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The germ line limited M element of *Tetrahymena* is targeted for elimination from the somatic genome by a homology-dependent mechanism

Christina A. Kowalczyk, Alissa M. Anderson, Maria Arce-Larreta and Douglas L. Chalker*

Biology Department, Washington University, St. Louis, MO 63130, USA

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ABSTRACT

A RNA interference (RNAi) like mechanism is involved in elimination of thousands of DNA segments from the developing somatic macronucleus of *Tetrahymena*, yet how specific internal eliminated sequences (IESs) are recognized remains to be fully elucidated. To define requirements for DNA rearrangement, we performed mutagenesis of the M element, a well-studied IES. While sequences within the macronucleus-retained DNA are known to determine the excision boundaries, we show that sequences internal to these boundaries are required to promote this IES's rearrangement. However, this element does not contain any specific sequence required in *cis* as removal of its entire left or right side was insufficient to abolish all rearrangement. Instead, rearrangement efficiency correlated with the overall size of the M element sequence within a given construct, with a lower limit of nearly 300 bp. Also, the observed minimal region necessary to epigenetically block excision supports this size limit. Truncated M element constructs that exhibited impaired rearrangement still showed full transcriptional activity, which suggests that their defect was due to inefficient recognition. This study indicates that IESs are targeted for elimination upon their recognition by homologous small RNAs and further supports the idea that DNA elimination is a RNAi-related mechanism involved in genome surveillance.

INTRODUCTION

Developmentally-regulated DNA rearrangements lead to massive genome reorganization within the somatic nuclei of ciliates [reviewed in Ref. (1)]. The extent and form of these rearrangements vary greatly among different species. In the most extreme cases, >90% of the germ line genome is eliminated. In *Tetrahymena thermophila*, ∼15% of the genome (∼15 Mbp) is removed from an estimated 6000 loci of the developing macronuclei (2,3). The DNA segments removed, called internal eliminated sequences (IESs) or deletion elements, range in size from 0.6 to >20 kbp and represent both unique sequence and the majority of repetitive sequences. The diversity of DNA segments removed has presented a challenge toward understanding how any particular sequence is recognized and targeted for elimination during development. The study of this process promises to provide unique insight into mechanisms eukaryotes use to target widely dispersed sequences for coordinate action by a cellular machinery.

Tetrahymena DNA rearrangements, like those of other ciliates, occur during mating (conjugation). This sexual stage is induced by pairing of pre-starved, mating-complementary cells that initiates meiosis of the germ line micronuclei within the two conjugates. Only one meiotic product is selected in each partner, which then undergoes an additional nuclear division leading to the exchange of haploid gametic nuclei between paired cells. Stationary and exchanged nuclei fuse to produce the diploid zygotic nuclei of each cell. After two subsequent mitoses, two of the four zygotic nuclei within each partner begin to differentiate into new somatic macronuclei from which the IESs are excised. In the other two nuclei, the germ line genome is preserved intact. Concurrently, the parental somatic nucleus becomes pycnotic and is resorbed and does not pass on its DNA [see Ref. (4) for a detailed cytological description of conjugation events]. Thus during conjugation, the genomes within three distinct types of nuclei are directed to achieve rather different fates.

Recent studies have discovered a role for homologous RNAs and a RNA interference (RNAi)-like mechanism in the elimination of germ line (micronucleus)-limited sequences from the differentiating somatic genome [reviewed in Refs (5,6)]. These homologous RNAs are generated by

*To whom correspondence should be addressed. Tel: +1 314 935 8838; Fax: +1 314 935 4432; E-mail: dchalker@biology2.wustl.edu
Present addresses:
Christina A. Kowalczyk, Department of Cell and Molecular Biology, Northwestern University, Chicago, IL 60611, USA
Alissa M. Anderson, Program of Molecular Biosciences, University of Chicago, Chicago, IL 60637, USA
Maria Arce-Larreta, University of Utah Medical School, Salt Lake City, UT 84132, USA

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bi-directional transcription of IES sequences that begins within meiotic micronuclei as early as 2 h after cells pair (7). These transcripts presumably form double-stranded RNAs that are processed within the micronuclei into 28–30 nt small RNAs by a Dicer-like ribonuclease encoded by the DCL1 gene (8,9). These small RNAs associate with the argonaute-family homologue encoded by TWI1 (10,11) and are directed to the differentiating macronuclei where they guide the di-methylation of IES chromatin on lysine 9 of histone H3 (H3K9me2) (9,12). It is believed that this and possibly other modifications placed on the IES-associated chromatin marks these sequences for their ultimate excision. The chromodomain containing protein Pdd1p (13), which is essential for DNA rearrangement (14), binds this modified chromatin (12) and likely recruits the remainder of the excision machinery.

The mechanistic connection between RNAi and the elimination of specific IESs is still poorly understood. Earlier studies of IESs identified *cis*-acting sequences involved in their excision. For the M deletion element, flanking polypurines tracks (5′-A_5G_5-3′) located ∼45 bp outside the IES were shown to be necessary and sufficient to determine the left and right boundaries of excision (15,16). Two copies of this sequence located 0.3 kbp apart on the left side directed two alternative outcomes of M element rearrangement, excision of either 0.6 or 0.9 kbp, with the right side boundary dictated by a single polypurine track (Figure 1A). Three other IESs, the R element, which is adjacent to the M element, MSE 2.9 and the Tlr1 element all were shown to have similarly positioned flanking boundary determinants; nevertheless, no other IES was found to contain the A_5G_5 track, nor did any two elements obviously share a common sequence motif in their flanking DNA (17–19). This diversity of these *cis*-acting determinants has presented a particular challenge to understanding how they define IES boundaries.

These flanking boundary determinants are not sufficient for DNA rearrangement. Sequences within the micronucleus-limited DNA appear to play an important role in promoting removal of IESs during development. For instance, a deletion of 395 bp from the right side of the M element was observed to severely impair this IES's rearrangement (15). Furthermore, several non-overlapping fragments of the 21 kbp repetitive Tlr element, ranging in size from 642 to 1317 bp, were each shown to be sufficient to direct their own elimination, even when placed in the context of macronucleus-retained DNA (20). In this study, specific flanking sequences appeared to be dispensable. The role that internal sequences play is likely shared between IESs as chimeric elements created by substitution of the MSE2.9 micronucleus-limited DNA with the internal regions of other elements, including the M element, were observed to be accurately eliminated using the MSE 2.9 boundaries (21). Together, these studies established the importance of sequences internal to the IES, but did not fully elucidate their function in the elimination of micronucleus-limited DNAs.

An initial clue that internal sequences may act in a homology-dependent mechanism was provided by the observation that the presence of an IES in the parental macronucleus blocked the efficient elimination of the corresponding element from the developing macronucleus during conjugation (22). This epigenetic effect was largely sequence specific

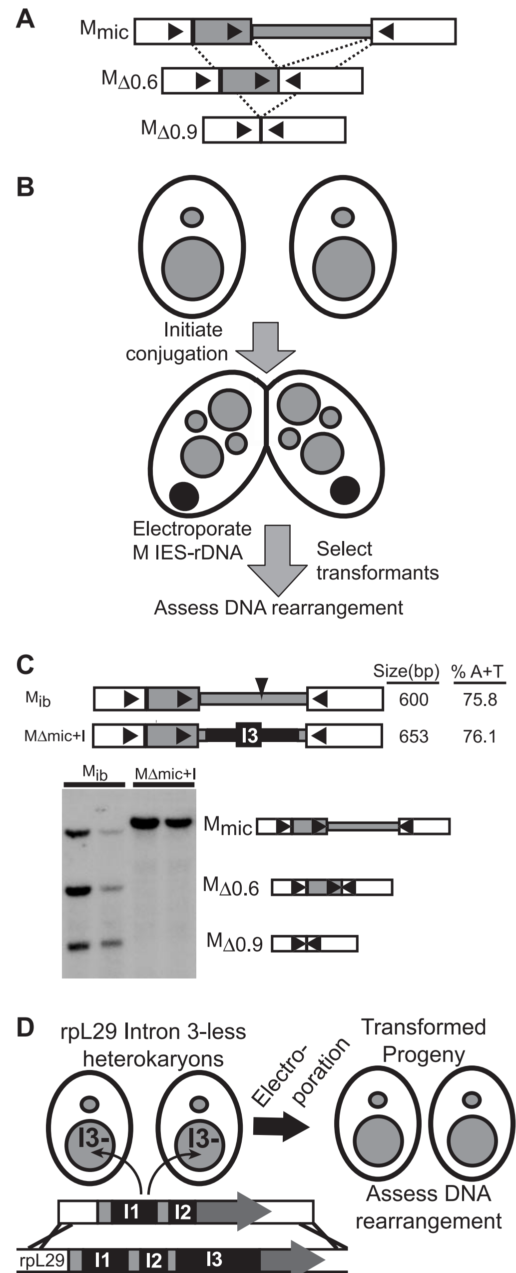

Figure 1. The micronucleus-limited region of the M element is required for DNA rearrangement. (**A**) Schematic of the M element illustrating the micronuclear locus (M_{mic}) and the two major, alternative rearranged forms ($M_{\Delta 0.6}$ and $M_{\Delta 0.9}$) found in the macronucleus. Narrow shaded bar, the 0.6 kbp micronucleus-limited region; wide shaded box, the 0.3 kbp alternatively eliminated segment; open boxes, Macronucleus-retained DNA; and the filled triangle, flanking A_5G_5 tracts that determine deletion boundaries. (**B**) Outline of rearrangement assay. *Tetrahymena* strains are crossed and transformed via conjugative electroporation with rDNA-based vectors containing mutated M element constructs. Total genomic DNA is recovered from the resulting paromomycin-resistant transformants and the rearrangement efficiency of each construct is assessed by Southern blot analysis. (**C**) Southern blot analysis assessing rearrangement of an M element construct, M_{ib}, tagged with a 21 bp unique sequence (the filled arrowhead) and a construct, MΔmic+I, for which the micronucleus-limited sequence is replaced with a macronuclear intron (the solid bar) of similar size. The size and %A+T of the micronucleus-limited region and the intron 3 (I3) fragment are indicated at the right. DNA from two transformants are shown for each. The positions of the unrearranged and rearranged forms are indicated. (**D**) Depiction of the removal of intron 3 of the rpL29 locus and subsequent electroporation with IES constructs.

as M element copies in the macronucleus primarily inhibited M element excision, while the presence of R element copies inhibited R element excision. The micronucleus-limited regions were sufficient for inhibition as high copy vectors maintained in the macronucleus containing the flanking boundary determinants alone failed to elicit this effect.

To understand how a particular IES is targeted for excision, we undertook an extensive mutational analysis of the M deletion element. As the M element has the smallest known (0.6 kbp) micronucleus-limited region and is one of the most extensively studied IES, it offered an advantageous system to dissect the minimum sequence requirements for DNA rearrangement. Sequences required in *cis* for excision can be easily identified using the well-established transformation-based rearrangement assay in which mutated IESs are introduced on an automomously replicating vector into conjugating cells after appearance of developing macronuclei, but prior to DNA rearrangement. This assay has previously facilitated the characterization of the flanking boundary determinants and indicated the additional role for internal sequences described above. Using this assay, we found that rearrangement efficiency correlated with the size of the IES-specific region present in each construct. The results described are consistent with a mechanism in which M element rearrangement is directed by its homology to developmental-specific small RNAs and not by the presence of any critically important *cis*-acting sequence. Therefore, the recognition of IESs shares mechanistic similarity to RNAi-mediated establishment of heterochromatin (23) and our data suggests a lower limit for efficient recognition of a genomic sequence by small RNAs.

MATERIALS AND METHODS

Strains

All *Tetrahymena* culture conditions were as previously described (24,25). Conventional lab strains CU427 [*chx1-1*/*chx1-1* (VI, cy-s)] and CU428 [*mpr1-1*/*mpr1-1* (VII, mp-s)] or transgenic strains derived from these were used for all transformation assays. Strains CU357 and CU367 nullisomic for micronuclear chromosome 4 were obtained from Drs P. Bruns and D. Cassidy-Hanley (Cornell University).

Plasmid construction

Tagged M element constructs were generated by two-step PCR using pDLCM1 as template (22). Appropriate primer sets were designed to separately amplify the left and right side of the M element plus its flanking DNA. The left and right outer primers were M004NK (5′-gcggccgcggtaccttaaacaaatgccatattgag) and M1410rNK (5′-gcggccgcggtacctatgtctttaagagtattga), respectively. A 21 bp unique sequence (5′-catcgcagtccggataacagt) containing a BspEI site was added to the 5′ end of the primers internal to the 0.6 kbp micronucleus region to tag the element. This sequence provided complementarity between the left and right side PCR products that allow these to anneal together in a second PCR step using only the initial outer primers M004NK and M1410rNK to regenerate a product starting at nt 4 and extending to nt 1410 of the published M element sequence (26), joined in the middle by the 21 bp tag. These flanking region primers added NotI and KpnI sites to both ends of the PCR product. The internal region primers contained 19–21 nt of M element complementarity downstream of the tag sequence. For generation of the full-length construct M_{ib}, the right side internal primer used in step one contained the tag sequence followed by M element sequence complementary to nt 816–797; the left side internal primer contained the tag followed by M element sequence nt 817–836. Removal of sequences near the left or right boundary were performed by similar two-step PCR starting with pCR2.1-M_{ib} as template and adding a 21 bp arbitrary sequence containing a SalI site. For constructs containing exclusively M element internal sequences, primers were again designed with 19–21 nt M element complementarity with NotI recognition sites added to the 5′ ends of each and used to amplify DNA from intact or previously altered templates. Sequences cloned and/or removed by these strategies are denoted within the figures.

All PCR products were initially cloned into pCR2.1 using the TOPO-TA cloning kit (Invitrogen) followed by verification of their sequence. The M element fragments were excised from pCR2.1 using NotI sites added during the PCR amplification and inserted into NotI digested rDNA vector pD5H8 (15). Tagged M element fragments were also inserted into p4T2 for integration into the M element locus. This vector contains the neomycin-resistance gene expressed from the *HHF1* promoter (27) and confers paromomycin resistance to transformants. First, a 714 bp fragment of genomic sequence located downstream from the M element (nt 1391–2105) was amplified from genomic DNA using oligonucleotide primers (5′-tggatccaatactcttaaagacatat) and (5′-tagcggccgcatatctagaaaattgtaaagt) and inserted into the BamHI and NotI sites of p4T2 downstream of the neo gene to create p4T2-3′M. M element fragments in pCR2.1 were excised by Acc65I and PacI digestion and ligated to Acc65I and EcoRV digested p4T2-3′M. Prior to biolistic transformation, these plasmids were cleaved at Acc65I and NotI sites at the upstream and downstream ends of the M element identity.

Intron 3 sequence of rpL29 from nt 1191 to 1843 (652 bp) (28) was amplified adding SalI sites to the fragment ends and cloned into the SalI sites added to construct MΔ84R79L to generate MΔmic+I. Intron 3 sequence from nt 1191 and 1468 was amplified with the addition of BspEI sites and cloned into the BspEI site of MΔ237L or MΔ306R. To remove intron 3 from the rpL29 gene, a plasmid containing a cycloheximide resistant rpL29 allele (28) was modified to replace the intron by PCR mutagenesis with oligos (5′-tctcagctggatgccacttttccatcaag) and (5′-tccagcgtactactacggaaagttgggtatg), generating a PvuII and AfeI sites at the intron/exon boundaries. After digestion with the corresponding enzymes, the resulting blunt ended fragments were ligated to remove the intron without altering the coding sequence.

Tetrahymena transformation

To introduce rDNA-based replicating vectors, $\sim 5 \times 10^6$ conjugating cells were electroporated with 5–20 μg of plasmid DNA between 8.5 and 9.5 h after mixing pre-starved populations using a BTX model ECM630 electroporator set to 250 V, 125 μF, 25 ohms as described previously (17,27). Cells were allowed to recover 16–24 h in 1 × SPP growth

medium distributed in 96 well microtiter plates followed by selection in 100 μg/ml paromomycin.

For removal of the rpL29 intron 3 sequence, plasmid pCHXΔI was digested with HindIII, coated onto 0.6 μm gold particles and introduced into macronuclei of CU427 and CU428 by particle bombardment (29,30). After 16 h recovery in growth medium, transformants were selected by addition of 12.5 μg/ml cycloheximide and distributed to 96 well plates. Transformants were serially subcloned into medium containing 25 μg/ml cycloheximide until complete replacement of the endogenous gene with the allele lacking intron 3 was achieved. This was confirmed by PCR analysis with a primer pair within exon 3 and 4 and by subsequent Southern blot analysis. Tagged M element constructs were similarly integrated into the macronuclear M locus and selected by growth in 1× SPP medium containing 80 μg/ml paromomycin. After verification of integration, transformants were serially subcloned into increasingly higher concentrations of paromomycin to drive cells to assort towards complete replacement of the endogenous locus with the tagged allele.

DNA analyses

Southern blotting and other analyses were performed by standard methodologies (31). Briefly, DNA was recovered from $\sim 1 \times 10^6$ *Tetrahymena* cells using a Wizard genomic purification kit (Promega) according to manufacturer's instructions. DNA (∼10 μg) was digested with appropriate restriction enzymes, electrophoresed on 1% agarose/ 1× Tris–Borate EDTA gels and transferred to Nylon membranes by downward capillary action. For analysis of DNA rearrangement, NotI digested DNA on membranes was hybridized to a radiolabled M3 probe (22) that hybridizes to macronucleus-retained DNA flanking the IES. Hybridization was quantified by phorphorimager analysis using a Personal FX imager and Quantity One software (Bio-Rad).

RNA analyses

RNA was isolated from *Tetrahymena* cells by RNAsol extraction (32), precipitated with isopropanol and resuspended in RNAse-free H_2O. Detection of M element small RNAs was performed as described (33). Strand-specific riboprobes were generated by run-off transcription of StuI digested pMint2 or pMint7 using T7 RNA polymerase with radiolabeled UTP (7). To detect transcripts originating directly from tagged M element constructs, RNA was isolated from mating cells at 3 and 5 h of conjugation and 25 μg from each time point was pooled and converted to cDNA using Superscript II reverse transcriptase and random hexamer or strand-specific primers. PCR were performed with 1 μg RNA equivalent and M element left side primer M740 (nt 740–759) together with a 21 nt tag primer insbpAr or right side primer M939r (nt 939–920) together with a 21 nt tag primer insbpA (30 cycles—94°C denaturation for 30 s, 58°C annealing for 30 s, 72°C extension for 1 min). PCR was also performed with control *ATU1* primers (28 cycles—94°C denaturation for 30 s, 55°C annealing for 30 s, 72°C extension for 1 min) (7). Identical rtPCR reactions were performed with reverse transcriptase omitted during cDNA conversion to control for the presence of contaminating DNA (data not shown). PCR products were electrophoresed on 1.8% agarose gels, transferred to membranes and hybridized with an radiolabeled M element-specific (M1) probe (7).

Epigenetic inhibition

Tetrahymena transformant lines carrying vectors with M element sequences were grown to maturity and crossed to generate progeny lines for DNA analysis. Progeny were identified by reversion to paromomycin sensitivity due to loss of the rDNA-based vector. The status of the endogenous M element locus was examined by PCR of *Tetrahymena* lysates using three primers that gives a semi-quantitative assessment of the extent of rearrangement (33). Failure of rearrangement was verified by Southern blot analysis (22).

Data presentation

Autoradiography was either captured on X-ray film or digitally during phosphorimager analysis. TIFF files created by Quantity One software or autoradiography films captured on a flatbed scanner (Epson) were cropped and scaled using Adobe Photoshop CS. All alterations to brightness or contrast of exposures were linearly applied to the entire data set. Where necessary, lanes were cut and the remaining data aligned in Photoshop files. All graphics were created and combined with digital data using Adobe Illustrator 10.

RESULTS

Micronucleus-limited sequences are required for DNA rearrangement

A RNAi-based mechanism has recently been found to be essential for DNA rearrangement, but its connection to identified or putative *cis*-acting sequences required for the elimination of specific IESs is poorly defined. Previous analysis of the M IES showed that flanking polypurine tracks were sufficient to specify the excision boundaries during macronuclear differentiation, but did not broadly investigate whether internal sequences, located within the micronucleus-limited DNA, participate in this DNA elimination event (15). Additional studies appeared to implicate sequences within this IES acting to promote its rearrangement [reviewed in Ref. (34)]. To determine whether sequences internal to the deletion boundaries (i.e. within the eliminated region) are required for M element rearrangement, we replaced most of the micronucleus-limited DNA with a macronuclear DNA segment of similar size and A+T content, intron 3 of the ribosomal protein L29 gene and examined its rearrangement activity (Figure 1). After introduction of this altered IES (MΔmic+I), carried on an rDNA-based vector, into conjugating *Tetrahymena*, transformants were selected, DNA recovered and rearrangement assessed by Southern blot analysis. In this transformation-mediated assay, a marked, full-length M element (M_{ib}) undergoes extensive rearrangement producing a nearly equal distribution of the two alternatively eliminated forms—MΔ0.6 kbp or MΔ0.9 kbp (Figure 1C); although rearrangement of all introduced M element copies is rarely observed. In contrast, the chimeric element, MΔmic+I, showed no detectable activity. Thus internal

sequences, acting with the flanking polypurine tracks, contribute to M element rearrangement.

One potential complication to interpreting this result is that the presence of DNA in the macronucleus that is homologous to an IES can interfere with the efficient elimination of that IES from developing macronuclei (22). We therefore could not rule out the possibility that this construct's failure to rearrange had resulted from the inhibitory action of the parental macronucleus, rather than from the lack of essential stimulatory sequences. To eliminate any such epigenetic inhibition, we used homologous recombination to replace the endogenous rpL29 locus with a copy in which we precisely eliminated intron 3 (Figure 1D). This altered rpL29 gene also contains a missense mutation that confers cycloheximide resistance to cells expressing this dominant allele (28). After transformation of two mating compatible *Tetrahymena* strains to cycloheximide resistance, cells were subcloned until lines were obtained for which all macronuclear rpL29 genes had been replaced by the intron-less allele (data not shown). These strains were then crossed and transformed with MΔmic+I, but still no rearrangement activity was detected (Figure 5A and data not shown). In addition, we replaced most of the 0.6 kbp region with a 0.58 kbp foreign DNA fragment, from the coding sequence of the *Aequorea* green fluorescent protein and observed no rearrangement of the chimeric element (data not shown). Thus, as assumed in initial experiments, constructs lacking the 0.6 kbp M element internal region are unable to undergo DNA rearrangement.

To identify the internal sequences required in *cis*, we removed increasing larger segments of the 0.6 kbp micronucleus-limited region. Starting at a central point (nt 817), we removed 66, 145, 225 or 306 bp of the rightmost region and examined the rearrangement of these modified IESs (Figure 2A). Because the absolute percentage of rearrangement for any construct can vary between individual transformants and between experiments, we normalized the activity of four or more transformants for each to that of the full-length M_{ib} element, with its activity set to 100%. All four right side deletion constructs showed detectable rearrangement in our transformation assay. Nevertheless, the three larger deletions all had activity levels significantly lower than the full-length M element (M_{ib}) (Figure 2B). Furthermore, activity decreased sequentially as more internal sequence was removed. In the complementary test, removing increasingly larger segments of the leftmost region resulted in a similar decrease in activity as was observed with the right side deletions (Figure 2). Construct MΔ143L that removed sequences on both sides of nt 817 was created to examine the possibility that an important sequence may span the centrally located tagged site. It however showed comparable activity to other similarly sized deletion constructs indicating that this specific region is not critical for rearrangement. In fact, as even the largest deletions that removed nearly the entire right side (MΔ306R) or left side (MΔ237L) exhibited some rearrangement, no single *cis*-acting sequence appears to be essential for M element excision.

One possible explanation for these results is that the internal region contains multiple *cis*-acting sequences that promote rearrangement additively. To investigate this possibility, we removed 79 and 84 nt segments that are located near the left or right boundary, respectively, of the 0.6 kbp

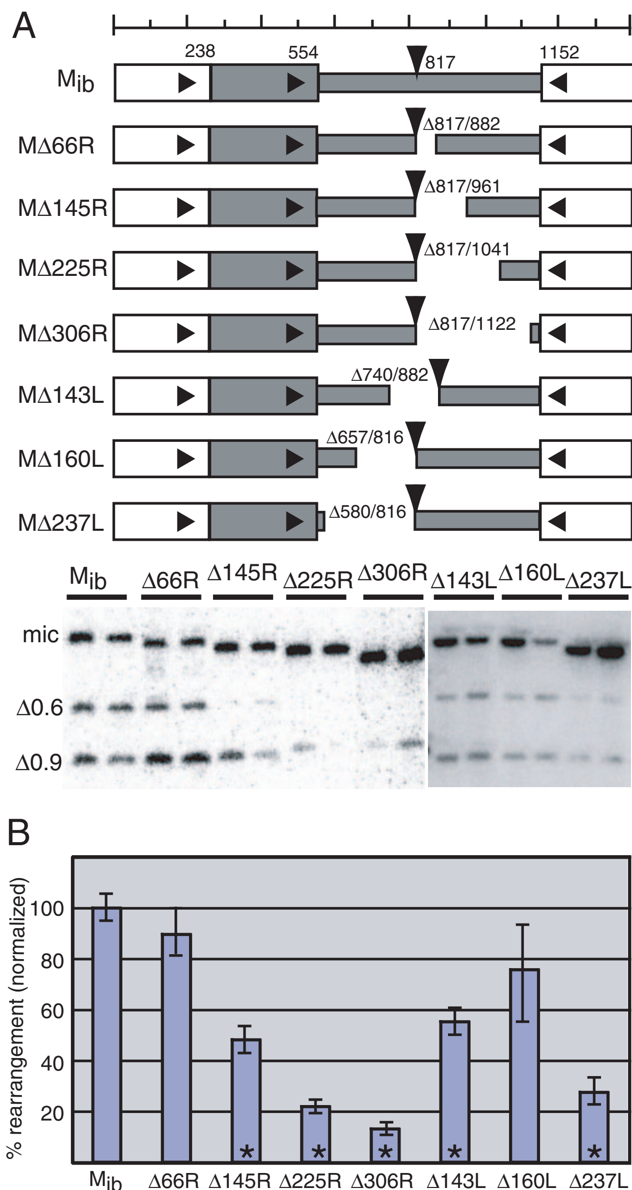

Figure 2. Decreasing the extent of micronucleus-limited sequences reduces rearrangement. (**A**) Diagrams of eight tested M region constructs indicate segments removed and the 21 bp sequence tag (filled arrowhead). The numbers given above the full-length element (M_{ib}) indicate the location of the deletion boundaries and sequence tag, based on the published sequence (26). The scale bar at the top has 100 bp increments. The name of each indicates the number of bp removed (coordinates are given above each gap) to the right (R) or left (L) of the tag. Southern blot analysis of representative transformants is show below the diagrams. The band corresponding to the unrearranged (mic) and the major rearranged forms (Δ0.6 and Δ0.9) are indicated to the right. (**B**) Quantification of the percent rearrangement for each construct normalized to M_{ib} activity set to 100%. The graph shows the mean activity based on four to twelve transformants of each taken from at least two independent electroporation experiments. Error bars indicate the standard error (SE). Asterisks denote constructs that exhibit activity significantly different from M_{ib} in a one-way ANOVA analysis (Bonferroni test).

micronucleus-limited DNA and assayed rearrangement of these constructs. The nucleotides deleted correspond to the DNA segments present in the constructs MΔ160L and MΔ225R, but missing from the largest left and right side

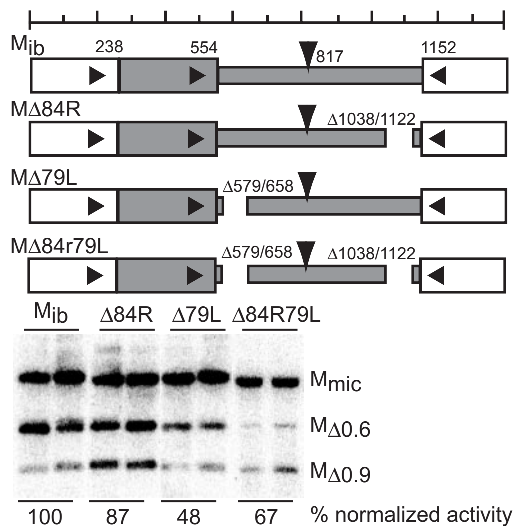

Figure 3. Rearrangement is promoted by dispersed sequences. Diagrams of tested constructs are shown at the top and coordinates of internal deletions are given above each gap. Southern blot analysis of two transformants of each construct is shown below the diagrams. The average percentage of rearrangement (of ⩾4 transformants) is given for each construct.

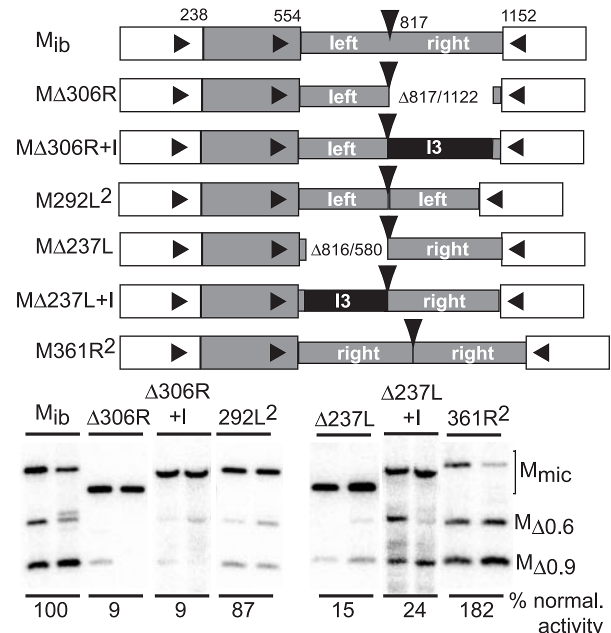

Figure 4. Duplication of either half restores full activity. Diagrams of tested constructs are shown above Southern blot analysis of two transformants for each. The left and right sections of micronucleus-limited region are labeled to indicate duplicated segments. The solid bar represents a ~300 bp rpl29 intron 3 sequence (I) used to restore the size of the altered element to near its wild-type length. The hybridizing fragments corresponding to the unrearranged and rearranged forms are indicated. The average percentage of rearrangement (of ⩾4 transformants) is given for each construct.

deletions, MΔ237L and MΔ306R, for which their removal resulted in decreased rearrangement in the context of these larger alterations. If either region contains a critically important *cis*-acting sequence, its absence should produce an observable drop in rearrangement. However, removal of either of these segments alone had, at most, a modest effect on DNA rearrangement when their activity was compared to the full-length element. Removing the left side segment (construct MΔ79L) appeared to reduce activity to a greater extent (Figure 3). This appears to be consistent with the analysis above for which removal of the same left side region, but in context of a larger deletion, has a more substantial decrease in activity than the right side deletion (compare the difference between MΔ160L and MΔ237L to that between MΔ225R and MΔ306R—Figure 2C). Therefore, some regions may contribute to rearrangement activity somewhat more than others. Removing both these left and right DNA segments (construct MΔ84R79L) did not further decrease rearrangement efficiency significantly (Figure 3). From these data, we conclude that removing sequences proximal to the boundaries does not alter rearrangement activity to a greater degree than removing more internal sequences. More importantly, none of these observations point to the obvious presence of a small number of *cis*-acting sequences that are particularly necessary for M element rearrangement.

The largest deletions of internal sequence exhibited the greatest decrease in rearrangement efficiency. This could result from the shorter distance between the flanking polypurine tracks, rather than removal of important internal sequence that promote M element elimination. To test this, we inserted a 277 bp segment of rpL29 intron 3 (+I) sequence into the largest right side (MΔ306R) and left side (MΔ237L) deletion constructs to restore the distance between the wild-type boundaries. Restoring the length of internal sequence with non-IES sequence was unable to return these constructs with decreased activity back to the levels of the full-length element (Figure 4). This is particularly notable with construct MΔ306R+I, whose parent construct, MΔ306R, exhibited consistently low rearrangement. Thus, it is the absence of M element specific sequences and not simply the decrease in size that resulted in decreased activity.

Internal DNA segments from both the right and left side contribute to DNA rearrangement. These two internal regions may contain distinct functional sequences that together are required to promote wild-type rearrangement activity or may be functionally equivalent. If the latter is the case, duplicating the sequence from either side, in the absence of the other, should restore activity of the mutant construct to wild-type levels. We made tandem duplications of the left or right side segments remaining in constructs MΔ306R and MΔ237L and examined their rearrangement. The M element construct containing a tandem duplication of 292 bp from the left internal region ($M292l^2$) exhibited activity near that of the intact element, M_{ib}. This is in marked contrast to its parent construct, MΔ306R, that contained a single copy of this region and showed 8- to 10-fold less rearrangement (Figure 4). Likewise, duplication of 361 bp from the right side internal region created a construct, $M361R^2$, that rearranged even more efficiently than the full-length element, whereas the construct MΔ237L, containing one copy of this region, exhibited severely impaired activity (Figures 2 and 4). These results further show that no individual sequence contained within the micronucleus-limited region is essential for full activity and that the two sides are functionally similar in their ability to stimulate rearrangement.

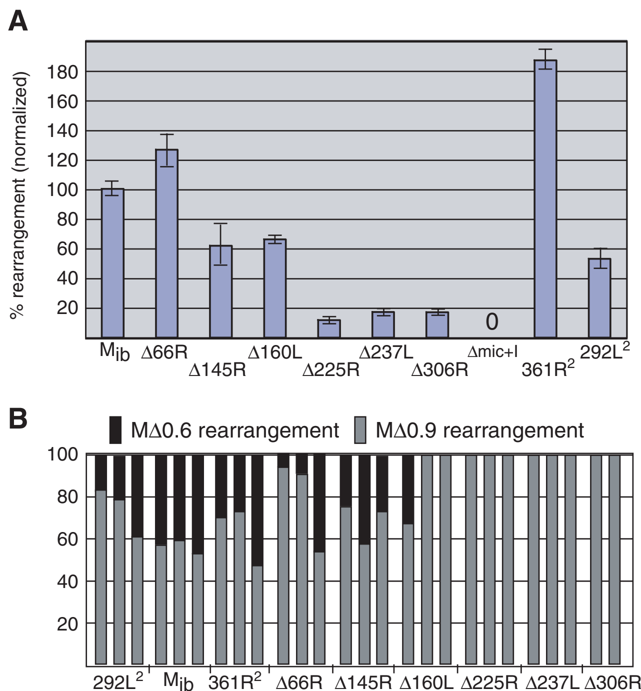

Figure 5. Rearrangement activity correlates with size. (**A**) Plot of the normalized rearrangement activity, relative to M_{ib}, arranged by increasing micronuclear sequence removed from construct. SE of the mean is shown. (**B**) Plot showing the relative distribution of the 0.6 (black bars) and 0.9 kb (gray bars) rearranged forms for each construct. The distribution for three independent transformants is shown for all except for MΔ306R as only two had exhibited detectable activity.

The extent of M element sequence correlates with rearrangement activity

We repeated our transformation assay for the M element internal deletions (Figure 2) and duplications (Figure 4) using the intron 3-less strains and compared the overall activity. As observed in initial trials, activity progressively decreased as more internal sequence was removed from each construct (Figure 5A). Interestingly, internal deletions of similar size, whether removed from the right or left side showed comparable activities—compare MΔ145R to MΔ160L and MΔ225R to MΔ237L. Internal sequence deletions of >306 bp decreased activity to undetectable levels (e.g. removal of 382 bp between nt 740 and 1122, data not shown). This suggests that a threshold of M element internal sequence is necessary to promote deletion. Above this threshold of ∼300 bp rearrangement activity increases as the length of specific sequence increases. This conclusion is further supported by construct $M361r^2$ that has 77 bp more internal sequence than intact construct M_{ib} and reproducibly shows greater rearrangement efficiency.

Polypurine tracks flanking the M element have been shown to direct this IES's excision boundaries (15,16). To assess whether mutation of internal sequences affects the choice of boundaries, we examined the frequency of the two alternative elimination events (MΔ0.6 or MΔ0.9 kbp) for each construct. In addition to the overall loss of rearrangement efficiency with decreasing amount of internal sequence, the selection of alternative boundaries was altered. Constructs lacking larger blocks of the micronucleus-limited region had their rearrangement skewed toward the larger 0.9 kbp elimination event (Figure 5B). Constructs missing >225 bp of internal sequence rarely performed DNA elimination between the proximal boundaries. Decreasing the distance between the flanking polypurine tracks either limits the ability of those that direct the smaller alternative to act together or favors the use of the more distal flanking regulatory sequence. This appears to be a physical size limitation as addition of a ∼300 bp intron 3 segment in place of the deleted sequence in constructs MΔ237L+I and MΔ306R+I restored the ability to perform the smaller 0.6 kb DNA rearrangement without significantly increasing total rearrangement (Figure 4).

A functional equivalence of internal sequences throughout the micronucleus-limited DNA is supported by the observations that similar sized deletions from the right or left displayed comparable levels of rearrangement (Figures 2 and 5A) and that duplication of either the right or left side sequences increased the activity of impaired constructs (Figures 4 and 5A). We find these data consistent with the hypothesis that the M element is targeted for elimination by a homology-dependent mechanism rather than by the action of specific *cis*-acting sequences. Small RNAs complementary to both strands of the M element are generated during development (33) and are required for DNA elimination (8,9). This mechanism would dictate that increasing the target size for these small RNAs should increase rearrangement efficiency as we observed above. The similar activity of left and right side sequences could be explained by the presence of small RNAs in developing cells that have complementarity throughout the micronucleus-limited region. To examine the distribution of small RNAs homologous to the M element, we fractionated total mating cell RNA on polyacrylamide gels, transferred these to membranes and hybridized to strand-specific probes that specifically detect minus-strand RNAs homologous to the left side or plus-strand RNAs from the right side of the M element. We detected 28–30 nt RNAs homologous with both probes. The accumulation of small RNAs that correspond to each side is consistent with the ability of multiple internal regions to similarly promote rearrangement (Figure 6).

We also isolated RNA from conjugating nulli 4 strains, which lack micronuclear chromosome 4 on which the M element locus resides. Homologous small RNAs were still present in cells lacking the M element locus. This observation shows that small RNAs that are believed to target rearrangement are likely produced from several loci and is consistent with Southern blot analysis that indicates that the M element micronucleus-limited DNA is a repetitive sequence (data not shown).

Removal of micronucleus-limited sequences does not impair IES transcription

The M element-specific small RNAs are the processed products of bi-directional transcripts synthesized during development (8,9). While the nature of this non-genic transcription is not fully understood, it clearly is important for efficient rearrangement (7). Other than serving to generate the precursors of the small RNAs, it is not known whether transcription of IESs participates in other steps in the DNA

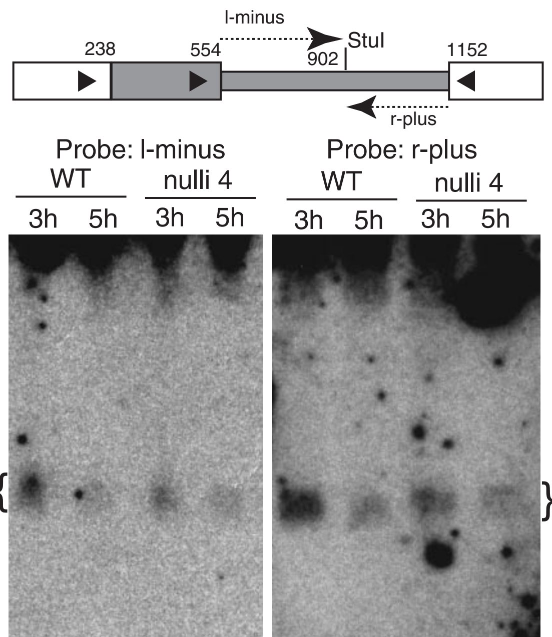

Figure 6. Small RNAs are homologous to multiple regions of M element. RNA isolated at either 3 or 5 h of conjugation from wild-type or nulli 4 cells was fractionated on polyacrylamide gels, blotted and hybridized to riboprobes (dashed arrows on diagram) detecting either the left side minus-strand RNAs (l-minus) or right side plus-strand (r-plus). 28–30 nt RNAs are denoted by brackets.

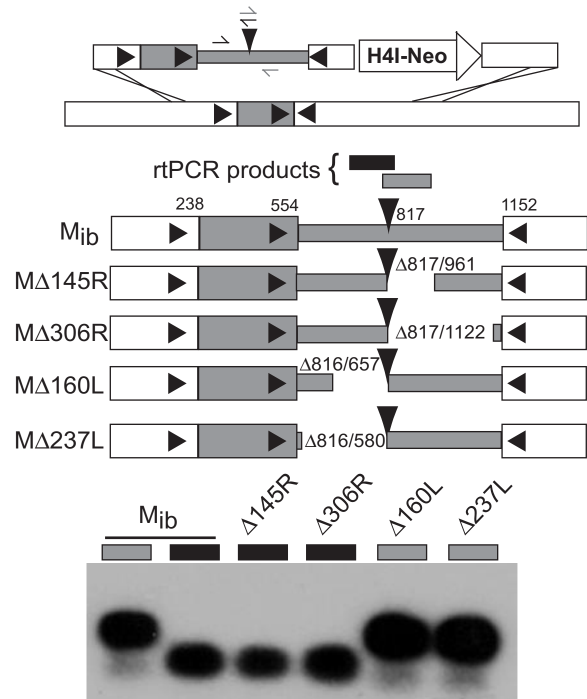

Figure 7. Internal deletions do not diminish transcriptional activity. Five tagged M element constructs were introduced into the macronuclear M locus and recombinants selected using the linked neomycin-resistance cassette (top). RNA was isolated from either 3 or 5 h mating cells and used for rtPCR (bottom) using primer sets (small lines with barbs), producing either right side (gray bar) or left side (black bar) specific products.

rearrangement pathway. It is possible that transcription of IESs in the developing macronucleus may be important for their recognition by the homologous small RNAs. We therefore wanted to determine whether mutations of the M element that impair rearrangement also impair the element's non-genic transcription.

Due to the transient character of the transformation assay, we could not directly examine the transcriptional activity of the rDNA-based constructs. Therefore, we selected constructs with progressively larger internal deletions and integrated these into the genome (Figure 7). Each was integrated into the endogenous M element locus via biolistics-mediated, homologous recombination using a linked neomycin-resistance (neo) cassette to allow for selection of transformants in paromomycin containing medium and subsequent assortment until all endogenous copies were replaced. The integrated constructs contained a 21 bp tag after M element nt 816 that served as a primer site for rtPCR analysis permitting specific detection of transcripts originating from the integrated constructs. We initially attempted to introduce these into the micronuclear genome, but were unsuccessful in multiple attempts. Since we had previously shown that copies of the typically micronucleus-limited M or R element exhibit conjugation specific bi-directional transcription even when present in the macronucleus (7), we introduce these constructs into the macronuclei of strains CU427 and CU428. Monitoring transcription of these integrated constructs should serve as a proxy for their activity in both nuclei during conjugation.

We integrated the tagged, full-length M element (M_{ib}) as well as the right side deletion constructs MΔ145R and MΔ306R and left-side deletions MΔ160L and MΔ237L and monitored their transcriptional activity at 3–5 h of conjugation. We could detect no significant impairment of transcription for any mutated element. The two right side deletion constructs and the two left side deletions produced equivalent transcript levels to each other and to the full-length M element (Figure 7). To ensure that transcription still occurred bi-directionally, we monitored transcription of M_{ib} as well as the two largest deletions, MΔ306R and MΔ237L, using strand-specific oligonucleotides to prime reverse transcription. We detected transcription of both strands for each integrated construct (data not shown). Therefore, the absence of micronucleus-limited DNA is unlikely to result in decreased rearrangement by negatively affecting transcription.

Inhibition of DNA rearrangement by M element sequences in the macronucleus

The most straightforward interpretation of the above data is that the M element is targeted for elimination by small RNAs that have complementarity throughout the micronucleus-limited sequence. In such a model, it would be expected that as the length of sequence complementarity is decreased by removal of internal DNA segments, the rearrangement efficiency should likewise decrease largely independent of the exact region deleted, just as we observed. If complementarity of the internal sequences to the small RNAs is the critical parameter, we should be able to block rearrangement by interfering with the action of M element specific RNAs. Previously we found that the presence of

the M element in the parental macronucleus blocks the efficient elimination of the endogenous M element from the developing macronucleus during the subsequent conjugation (22). The homologous sequence in the macronucleus is hypothesized to inhibit rearrangement by sequestering the complementary small RNAs (10,33). If this is the case, we reasoned that we could further test the importance of internal region/small RNA complementarity by ascertaining the minimal segment of M element sequence required to be in the macronucleus to block rearrangement. In our transformation assay, the M element constructs that contained <300 bp of the 598 bp internal region exhibited little or no rearrangement (Figures 2 and 5B, data not shown). Conversely, we predicted that we would need to minimally sequester small RNAs to at least a 300 bp region to effectively block rearrangement.

We took advantage of the cell lines obtained from our transformation assays that had the lowest level of rearrangement of each introduced construct as these retained relatively high copy numbers of micronucleus-limited M element segments within their macronuclei. After subcloning and outgrowing these cell lines to maturity (i.e. mating competence) and confirming the retention of unrearranged copies (data not shown), we crossed two mating compatible transformants. Successful conjugation produced progeny that were identified by their reversion to paromomycin sensitivity due to loss of the transforming rDNA upon nuclear differentiation.

We examined the ability of macronuclear copies of the unrearranged constructs MΔ145R, MΔ306R, MΔ160L and MΔ237L, which retained 453, 292, 438 and 361 bp, respectively, of normally 0.6 kbp micronucleus-limited M element DNA, to block rearrangement of the endogenous M element locus as assessed by PCR and confirmed by Southern blot analysis (Figure 8). Of these, all but MΔ237L showed some ability to inhibit DNA rearrangement. The two constructs, MΔ145R and MΔ160L, that retained the greatest amount of M element sequence showed the greatest degree of inhibition, blocking M element rearrangement in 31 and 21% of their progeny, respectively (Figure 8A). Transformants containing the entire 0.6 kbp region blocked rearrangement more effectively than these deletion constructs [(22) and data not shown]. The inhibition rarely affected all copies of the M element. Rearrangement occurs when the developing macronucleus has between four to eight copies of each chromosome. Typically half or less of these copies failed to be eliminated in any of the progeny (Figure 8B). The observation that larger segments of the M element more effectively blocked rearrangement is again consistent with the role of homologous small RNAs in targeting this element's elimination.

To further determine the minimal region necessary to block rearrangement, we cloned 300–402 bp fragments of M element sequence into the rDNA-based vector, pD5H8 and introduced these into *Tetrahymena*. Mature transformants were crossed and M element rearrangement was assessed in their progeny. M element fragments of 402 bp (M402), 399 bp (M465Δ66r) and 320 bp (M465Δ145R) placed in the macronucleus inhibited rearrangement of the endogenous locus in a small fraction, 4.5–8.3%, of the progeny. Fragments of 350 and 300 bp did not block rearrangement, nor did a tandem duplication of this 300 bp fragment. This fits the prediction that inhibition should only increase when the

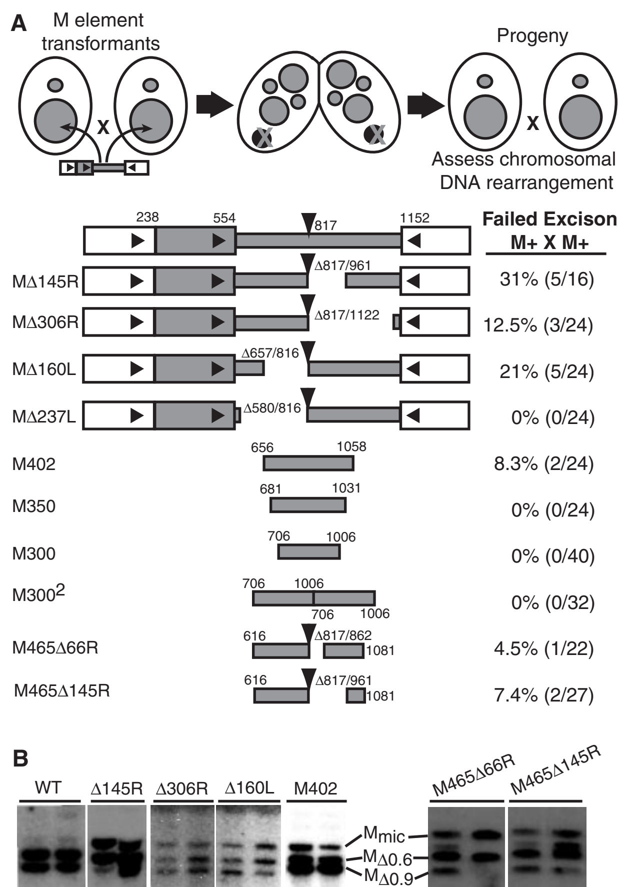

Figure 8. Large fragments of micronuclear sequence are needed in the macronucleus to inhibit DNA rearrangement. (**A**) Diagram at top shows scheme to assess affect of macronucleus-located M element constructs. Below is shown diagram of the M element subclones introduced on rDNA vectors. To the right the percentage of progeny showing detectable failure of chromosomal M locus rearrangement is indicated with observed/total given in parentheses. End coordinates or deleted regions are indicated. (**B**) Southern blot analysis of DNA from representative progeny lines that showed failure of DNA rearrangement. The rearranged and unrearranged products are marked as in figures above. One sample showing failed rearrangement for construct M465Δ66 was derived from a cross of one transformed line to CU428 and thus in not reported in the column in (A). In general, crossing transformant lines to wild-type strains produced progeny with lower or undetectable failure rates (data not shown).

extent of M element DNA in the macronucleus increases, not simply due to an increase in the copy number of a limited region.

Interestingly, segments that contained more left side sequences appeared to be more effective at blocking rearrangement. For instance, MΔ306R and M465Δ145R that contained only 292 and 320 bp of internal sequence showed some inhibition whereas MΔ237L that retained 361 bp did not. One explanation for this observation may be that sequences homologous to the right side can be found in the macronuclear genome. A BLAST search of the *Tetrahymena* Genome Database (www.ciliate.org) with the M element sequence reveals that a ~184 bp block from the right side (nt 877–1061) is >90% identical to several contigs found in the database (C. D. Malone and D. L. Chalker, unpublished data). Additional copies introduced on our rDNA vectors may thus be no more effective at sequestering small RNAs

to this region than these genomic sequences. This finding further supports the repetitive nature of the M element and may suggest that it was originally derived from a mobile genetic element.

DISCUSSION

The germ line, micronucleus-limited sequences that are removed during macronuclear differentiation vary dramatically in size and sequence and it has been an enigma how thousands of diverse sequences might be recognized by the a common rearrangement machinery. Recent data has revealed that homologous RNAs and a RNAi-like mechanism is essential for DNA elimination [see Refs (5,6)]. The findings above further resolve this enigma by providing strong evidence that the M deletion element is targeted for elimination by its recognition by homologous RNAs. By mutagenizing the M element and examining the ability of the altered constructs to undergo DNA rearrangement, we were unable to identify any critically important *cis*-acting sequence. Instead we found that several non-overlapping regions could promote M element elimination. In general, increasing the length of M element specific DNA increased rearrangement efficiency, which is consistent with a homology-dependent recognition mechanism. In such a system, the larger the target sequence the more effectively it will be recognized by the available pool of homologous small RNAs. In support of this idea, we show that M element small RNAs are complementary to both the right and left regions and thus appear to be distributed throughout most of the eliminated region (Figure 6, data not shown).

While previous studies of the M element revealed that flanking polypurine tracks are sufficient to determine the boundaries of the deletion event (15,16), here we show that sequences within the eliminated region are essential to target the element for excision. We suggest that the action of the flanking and the internal sequences are separable. The internal sequences must first be recognized by homologous RNAs, which leads to the marking of the associated chromatin by histone H3 K9 di-methylation (12). The modified chromatin can then associate with DNA rearrangement proteins, including the chromodomain containing Pdd1p (13), that recruit any additional excision machinery. The flanking boundary determinants either act concurrently with or after the recognition of an IESs to limit the extent of DNA eliminated by an, as yet, unknown mechanism. We favor models that suggest that the boundary determinants flanking the IES act by perturbing chromatin structure during the chromatin modification process, thereby limiting the spread of this mark and/or ensuring that the boundaries are accessible to the rearrangement machinery. These flanking sequences may function by binding specific DNA rearrangement proteins, but one must consider the observation that none of the 10 or more IESs sequenced contain obviously conserved regulatory sequences in their flanking DNA. Thus such a mechanism would seem to require a very large number of unique factors that serve to define the boundaries of the thousands of IESs.

In the transformation-based rearrangement assay, the M element rearranges with rather low efficiency. Rarely does even the full-length element rearrange completely. This is in marked contrast to the endogenous locus that shows extremely efficient rearrangement or even other IESs using this assay (20,21). One explanation for this observation may be that the chromatin conformation or nuclear organization of the endogenous locus provides a more favorable environment for recognition by the RNA-guided excision machinery. Alternatively, the fact that the transformed constructs are introduced into conjugation cells a few hours after appearance of the developing macronuclei allows less time for the vector-borne element to be recognized by homologous small RNAs than the endogenous locus. Thus the observation that mutated constructs that have smaller homologous regions rearrange even less efficiently is consistent with the view that the time necessary for even the wild-type element to be targeted, given the available concentration of small RNAs in each cell, is limiting. Upon transformation, it is likely that each construct must first be packaged into chromatin that can then be subsequently modified by a putative small RNA-directed histone H3 K9 methyltransferase. Introduced IESs may be poor targets relative to the genome.

Although we found no evidence for specific, *cis*-acting sequences within the IES, transformation of altered constructs indicated that this IES has a lower size threshold ~300 bp. Constructs with smaller blocks of internal sequence failed to rearrange. The fact that numerous loci in the macronucleus retain a ~180 bp block of M element related sequence with >90% identity further supports that an IES size threshold exists (C. D. Malone and D. L. Chalker, unpublished data). This threshold may simply indicate the kinetic limit of the small RNA homology search. The small RNA pool has a finite time to recognize the estimated 15 Mbp of DNA that must be eliminated from the developing macronucleus and a sequence that falls <300 bp in length may be an inadequate target. The known involvement of histone methylation in DNA rearrangement allows for an alternative explanation for a 300 bp size limit. It is possible that this limit is indicative of the need for two adjacent modified nucleosomes to guide efficient elimination. As a core nucleosome covers 146 bp, 300 bp is the minimal size for a di-nucleosome. IES constructs with <300 bp between boundaries can only be packaged into a single nucleosome. The current study does not allow us to distinguish between these two possibilities; however, it is interesting to note that the micronucleus-limited TEC elements of *Euplotes crassus* adopt a unique chromatin configuration that has ~300 bp repeating units upon nuclease digestion (35).

The 300 bp size limitation cannot be extrapolated as a requirement for all IES excision in other ciliates, which can contain thousands of IESs in their genomes with sizes on the order of tens of bps not hundreds [reviewed in Refs (1,36–38)]. It is unclear whether homologous RNAs play a role in the elimination of these smaller IESs. Studies on IES excision in *Paramecium* indicate that two classes of IES can exist in the same species, those that are controlled by homology-dependent effects and those that are not (39,40). Larger, transposon-like sequences that are imprecisely excised are subject to homology-dependent regulation by the maternal macronucleus whereas many smaller, precisely excised 'TA' IES are immune to such effects. The imprecisely excised sequences bear the strongest resemblance to *Tetrahymena* IESs and could have similar size

requirements; however, TA IES of *Paramecium* <300 bp are subject to homology-dependent regulation (39). A class of developmentally expressed small RNAs has been identified in the stichotrichous ciliate *Stylonychia*, which does suggest to us that recognition of IESs using homologous RNAs may be wide-spread (41).

Cis-requirements for efficient M element rearrangement have striking parallels to requirements observed for meiotic silencing in *Neurospora*. In this fungus, the presence of unpaired DNA during meiosis silences the unpaired gene and any homologous copies. Several studies have indicated that this silencing is mediated by RNA (42–44). Efficient silencing occurs only when the transcribed region is unpaired and requires ∼700 bp of unpaired sequence as a trigger (45). Larger unpaired regions consistently are more effective at silencing. Efficient silencing of gene copies in *trans* (i.e. silencing of homologous gene copies unlinked to the unpaired locus) requires even larger unpaired regions than that required to silence the unpaired (i.e. *cis*) copy (45). While the exact length requirements for M element excision and meiotic silencing differ somewhat, both exhibit a lower size threshold with efficiency increasing as function of size. It is not surprising that homology-dependent regulatory mechanisms are sensitive to the length of complementarity available for recognition.

We also assessed the minimal region of the M element that when present in the macronucleus is sufficient to block rearrangement at the endogenous locus. If this epigenetic inhibitory effect is mediated by the sequestration of the homologous RNAs, we predicted that larger regions present in the parental macronucleus should more effectively block rearrangement. This was indeed the case. Furthermore, the segments needed in the macronucleus to elicit a detectable inhibition were predominantly larger than half of the M element and, as such, were sufficiently large to limit the availability of small RNAs to what our transformation assay shows is a critically small target. These results provide additional support to show that the M element has a lower size limit and is recognized as an IES via its homology to the germ line derived small RNAs.

The mutated IES constructs that rearranged poorly still had developmentally-induced transcriptional activity that was comparable to the full-length element. This transcription occurs even though the tagged elements that we monitored are integrated into the parental macronucleus. This observation indicates that the M element has the ability to induce its own transcription. We have integrated portions of the micronucleus-limited M element upstream of the rpL29 gene as well and have found that they can induce the developmental transcription of this intergenic locus that is not normally transcribed (A. M. Anderson, M. Arce-Larreta and D. L. C Chalker, unpublished data). Even though we did not identify any specific sequence acting in *cis* that is necessary for the M element to be targeted by the small RNAs, we cannot dismiss the possibility that specific sequences within the M element are required for this unique transcriptional phenomenon. As the homologous RNAs proposed to mark the IES can be provided by the endogenous locus, we likely can only examine requirements for targeting when employing the transformation assay. We are attempting to introduce mutated elements into the micronucleus, which should allow us to identify any role for *cis*-acting sequences in early steps leading to DNA rearrangement.

Our extensive mutagenesis of the M element fully supports the idea that DNA rearrangement is directed by a homology-dependent mechanism. The observation that multiple, non-overlapping fragments of the Tlr element are efficiently eliminated despite sharing no apparent similarity to one another could be explained simply by the existence of small RNAs with complementarity throughout this repetitive sequence (20). It also accounts for data showing that endogenous IESs have frequent insertion and/or deletion of sequence among different strains (46,47). Alterations in IES composition should only disrupt their efficient elimination if they remove their homology to the developmentally produced small RNAs or shorten them below the recognition threshold. Endogenous IES excision is likely part of a broader genome surveillance system that is able to eliminate foreign sequences, such as transposable elements, from the developing somatic genome. In support of this idea, transgenes introduced into the micronucleus can be identified as foreign and eliminated during nuclear differentiation of the macronucleus (48,49). Further studies of RNA-directed DNA elimination should provide novel insights into the role of homologous RNAs in shaping the eukaryotic genome.

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Conflict of interest statement. None declared.

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