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*

Kathleen Karrer was affiliated with the University of Rochester and Marquette University at the time of publication.
Elimination of Foreign DNA during Somatic Differentiation in Tetrahymena thermophila Shows Position Effect and Is Dosage Dependent†

Yifan Liu,‡ Xiaoyuan Song, Martin A. Gorovsky, and Kathleen M. Karrer

Department of Biology, University of Rochester, Rochester, New York, and Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin

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 macro- nucleus 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 macro- nuclei 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-
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. Mo-chizuki and M. A. Gorovsky, unpublished observations). A conjugation-specific gene, \( TWI \), encodes an Argonaute homo-
logue that is required for the accumulation of the small RNAs (50). Methylation of lysine 9 of histone H3 and an abundant
logue that is required for the accumulation of the small RNAs
conjugation-specific gene,
by a Dicer-like enzyme to produce the small RNAs (K. Mo-
LIU ET AL. EUKARYOT. CELL
422
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 macronuclear-des-
tined 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 Tr
elements, where at least seven different regions varying con-
siderably 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 “cosup-
pression” 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 tran-
scriptional 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 paro-
momycin resistance in transformants (37). Germ line hetero-
karyon 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 heterokary-
on 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 va-
riety 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 macronuclear
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 \( 2 \times 10^7 \)
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 \( neo2 \) cas-
sette (29), which confers paromomycin resistance in \( T. thermophila \). The \( neo2
 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 replac-
ing 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 \( HHT2 \) locus contained an insertion of the \( neo2 \) 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 \( neo3 \) cassette, which confers cadmium-dependent paromomycin resis-
tance 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 conjugat-
ing 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 \( neo3 \) cassette instead of the \( neo2
 cassette. \( neo3 \) differs from \( neo2 \) in that \( neo \) transcription is under the control of

Downloaded on May 28, 2014 by Marquette University Libraries from fec.asm.org/
FIG. 1. neo is eliminated from the ngoA locus during conjugation of ΔngoA 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 ΔngoA germ line knockout heterokaryons. Genomic DNA samples isolated at the indicated times after initiation of conjugation were used as templates for PCR amplification. The primers used for the PCR analysis amplify the micronuclear-specific M element as a loading control were M3-fwd (5′-GCA CGC GGA TTC TCC GGC CGC TTG-3′) and M3-rev (5′-GCC GCC GCA TTG CAT CAG CCA TG-3′).

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 (nucleotide positions 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 (5′-GCA CGC GGA TTC TCC GGC CGC TTG-3′) and M3-rev (5′-GCC GCC GCA TTG CAT CAG CCA TG-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 GGA 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 cytotologically.

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 the 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. 2C). Thus, there is significant position effect variation in the efficiency with which a foreign sequence is eliminated.

**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 ΔHHT1 ΔHHT2/HHF2 ΔHHF1 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× ΔHHT1 ΔHHT2 ΔHHF1 strains (Fig. 4B) in which significant neo elimination occurred. Little neo gene transcription was detected in the conjugation of 1× ΔHHT1 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-
The crosses analyzed were (from left to right): 1 neoing heterokaryon strains with different number of generating 1.1-kb, 2.2-kb, and 0.4-kb fragments, respectively. (B) Southern indicated primers can amplify PCR products from all three loci, gen-

flanked by a Bgl II (B) and a ClaI (C) site. There was one HindIII with 1 locus, the knockout locus (containing the during conjugation. (A) Schematic representation of the endogenous

HHT1 locus with 0 /H9004 HHT1 with 3 /H11003 [HHT1 with 0 /H9004 HHT1

with 0 /H9004 HHT1/HHF1neo processed knockout locus B

HHT1::neo HHT1::neo HHT1::neo HHT1::neo

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): 1X crossed with 1X [(ΔHHT1) crossed with (ΔHHT1)], 2X crossed with 2X [(ΔHHT1 ΔHHT2) crossed with (ΔHHT1 ΔHHT2)], and 3X crossed with 0X [(ΔHHT1 ΔHHT2 ΔHHF1) 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

rectly related to the copy number of the neo gene, matings were done between strains with one, two, or three loci homozy-

gous 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 micronu-

cleus. neo elimination in the HHT1 locus was barely detectable in crosses between 1X neo homozygous heterokaryon strains (diploid for the neo gene in their micronuclei), but increased in a mating between two 2X neo (ΔHHT1 ΔHHT2 ΔHHF1) strains (Fig. 2B) and increased still more in the 3X neo (ΔHHT1 ΔHHT2 ΔHHF1) 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 estab-

lish 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 micro-

nuclei were homozygous for a version of the HHT2 locus with the neo2 cassette in its 3'-flanking region (1X neo, Fig. 5A) and therefore retain functional HHF2 and HHIT2 genes. The 1X neo strains were combined genetically with germ line knockout heterokaryon ΔHHT1 strains to produce 2X neo strains (see Materials and Methods). Finally, 2X neo strains were com-

bined genetically with the ΔHHF3 (histone H3.3) strain to produce 3X 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

neo elimination product was only detected in the 2X crossed with 2X 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, ΔHHF1 strain; lane 3, ΔHHT1 ΔHHT2 strain; lane 4, ΔHHT1 ΔHHT2 ΔHHF1 strain; lane 5, another ΔHHT1 ΔHHT2 ΔHHF1 strain. Lanes 6 to 8 show PCR products with DNA isolated from mass mating cells at 24 hr as the template. Lane 6, 2X crossed with 1X [(ΔHHT1 ΔHHT2) crossed with (ΔHHF1)]; lane 7, 3X crossed with 0X [(ΔHHT1 ΔHHT2 ΔHHF1) crossed with wild-type CU428]; lane 8, 3X crossed with 3X [(ΔHHT1 ΔHHT2 ΔHHF1) crossed with (ΔHHT1 ΔHHT2 ΔHHF1)]. All three forms of the HHT1 locus should be detected in the undi-

gested 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 de-

tected 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.
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 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 (ΔHHT1 ΔHHT2 ΔHHF1) 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 ΔHHT1 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...
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 HHT1 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 ΔHHT1 ΔHHT2 ΔHHF1 strain and a ΔHTA1 ΔHTA2 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× ([ΔHHT1 ΔHHT2] crossed with (ΔHHT1 ΔHHT2 ΔHHF1)] crossed with (ΔHHF1]); lane 2, 3× crossed with 1× ([ΔHHT1 ΔHHT2 ΔHHF1]) crossed with (Δ HHT1); lane 3, 3× crossed with 1× ([ΔHHT1 ΔHHT2 ΔHHF1]) crossed with (ΔHHT1 ΔHTA2]); lane 4, 3× crossed with 2× ([ΔHHT1 ΔHHT2 ΔHHF1]) crossed with (ΔHHT1 ΔHHF1); lane 5, 3× crossed with 2× ([ΔHHT1 ΔHHT2 ΔHHF1]) crossed with (ΔHHT1 ΔHTA2]; lane 6, 3× crossed with 3× ([ΔHHT1 ΔHHT2 ΔHHF1]) crossed with (ΔHHT1 ΔHHT2 ΔHHF1]). 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 micronuclear 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 *HH1†* 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 macronucleus, 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 micronuclear 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 *HH1†* 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 *HH1†* 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 macronuclear 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 *TW1†*, 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 macronuclear transposon-like sequences are mobilized in *PDD1* and *TW1†* 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, 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 S. pombe (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 chromo-domain 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 Marla A. Gorovsky and by grant MCB-0974885 from the National Science Foundation to Kathleen M. Karrer. K.M.K. was supported in part by a Sabbatical Fellowship from Marquette University.

REFERENCES


