Marquette University
e-Publications@Marquette

Biological Sciences Faculty Research and Publications

Biological Sciences, Department of

2-1-2005

Elimination of Foreign DNA during Somatic Differentiation in *Tetrahymena thermophila* Shows Position Effect and Is Dosage Dependent

Yifan Liu
University of Rochester

Xiaoyuan Song
University of Rochester

Martin A. Gorovsky
University of Rochester

Kathleen M. Karrer
Marquette University, kathleen.karrer@marquette.edu

Published version. *Eukaryotic Cell*, Vol. 4, No. 2 (2005): 421-431. DOI. © American Society for Microbiology 2005. Used with permission.
Kathleen Karrer was affiliated with the University of Rochester and Marquette University at the time of publication.

EUKARYOTIC CELL, Feb. 2005, p. 421–431 Vol. 4, No. 2
1535-9778/05/$08.00+0 doi:10.1128/EC.4.2.421–431.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Elimination of Foreign DNA during Somatic Differentiation in *Tetrahymena thermophila* Shows Position Effect and Is Dosage Dependent†

Yifan Liu,[1]‡ Xiaoyuan Song,[1] Martin A. Gorovsky,[1] and Kathleen M. Karrer[1,2]*

Department of Biology, University of Rochester, Rochester, New York,[1] and Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin[2]

Received 14 October 2004/Accepted 23 November 2004

In the ciliate *Tetrahymena thermophila*, approximately 15% of the germ line micronuclear DNA sequences are eliminated during formation of the somatic macronucleus. The vast majority of the internal eliminated sequences (IESs) are repeated in the micronuclear genome, and several of them resemble transposable elements. Thus, it has been suggested that DNA elimination evolved as a means for removing invading DNAs. In the present study, bacterial *neo* genes introduced into the germ line micronuclei were eliminated from the somatic genome. The efficiency of elimination from two different loci increased dramatically with the copy number of the *neo* genes in the micronuclei. The timing of *neo* elimination is similar to that of endogenous IESs, and they both produce bidirectional transcripts of the eliminated element, suggesting that the deletion of *neo* occurred by the same mechanism as elimination of endogenous IESs. These results indicate that repetition of an element in the micronucleus enhances the efficiency of its elimination from the newly formed somatic genome of *Tetrahymena thermophila*. The implications of these data in relation to the function and mechanism of IES elimination are discussed.

Eukaryotic cells have a variety of strategies for protecting themselves from foreign elements that invade the genome. An especially important mechanism is gene silencing mediated by small RNAs complementary to the invading virus or transgene. These small RNAs (usually 20 to 26 nucleotides in size) are generated through cleavage of double-stranded RNAs by an RNase III-related enzyme, Dicer (75). Small RNAs can silence genes at both the transcriptional and posttranscriptional levels. In posttranscriptional gene silencing, which is often referred to as RNA interference, small interfering RNAs are incorporated into a ribonucleoprotein complex, the RNA-induced silencing complex. In addition to small interfering RNAs, another conserved component of the RNA-induced silencing complex is an Argonaute protein (9), a member of the PAZ/Piwi domain (PPD) family of proteins that can bind to small interfering RNAs (43, 70, 82). Alternatively, transcriptional gene silencing may result when small RNAs target sequence-specific chromatin modifications and/or secondary modifications of the DNA, leading to inhibition of transcription (30, 59, 78). A RITS (RNA interference-induced initiator of transcriptional gene silencing) complex that is required for transcriptional silencing has recently been identified in *Schizosaccharomyces pombe* (77). Its components include an Argonaute homologue (*Ago1*) and a chromodomain-containing protein (*Chp1*) that may recognize specific chromatin modifications. Thus, though they work at different levels, there may be overlap in the mechanisms of posttranscriptional and transcriptional gene silencing (58).

In the ciliated protozoan *Tetrahymena thermophila*, transposon-like elements are silenced by deletion from the somatic genome. Recent studies have shown that developmentally programmed DNA elimination in *T. thermophila* is a small-RNA-dependent event that is mechanistically related to transcriptional gene silencing in other systems (52). Like most ciliates, *T. thermophila* has two morphologically and functionally distinct nuclei: a large, transcriptionally active somatic macronucleus and a small germ line micronucleus which is transcriptionally inert during vegetative growth (26, 27). During conjugation, the sexual phase of the *T. thermophila* life cycle, the old macronuclei are destroyed and new micro- and macronuclei are derived from mitotic products of the zygotic micronuclei. The development of the new macronucleus involves the elimination of about 15% of the genome (17, 34, 39). Most of this DNA is removed by deletion of about 6,000 micronucleus-specific DNA elements, ranging in size from 0.5 kb to more than 22 kb, followed by ligation of the flanking sequences. These excised DNAs, referred to as internal eliminated sequences (IESs), are efficiently excised, although the boundaries of some IESs can vary over several hundred base pairs (2, 17).

The bulk of the IESs in *T. thermophila* consist of moderately repeated sequences (31, 32, 38, 85). Some of the IESs resemble transposable elements (16, 21, 80). It has been suggested that IES processing evolved to eliminate foreign DNA elements that invaded the silent, germ line micronuclear genome, preventing their entry into the somatic macronuclear genome, where their transcription could be detrimental (17).

Small RNAs with molecular characteristics similar to small interfering RNAs in other systems accumulate specifically during conjugation in *T. thermophila*, and are enriched in micronucleus-specific sequences (50). These RNAs are likely de-

* Corresponding author. Mailing address: Department of Biological Sciences, Marquette University, Milwaukee, WI 53201-1881. Phone: (414) 288-1474. Fax: (414) 288-7357. E-mail: kathleen.karrer@marquette.edu.

† Supplemental material for this article may be found at http://ec.asm.org/.

‡ Present address: Laboratory of Chromatin Biology, Rockefeller University, New York, N.Y.

rived from double-stranded transcripts of IESs synthesized in micronuclei during conjugation (14), which are then processed by a Dicer-like enzyme to produce the small RNAs (K. Mochizuki and M. A. Gorovsky, unpublished observations). A conjugation-specific gene, *TWI1*, encodes an Argonaute homologue that is required for the accumulation of the small RNAs (50). Methylation of lysine 9 of histone H3 and an abundant chromodomain protein (Pdd1p) that specifically recognizes this modification are associated with IESs in the developing macronuclei (45, 73). Lysine 9 methylation and the genes *PDD1* (encodes Pdd1p) and *TWI1* are all required for IES elimination (18, 44, 45, 50, 73). Direct evidence for a small-RNA-based model for DNA elimination was provided by the finding that microinjection of double-stranded RNA into conjugating cells induced deletion of the corresponding DNA sequence from the developing macronucleus (84). These observations all point to a pathway in which small RNAs target histone methylation and heterochromatin formation to IESs, which eventually leads to DNA elimination.

Little is known about the mechanism that initially targets a specific sequence for silencing. In *T. thermophila*, an in vivo assay has been used in attempts to identify *cis*-acting sequences required for developmentally regulated DNA rearrangement. The deleted element, together with some macronucleus-destined flanking sequence, is placed on an rRNA gene-based vector. When the constructs are introduced into the developing macronucleus, the element is deleted in a manner that closely mimics the deletion of the element from the chromosome (25). Experiments with these processing vectors suggested that two types of sequences are important for the deletion of IES in *T. thermophila*: sequences internal to the element that promote deletion, and sequences in the flanking DNA that determine the boundaries of the deletion. With regard to the internal promoting sequences in the short M element, it was concluded that multiple sequences within the element promoted deletion (83). A similar result was obtained with the larger, 22-kb Tlr elements, where at least seven different regions varying considerably in sequence, structure, and GC content promoted efficient deletion. Thus, it was suggested that something other than primary sequence was recognized to promote deletion, possibly the repeated nature of the IES (81).

Copy number has been implicated as a factor in processes involving small RNAs. In fact, the first indications of RNA silencing came from experiments designed to intensify petunia color by creating strains with additional copies of the chalcone synthase gene. Unexpectedly, instead of exhibiting enhanced color, some flowers became white, due to silencing or "cosuppression" of both the transgene and the endogenous gene (56, 76). This transgene silencing was shown to be related to the strength of the promoter and highly sensitive to copy number (62). Copy number is also an important factor in the related process of "quelling" in *Neurospora crassa* (65), and both transcriptional and posttranscriptional gene silencing in *Drosophila melanogaster* (58). One possible explanation of these data is that increasing the gene copy number raises the small-RNA abundance above a critical threshold required for transgene silencing (58). This hypothesis is supported by the observation that overexpression of a gene encoding an RNA-dependent RNA polymerase in *Neurospora crassa* reduces the number of gene copies necessary to induce quelling (22).

DNA-mediated transformation in *T. thermophila* occurs largely if not exclusively by homologous recombination (23), and mass transformation methods have been developed to achieve gene knockout and gene replacement in either the micronucleus or the macronucleus (10). A heterologous gene, *neo*, is commonly used as a selectable marker to confer paromomycin resistance in transformants (37). Germ line heterokaryon strains have been created in which the target gene(s) in micronuclei has been disrupted or flanked by the *neo* gene, but the macronuclei contain the wild-type gene(s) and no *neo* gene (29). In these strains, the *neo* gene is a newly introduced, germ line-specific foreign gene, not unlike a selfish DNA element that has recently invaded the (germ line) genome.

Here we show that *neo* genes in these germ line heterokaryons can be eliminated during macronuclear formation. The elimination of the *neo* sequences occurred at the same time as the elimination of an endogenous IES, suggesting that they were eliminated by the same mechanism. Micronuclear *neo* sequences were eliminated at different efficiencies from a variety of different loci, indicating that chromosomal position has a strong influence on the efficiency of elimination. Strikingly, in two cases tested, there was a clear relationship between the repetitiveness of the *neo* gene in the micronucleus and the efficiency with which it was eliminated during macronucleus formation. These results provide additional evidence that IES elimination in *T. thermophila* is similar to posttranscriptional gene silencing in plants and fungi and strongly support the hypothesis that the IES elimination mechanism in ciliates evolved as a mechanism to defend the genome against invading foreign genetic elements.

MATERIALS AND METHODS

***T. thermophila* strains and culture conditions.** Wild-type CU428 and B2086 strains of *T. thermophila* were provided by Peter J. Bruns (Cornell University). Cells were grown in SPP medium (28) at 30°C. To initiate conjugation, log-phase growing cells of different mating types were starved in 10 mM Tris buffer (pH 7.4) for 16 to 24 h at 30°C and mixed together at a concentration of 2×10^5 cells/ml.

Creation of *T. thermophila* knockout and gene replacement strains. The *HHT2* locus contains two divergently transcribed genes, *HHT2* and *HHF2*, separated by ≈400 bp. Both genes were simultaneously disrupted and were treated as a single locus. The knockout constructs for the *HHT1* and *HHT2* loci were made by replacing the coding regions of the corresponding gene with a single *neo*2 cassette (29), which confers paromomycin resistance in *T. thermophila*. The *neo*2 cassette was flanked by 1 to 4 kb of noncoding sequence on both ends to facilitate homologous recombination. The *HHF1* knockout construct was made by replacing the coding region with the *neo* coding region (37), placing the *neo* gene under the control of the endogenous *HHF1* promoter. The replacement construct for the *HHT2* gene contained an insertion of the *neo*2 cassette into the 3′-flanking region of the *HHT2* gene, about 0.6 kb downstream of the stop codon. The knockout construct for the *ngoA* locus was made by replacing its coding region with the *neo*3 cassette, which confers cadmium-dependent paromomycin resistance in *T. thermophila* (67).

To obtain germ line transformants in which a single locus had been knocked out (or replaced), the individual constructs were introduced into 2.5-h conjugating CU428 and B2086 cells with the Biolistic PDS-1000/He particle delivery system (Bio-Rad), as described previously (10). Strains with *neo* sequences at multiple loci were constructed by crossing single gene germ line knockout or replacement strains and further genetic manipulation as described previously (7, 29). Germ line knockout (replacement) heterokaryons with a mutated gene in their micronuclei and wild-type copies in their macronuclei were created by phenotypic assortment (29). Strains in which the *ngoA* gene was disrupted were constructed by a similar strategy with the *neo*3 cassette instead of the *neo*2 cassette. *neo3* differs from *neo*2 in that *neo* transcription is under the control of

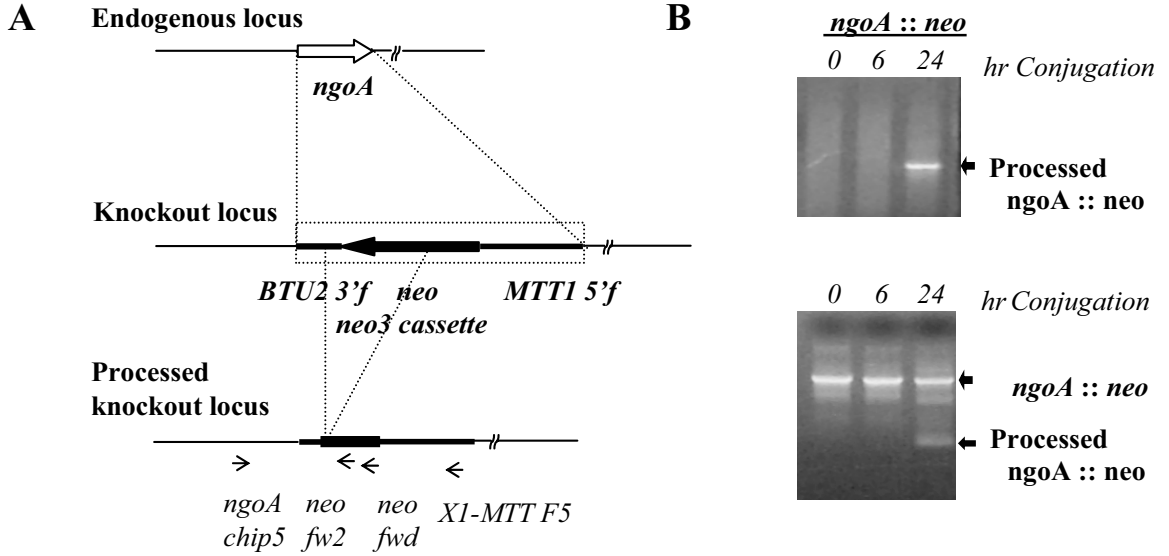

FIG. 1. *neo* is eliminated from the *ngoA* locus during conjugation of Δ*ngoA* homozygous (1×) heterokaryon strains. (A) Schematic representation of the endogenous *ngoA* locus, the knockout locus (containing the *neo3* cassette), and the locus after *neo* elimination. The indicated primers were used for PCR analysis in B. (B) PCR analysis of *neo* elimination from the *ngoA* locus in the conjugation progeny of Δ*ngoA* germ line knockout heterokaryons. Genomic DNA samples isolated at the indicated times after initiation of conjugation were used as templates for PCR amplification. In the upper panel, the primer set used was *ngoA* chip5 and X1-*MTT* F5. The intact Δ *ngoA*::*neo* (3.3-kb) fragment was not amplified, probably due to its large size. A prominent processed *neo* product (processed Δ*ngoA*::*neo*, 2.3 kb) was amplified only from the late (24 h) conjugation DNA; its size indicated that at least part of the *neo* coding region was eliminated. The lower panel shows the analysis of the same genomic DNA samples by nested PCR with a different set of primers. First-round PCR was amplified by *ngoA* chip5 and *neo* fwd, and second-round PCR was amplified by *ngoA* chip5 and *neo* fw2. Bands corresponding to the intact (Δ*ngoA*::*neo*, 1.3 kb) and processed *neo* (processed Δ*ngoA*::*neo*, 0.3 kb) PCR products in the *ngoA* locus are marked by arrows beside the panels. Sequencing of the processed *neo* PCR product showed that 982 bp of the *neo3* cassette (most of the *neo* coding region and some of the *BTU2* 3′-flanking region) were eliminated from the *ngoA* locus in late (24 h) conjugation but not during starvation (0 h) or early conjugation (6 h) (see Fig. A in the supplemental material).

the 5′-flanking region of the *MTT1* (metallothionein) promoter instead of the *HHF1* promoter (67).

PCR and Southern analysis. For PCR and Southern blot analyses, genomic DNA was isolated from *T. thermophila* by concentrating 10^7 cells to 0.5 ml in Tris buffer (10 mM, pH 7.4), adding 4 ml of lysis buffer (0.7 M NaCl, 20 mM Tris, pH 7.4, 20 mM EDTA, 2% sodium dodecyl sulfate), and incubating at room temperature for 15 min, followed by phenol-chloroform extraction and isopropanol precipitation.

The accession number of the *HHT1* gene is M87304. The primers used for the PCR analysis to detect *neo* elimination in the *HHT1* locus were *HHT1*-fwd (nucleotide positions 16 to 46) and *HHT1*-rev (1130 to 1099). The probe used for Southern analysis was a fragment of the *HHT1* 3′-flanking region (590 to 1283). The accession number of the M element is M21936. The primers used to PCR amplify the micronuclear-specific M element as a loading control were M3-fwd (808 to 836) and M3-rev (1000 to 977). The primers used for the PCR analysis of IES processing in the M element were M5′-1 (2 to 25) and M3′-1 (1194 to 1172).

The primers used for the PCR analysis of *neo* elimination in the *ngoA* locus were *ngoA* chip 5 (5′-GAT TAT TCT CTT CTA AAA TAT GGA GG-3′), *neo* fwd (5′-GCA CGC AGG TTC TCC GGC CGC TTG-3′), *neo* fw2 (5′-CTG GGC ACA ACA GAC AAT CG-3′), and X1 *MTT* F5 (5′-AAT CTA GAT CTG TCA TTT ATC ATT TCT GT-3′).

Reverse transcription-PCR analysis. RNA was isolated from 2×10^6 starved or conjugating cells with Trizol reagent (Invitrogen) and treated with RQ1 RNase-free DNase I (Promega) to remove contaminating DNA. RNA was reverse transcribed with Stratascript reverse transcriptase (Stratagene) with a gene-specific, strand-specific primer (either *neo*-fwd, or *neo*-rev), and the reaction was inactivated by incubation at 90°C for 5 min. cDNA was detected by 35 rounds of PCR amplification with the specified primers. The primers used for the reverse transcription-PCR analysis of *neo* transcription were *neo*-fwd (5′-GCA CGC AGG TTC TCC GGC CGC TTG-3′) and *neo*-rev (5′-GCC GCC GCA TTG CAT CAG CCA TG-3′).

RESULTS

Foreign genes introduced into the micronucleus show position effects in their elimination from the developing macronucleus. While this work was in progress, Yao et al. (84) demonstrated that when strains containing single copies of the *neo* gene in their micronuclei were mated, the *neo* gene could be partially eliminated during formation of the new macronucleus. We also found cases in which a *neo* gene homozygous at a single locus was sufficient to promote elimination. *ngoA* is a gene of unknown function that is expressed in stationary-phase or starved cells but not in growing cells (48). *ngoA* knockout heterokaryon strains were produced by replacing the putative coding region with a *neo3* cassette (Fig. 1A). When two homozygous germ line knockout heterokaryon strains were mated, a PCR product smaller than the full-length *neo* replacement product was detected at late conjugation but not during starvation or early in conjugation (Fig. 1B). Sequencing of the PCR product confirmed that about 1 kb of DNA, including a large part of the *neo* coding region, was deleted from the *neo3* cassette (Fig. 1A and Fig. A in the supplemental material). Similar results (data not shown) were observed with knockout heterokaryons for two other genes, *RFT2* (encoding a protein involved in intracilium protein transport) (C.-C. Tsao and M. A. Gorovsky, unpublished results) and *TWI1* (a gene involved in small-RNA generation and DNA elimination) (50).

In contrast to the observations described above, when we

mated germ line heterokaryon strains whose micronuclei were homozygous for a knockout of the *HHT1* gene, *neo* elimination in the *HHT1* locus was barely detectable in crosses between homozygous heterokaryon strains (diploid for the *neo* gene in their micronuclei; Fig. 2B), These strains are referred to as 1× *neo*, since they contain a single *neo* gene per haploid chromosome set. Similarly, little or no elimination was detected by Southern blot analysis matings between two 1× homozygous heterokaryon strains containing a *neo2* cassette flanking the *HHF2-HHT2* locus (see Fig. 5). Thus, there is significant position effect variation in the efficiency with which a foreign sequence is eliminated.

Elimination is enhanced by increasing the number of foreign genes in the germ line micronucleus. The observation that elimination occurred inefficiently from some loci provided an opportunity to test the effects of increasing the number of foreign genes on the elimination process. To determine whether a repeated foreign DNA sequence would be eliminated from the developing macronucleus, strains were built with multiple copies of the *neo* gene in the micronucleus. The 1× *HHT1*::*neo* strains were combined genetically with a strain in which the neo cassette replaced the *HHT2*/*HHF2* locus to produce 2× *neo* strains containing two *neo* genes per haploid chromosome set. The 2× *neo* strains were then combined genetically with the *HHF1*::*neo* (histone H4.1) strain to produce 3× *neo* strains containing three *neo* genes per haploid chromosome set.

To determine whether *neo* sequences at a locus that is eliminated poorly would behave like IESs when the *neo* gene was repeated, heterokaryon strains with multiple copies of *neo* were crossed with each other. Although no viable progeny were produced due to the absence of a functional histone H4 gene in the developing macronuclei, the cells did complete development through the stage of DNA elimination. The ability of macronuclei completely lacking genes encoding a major histone to develop past the point of IES elimination has been demonstrated previously (44). Because the rate at which these multiple knockout strains progress through mating varies considerably, the matings were monitored cytologically.

DNA was extracted from the population of mating cells when the majority of the cells reached the stage of late anlagen development or exconjugants. PCR amplification was conducted with primers specific for the flanking DNA of the *HHT1* locus (Fig. 2C). The PCR products from DNA of the two parental cell lines contained a major band at 1,143 bp, as expected for amplification of the wild-type allele of the *HHT1* locus in the macronuclear DNA. Only a very faint band was detected at 2,203 bp, the size expected for amplification of the knockout version of the *HHT1* locus in the micronucleus. This was probably due to the lower copy number of the micronuclear DNA and the larger size of the PCR product.

No processing of the *HHT1* locus was detected in crosses of a 1× *neo* strain with 2× *neo* or in a cross between 3× *neo* and wild-type cells. However, PCR amplification of the DNA from the 3× *neo* cross produced the 1,143-bp band expected from the macronuclear DNA of unpaired cells in the cross and an additional, smaller band. The small PCR product was cloned and sequenced and determined to be a deletion product of the *neo* cassette region. This showed that the *neo* cassette could be eliminated efficiently from the macronucleus in crosses between cell lines with multiple copies of the *neo* gene in the micronucleus, even though it was poorly eliminated when present in low copy number. However, deletion of the *neo* cassette was imprecise. One deletion boundary was near the 3′ end of the *neo* cassette and the other was 372 bp into the flanking DNA at the *HHT1* locus (Fig. 2; Fig. B in the supplemental material).

The fact that only a single new PCR product was observed in the progeny from mated cells suggested that deletion of the *HHT1* locus was a highly reproducible event with defined boundaries. However, deletions with alternative boundaries distal to the primer sequences would not have been detected in the PCR experiment. Southern blot analysis confirmed that the *neo* gene in the *HHT1* locus was eliminated during late conjugation to produce only one detectable form of the processed product (Fig. 3B). Thus, this elimination resembles deletion of the R element (2, 12) in that the boundaries of the deleted element are fairly constant. The structure of the deletion product showed that while foreign DNA sequences can initiate elimination, the deletion event can include adjacent chromosomal sequences.

Elimination of foreign *neo* genes and the endogenous IES probably occurs by the same mechanism. If DNA elimination evolved in ciliates as a mechanism for removing invading sequences, the elimination process operating on newly "invading" sequences should resemble the normal elimination process. The elimination of the foreign *neo* genes was similar to the elimination of endogenous IESs in two respects. The first of these was the kinetics of elimination. Cytological analysis suggested that the progression of mating between 3× *neo* strains was slow relative to that of the wild type. This is likely attributable to the slowdown of the conjugation progress caused by the lack of newly synthesized histones from the developing macronuclei. PCR and Southern analysis (Fig. 3A and B) confirmed that *neo* elimination in the conjugation progeny of Δ*HHT1* Δ*HHT2*/*HHF2* Δ*HHF1* homozygous heterokaryon strains occurred later than processing of normal IES in wild-type cells, which occurs at about 12 h of mating. However, the time course of elimination of the M element, a well-studied endogenous IES, was similarly delayed in the matings between 3× *neo* strains (data not shown), supporting the conclusion that elimination of *neo* and the endogenous IES occurred by the same mechanism.

Second, Chalker and Yao (14) showed that endogenous IESs are bidirectionally transcribed during conjugation. If *neo* is eliminated by the same mechanism, it should be possible to detect developmentally programmed bidirectional transcription of *neo*. With reverse transcription-PCR, transcripts were detected from both strands of the *neo* gene in conjugating 3× Δ*HHT1* Δ*HHT2* Δ*HHF1* strains (Fig. 4B) in which significant *neo* elimination occurred. Little *neo* gene transcription was detected in the conjugation of 1× Δ*HHT1* heterokaryon strains (Fig. 4C), in which no *neo* elimination was detected. Thus, there is bidirectional, developmentally regulated transcription of the *neo* genes, similar to that of endogenous IESs. The RNA was readily detected in a cross where there is efficient elimination of *neo*. This suggests that the elimination of *neo*, like that of the endogenous IES, is an RNA-mediated event.

To determine whether the efficiency of elimination was di-

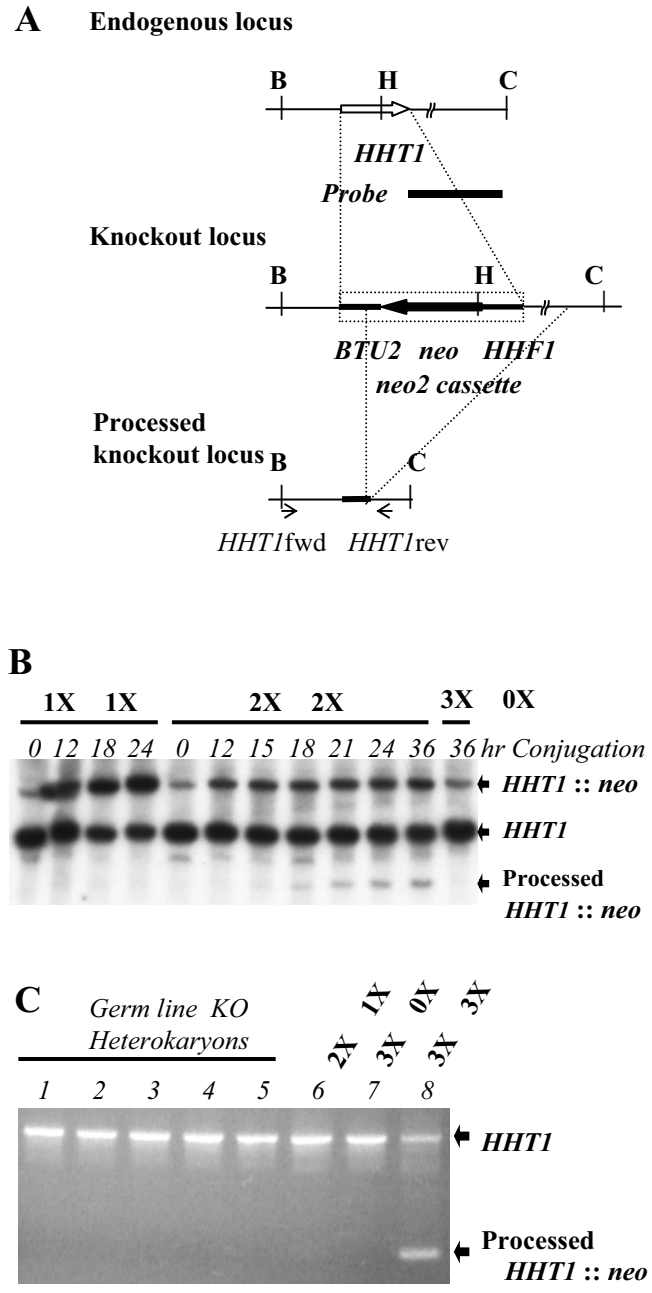

FIG. 2. Dosage-dependent elimination of *neo* from the *HHT1* locus during conjugation. (A) Schematic representation of the endogenous *HHT1* locus, the knockout locus (containing the *neo2* cassette), and the locus after *neo* elimination (cloned and sequenced). The locus was flanked by a Bgl II (B) and a ClaI (C) site. There was one HindIII (H) site in the *HHT1* coding region and in the *neo2* cassette (23). The indicated primers can amplify PCR products from all three loci, generating 1.1-kb, 2.2-kb, and 0.4-kb fragments, respectively. (B) Southern blot analysis of *neo* elimination from the *HHT1* locus in crosses involving heterokaryon strains with different number of *neo* genes in their micronuclei. The crosses analyzed were (from left to right): 1× crossed with 1× [(Δ*HHT1*) crossed with (Δ*HHT1*)], 2× crossed with 2× [(Δ*HHT1* Δ*HHT2*) crossed with (Δ*HHT1* Δ*HHT2)*], and 3× crossed with 0× [(Δ*HHT1* Δ*HHT2* Δ*HHF1*) crossed with wild-type CU427]. Genomic DNA was isolated at the indicated times after the initiation of conjugation and digested with BglII and ClaI. A DNA fragment containing the *HHT1* 3′-flanking region (see panel A), which can detect all three forms of the *HHT1* locus, was used as the probe. The *neo* elimination product was only detected in the 2× crossed with 2× cross. (C) PCR analysis of *neo* elimination from the *HHT1* locus in crosses with different numbers of *neo* genes in the parental cells. Lanes 1 to 5 show the PCR products obtained with DNA from the parental heterokaryons as the template. Lane 1, wild-type strain CU428; lane 2, Δ*HHF1* strain; lane 3, Δ*HHT1* Δ*HHT2* strain; lane 4, Δ*HHT1* Δ*HHT2* Δ*HHF1* strain; lane 5, another Δ*HHT1* Δ*HHT2* Δ*HHF1*) strain. Lanes 6 to 8 show PCR products with DNA isolated from mass mating cells at 24 h as the template. Lane 6, 2× crossed with 1× [(Δ*HHT1* Δ*HHT2*) crossed with (Δ *HHF1*)]; lane 7, 3× crossed with 0× [(Δ*HHT1* Δ*HHT2* Δ *HHF1*) crossed with wild-type CU428]; lane 8, 3× crossed with 3× [(Δ*HHT1* Δ*HHT2* Δ*HHF1*) crossed with (Δ*HHT1* Δ*HHT2* Δ*HHF1*)]. All three forms of the *HHT1* locus should be detected in the undigested genomic DNA template, though the large fragment from the intact *neo* insertion locus was always poorly amplified when competing with smaller PCR products. No *neo* elimination products were detected from crosses with small numbers of *neo* genes in parental cells, even when one of the parental cells contained three copies of *neo* per haploid chromosome set.

rectly related to the copy number of the *neo* gene, matings were done between strains with one, two, or three loci homozygous for the *neo* gene in the micronucleus. The efficiency of elimination of the *neo* gene from the *HHT1* locus was found to be sensitive to the number of *neo* sequences in the micronucleus. *neo* elimination in the *HHT1* locus was barely detectable in crosses between 1× *neo* homozygous heterokaryon strains (diploid for the *neo* gene in their micronuclei), but increased in a mating between two 2× *neo* (Δ*HHT1* Δ*HHT2)* strains (Fig. 2B) and increased still more in the 3× *neo* (Δ*HHT1* Δ*HHT2* Δ*HHF1*) mating. (Compare the signal ratio of the processed neo product and unprocessed neo product in Fig. 2B and 3B.)

The deleted version of the *HHT1* locus was not detected or was present at very low levels in matings between strains with a total of three or fewer homozygous *neo* loci in the mating pair (Fig. 2B and C) but was consistently observed in experiments with mating pairs containing more than three copies of the *neo* gene (Fig. 2B and 3B). However, it was not possible to establish a simple quantitative relationship between copy number and the efficiency of the *neo* deletion due to variability among matings and the nonquantitative nature of the PCR assay. Thus, an experiment was designed to examine neo deletion in matings that produced viable progeny.

Germ line heterokaryon strains were created whose micronuclei were homozygous for a version of the *HHT2* locus with the *neo2* cassette in its 3′-flanking region (1× *neo*, Fig. 5A) and therefore retain functional *HHF2* and *HHT2* genes. The 1× *neo* strains were combined genetically with germ line knockout heterokaryon Δ*HHT1* strains to produce 2× *neo* strains (see Materials and Methods). Finally, 2× *neo* strains were combined genetically with the Δ*HHT3* (histone H3.3) strain to produce 3× *neo* strains. To determine whether *neo* sequences would be eliminated in these strains, the heterokaryons were crossed to each other. Clonal progeny were isolated from the crosses and grown in drug-free medium. Genomic DNA (predominantly macronuclear DNA due to the polyploidy of the macronucleus) from several independent progeny strains was isolated and examined by Southern blotting for the elimination of the micronucleus-specific *neo* sequence flanking the *HHT2* gene. Probes were used that recognized mainly the 3′-flanking region (Fig. 5B) or the coding region of the gene (Fig. 5C). Note that because both probes contained portions of the

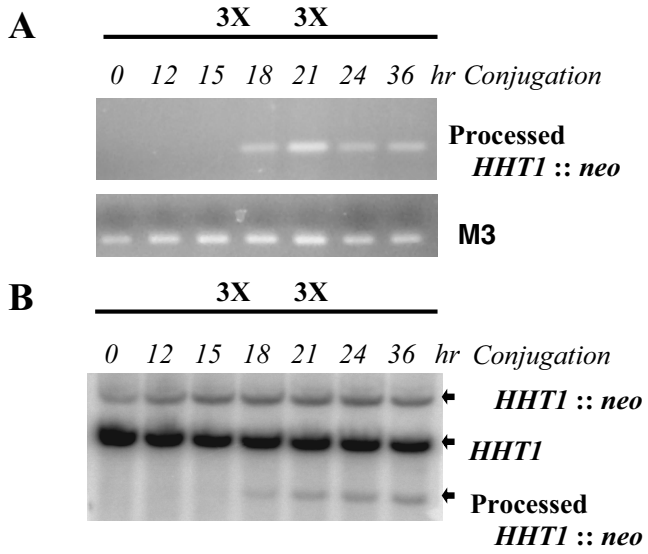

FIG. 3. Kinetics of *neo* elimination. (A) PCR analysis of *neo* elimination from the *HHT1* locus during the conjugation of 3× crossed with 3× (Δ*HHT1* Δ*HHT2* Δ*HHF1*) heterokaryons. Genomic DNA was isolated at different times after initiation of conjugation, digested by HindIII to destroy the PCR template from the *HHT1* endogenous and knockout loci, and PCR amplified. The upper panel (*HHT1 neo*) shows that a 0.4-kb PCR product was detected late in conjugation, indicating *neo* elimination from the *HHT1* locus. The PCR amplification product of a micronucleus-specific sequence (M3) served as a control for DNA template loading and integrity. (B) Southern blot analysis of *neo* elimination from the *HHT1* locus during the conjugation of 3× crossed with 3× (Δ*HHT1* Δ*HHT2* Δ*HHF1*) heterokaryons. Genomic DNA was isolated at different times after the initiation of conjugation and digested by BglII and ClaI. A DNA fragment containing the *HHT1* 3′-flanking region, which can detect all three loci, was used as the probe (see Fig. 2A). The processed product from which *neo* had been eliminated was detected at late conjugation.

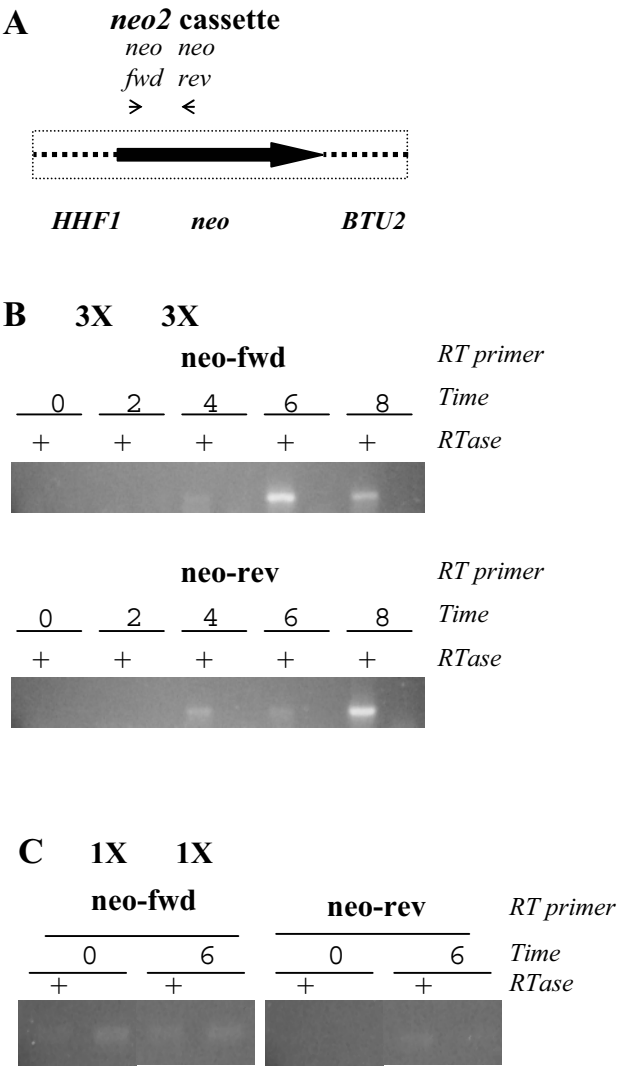

FIG. 4. Both strands of the *neo* gene were transcribed during conjugation of 3× crossed with 3× (Δ*HHT1* Δ*HHT2* Δ*HHF1*) germ line heterokaryon strains. (A) Schematic representation of the *neo2* cassette, in which the coding region of the *neo* gene is flanked by the 5′ untranslated region of the *HHF1* gene and the 3′ untranslated region of the *BTU2* gene. The PCR primers (*neo*-fwd and *neo*-rev) homologous to the coding region of *neo* gene are indicated. (B) Reverse transcription-PCR analysis of *neo* transcripts during conjugation of 3× (Δ*HHT1* Δ*HHT2* Δ*HHF1*) germ line knockout heterokaryons. Total RNA was isolated at the indicated times after the initiation of conjugation and digested by DNase I. The PCRs were performed either with or without prior reverse transcription of RNA by a strand-specific primer (*neo*-fwd or *neo*-rev). Transcripts of antisense (upper panel) and sense (lower panel) were detectable as early as 4 h after the cells were mixed. (C) Reverse transcription-PCR analysis of *neo* transcripts during conjugation of Δ*HHT1* germ line knockout heterokaryons. No bands dependent on reverse transcription, representing *neo* transcripts, were detected.

HHT2 coding region, all three highly conserved *HHT* genes are visualized by this method.

As noted previously, little or no sequence elimination of the *neo2* gene from the 3′-flanking region was detected when the progeny of 1× *neo* strains were analyzed (Fig. 5B). However, faint bands that were significantly smaller than the *neo*-containing 3′ fragment were detected in progeny of matings between two 2× *neo* strains and became the major type of 3′-flanking sequence detected in matings of 3× *neo* strains. The appearance of these bands was accompanied by a reciprocal decrease in the intensity of the intact *HHT2* + *neo* band, indicating that they result from deletions of the *neo* cassette from the *HHT2* locus. The presence of multiple smaller bands in these blots indicated that there were multiple *neo* deletion products. This is similar to the variability observed in the boundaries of several IESs (3, 15, 60). Thus, in this experiment there was a direct correlation between the number of *neo* genes in the micronucleus and the efficiency of *neo* deletion in the viable progeny of the crosses. In addition, these results show that increasing the copy number of the foreign *neo* gene increases the efficiency of deletion at a second locus.

We next addressed whether the number of *neo* genes in the micronucleus from one or both members of a mating pair was counted. When a 3× *neo* (Δ*HHT1* Δ*HHT2* Δ*HHF1*) homozygous heterokaryon strain was crossed with wild-type cells, only trace amounts of *neo* elimination product were observed (Fig. 2B, last lane, and 2C, lane 7). However, the processed Δ*HHT1* product was readily detectable when two 2× *neo* strains were crossed (Fig. 2B and Fig. 6, lane 1). That is, in a cross where the number of copies per haploid genome in each parental cell

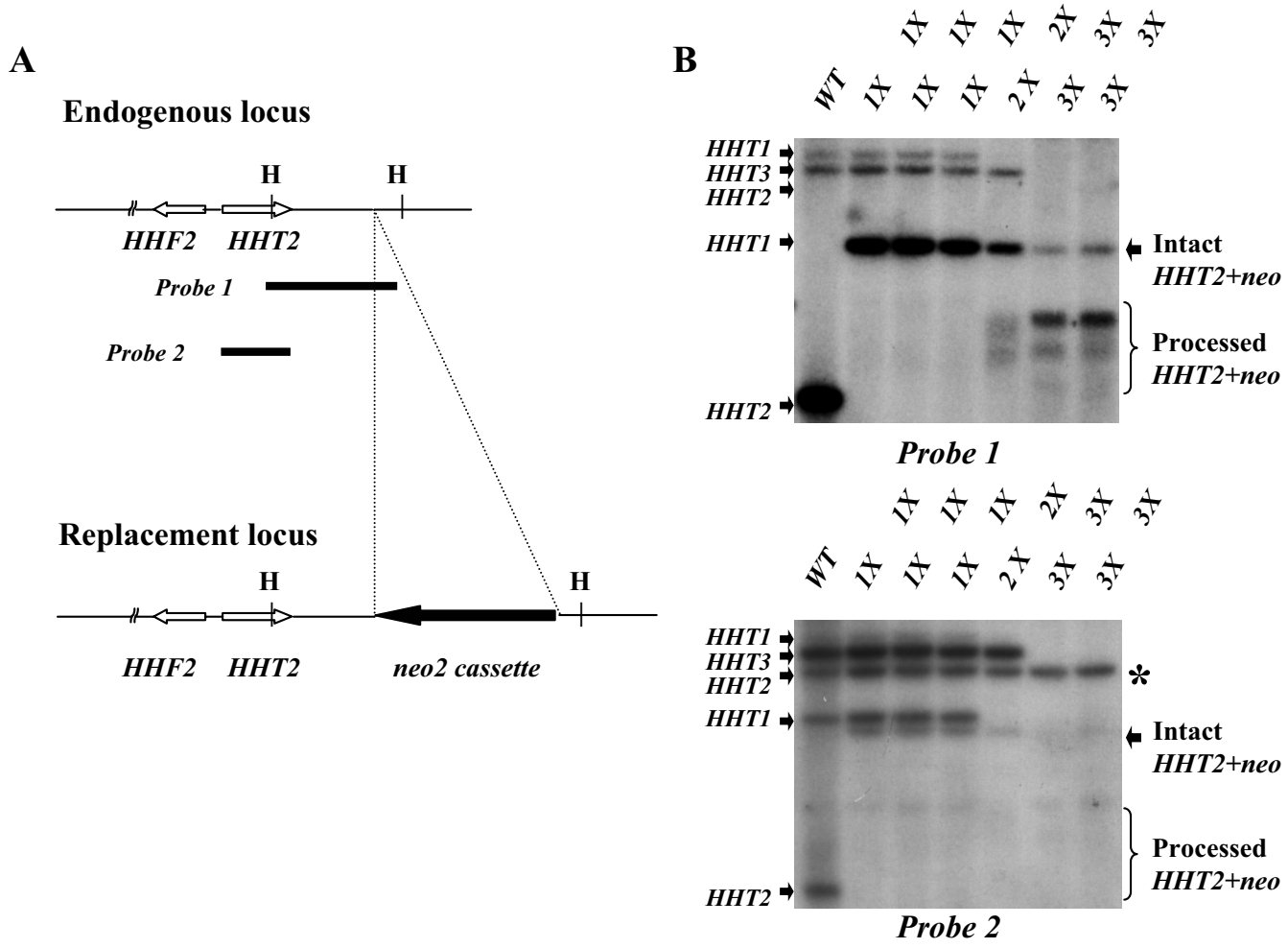

FIG. 5. *neo* elimination from the *HHT2* locus is enhanced with increasing number of *neo* genes. (A) Schematic representation of the endogenous *HHT2* locus and the locus after *neo* insertion. The *neo2* cassette was inserted between two HindIII sites (H) in the *HHT2* locus. There were no HindIII sites in this *neo2* cassette (68, 86). (B) Southern blot analysis of *neo* elimination from the *HHT2* locus in conjugation progeny of the heterokaryons. The cells analyzed were (from left to right): wild-type CU428 (WT), three independent 1× crossed with 1× [(*HHT2* + *neo*) crossed with (*HHT2* + *neo*)] progeny strains, one 2× crossed with 2× [(*HHT2* + *neo* Δ*HHT1*) crossed with (*HHT2* + *neo* Δ*HHT1*)] progeny strain, and two independent 3× crossed with 3× [(*HHT2* + *neo* Δ*HHT1* Δ*HHT3*) crossed with (*HHT2* + *neo* Δ*HHT1* Δ*HHT3*)] progeny strains. HindIII-digested DNA was analyzed by Southern blotting with probe 1 (upper panel); the blot was stripped and reprobed with probe 2 (lower panel). Bands corresponding to the three endogenous loci encoding histone H3 are marked by arrows on the left side of the panels. The patterns confirm the genotypes of the strains. Bands corresponding to the intact (arrows) and processed *neo* gene (brackets) in the *HHT2* locus are indicated on the right side of each panel. Comparison of the ratio of intact and processed bands provides an estimate of the efficiency of *neo* elimination in different strains. The asterisk on the right side of the lower panel marks the position of the HindIII fragment containing the N-terminal half of the *HHT2* coding region and the upstream sequence. It was detected uniformly in all strains, indicating that processing is limited to the *neo*-containing region of the *HHT2* locus.

was less than three, but the total number of copies in the mating pair was four, elimination occurred at relatively high efficiency. This suggests that the number of *neo* genes from both parental cells is counted to determine its elimination efficiency. Additional crosses with parental cells with different numbers of *neo* genes in their micronuclei indicated that *neo* elimination at the *HHT1* locus was detected in significant amounts only when the combined number of *neo* genes from both parental cells reached four copies per haploid chromosome set (Fig. 2C and Fig. 6).

The observation that a *neo* gene in the *HHF1* locus (encoding histone H4.1), nonallelic and nonhomologous to the *HHT1* gene, can affect the efficiency of *neo* elimination from the *HHT1* gene (Fig. 6, lane 2) suggested that *neo* genes in any locus can participate in the counting. This was confirmed by crossing a Δ*HHT1* Δ*HHT2* Δ*HHF1)* strain and a Δ*HTA1* Δ*HTA2* strain (both major H2A genes replaced with *neo2* cassettes), which also resulted in significant *neo* elimination from the *HHT1* locus (Fig. 6, lane 4). Thus, the repetitiveness of a micronucleus-specific sequence (the *neo* gene in this case) enhances its elimination, and the number of *neo* sequences from both parental cells is counted in a relatively locus-independent manner. These observations strongly suggest that a molecule originating in the micronuclei and able to diffuse

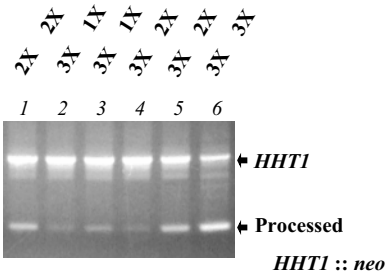

FIG. 6. Sum of *neo* genes in both cells of a mating pair is counted. The crosses were: lane 1, 2× crossed with 2× [(Δ*HHT1* Δ*HHT2*) crossed with (Δ*HHT1* Δ*HHT2*)]; lane 2, 3× crossed with 1× [(Δ*HHT1* Δ*HHT2* Δ*HHF1*) crossed with (Δ*HHF1*)]; lane 3, 3× crossed with 1× [(Δ*HHT1* Δ*HHT2* Δ*HHF1*) crossed with (Δ *HHT1*)]; lane 4, 3× crossed with 2× [(Δ*HHT1* Δ*HHT2* Δ*HHF1*) crossed with (Δ*HTA1* Δ*HTA2*)]; lane 5, 3× crossed with 2× [(Δ*HHT1* Δ*HHT2* Δ*HHF1*) crossed with (Δ*HHT1* Δ*HHT2*)]; lane 6, 3× crossed with 3× [(Δ*HHT1* Δ*HHT2* Δ*HHF1*) crossed with (Δ*HHT1* Δ*HHT2* Δ*HHF1*)]. The progress of conjugation was monitored by fluorescence microscopy, and DNA was isolated after 22 to 48 h of conjugation, when the maximum number of exconjugants was present. The *neo* elimination product was detected in all crosses containing at least four copies of *neo* in the conjugating pair.

between the paired conjugating cells determines the efficiency of IES elimination. The most likely candidates are the double-stranded RNAs synthesized in the micronuclei of conjugating cells and the small RNAs derived from them.

DISCUSSION

Scan RNA model for DNA elimination in *T. thermophila*. The known IESs in *T. thermophila* have little in common at the sequence level. This suggests that something other than specific *cis*-acting elements promote the developmentally regulated deletion (81). The situation is further complicated by the presence of an epigenetic effect on DNA elimination observed in both *T. thermophila* (13) and *Paramecium* (49), in which the abnormal presence of an IES sequence in the macronucleus specifically inhibits the elimination of that IES sequence in the subsequent conjugation. Since the majority of micronucleus-limited DNA sequences are repeated, it was proposed that repetition of a sequence in the micronuclear genome together with its absence from the macronuclear genome was sufficient to induce deletion.

A scan RNA model has been proposed to explain the involvement of small RNAs and the epigenetic regulation of IES processing (50). This model is based in part on the observation that Twi1p first accumulates in the parental macronucleus and is then transferred to the developing macronuclei. It is hypothesized that scan RNAs, in association with Twi1p, scan the macronuclear genome, and those complexes homologous to macronuclear DNA sequences are destroyed. The remaining scan RNAs, which are complementary to micronucleus-limited sequences, are then transferred to the developing macronucleus, where they mark homologous sequences for heterochromatin formation and DNA elimination. Thus, a small-RNA-mediated genome comparison event determines the DNA sequences to be eliminated. Recent studies testing a number of its predictions have supported this model (52). A corollary of the model is that any foreign DNA sequence introduced into the micronucleus would become an IES (50). It predicts that any invading DNA inserted into the micronucleus can be eliminated from the macronucleus of the next sexual generation provided that it produces double-stranded RNA transcripts, and no homologous sequence is present in the parental macronucleus.

The predictions of the scan RNA model were tested by studying the elimination of micronucleus-specific *neo* genes inserted into non-IES, gene-coding loci. *neo* elimination was detected from five different loci. Elimination from three loci can occur with a low copy number of *neo* genes, while elimination from two loci is easily detectable only when multiple copies of the *neo* gene are in the micronucleus. The variation in the efficiency of *neo* elimination and the dependence on gene number can be explained if the transcription levels from different loci vary and a threshold level of transcripts must be achieved for elimination to occur, as in posttranscriptional gene silencing in *Drosophila melanogaster* (58). Alternatively, some loci may require higher levels of small RNAs for the elimination machinery to overcome the unfavorable state of chromatin in a transcriptionally active gene. In *Drosophila melanogaster*, it has been found that, under otherwise similar conditions, a weak promoter (*white*) can be repressed by transcriptional gene silencing, while a strong promoter (*Adh*) can only be silenced posttranscriptionally, requiring much higher levels of small RNAs (58).

The positive correlation between the repetitiveness of the *neo* gene and the efficiency of its elimination provides an explanation for the predominance of repeated sequences in IESs and the lack of these sequences in the macronuclear genome. This constitutes an interesting host-parasite interaction between the organism and the invading DNA with similarities to other systems. In *Drosophila melanogaster*, when transposable elements invade a virgin genome, high-frequency transposition occurs transiently. As the copy number of the transposable element increases, transposition activity decreases and eventually ceases (11, 35). A clear correlation has also been established between the copy number of a dispersed transgene (*w-Adh*) and its transcriptional silencing, in a manner dependent on RNA interference-related mechanisms (58). Similar small-RNA-induced heterochromatin formation probably occurs in other eukaryotic systems (5, 20, 30, 46, 53, 78, 87), in which the repetitiveness of "junk DNAs" ensures that they are always packaged in heterochromatin and transcriptionally repressed. Actually, a certain dosage (repetitiveness) of these junk DNAs may be required to silence effectively them in the genomes of higher eukaryotes (5, 36).

Our results show that the total number of *neo* genes from both conjugation partners is counted to affect the efficiency of *neo* elimination. This suggests that a molecule that is diffusible through the cytoplasm is involved. Earlier studies showed that Twi1p is required for the accumulation of scan RNAs in *T. thermophila* and that it diffuses between conjugating partners, suggesting that the scan RNAs (or their double-stranded precursors), probably in a complex with Twi1p, are likely to be the diffusible factor (50, 51). This result is also compatible with the observation that the epigenetic influence of the old macronucleus on IES processing exhibits cytoplasmic dominance (13), similar to the maternal inheritance of transposition inhibition

in *Drosophila melanogaster* (11, 35). Our results do not support the suggestion that direct homologous DNA interactions between repeated sequences or "pairing" plays a dominant role in determining the elimination efficiency (79) because the multiple copies of the *neo* gene required for elimination from the *HHT1* locus can be supplied by *neo* genes at five different loci (Fig. 6, lane 4).

Our experiments also strengthen the argument that IES elimination is an RNA-mediated process. Transcripts from both strands of the *neo* gene were present at detectable levels during conjugation in crosses that resulted in significant *neo* elimination. Therefore, there is a positive correlation between the presence of specific double-stranded RNAs and the efficiency of DNA elimination.

Early studies of endogenous IES transcripts show that their levels peak at 6 to 7 h into conjugation (14), after the period (2 to 3 h into conjugation) when transcriptional activity can be detected autoradiographically in the micronucleus (47, 71). This suggests that there may be secondary amplification of the double-stranded RNAs by an RNA-dependent RNA polymerase-like enzymatic activity (1, 69). The presence of such a positive feedback loop may also help to explain the dramatic difference in the level of transcripts and *neo* elimination when the template number is changed only threefold.

What about foreign DNAs in macronuclei? Based on the studies described here, it seems likely that when a foreign DNA first invades the micronucleus, its elimination from the macronucleus of the next sexual generation will be incomplete. The persistence of the foreign DNA in the macronuclear genome will in turn further inhibit its elimination from the cell's sexual progeny (13). Fortunately, there is a mechanism that will prevent the initial invasion of foreign DNA into macronuclei from becoming permanent, at least in some of the clonal progeny of the invaded cell. Because the polyploid macronucleus divides amitotically, randomly assorting chromosomes to sister cells, there can be significant changes in the allele composition in the macronucleus as *T. thermophila* propagates vegetatively (57). Conjugation progeny with heterozygous macronuclei (as will be the case for the first sexual generation after the cross of cells with the micronucleus heterozygous for a foreign DNA) will give rise to strains assorted to homozygosity in about 100 divisions (2), which is similar to the amount of time it takes for the progeny to mature sexually for the next round of conjugation (64). Thus, while a foreign sequence invading the macronucleus will become enriched in some clonal progeny of the invaded cell, it will be eliminated from other progeny by random assortment.

Because of the epigenetic influences of the parental macronuclei on IES processing, only cells that have assorted to lose the invading element from the macronucleus can eliminate it from the macronucleus of subsequent sexual progeny. If insertion of the element has a deleterious effect, a selective advantage would be conferred on the progeny that are able to delete it. Assortment probably also protects macronuclei from direct invasion by foreign genetic elements during the long periods of vegetative growth that can occur between successive conjugations. To become fixed in the *T. thermophila* germ line genome and transmitted to the somatic macronuclear genome of the next sexual generation, an invading genetic element would have to escape both elimination during macronuclear development and assortment during vegetative growth, an unlikely prospect.

Determining the boundaries of IES elimination. In *T. thermophila*, the boundaries of some IESs are relatively precise in different clonal progeny from the same conjugating parental cells, while others may have alternative sites at one or both ends (34). Elimination of the *neo* gene described here showed similar properties. In the case of elimination of *neo* from the disrupted *HHT1* locus in 3× heterokaryons, analysis of the predominant processed product indicated that, besides the *neo* coding region, the *HHF1* promoter and a significant length of *HHT1* 3′-flanking sequence, along with most of the 3′ *BTU2* sequence of the *neo* cassette, were also deleted. Importantly, the eliminated sequences flanking the *neo* coding region are endogenous sequences present in the old macronucleus during conjugation, and according to the scan RNA hypothesis, small RNAs specific to them should not accumulate. This makes it unlikely that the boundaries of IES elimination are determined by the small RNAs. More likely, the small RNAs serve only to initiate heterochromatin formation, as is the case in *Schizosaccharomyces pombe* (30, 78).

The extent of heterochromatin spreading is probably determined by other features of chromatin structure, such as the boundary elements in the silent mating type locus of *Schizosaccharomyces pombe* and the insulators in the chicken β-globin locus (8). Thus, the phenomenon of alternative boundaries for DNA deletion in *T. thermophila* might be explained as cases where altered chromatin structure can sometimes spread through an inefficient boundary and then be halted at the next suitable boundary in the adjacent chromatin. Alternatively, the boundaries of deletion could reflect the preference of the DNA-splicing enzymes, which seem to prefer short, direct repeats (34, 39). These results on removal of the newly introduced *neo* sequences are also consistent with earlier studies showing that IES boundaries are at least partly determined by their flanking sequences (12, 21, 24, 25) but that no specific flanking sequences are required for deletion (81).

Relationship between IES elimination and gene silencing. Sequence elimination in *T. thermophila* can be considered an extreme form of gene silencing. The proposed mechanism of IES processing applied to foreign DNAs that invade the micronuclear genome provides a defense protecting the whole macronuclear genome by a mechanism similar to RNA silencing in other organisms (61). In *Caenorhabditis elegans*, some mutations affecting the RNA interference pathway also exhibit desilencing and mobilization of transposons (41, 72). Tandem arrays of transposons in *Caenorhabditis elegans*, like in many other organisms, are silenced by a chromatin-based mechanism (40), which is affected by homologues to *Enhancer of Zeste* and *extra sex combs* (74), both components of a histone methyltransferase complex important for heterochromatin formation and gene repression in *Drosophila melanogaster* (19, 54). In *T. thermophila*, two genes required for the accumulation of small RNAs, *PDD1* and *TWI1*, are required for elimination of IES elements, including some putative transposable elements, from the macronuclear genome (18, 45, 50). It will be interesting to determine whether micronuclear transposon-like sequences are mobilized in *PDD1* and *TWI1* knockout cells.

A common pathway is emerging from recent studies of transcriptional gene silencing and IES elimination. First, double-

stranded RNAs are processed by an RNA interference-like mechanism to generate small RNAs (14, 30, 50, 63, 73, 78). Small RNAs target histone H3 methylation at lysine 9 (30, 50, 73, 78) and/or lysine 27 (19, 54) to loci with homologous sequences. Histone methylation recruits chromodomain proteins, such as Pdd1p (45, 73) and HP1 (4, 42) and polycomb proteins (19) in *Drosophila melanogaster* and *Swi6* (55) in *Schizosaccharomyces pombe*, which lead to the formation and spreading of heterochromatin that is ultimately limited by some features of chromatin structure (8). Pdd1p is a chromodomain protein that can interact with K9-methylated H3, which is specifically marks IES sequences in *T. thermophila* (45, 73). Some organisms appear to process the heterochromatin further. In vertebrates and plants, DNA methylation is likely dependent on histone methylation (6, 33) and probably reinforces silencing. In *Neurospora crassa*, DNA methylation further induces repeat-induced point mutation (66). In *T. thermophila*, the DNA is eliminated from the macronuclear genome (17). Thus, the pathway to heterochromatin formation appears to be highly conserved, but subsequent consequences may vary.

ACKNOWLEDGMENTS

We thank Meng-Chao Yao for communication of unpublished data and discussion.

This work was supported by grant GM-21793 from the National Institutes of Health to Martin A. Gorovsky and by grant MCB-9974885 from the National Science Foundation to Kathleen M. Karrer. K.M.K. was supported in part by a Sabbatical Fellowship from Marquette University.

REFERENCES

1. **Ahlquist, P.** 2002. RNA-dependent RNA polymerases, viruses, and RNA silencing. Science **296:**1270–1273.
2. **Austerberry, C. F., and M. C. Yao.** 1987. Nucleotide sequence structure and consistency of a developmentally regulated DNA deletion in *Tetrahymena thermophila.* Mol. Cell. Biol. **7:**435–443.
3. **Austerberry, C. F., and M. C. Yao.** 1988. Sequence structures of two developmentally regulated, alternative DNA deletion junctions in *Tetrahymena thermophila.* Mol. Cell. Biol. **8:**3947–3950.
4. **Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas, R. C. Allshire, and T. Kouzarides.** 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature **410:**120–124.
5. **Birchler, J. A., M. P. Bhadra, and U. Bhadra.** 2000. Making noise about silence: repression of repeated genes in animals. Curr. Opin. Genet Dev. **10:** 211–216.
6. **Bird, A. P., and A. P. Wolffe.** 1999. Methylation-induced repression–belts, braces, and chromatin. Cell **99:**451–454.
7. **Brown, J. M., C. Marsala, R. Kosoy, and J. Gaertig.** 1999. Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in *Tetrahymena.* Mol. Biol. Cell **10:**3081–3096.
8. **Burgess-Beusse, B., C. Farrell, M. Gaszner, M. Litt, V. Mutskov, F. Recillas-Targa, M. Simpson, A. West, and G. Felsenfeld.** 2002. The insulation of genes from external enhancers and silencing chromatin. Proc. Natl. Acad. Sci. USA **99(Suppl. 4):**16433–16437.
9. **Carmell, M. A., Z. Xuan, M. Q. Zhang, and G. J. Hannon.** 2002. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. **16:**2733–2742.
10. **Cassidy-Hanley, D., J. Bowen, J. Lee, E. S. Cole, L. A. VerPlank, J. Gaertig, M. A. Gorovsky, and P. J. Bruns.** 1997. Germline and somatic transformation of mating *Tetrahymena thermophila* by particle bombardment. Genetics **146:**135–147.
11. **Chaboissier, M. C., A. Bucheton, and D. J. Finnegan.** 1998. Copy number control of a transposable element, the I factor, a LINE-like element in *Drosophila.* Proc. Natl. Acad. Sci. USA **95:**11781–11785.
12. **Chalker, D. L., A. La Terza, A. Wilson, C. D. Kroenke, and M. C. Yao.** 1999. Flanking regulatory sequences of the *Tetrahymena* R deletion element determine the boundaries of DNA rearrangement. Mol. Cell. Biol. **19:**5631–5641.
13. **Chalker, D. L., and M. C. Yao.** 1996. Non-mendelian, heritable blocks to DNA rearrangement are induced by loading the somatic nucleus of *Tetrahymena thermophila* with germ line-limited DNA. Mol. Cell. Biol. **16:**3658–3667.
14. **Chalker, D. L., and M. C. Yao.** 2001. Nongenic, bidirectional transcription precedes and may promote developmental DNA deletion in *Tetrahymena thermophila.* Genes Dev. **15:**1287–1298.
15. **Chau, M. F., and E. Orias.** 1996. Developmentally programmed DNA rearrangement in *Tetrahymena thermophila*: Isolation and sequence characterization of three new alternative deletion systems. Biol. Cell **86:**111–120.
16. **Cherry, J. M., and E. H. Blackburn.** 1985. The internally located telomeric sequences in the germ-line chromosomes of *Tetrahymena* are at the ends of transposon-like elements. Cell **43:**747–758.
17. **Coyne, R. S., D. L. Chalker, and M. C. Yao.** 1996. Genome downsizing during ciliate development: nuclear division of labor through chromosome restructuring. Annu. Rev. Genet. **30:**557–578.
18. **Coyne, R. S., M. A. Nikiforov, J. F. Smothers, C. D. Allis, and M. C. Yao.** 1999. Parental expression of the chromodomain protein Pdd1p is required for completion of programmed DNA elimination and nuclear differentiation. Mol. Cell **4:**865–872.
19. **Czermin, B., R. Melfi, D. McCabe, V. Seitz, A. Imhof, and V. Pirrotta.** 2002. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell **111:**185–196.
20. **Dernburg, A. F., and G. H. Karpen.** 2002. A chromosome RNAissance. Cell **111:**159–162.
21. **Fillingham, J. S., D. Bruno, and R. E. Pearlman.** 2001. Cis-acting requirements in flanking DNA for the programmed elimination of mse2.9: a common mechanism for deletion of internal eliminated sequences from the developing macronucleus of *Tetrahymena thermophila.* Nucleic Acids Res. **29:**488–498.
22. **Forrest, E. C., C. Cogoni, and G. Macino.** 2004. The RNA-dependent RNA polymerase, QDE-1, is a rate-limiting factor in post-transcriptional gene silencing in *Neurospora crassa.* Nucleic Acids Res. **32:**2123–2128.
23. **Gaertig, J., L. Gu, B. Hai, and M. A. Gorovsky.** 1994. High frequency vector-mediated transformation and gene replacement in *Tetrahymena.* Nucleic Acids Res. **22:**5391–5398.
24. **Godiska, R., C. James, and M. C. Yao.** 1993. A distant 10-bp sequence specifies the boundaries of a programmed DNA deletion in *Tetrahymena.* Genes Dev. **7:**2357–2365.
25. **Godiska, R., and M. C. Yao.** 1990. A programmed site-specific DNA rearrangement in *Tetrahymena thermophila* requires flanking polypurine tracts. Cell **61:**1237–1246.
26. **Gorovsky, M. A.** 1970. Studies on nuclear structure and function in *Tetrahymena pyriformis.* II. Isolation of macro- and micronuclei. J. Cell Biol. **47:** 619–630.
27. **Gorovsky, M. A., and J. Woodard.** 1967. Histone content of chromosomal loci active and inactive in RNA synthesis. J. Cell Biol. **33:**723–728.
28. **Gorovsky, M. A., M.-C. Yao, J. B. Keevert, and G. L. Pleger.** 1975. Isolation of micro- and macronuclei of *Tetrahymena pyriformis.* Methods Cell Biol. **IX:** 311–327.
29. **Hai, B., and M. A. Gorovsky.** 1997. Germ-line knockout heterokaryons of an essential a-tubulin gene enable high-frequency gene replacement and a test of gene transfer from somatic to germ-line in *Tetrahymena thermophila.* Proc. Natl. Acad. Sci. USA **94:**1310–1315.
30. **Hall, I. M., G. D. Shankaranarayana, K. Noma, N. Ayoub, A. Cohen, and S. I. Grewal.** 2002. Establishment and maintenance of a heterochromatin domain. Science **297:**2232–2237.
31. **Howard, E. A., and E. H. Blackburn.** 1985. Reproducible and variable genomic rearrangements occur in the developing somatic nucleus of the ciliate *Tetrahymena thermophila.* Mol. Cell. Biol. **5:**2039–2050.
32. **Huvos, P. E., M. Wu, and M. A. Gorovsky.** 1998. A developmentally eliminated sequence in the flanking region of the histone H1 gene in *Tetrahymena thermophila* contains short repeats. J. Eukaryot. Microbiol. **45:**189–197.
33. **Jackson, J. P., A. M. Lindroth, X. Cao, and S. E. Jacobsen.** 2002. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature **416:**556–560.
34. **Jahn, C. L., and L. A. Klobutcher.** 2002. Genome remodeling in ciliated protozoa. Annu. Rev. Microbiol. **56:**489–520.
35. **Jensen, S., M. P. Gassama, and T. Heidmann.** 1999. Taming of transposable elements by homology-dependent gene silencing. Nat. Genet. **21:**209–212.
36. **Jenuwein, T.** 2002. Molecular biology. An RNA-guided pathway for the epigenome. Science **297:**2215–2218.
37. **Kahn, R. W., B. H. Andersen, and C. F. Brunk.** 1993. Transformation of *Tetrahymena thermophila* by microinjection of a foreign gene. Proc. Natl. Acad. Sci. USA **90:**9295–9299.
38. **Karrer, K. M.** 1983. Germ line-specific DNA sequences are present on all five micronuclear chromosomes in *Tetrahymena thermophila.* Mol. Cell. Biol. **3:**1909–1919.
39. **Karrer, K. M.** 2000. *Tetrahymena* genetics: two nuclei are better than one. Methods Cell Biol. **62:**127–186.
40. **Kelly, W. G., and A. Fire.** 1998. Chromatin silencing and the maintenance of a functional germline in *Caenorhabditis elegans.* Development **125:**2451–2456.

41. **Ketting, R. F., T. H. Haverkamp, H. G. van Luenen, and R. H. Plasterk.** 1999. Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell **99:**133–141.
42. **Lachner, M., D. O'Carroll, S. Rea, K. Mechtler, and T. Jenuwein.** 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature **410:**116–120.
43. **Lingel, A., B. Simon, E. Izaurralde, and M. Sattler.** 2003. Structure and nucleic-acid binding of the *Drosophila* Argonaute 2 PAZ domain. Nature **426:**465–469.
44. **Liu, Y., K. Mochizuki, and M. A. Gorovsky.** 2004. Histone H3 lysine 9 methylation is required for DNA elimination in developing macronuclei in *Tetrahymena.* Proc. Natl. Acad. Sci. USA **101:**1679–1684.
45. **Madireddi, M. T., R. S. Coyne, J. F. Smothers, K. M. Mickey, M. C. Yao, and C. D. Allis.** 1996. Pdd1p, a novel chromodomain-containing protein, links heterochromatin assembly and DNA elimination in *Tetrahymena.* Cell **87:** 75–84.
46. **Maison, C., D. Bailly, A. H. Peters, J. P. Quivy, D. Roche, A. Taddei, M. Lachner, T. Jenuwein, and G. Almouzni.** 2002. Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. Nat. Genet. **30:**329–334.
47. **Martindale, D. W., C. D. Allis, and P. J. Bruns.** 1985. RNA and protein synthesis during meiotic prophase in *Tetrahymena thermophila.* J. Protozool. **32:**644–649.
48. **Martindale, D. W., and P. J. Bruns.** 1983. Cloning of abundant mRNA species present during conjugation of *Tetrahymena thermophila*: Identification of mRNA species present exclusively during meiosis. Mol. Cell. Biol. **3:** 1857–1865.
49. **Meyer, E., and S. Duharcourt.** 1996. Epigenetic programming of developmental genome rearrangements in ciliates. Cell **87:**9–12.
50. **Mochizuki, K., N. A. Fine, T. Fujisawa, and M. A. Gorovsky.** 2002. Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in *Tetrahymena.* Cell **110:**689–699.
51. **Mochizuki, K., and M. A. Gorovsky.** 2004. Conjugation-specific small RNAs in *Tetrahymena* have predicted properties of scan (scn) RNAs involved in genome rearrangement. Genes Dev. **18:**2068–2073.
52. **Mochizuki, K., and M. A. Gorovsky.** 2004. Small RNAs in genome rearrangement in *Tetrahymena.* Curr. Opin. Genet. Dev. **14:**1–7.
53. **Muchardt, C., M. Guilleme, J. S. Seeler, D. Trouche, A. Dejean, and M. Yaniv.** 2002. Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1alpha. EMBO Rep. **3:**975–981.
54. **Muller, J., C. M. Hart, N. J. Francis, M. L. Vargas, A. Sengupta, B. Wild, E. L. Miller, M. B. O'Connor, R. E. Kingston, and J. A. Simon.** 2002. Histone methyltransferase activity of a *Drosophila* Polycomb group repressor complex. Cell **111:**197–208.
55. **Nakayama, J., J. C. Rice, B. D. Strahl, C. D. Allis, and S. I. Grewal.** 2001. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science **292:**110–113.
56. **Napoli, C., C. Lemieux, and R. Jorgensen.** 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell **2:**279–289.
57. **Orias, E., and M. Flacks.** 1975. Macronuclear genetics of *Tetrahymena.* I. Random distribution of macronuclear genecopies in *T. pyriformis*, syngen 1. Genetics **79:**187–206.
58. **Pal-Bhadra, M., U. Bhadra, and J. A. Birchler.** 2002. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila.* Mol. Cell **9:**315–327.
59. **Pal-Bhadra, M., B. A. Leibovitch, S. G. Gandhi, M. Rao, U. Bhadra, J. A. Birchler, and S. C. Elgin.** 2004. Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. Science **303:** 669–672.
60. **Patil, N. S., P. M. Hempen, R. A. Udani, and K. M. Karrer.** 1997. Alternate junctions and microheterogeneity of Tlr1, a developmentally regulated DNA rearrangement in *Tetrahymena thermophila.* J. Eukaryot. Microbiol. **44:**518–522.
61. **Plasterk, R. H.** 2002. RNA silencing: the genome's immune system. Science **296:**1263–1265.
62. **Que, Q., and R. A. Jorgensen.** 1998. Homology-based control of gene expression patterns in transgenic petunia flowers. Dev. Genet. **22:**100–109.
63. **Reinhart, B. J., and D. P. Bartel.** 2002. Small RNAs correspond to centromere heterochromatic repeats. Science **297:**1831.
64. **Rogers, M. B., and K. M. Karrer.** 1985. Adolescence in *Tetrahymena thermophila.* Proc. Natl. Acad. Sci. USA **82:**436–439.
65. **Romano, N., and G. Macino.** 1992. Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. Mol. Microbiol. **6:**3343–3353.
66. **Selker, E. U.** 1997. Epigenetic phenomena in filamentous fungi: useful paradigms or repeat-induced confusion? Trends Genet. **13:**296–301.
67. **Shang, Y., X. Song, J. Bowen, R. Corstanje, Y. Gao, J. Gaertig, and M. A. Gorovsky.** 2002. A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in *Tetrahymena thermophila.* Proc. Natl. Acad. Sci. USA **99:**3734–3739.
68. **Shen, X., L. Yu, J. W. Weir, and M. A. Gorovsky.** 1995. Linker histones are not essential and affect chromatin condensation in vivo. Cell **82:**47–56.
69. **Sijen, T., J. Fleenor, F. Simmer, K. L. Thijssen, S. Parrish, L. Timmons, R. H. Plasterk, and A. Fire.** 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107:**465–476.
70. **Song, J. J., J. Liu, N. H. Tolia, J. Schneiderman, S. K. Smith, R. A. Martienssen, G. J. Hannon, and L. Joshua-Tor.** 2003. The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nat. Struct. Biol. **10:**1026–1032.
71. **Sugai, T., and K. Hiwatashi.** 1974. Cytologic and autoradiographic studies of the micronucleus at meiotic prophase in *Tetrahymena pyriformis.* J. Protozool. **21:**542–548.
72. **Tabara, H., M. Sarkissian, W. G. Kelly, J. Fleenor, A. Grishok, L. Timmons, A. Fire, and C. C. Mello.** 1999. The rde-1 gene, RNA interference, and transposon silencing in *C. elegans.* Cell. **99:**123–132.
73. **Taverna, S. D., R. S. Coyne, and C. D. Allis.** 2002. Methylation of histone H3 at lysine 9 targets programmed DNA elimination in *Tetrahymena.* Cell **110:** 701–711.
74. **Tie, F., T. Furuyama, and P. J. Harte.** 1998. The *Drosophila* Polycomb group proteins ESC and E(Z) bind directly to each other and co-localize at multiple chromosomal sites. Development **125:**3483–3496.
75. **Tijsterman, M., R. F. Ketting, and R. H. Plasterk.** 2002. The genetics of RNA silencing. Annu. Rev. Genet. **36:**489–519.
76. **van der Krol, A. R., L. A. Mur, M. Beld, J. N. Mol, and A. R. Stuitje.** 1990. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:**291–299.
77. **Verdel, A., S. Jia, S. Gerber, T. Sugiyama, S. Gygi, S. I. Grewal, and D. Moazed.** 2004. RNAi-mediated targeting of heterochromatin by the RITS complex. Science **303:**672–676.
78. **Volpe, T. A., C. Kidner, I. M. Hall, G. Teng, S. I. Grewal, and R. A. Martienssen.** 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science **297:**1833–1837.
79. **Wolffe, A. P., and M. A. Matzke.** 1999. Epigenetics: Regulation through repression. Science **286:**481–486.
80. **Wuitschick, J. D., J. A. Gershan, A. J. Lochowicz, S. Li, and K. M. Karrer.** 2002. A novel family of mobile genetic elements is limited to the germline genome in *Tetrahymena thermophila.* Nucleic Acids Res. **30:**2524–2537.
81. **Wuitschick, J. D., and K. M. Karrer.** 2003. Diverse sequences within Tlr elements target programmed DNA elimination in *Tetrahymena thermophila.* Eukaryot. Cell **2:**678–689.
82. **Yan, K. S., S. Yan, A. Farooq, A. Han, L. Zeng, and M. M. Zhou.** 2003. Structure and conserved RNA binding of the PAZ domain. Nature **426:** 468–474.
83. **Yao, M. C.** 1996. Programmed DNA deletions in *Tetrahymena*: mechanisms and implications. Trends Genet. **12:**26–30.
84. **Yao, M. C., P. Fuller, and X. Xi.** 2003. Programmed DNA deletion as an RNA-guided system of genome defense. Science **300:**1581–1584.
85. **Yao, M. C., and M. A. Gorovsky.** 1974. Comparison of the sequences of macro- and micronuclear DNA of *Tetrahymena pyriformis.* Chromosoma **48:** 1–18.
86. **Yu, L., and M. A. Gorovsky.** 1997. Constitutive expression, not a particular primary sequence, is the important feature of the H3 replacement variant hv2 in *Tetrahymena thermophila.* Mol. Cell. Biol. **17:**6303–6310.
87. **Zilberman, D., X. Cao, and S. E. Jacobsen.** 2003. ARGONAUTE4 control of locus-specific small interfering RNA accumulation and DNA and histone methylation. Science **299:**716–719.