorescent staining was used to localize Twi1p during mating of HA-*TWI1* and wild-type cells. HA-Twi1p was observed from early to late conjugation stages (Figure 4), consistent with the Western blot analyses (Figure 3D).

Twi1p was first observed in the cytoplasm at early crescent (early meiosis I) stage (Figure 4A). Because only one of the two mating cells in each pair had a HA-*TWI1* gene in this experiment, HA-Twi1p was first observed only in one member of each pair. As mating progressed, HA-Twi1p staining gradually appeared in the other cell and localized to the macronuclei, but not to the micronuclei, of both cells (Figures 4B and 4C). After meiosis, Twi1p localized to the old macronucleus, and cytoplasmic HA-Twi1p became undetectable (Figure 4D). By this stage, staining of both cells in a pair had become almost equal. HA-Twi1p continued to localize in the parental macronucleus until the new macronucleus formed (Figure 4E), at which time it localized to the anlagen (newly formed macronucleus), while staining of the old macronucleus rapidly disappeared (Figure 4F). Because *TWI1* mRNA is present largely, if not exclusively, during early stages of conjugation (Figure 1C), it is likely that Twi1p is transferred from the old macronucleus to the new macronucleus.

Excision of IESs Is Severely Affected in *TWI1* Knockout Cells

The lack of production of viable conjugation progeny in knockouts and the localization of Twi1p described above are similar to the properties of *PDD1* and *PDD2*, two other genes expressed during conjugation (Smothers et al., 1997; Coyne et al., 1999; Nikiforov et al., 1999). Both *PDD1* and *PDD2* knockout cells arrest at the 2 Mac-2 Mic stage and do not produce viable progeny. Pdd1p and Pdd2p localize to the old macronucleus and

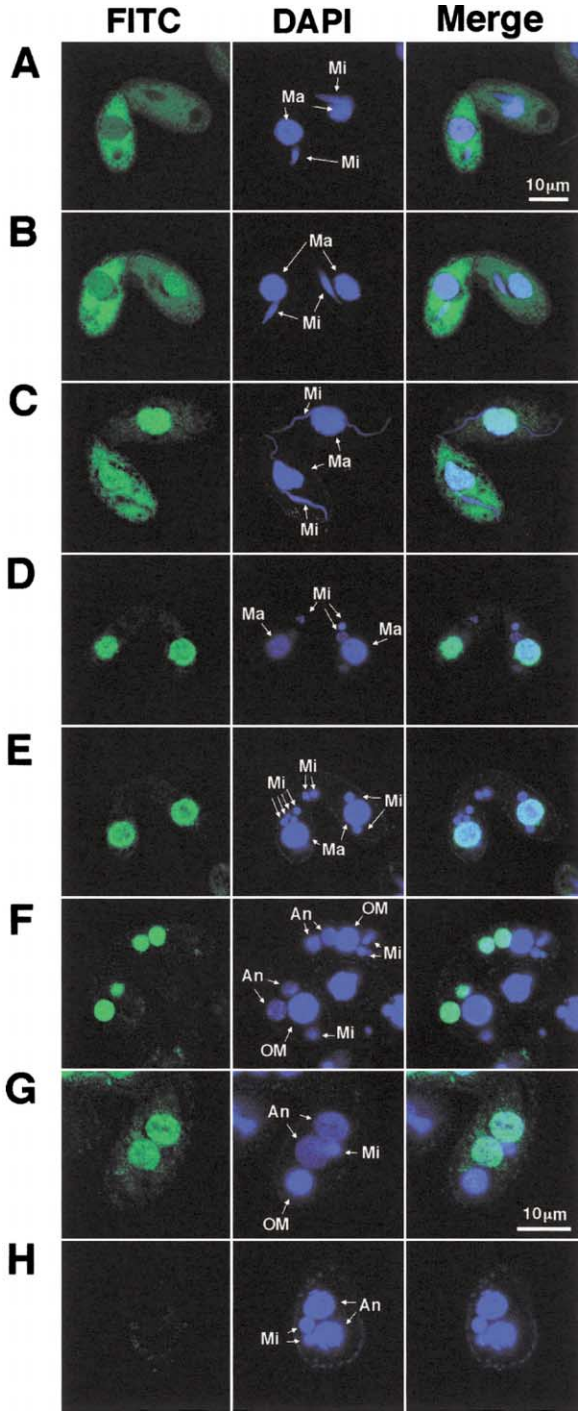


Figure 4. Localization of HA-Twi1p

HA-*TWI1* cells and wild-type cells (B2086) were mated. The cells collected at 3 hr (A–C), 5 hr (D), 7 hr (E–F), 9 hr (G), or 12 hr (H) postmixing were fixed and processed for immunofluorescence staining using anti-HA primary and FITC-conjugated secondary antibody (left column). Cells were also stained with DAPI (middle column). Ma, macronuclei; Mi, micronuclei; An, Anlagen/new macronuclei; OM, parental/old macronuclei. Cells were in Crescent Stage (A–C), Meiosis I Completed (D), Pronuclear Exchange (E), Anlagen (F), Pair Separation (G) and Old Mac Elimination (H). (A)–(F) share common scale bars, as do (G) and (H).

then relocalize to new macronuclei. In addition, Pdd1p and Pdd2p colocalize to a putative DNA degradation structure in macronuclear anlagen, and each is essential for elimination of IESs. To determine if Twi1p also functions in IES elimination, we used single-cell PCR to analyze IES excision at two regions (M and R; Yao et al., 1987), in mating *TWI1* knockout cells. Primers were prepared that flank each deletion element (Figure 5A). In wild-type exconjugants, only processed PCR products were detected in both regions (Figures 5B and 5C, WT lanes). This likely reflects both the greater amount of macronuclear than micronuclear DNA and that the smaller, macronuclear DNA, lacking IESs, can amplify more efficiently than the larger, micronuclear DNA that retains them. Despite the likely bias for detecting smaller products, progeny of *TWI1* knockout cells showed only the unprocessed PCR products for both regions M and R (Figure 5B and 5C, KO lanes). These results demonstrate that DNA processing in these regions did not occur in *TWI1* knockouts and that DNA in the old macronucleus was completely degraded. We conclude that, like *PDD1* and *PDD2*, *TWI1* is required for IES processing.

Chromosome Breakage Is Affected in *TWI1* Knockout Cells

In addition to IES elimination, *PDD2* is essential for chromosome breakage (Nikiforov et al., 1999). This led us to analyze chromosome breakage and telomere addition in the progeny of *TWI1* knockout cells. Primers that flank a BES and one complementary to the telomere DNA sequence were used to amplify BES-containing (Mic form) and/or processed (Mac form) DNA of Tt819 site (Figure 5D, Yao et al., 1987) from single exconjugants. Two shorter DNAs, corresponding to the left (about 260 bp) and the right (about 140 bp) arms of the processed end, were amplified in progeny of wild-type cells (Figure 5E, WT lanes). In contrast, a longer, unprocessed form of DNA (425 bp) was amplified in *TWI1* knockout progeny (Figure 5E, KO lanes). In addition, a processed DNA corresponding to the left, but not the right, arm of the breakage site was observed in two of ten exconjugants of *TWI1* knockout cells. These sequences do not reflect a failure to degrade the old macronucleus, because both arms are not represented and the neomycin resistance genes that were present only in the old macronucleus were not amplified from these progeny (Figure 5E, lower panel). Thus, in the progeny of *TWI1* knockout cells, chromosome breakage was severely inhibited, but was not completely blocked.

A Small RNA Enriched in Micronuclear-Limited Sequences Is Expressed Specifically During Conjugation

Some PPD family genes are involved in posttranscriptional and/or transcriptional gene silencing. During these processes, small RNAs (20–26 nt) homologous to the target RNA or DNA have important roles (Vaucheret et al., 2001; Zamore, 2001). This led us to investigate whether *TWI1* might function in DNA rearrangement via small RNAs.

In RNA extracted from wild-type cells, a rapidly migrating band of ~28 nt was detected by ethidium bromide staining during conjugation but not in growing or

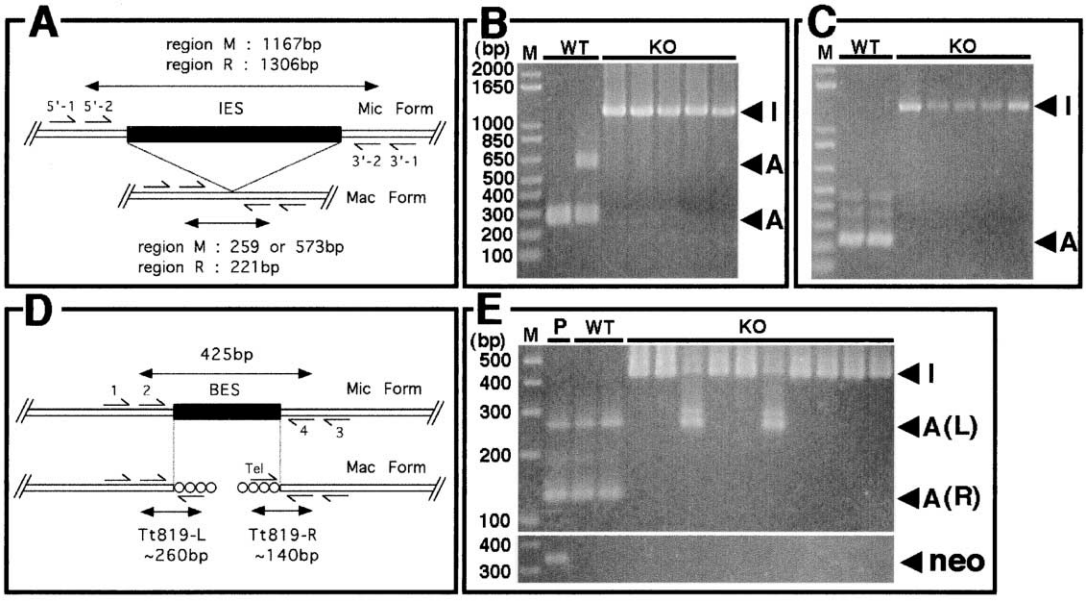


Figure 5. DNA Elimination in the Progeny of *TWI1* Knockout Cells

(A) Schematic drawing of the IES elimination assay. Double horizontal lines indicate DNA retained in the macronucleus, and filled box indicates an IES. Four primers (arrows on the horizontal lines) were used for nested PCR. The lengths of the expected products are shown at top and bottom.

(B and C) The result of the IES elimination assay for region M (B) and region R (C).

(D) A schematic representation of the chromosome breakage assay. Double horizontal lines indicate DNA retained in the macronucleus, the filled box indicates BES, and circles indicate telomeres. Four primers that flank the BES of the Tt819 region and a primer that can anneal to the telomere sequence were used for the assay. The sizes of expected products are shown at the top and bottom.

(E) The result of the chromosome breakage assay. The neomycin resistance genes that are in parental macronuclei of *TWI1* knockout cells were also amplified as an indicator of the degradation of the old macronuclei (lower panel). In (B), (C), and (E), the sizes of the processed (Mac form) and unprocessed (Mic form) products are marked by arrowheads with "A" and "I," respectively. M, molecular weight marker; WT, products from the progeny of wild-type cells; KO, products from the progeny of *TWI1* knockout cells. P, PCR products from parental *TWI1* knockout cells (Δ*TWI1*-S2).

starved cells (Figure 6A). This band was degraded by RNase but not by DNase (Figure 6B), arguing that it is RNA. Labeling of these RNAs with polynucleotide kinase and [γ-^{32}P]-ATP was increased by alkaline phosphatase treatment (Figure 6C), indicating that there is at least one 5′ terminal phosphate. 5′, 3′ biphosphate cytosine (pCp) could be ligated to the RNA using T4 RNA ligase (Figure 6D), indicating a 3′ hydroxyl. These ends suggest that these RNAs were produced by an *E. coli* RNase III-like enzyme (Robertson, 1990). Analysis of the RNA on a long sequencing gel revealed that the small RNAs were heterogeneous, ranging from ~26 nt to 31 nt (Figure 6E).

The small RNAs were detectable by 2 hr, were present in large amounts from 4–10 hr, and decreased after 12 hr postmixing. Expression of these small RNAs was similar to that of Twi1p (compare Figure 3D and Figure 6A) and precedes the timing of DNA rearrangement, which occurs 12–15 hr postmixing, just before the 2 Mac-1 Mic stage (Austerberry et al., 1984).

To determine whether they were related to micronuclear-specific (IES or BES) sequences, small RNAs purified from wild-type cells at 10 hr postmixing were isolated, labeled, and used to probe macro- and micronuclear DNA (Figure 6F). They hybridized with heterogeneous EcoRI digested genomic DNA fragments from both nuclei. Strikingly, they hybridized ~30× more with micronuclear DNA than with macronuclear DNA. These results indicate that the small RNAs are transcribed from many different sites and are homologous to micronucleus-specific sequences.

Accumulation of Small RNAs Is Dependent on *TWI1* and *PDD1*

Small RNAs were not detected in *TWI1* knockout cells (Figure 6G). Because the number of mating pairs formed by *TWI1* knockout cells was two to three times lower than wild-type (Figure 2D), five times more RNA was analyzed from these cells (Figure 6G) than from wild-type cells (Figure 6A). We conclude that the small RNAs either were not synthesized or were not stable in *TWI1* knockout cells.

In contrast to the *TWI1* knockout cells, the small RNAs were observed in both *PDD1* (Figure 6H) and *PDD2* (data not shown) knockout cells. However, in *PDD1* knockout cells, expression was not observed until much later (8 hr post mixing) in conjugation (Figure 6H). It is known that *PDD1* mRNA is transcribed both from parental macronuclei early in conjugation and from new macronuclei in later stages (from 7.5 hr postmixing; Coyne et al., 1999). Because *PDD1* is knocked out only in the macronucleus, *PDD1* genes in the micronucleus and in the new macronucleus derived from it are intact in *PDD1* knockout progeny cells, so these cells can express Pdd1p in later stages. Thus, the temporal pattern of

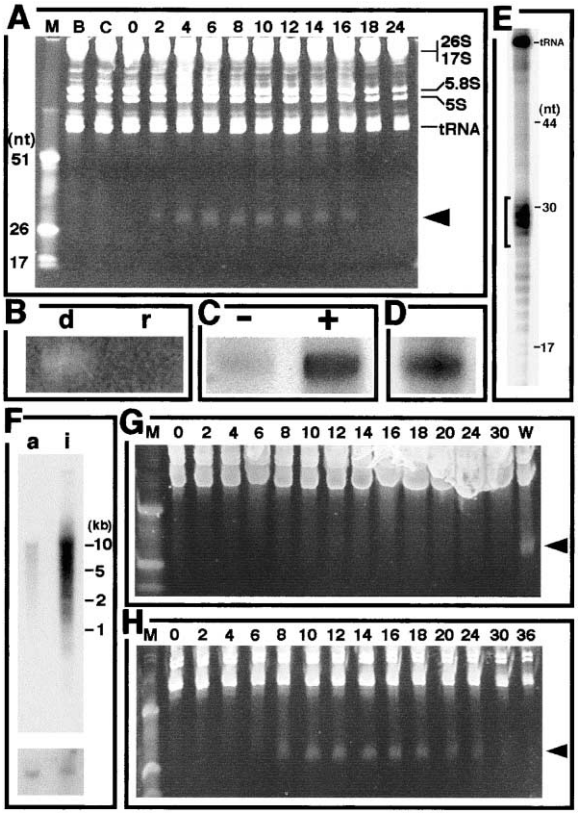


Figure 6. Expression and Characterization of Small RNAs

(A) Total RNA from wild-type cells was extracted from log phase growing (lane B, B2086; lane C, CU428) or mating cells (numbers on lanes indicate the hours after mixing). RNA from 5×10^4 cells was fractionated in 12% acrylamide-urea gels and stained by ethidium bromide. In vitro transcribed RNAs were used as size markers (lanes M). Note that these marker RNAs have 5′-triphosphate and migrate slightly faster than the same size RNAs that have 5′-monophosphate. The position of the small RNA is marked by arrowheads.

(B–D) Total RNA from WT cells 10 hr postmixing: treated with either DNase (d) or RNase (r) (B); incubated with (+) or without (−) alkaline phosphatase and labeled with [γ-^{32}P] ATP and polynucleotide kinase (C); labeled with [5′-^{32}P] pCp and T4 RNA ligase (D). The RNAs were visualized by ethidium bromide staining (B) or by autoradiography (C and D). In (B), (C), and (D), only the small RNA was shown.

(E) Analysis of the length of the small RNAs. Total RNAs from WT cells 10 hr postmixing labeled as in Figure 6C (+) were fractionated in 15% acrylamide-urea gels and were detected by autoradiography. The sizes of similarly labeled markers are indicated. The bands corresponding to 26–31 nt are bracketed.

(F) EcoRI-digested macro- (a) or micronuclear (i) DNA was probed with labeled small RNA from 10 hr postmixing (upper panel). The membrane was stripped and reprobed with *TWI1* probe to normalize loading (lower panel).

(G and H) Total RNA from knockout cells of *TWI1* (2.5×10^5 cells; G) or *PDD1* (5×10^4 cells; H) genes were analyzed as in (A). In (G), total RNA from wild-type cells (10 hr postmixing, lane W) was used as control.

expression of the small RNA molecules in *PDD1* knockout cells correlates with the expression of Pdd1p in the new macronucleus, arguing that Pdd1p is required for accumulation of the small RNAs. Pdd1p expression from the new macronucleus is not sufficient to restore the deficiency in IES elimination caused by the absence of Pdd1p expression from the old macronucleus (Coyne et al., 1999). We hypothesize that this inability is related to the delayed expression of the small RNAs in these *PDD1* knockout cells.

Discussion

We have identified *TWI1* as a *Tetrahymena piwi*-related gene and shown that its parental expression is essential to produce viable conjugation progeny. This is likely to be due, wholly or in part, to defects in the elimination of IESs and in chromosome breakage (Figure 5) during macronuclear development. We also identified small RNAs that are only expressed during conjugation (Figure 6A). We propose that these small RNAs function in DNA rearrangement events that eliminate micronuclear-specific sequences because: (1) they are highly enriched in micronuclear specific sequences; (2) they are not expressed in the absence of *TWI1* genes in the parental macronucleus; and (3) their expression requires the product of *PDD1*, another gene that is essential for IES elimination (Figures 6F–6H).

scan RNA model

Combining the data described here with previous studies on IES elimination and IES associated proteins, we propose a model for how IESs, which lack obvious consensus sequences, are recognized during macronuclear development (Figure 7). In *Tetrahymena*, it is known that both strands of the micronucleus-specific IESs (and their flanking sequences) are transcribed early in conjugation (Chalker and Yao, 2001). The significance of this nongenic transcription is not understood, and it is not known whether it includes the entire genome, or is restricted to regions around micronucleus-specific sequences. We propose this transcription as step 1 in our model. Because Pdd1p localizes first to meiotic micronuclei (Coyne et al., 1999), it could become associated with these transcripts (or their cleavage products) in the micronucleus. However, this association cannot be essential for some of the subsequent steps in this process, since these occur even when the synthesis of Pdd1p is delayed until new macronuclei form.

Because both strands are transcribed, micronuclear transcripts could form double-stranded RNA (step 2), which, as in RNAi-related systems, is processed to short fragments (step 3) by a Dicer-like protein. Dicer is an RNase III-like enzyme required for RNAi in *Drosophila*, and Dicer-related proteins are also essential for RNAi in *C. elegans* (see Ambros, 2001). *Arabidopsis* also has at least one ortholog of Dicer (Jacobsen et al., 1999). Although the length of the small RNAs ($\sim$28 nt) is longer than other small RNAs (20–26 nt) observed in PTGS systems, their 5′ phosphate and 3′ hydroxyl ends (Figures 6C and 6D) are consistent with their production by a Dicer-like activity.

We propose that the micronuclear transcripts or the small RNAs derived from them are transferred through the cytoplasm to the parental macronucleus, where they accumulate in early stages of conjugation, possibly in association with Pdd1p, which also localizes to the macronucleus subsequent to its localization in the micronucleus. In the cytoplasm, they also could become associated with Twi1p, which accumulates first in the

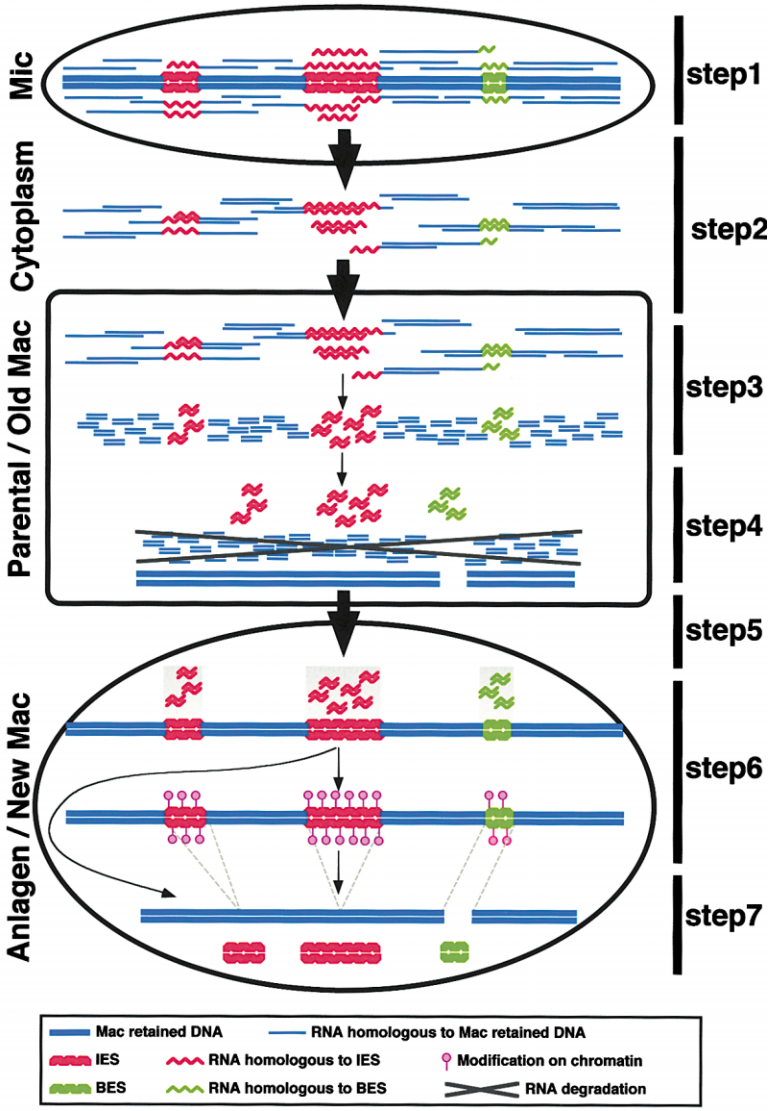


Figure 7. scan RNA Model
See text for details.

cytoplasm and then in the parental macronucleus. PPD genes, including *piwi*, are essential for RNAi, PTGS, and/or TGS, although their function in these processes is not understood. We call these short RNAs scan RNAs (scnRNAs) because we believe that they are required to recognize IESs, and possibly BESs, by scanning old macronuclei and developing macronuclei for DNA sequences that are in the micronucleus but not in the old macronucleus (see below). They could also recognize viruses and transposons that have been integrated into the micronuclear genome since the preceding conjugation.

The normal expression pattern of the small RNAs we have identified and its changes in mutations that affect DNA elimination are consistent with this RNA being the scnRNA. The processing of scnRNA could occur in the cytoplasm or in the macronucleus. We prefer the latter possibility because, in *PDD1* somatic knockout cells, accumulation of the small RNAs is delayed until the midstage of conjugation when Twi1p, which is essential for their accumulation, is localized in the macronucleus.

We hypothesize that, if the sequence of scnRNA is homologous to any DNA in the old macronucleus, it will be degraded (step 4). An RNase H-like enzyme that can act on DNA-RNA hybrids could be involved in this process. The result of this step is that only scnRNAs homologous to the micronuclear-specific sequences remain in the macronucleus. Our finding that the small RNAs isolated from cells in the late stages of conjugation hybridized more strongly with micronuclear DNA than macronuclear DNA (Figure 6F) supports a scnRNA function for the small RNAs.

We further hypothesize that the selected scnRNAs are then transferred to the new macronucleus (step 5), possibly in association with Twi1p, Pdd1p, and Pdd2p, all of which show the remarkable property of localizing to the new macronucleus after they appear in the old one. Interestingly, Pdd1p contains three chromodomains (Callebaut et al., 1997) and Pdd3p (another protein associated with IESs during macronuclear development) contains one (Nikiforov et al., 2000), and chromodomains can interact with RNA (Akhtar et al., 2000).

In the new macronucleus, the selected scnRNAs are hypothesized to target the DNAs to which they are homologous for elimination (step 6, 7). The scnRNAs (and proteins complexed with them) could participate in the elimination process directly, or change some state of the chromatin region with which they interact (step 6) by chemical (DNA methylation or histone modifications) or structural (chromatin remodeling) modification. Enzymes required for IES excision could then recognize one of these "marked" sequences and excise it (step 7).

This model can explain how the parental macronucleus functions to guide DNA processing in the new macronucleus but cannot completely account for two features of DNA elimination in *Tetrahymena*. One is that some mutations in the IES flanking sequences inhibit IES elimination (see Yao et al., 2002). The other is that, in knockout heterokaryon strains, transgenes present only in the micronucleus can be inherited by the new macronucleus (Hai et al., 2000). There are three possible explanations for these observations. The first is that the enzymatic machinery required for excision and rejoining of IES regions could have some sequence specificity in addition to that required to initially identify the IESs. The second is that the flanking sequences of IESs could be required for the micronuclear-specific, nongenic transcription in step 1. Last is that the flanking sequences of IESs could have a role as boundaries of chromatin modification (step 6) that inhibit spreading of modifications into or out of the chromatin containing the IESs.

The well-conserved Cbs sequence suggests that a different, Cbs-mediated, mechanism for recognizing BESs may occur. However, the observations that the disruption of either *TWI1* or *PDD2* caused defects in both IES and BES elimination (this study; Nikiforov et al., 1999) suggests that there may be common features to both IES and breakage-associated BES elimination.

Relationship of scnRNA to Other Small RNA-Mediated Systems

Evolutionarily, this system could have arisen as a host defense against viruses and transposable elements because these foreign DNAs, while harmless with regard to cell phenotype when in the transcriptionally inactive micronucleus, could be detrimental in the transcriptionally active macronucleus. This agrees with the proposed origin of IESs from transposons (see Yao et al., 2002) and is consistent with the similarities of the scnRNA mechanism to the TGS triggered by viruses, transposons, and other transgenes in plants, although it is not known whether small RNAs are involved in TGS of plants. Recently, transgene-triggered TGS was also reported in animals. Strikingly, *piwi* was required for TGS induced by high copy number of *Adh* genes in *Drosophila* (Pal-Bhadra et al., 2002). In *piwi* mutants, small RNAs homologous to the *Adh* gene, which were induced during normal silencing in high copy number strains, were greatly reduced. Thus, TGS mediated by small RNA may be an evolutionarily conserved phenomenon, and PPD proteins are likely to have important roles in this process. In contrast to small RNA-mediated PTGS, the mechanisms of TGS are not well understood. Inasmuch as both TGS and DNA elimination occur at the DNA level, further analysis of small RNAs associated with DNA rearrangement in *Tetrahymena* may help us to understand the general mechanisms of TGS.

Experimental Procedures

Tetrahymena Strains and Culture Conditions

Wild-type B2086 and CU428 strains of *Tetrahymena thermophila* were provided by Dr. Peter. J. Bruns (Cornell University). *PDD1* knockout strains *pdd1*::*neo2*-CU427 and *pdd1*::*neo2*-CU428, and *PDD2* knockout strains BC1 and BC4, were described previously (Coyne et al., 1999; Nikiforov et al., 1999). These strains were provided by Dr. C. David Allis (University of Virginia). Cells were grown in SPP medium (Gorovsky et al., 1975) at 30°C. For conjugation, log-phase growing cells of different mating types were washed, starved (16–24 hr at 30°C), and mixed in 10 mM Tris-HCl (pH 7.4).

Isolation of *TWI1*

A fragment of *TWI1* was isolated by RT-PCR using total RNA extracted with TRI-zol Reagent (Invitrogen) from mating B2086 and CU428 cells at 6 hr postmixing. Degenerate primers were from two different regions within the Piwi domain conserved among Piwi-related proteins: Piwi-FW (amino acid sequence QIIIYRDG); 5′-CA(AG)AT(ACT)AT(ACT)AT(ACT)TA(CT)(AC)GIGA(CT)GG-3′,Piwi-RV (amino acid sequence YAHCLCN); 5′-TT(AG)CA(ACGT)A(AG)(AG) CA(AG)TG(ACGT)GC(AG)TA-3′; "I" stands for inosine. RT-PCR was performed as described (Mochizuki et al., 2000). The sequence of the macronuclear *TWI1* locus, including the entire coding region, was determined as described by Liao et al. (1997). Genomic DNA was prepared from B2086 cells as described (Karlinsey et al., 1989). The full-length cDNA sequence was determined as described (Mochizuki et al., 2000).

Construction of Somatic Knockout Strains

The *neo3* cassette conferring paromomycin resistance in *Tetrahymena* cells grown in the presence of Cd^{2+} (Shang et al., 2002) was flanked with the 5′- (bp 1–853) and 3′- (bp 3531–4433) non-protein coding regions of the *TWI1* gene.

B2086 and CU428 cells were mated and transformed with the construct at 3–4.5 hr postmixing as described (Cassidy-Hanley et al., 1997). To select transformants, cells were subjected to stepwise selection in increasing concentrations of paromomycin sulfate (pm, Sigma) in the presence of 1 μg/ml $CdCl_2$, starting from 120 μg/ml to a final concentration of 50 mg/ml, above which the cells failed to grow.

Viability Test

At 12–13 hr postmixing, individual pairs were placed into SPP drops and cultured at 30°C. Two hours later, drops were examined for swimming cells to exclude from the analysis any cells that had been killed during pair isolation. At 48 hr after cloning, drops were examined for growth of cells. The phenotypes of growing cells ($>\sim$50 cells/drop) were then tested to determine whether the cells had completed or aborted conjugation. To determine the completion of conjugation after mating, cells were incubated with 120 μg/ml pm and 1 μg/ml $CdCl_2$ in SPP and scored for sensitivity to pm. This assay depends on the fact that, in all cases examined, one or both of the cells of a conjugating pair contained genes in its macronucleus that conferred pm resistance, but both members of the pair contained pm-sensitive alleles in their micronuclei. Thus, pm-sensitive cells can be obtained only if conjugation is successful. To check for completion of conjugation in wild-type cells (B2086 × CU428), cells were incubated with 15 μg/ml 6-methylpurine (6-mp, Sigma) in SPP and scored for cells resistant to 6-mp. This assay depends on the fact that CU428 cells contain a gene in their micronuclei that confers 6-mp resistance, but both strains contain 6-mp-sensitive alleles in their macronuclei.

Construction of Transgenes Encoding HA-Tagged Twi1p

Genomic DNA of *TWI1* (bp 1–4849) was amplified by PCR with Pfx DNA polymerase (Invirogen). The amplified product was cloned into plasmid vector and a *neo3* cassette was introduced into the EcoRV site in the 3′-nontranscribed sequence of *TWI1*. A HA coding sequence was added to the N terminus by amplifying the whole con-

struct with Pfx DNA polymerase using 5′-ATGTATCCTTATGATGTTCCTGATTATGCTTCTAACAAAGGCCTTGTCTA-3′ (HA coding sequence is underlined) and 5′-CGAAGCTTGGATGTTAATTATATCGCTT-3′. The amplified product was circularized by self-ligation and used for transformation.

Western Blotting

Whole-cell protein from 2.5×10^3 cells was separated by SDS-PAGE and transferred to PVDF membranes. Blots were incubated with 1:500 diluted mouse anti-HA antibody (16B12; Covance, Berkeley, CA) in blocking solution (3% BSA, 0.1% Tween 20 in PBS), and visualized by incubation with 1:2000 dilution of HRP conjugated anti-mouse IgG antibody (Zymed Labs Inc., South San Francisco, CA) in blocking solution followed by reaction with Western Blot Chemiluminescence Reagent (NEN).

Indirect Immunofluorescence Analysis

Cells were fixed in Lavdowsky's fixative (ethanol:formalin:acetic acid:water = 50:10:1:39) overnight at 4°C and immobilized on poly-L-lysine (Sigma) coated cover glasses. Samples were incubated with 1:100 dilution of anti-HA antibody in the blocking solution plus 10% normal goat serum (Invitrogen), followed by incubation with 1:300 diluted, FITC conjugated anti mouse IgG (Zymed Labs) in blocking solution. The samples were incubated with 1 μg/ml DAPI (Roche) in PBS, mounted, and observed by confocal microscopy.

Analysis of IES, BES Elimination, and Telomere Addition

Mating pairs were cloned into drops of 10 mM Tris-HCl (pH 7.4) at 12 hr postmixing and incubated for 24 hr at 30°C. One of the separated exconjugants was transferred to 1 μl lysis buffer (Coyne et al., 1999), incubated at 37°C for 30 min, and boiled for 3 min. The other exconjugant was transferred to SPP and tested for viability and drug resistance phenotypes. For the assay of *TWI1* knockout cells, the cell lysate was used for PCR analysis only if the partner exconjugant was not viable. For B2086 × CU428 matings, the sample was analyzed when the partner was 6-mp resistant.

The cell lysate (1 μl) was used for the first PCR (40 cycles) at final volume of 25 μl, and 1 μl of the first reaction was used for a second PCR (40 cycles). The following primers were used (nucleotide positions and directions are in parentheses): M5′-1 (2–25, sense); M5′-2 (28–51, sense); M3′-1 (1172–1194, antisense); M3′-2 (1168–1194, antisense); R5′-1 (1–24, sense); R5′-2 (168–190, sense); R3′-1(1461–1483, antisense); R3′-2 (1453–1473, antisense); 819-1 (481–503, antisense); 819-2 (453–476, antisense); 819-3 (1–26, sense); 819-4 (52–77, sense); Tel: 5′-CCCCAACCCCAACCCCAA-3′, neoFW1 (192–211, sense); neoFW2 (219–238, sense); neoRV1 (606–626, antisense); neoRV2 (546–565, antisense). The accession numbers of M, R, Tt819, and neo genes are M21936, U12972, M15711, and V00618, respectively.

For the IES elimination assay, M5′-1+M3′-1 or R5′-1+R3′-1 were used for the first PCR of regions M and R, respectively, and M5′-2+M3′-2 or R5′-2+R3′-2 were used for the second PCR. For the chromosome breakage assay, 819-1, 819-3, Tel, neoFW1, and neoRV1 were used for the first PCR. The second PCR was performed in two independent tubes either with 819-2, 819-4, and Tel or with neoFW2 and neoRV2.

Analyses of Small RNAs

Total RNA extracted with TRI-zol regent was dissolved with formamide directly. For enzymatic reactions, purified RNA was reextracted with TRI-zol reagent and dissolved in water. RNA was separated on 12% or 15% polyacrylamide-urea DNA sequencing gels and visualized by soaking gels in 1.5 μg/ml ethidium bromide or by autoradiography. Intensity of the signals was measured by NIH-Image 1.61.

For nuclease sensitivity analyses, denatured RNA was incubated with 1.0 U/μl DNase I (Roche) or 0.1 μg/μl RNase A (Amersham Pharmacia Biotech) in 100 mM sodium acetate (pH 5.0), 5 mM $MgCl_2$, for 1 hr at 37°C.

For 5′-terminal analysis, 5 μg of total RNA was incubated with or without 5 U of calf intestinal alkaline phosphatase (Promega) at 37°C for 1 hr and phosphorylated with 3.3 pmol (20 μCi) of [γ-^{32}P]-ATP and 10 U of T4 poly nucleotides kinase (NEB) (Sambrook and Russell, 2001). For 3′-terminal analysis, 5 μg of denatured total RNA was treated with 1.7 pmol (~10 μCi) of [5′-^{32}P] pCp and 60 U of T4 RNA ligase (Takara, Japan) at 4°C for 36 hr (Butler et al., 1997).

Southern Hybridization with Small RNA Probe

The small RNA from 10 hr postmixing was purified from acrylamide-urea gels (Elbashir et al., 2001). The 5′-end of the RNA was labeled as described above. Macro- and micronuclei of CU428 were isolated (Gorovsky et al., 1975) and DNA was extracted as described above. 10 μg of DNA was digested with EcoRI and was separated in agarose gel followed by blotting to nylon membrane. The membrane was probed with the labeled small RNA in 6× SSC, 20% formamide, 5× Denhart's solution, 0.1% SDS, and 1μg/ml polyA$^-$ RNA of starved *Tetrahymena* at 37°C and washed in 1 x SSC, 0.1% SDS at 42°C. The localization of the probes was visualized by autoradiography.

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Accession Numbers

The GenBank accession number of the *TWI1* gene is AB084111.