Zygotic Expression of the Double-Stranded RNA Binding Motif Protein Drb2p Is Required for DNA Elimination in the Ciliate Tetrahymena thermophila

Jason A. Motl and Douglas L. Chalker*
Department of Biology, Washington University in St. Louis, Campus Box 1137, One Brookings Dr., St. Louis, Missouri 63130-4899

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Double-stranded RNA binding motif (DSRM)-containing proteins play many roles in the regulation of gene transcription and translation, including some with tandem DSRMs that act in small RNA biogenesis. We report the characterization of the genes for double-stranded RNA binding proteins 1 and 2 (DRB1 and DRB2), two genes encoding nuclear proteins with tandem DSRMs in the ciliate Tetrahymena thermophila. Both proteins are expressed throughout growth and development but exhibit distinct peaks of expression, suggesting different biological roles. In support of this, we show that expression of DRB2 is essential for vegetative growth while DRB1 expression is not. During conjugation, Drb1p and Drb2p localize to distinct nuclear foci. Cells lacking all DRB1 copies are able to produce viable progeny, although at a reduced rate relative to wild-type cells. In contrast, cells lacking germ line DRB2 copies, which thus cannot express Drb2p zygotically, fail to produce progeny, arresting late into conjugation. This arrest phenotype is accompanied by a failure to organize the essential DNA rearrangement protein Pdd1p into DNA elimination bodies and execute DNA elimination and chromosome breakage. These results implicate zygotically expressed Drb2p in the maturation of these nuclear structures, which are necessary for reorganization of the somatic genome.

Proteins containing a double-stranded RNA (dsRNA) binding motif (DSRM) participate in diverse biological pathways in a wide range of organisms. This motif was first identified in the developmentally essential gene Staufen of Drosophila melanogaster and has since been recognized to be encoded in the genomes in all three domains of living organisms, as well as in viruses (63; reviewed in references 20 and 67). DSRM proteins commonly act in developmental pathways (e.g., RNA localization by the Staufen family and developmental transcriptional regulation by the DIP1 family) (5, 18, 62, 68) but also have ubiquitous roles in transcriptional and translational regulation (e.g., PKR family and PKR-associated proteins) (26, 45, 55, 58). Proteins vital for RNA interference (RNAi) also contain DSRMs. These include members of the RNase III family (e.g., Dicer and Drosha family proteins) and their tandem DSRM-containing partner proteins (e.g., RDE-4 of Caenorhabditis elegans, Pasha, R2D2, and Loqs in D. melanogaster, and their homologues in Homo sapiens) (4, 12, 17, 23, 27, 36, 37, 57, 65).

In the ciliate Tetrahymena thermophila, the DSRM-containing protein Dicer-like 1 (DCLI) has been shown to play a pivotal role in a process linking RNAi to heterochromatin formation and developmentally regulated DNA elimination (42, 49). Like all ciliates, T. thermophila is unicellular yet contains two distinct types of nuclei, the somatic macronucleus and the germ line micronucleus (reviewed in references 46 and 56). The polyploid micronucleus (~50C) acts as a transcriptionally active somatic nucleus during vegetative growth, while the diploid, germ line micronucleus is transcriptionally silent (19, 70; reviewed in references 46 and 56). Under optimal growth conditions T. thermophila undergoes asexual, binary fission; however, when starved T. thermophila reproduces through the sexual process of conjugation, generating new micronuclei and macronuclei from the parental germ line micronucleus (reviewed in references 46 and 56). During the maturation of the zygotic macronucleus, the macronuclear chromosomes are fragmented at ~180 sites, lose ~15% of their overall genomic content, and are amplified to ~50C (1, 7, 14, 19, 29, 69, 70). The loss of genome complexity is the result of programmed DNA rearrangements that remove specific DNA sequences, called internal eliminated sequences (IESs), from thousands of chromosomal sites (46, 56).

DNA elimination has been shown to be guided by an RNAi-related mechanism (11, 42, 47, 49). Bidirectional transcription of the germ line genome in meiotic micronuclei provides an abundant source of IES-specific dsRNA (11, 44). The resulting noncoding RNAs (ncRNAs) are processed into 27- to 30-nucleotide (nt) sRNA species, called scan RNAs (scnRNAs), by Dcl1p in the meiotic micronucleus (42, 49). These scnRNAs are exported into the cytoplasm, where they are bound by a PIWI homologue, Twi1p (47). Twi1p/scnRNA complexes are transported into the parental macronucleus, where these complexes scan macronuclear ncRNAs, and possibly mRNAs. The Twi1p/scnRNA complexes homologous to the parental macronucleus are removed from the pool of active complexes, and the remaining complexes are transported to the zygotic macronuclei upon their emergence, where they guide H3K9 and H3K27 methylation of IES-associated histones by the E(z) homologue Ezl1p (38, 47, 48). Methyalted histones in zygotic macronuclei are bound by the chromo domain-containing proteins Pdd1p and Pdd3p, which along with other associated...
proteins form large nuclear structures called DNA elimination bodies late in conjugation (38, 40, 51, 66). DNA elimination in these bodies is catalyzed by the domesticated PiggyBac transposase Tbp2p, resulting in removal of IESs from zygotic macronuclei (13).

A second endogenous RNAi pathway that acts to silence genes and/or pseudogenes is evidenced by a class of 23- to 24-nt sRNAs that accumulate during vegetative growth (33). These sRNAs are homologous to loci clustered at ~12 genomic positions and exhibit biased polarity, mapping to only one strand. They are produced by the essential Dicer protein Der2p in a coupled reaction with an RNA-dependent RNA polymerase, Rdr1p (34). This coupling likely accounts for the strand specificity observed.

As dsRNA has clear roles in regulating genome structure and activity, we characterized the two putative tandem DSRM-containing proteins, double-stranded RNA binding proteins 1 and 2 (Dbp1p and Dbp2p), encoded in the T. thermophila genome (21, 64). We show that both are nuclear proteins that exhibit distinct subnuclear organization. By knocking out the gene for each, we found that Dbp2p is essential both during vegetative growth and also late in conjugation, where it facilitates DNA elimination body formation and subsequent RNAi-dependent DNA elimination. Dbp1p, in contrast, is dispensable but is nonetheless important for efficient prezygotic development. Our data do not support that either protein acts as an essential Dicer partner protein as do tandem DSRM proteins in other eukaryotes, but instead our data suggest that these proteins have diverse roles during the life cycle and expose a role for dsRNA late in macronuclear development (4, 12, 17, 23, 27, 37, 57, 65).

MATERIALS AND METHODS

Tetrahymena strains and growth conditions. Standard wild-type, laboratory T. thermophila strains CU427 (Chx/Chx [VII, cy-s]) was generated through genomic exclusion B2086 (II), and micronucleus-defective strains B*VI (VI) and B*VII (VII) were generated by mixing equal numbers of mating-compatible strains. Optical densities of cell populations were used to estimate cell numbers prior to 6 h to overnight in 10 mM Tris (pH 7.5) prior to mixing to initiate conjugation.

Drb1p and Drb2p were grown and maintained as previously described (25, 52). Strains were starved to near depletion (4, 12, 17, 23, 27, 37, 57, 65). Knockouts of the genes for each, we found that Drb2p is essential both during vegetative growth and also late in conjugation, where it facilitates DNA elimination body formation and subsequent RNAi-dependent DNA elimination. Drb1p, in contrast, is dispensable but is nonetheless important for efficient prezygotic development. Our data do not support that either protein acts as an essential Dicer partner protein as do tandem DSRM proteins in other eukaryotes, but instead our data suggest that these proteins have diverse roles during the life cycle and expose a role for dsRNA late in macronuclear development (4, 12, 17, 23, 27, 37, 57, 65).

Cloning of T. thermophila genes for protein localization. Oligonucleotide primers (Table 1) were used to amplify the entire DRB1 or DRB2 coding sequences from genomic DNA by PCR. The resulting products were cloned into the Gateway way recombination-compatible pENTR-D (Invitrogen) to create pENTR-D-DRB1 and pENTR-D-DRB2, respectively. Plasmids containing the DRB1 and DRB2 coding sequences were sequenced to verify coding sequence integrity. LR recombination of pENTR-D-DRB1 with plcY-GTW and of pENTR-D-DRB2 with plpCC-GTW using LR clonase II (Invitrogen) fused the coding regions to yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) in plcY-GTW and plpCC-GTW, respectively (71). Plasmids plcY-DRB1 and plpCC-DRB2 were introduced into mating wild-type cells (CU427 × CU428) by conjugative electroporation (24). Similarly, the entire PDD1 coding sequence was amplified from genomic DNA (primers are listed in Table 1) and cloned into pENTR-D to create donor plasmid pENTR-D-PDD1. LR recombination of pENTR-D-PDD1 with plcY-GTW using LR clonase II (Invitrogen) to create plcY-PDD1. Cells were harvested by low-speed centrifugation (1,000 × g at 4 h, 10 h, and 14 h postmixing, with 4.6-diamidino-2-phenylindole (DAPI; 1 μg/ml) and immobilized in 5 μl 2% methylcellulose. DIC, CFP fluorescence, YFP fluorescence, and DAPI fluorescence images were captured using a Qimaging RetigaxE charge-coupled-device camera (Barnaby, British Columbia, Canada) and Openlab software (PerkinElmer). Images were cropped and their brightness and contrast uniformly adjusted using Adobe Photoshop CS3.

Generation of DRB1, DRB2, and PDD1 knockout strains. Genomic sequencing was performed on April 25, 2021 by guest http://ec.asm.org/ Downloaded from
## TABLE 1. Oligonucleotides used in the course of this study

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<th>Primer purpose and name</th>
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<td>For gene amplification of coding sequence</td>
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Conjugation stage of each mating at 30 h was determined by comparison of
Burnaby, British Columbia, Canada) and Openlab software (PerkinElmer). The
were captured using a Qimaging RetigaEX charge-coupled-device camera
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1,000
knockout cells 30 h into conjugation were harvested by low-speed centrifugation
B*VII
fied by their resistance to both paromomycin/CdCl₂ and 6-methylpurine (resis-
tance gene from the micronucleus of CU428). Genomic exclusion crosses of
homozygous mutants that were subsequently crossed to produce complete PDD1
knockouts ∆PDD1 39.1 and ∆PDD1 W3.3, missing all copies of the gene from
both the micro- and macronucleus.

Southern blotting and PCR analyses. T. thermophila genomic DNA was iso-
lated using a Wizard genomic DNA purification kit (Promega). Gel electropho-
resis, blotting, and hybridization were performed as previously described, except
blots were washed with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium
citrate–1% SDS after hybridization (42). The probe for DRB2 was a radiolabeled
KpnI and BsrGI restriction fragment of pDONR-R2-DRB2Down-L3. The DRB1
probe was a labeled BsrGI and XmnI restriction fragment from pDONR-R2-
DRB1Down-L3. Genomic DNA from heterozygous, homozygous, and homozy-
gous micronuclear DRB1 knockouts was digested with XmnI and separated on a
1.0% agarose gel. Genomic DNA from heterozygous DRB2 knockouts was di-
gested with ClaI and SacI and fractionated on a 0.8% agarose gel prior to
Southern blotting and PCR analyses. T. thermophila genomic DNA was iso-
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1.0% agarose gel. Genomic DNA from heterozygous DRB2 knockouts was di-
gested with ClaI and SacI and fractionated on a 0.8% agarose gel prior to
 blotting.

Chromosome breakage was assayed in DRB2 mic knockouts by using genomic
DNA from CU428 × B2086, ∆PDD1 39.1 × ∆PDD1 W3.3, ∆DCL1 1.86 ×
∆DCL1 4.2, and B*VI[^12][^12] × ∆B*VI[^12][^12] 3 h after mixing and then
digestion with EcoRI and separated on a 0.8% agarose gel. The Southern
blotting probe for chromosome breakage was created using a 0.8-kbp probe
fragment that spans the EcoRI site at position 335013 of chromosomal scaffold
CH445662 (GenBank accession number gi62422284). DNA rearrangement of
IES B and the M IES was assayed by PCR using CU428 × B2086, ∆DCL1
1.86 × ∆DCL1 4.2, B*VI[^12][^12] × ∆B*VI[^12][^12] 1, and B*VI[^12][^12] 6 ×
B*VI[^12][^12] 2 30-h genomic DNA and primers flanking each IES (Table 1).

Analysis of nuclear morphology postconjugation. Wild-type or the indicated
knockout cells 30 h into conjugation were harvested by low-speed centrifugation
(1,000 × g), DAPI stained (1 μg/ml), and immobilized in 5 μl 2% methylcel-
llose. Differential interference contrast (DIC) and DAPI fluorescence images
were captured using a Qimaging RetigaEX charge-coupled-device camera
(Burnaby, British Columbia, Canada) and Openlab software (PerkinElmer).
The conjugation stage of each mating at 30 h was determined by comparison of
images with previously described wild-type stages of conjugation (44). Images
were cropped and their brightness and contrast uniformly adjusted using Adobe
Photoshop CS3.

RESULTS

The T. thermophila macronuclear genome encodes two pro-
teins with tandem DSRMs. For optimal sRNA production and
protein localization, Dicer and Drosha homologues in C. el-
egans, D. melanogaster, and H. sapiens require association with a
tandem DSRM-containing protein (4, 12, 17, 23, 27, 37, 53,
57, 65). Bioinformatic analysis (BLAST, Pfam, and ClustalW)
of the T. thermophila macronuclear genome identified two
genes, DRB1 and DRB2, encoding tandem DSRM-containing
proteins (Fig. 1A). Alignment of their putative DSRMs with
other DSRM-containing proteins indicated conservation in the
regions where key residues known to be important for DSRM
structure and function are located. The homologies of these
proteins with other tandem DSRM proteins did not extend
beyond these domains (Fig. 1A and data not shown). However,
alignment of full-length DRB1 and DRB2 revealed additional
regions of similarity outside the DSRMs; one in the N-terminal
region (NTR) and two in the C-terminal regions (CT1 and
CT2) of each protein (data not shown). In the ciliate Parame-
cium, only DRB1 homologues are evident, which suggests that
the duplication and diversification of these proteins occurred
after these two ciliates diverged.

RT-PCR and Northern blot analysis demonstrated that
DRB1 and DRB2 are both expressed throughout much of the

![FIG. 1. T. thermophila contains two predicted tandem double-stranded RNA binding motif proteins. (A) Genomic locus, conserved motifs, and length of the putative tandem double-stranded RNA binding motif proteins Drb1p and Drb2p. Splice sites are indicated by small connected gaps in the gene. aa, amino acids. (B) ClustalW alignment of DSRMs from Dicer family tandem DSRM-containing partner proteins. #, sites determined to be essential for structure and function of DSRMs; ^, sites that have been mutated in DSRM-containing proteins and shown to cause loss of RNA binding. Conserved aa (white text) are shaded in black (identical aa) or gray (similar aa). (C and D) RT-PCR analysis of DRB1 and DRB2 expression relative to α-tubulin (ATU1). RNA samples were isolated from CU428 cells growing vegetatively (V), after an 18-h starvation (S), and from CU428 × B2086 conjugating cells at 2-h intervals and were used to monitor the expression of each gene.](http://ec.asm.org/Downloaded from http://ec.asm.org/ on April 25, 2021 by guest)
T. thermophila life cycle (Fig. 1C and D and data not shown). DRB1 mRNA levels are low in growing and starved cells but increase significantly during meiosis (2 to 4 h into conjugation, when scnRNA production occurs) and again after the appearance of the zygotic macronuclei (8 h), a pattern that parallels DCL1 expression (Fig. 1C) (42, 49). Its decrease in expression at 6 h coincides with the drop in ATU1 RNA levels, which may simply reflect the switch between parental and zygotic expression.

DRB2 expression is higher during vegetative growth but also shows less dramatic induction during conjugation relative to DRB1. After decreased expression during starvation, DRB2 is induced starting at 2 h of conjugation and peaks at 8 h, shortly after the appearance of the zygotic macronuclei (Fig. 1D). This profile suggests possible roles for Drb2p during both growth and development.

DRB1 and DRB2 encode nuclear proteins that localize to distinct structures. Ectopic expression of Drb1p and Drb2p tagged with YFP or CFP, respectively, on their C termini showed that both are nuclear proteins visible in small foci throughout the macronucleus during vegetative growth (data not shown), whereas green fluorescent protein (GFP) alone expressed in cells is uniformly distributed (42, 43). During early conjugation, both proteins localize to the parental macronucleus in distinct foci (Fig. 2). Later, at the beginning of zygotic macronuclear differentiation (10 h), all Drb1p-YFP and most Drb2p-CFP disappeared from the parental macronucleus and then appeared in zygotic macronuclei (Fig. 2A and B, middle rows). Whether the foci seen in the parental macronucleus are functionally related to those observed in zygotic macronuclei could not be determined (Fig. 2A and B, compare top and middle rows). Near completion of zygotic macronuclear development (14 h into conjugation), Drb1p-YFP localization was primarily diffuse (Fig. 2A, bottom row). In contrast, the small Drb2p-CFP foci coalesced into larger foci, although low-level diffuse localization remained throughout the zygotic macronucleus as well (Fig. 2B, bottom row).

Upon initial inspection, the size and number of nuclear foci of Drb1p and Drb2p in parental macronuclei appeared rather different. To better compare their localizations, Drb1p-YFP and Drb2p-CFP were coexpressed and visualized 4 h into conjugation. Their nuclear foci were distinct, with only a small degree of overlapping localization (Fig. 2C). DRB1 and DRB2

**FIG. 2.** Nuclear localization of Drb1p and Drb2p during conjugation. (A and B) Nuclear localization of Drb1p-YFP (A) and Drb2p-CFP (B) at 4, 10, and 14 h into conjugation. White arrowheads, micronuclei; black arrowheads, parental macronuclei; white arrows, zygotic macronuclei. (C) Simultaneous localization of Drb1p-YFP and Drb2p-CFP in the parental macronucleus 4 h into conjugation. (Top) Drb1p-YFP and Drb2p-CFP foci are predominantly distinct in the macronucleus early during conjugation. Yellow arrowheads, Drb1p-YFP foci only; blue arrowheads, Drb2p-CFP foci only; green arrowheads, Drb1p-YFP and Drb2p-CFP foci. (Bottom) The number of Drb1p-YFP foci, Drb2p-CFP foci, and Drb1p-YFP/Drb2p-CFP colocalization foci, and the total number of foci in the parental macronucleus above.
were best reciprocal hits in a BLASTp analysis of the CT2 regions, which could explain the small overlap in localization through partially redundant protein function. Despite this, it seems that both Drb1p and Drb2p have distinct primary functions based on their localizations and divergent protein sequences outside their DSRMs and CT2. In addition to its abundant macronuclear localization, Drb1p-YFP also localized to the micronucleus just prior to and during crescent formation (prophase meiosis I) (Fig. 2A, top row, and data not shown). Drb1p-YFP was observed specifically at the poles of these nuclei, at either one or both ends depending on the developmental stage. This micronuclear localization pattern is quite distinct from that of Dcl1p, which is found throughout the nucleoplasm of the crescent micronucleus, and suggests that Drb1p may not be a critical Dcl1p protein partner (42, 49). Point localization of Drb1p-YFP was seen early in conjugation once the micronucleus began to elongate at one end, and later, after the crescent micronucleus fully elongated, it was seen at both ends of the micronucleus (Fig. 2A, top row, and data not shown). Upon anaphase of meiosis I, Drb1p-YFP micronuclear localization is lost. While it is likely that DRB1 and DRB2 arose from an ancient gene duplication, differential localization and expression patterns indicate that each DSRM-containing protein has specific cellular roles.

**DRB2, but not DRB1, is essential for growth and development.** We created strains lacking each gene to establish whether and when each protein functions during the *T. thermophila* life cycle. Constructs containing the NEO3 selectable marker, flanked by up- and downstream homology regions to either DRB1 or DRB2, were biolistically transformed into cells during conjugation to generate heterozygous micronuclear/macronuclear knockout strains. By taking advantage of the random assortment of alleles during amitotic macronuclear division, we obtained strains for which all wild-type DRB1 gene copies in the macronucleus were replaced with the knockout allele, which revealed that Drb1p is not required for vegetative growth (Fig. 3A and B and data not shown).

To further verify that DRB1 is not essential, homozygous micronuclear knockout strains were crossed to produce complete DRB1 knockout cell lines. Southern blot analysis of genomic DNA isolated from these strains detected only the DRB1 knockout allele (Fig. 3A). RT-PCR of the DRB1 knockout strains during conjugation confirmed loss of all DRB1 expression (Fig. 3B). While these complete DRB1 knockout strains showed no growth defects, matings between two DRB1 knockout strains generated progeny at a reduced rate relative to crosses of wild-type strains (Table 2). The DRB1 knockout cells that were able to complete conjugation arrested with two new macronuclei and a single micronucleus, as do wild-type conjugants, until they were returned to growth medium and started vegetative growth (Fig. 4). The observation that only a fraction of mated DRB1 knockout cells progressed to zygotic development suggests that Drb1p is important, but not essential, for prezygotic development. The lack of Drb1p during this stage(s) of early conjugation resulted in substantial premature abortion of conjugation (data not shown).

Unlike our experience with DRB1, we were unable to identify strains in which all macronuclear copies of DRB2 were disrupted, which indicates that vegetative DRB2 expression is essential (Fig. 3C and D). To verify this, we first performed genomic exclusion crosses between the original heterozygous micronuclear knockout strains and “star” strains (B’VI and B’VII) to create strains homozygous for the knockout cassette in the micronucleus while maintaining wild-type copies of DRB2 in the macronucleus to support growth (see Materials and Methods for details). These homozygous micronuclear knockout strains were then crossed in an attempt to generate strains homozygous for the knockout cassette in both the micro- and macronucleus, thus eliminating all wild-type DRB2 gene copies. Despite each individual DRB2 micronuclear knockout strain being able to produce progeny when complemented by crossing to wild-type strains, when these lines were crossed to each other no viable progeny emerged (Table 3).

Further analysis revealed that DRB2 micronuclear knockout strains are unable to reach the terminal stage of conjugation with 2 macronuclei and 1 micronucleus even 30 h after pairing, but instead arrest with 2 macronuclei and 2 micronuclei (Fig. 4). Thus, not only is DRB2 expression necessary for vegetative growth, but zygotic DRB2 expression is essential for completion of conjugation as well (Fig. 3C and 4). As observed in other mutants that arrest at the 2-macronuclei, 2-micronuclei stage, conjugating DRB2 mic knockouts underamplified their macronuclear DNA relative to zygotic macronuclei of wild-type conjugants at their terminal stage prior to refeeding (15, 42, 47, 49). Although DRB2 mic knockout strains only lack zygotic expression of DRB2, the majority of conjugants arrest at the 2-macronuclei, 2-micronuclei stage, while the remainder arrest after elimination of one of the remaining micronuclei (Fig. 4, bottom). RT-PCR analysis of DRB2 mic knockout matings showed reduced, but not complete loss of expression after 12 h of conjugation relative to wild-type cells, when zygotic DRB2 expression normally should predominate (Fig. 3D). Unmated cells as well as parentally expressed DRB2 mRNA in the DRB2 mic knockout mating population accounted for the DRB2 mRNA detected. The residual, parental expressed DRB2 transcripts may enable a fraction of cells to proceed further into conjugation and eliminate one micronucleus.

**DRB2 mic knockouts fail to remodel chromosomes late in conjugation.** The DRB2 conjugation arrest phenotype is commonly observed in knockouts of genes necessary for genome rearrangement in *T. thermophila*, including DCL1, TWI1, and PDD1 (15, 42, 47, 49). To determine whether the DRB2 mic knockout arrest is accompanied by failure of RNA-directed DNA elimination or due to some other perturbation during conjugation, we monitored the rearrangements of several IESs. Genomic DNA was isolated from mated cell populations 30 h after initiating conjugation, when all genome reorganization should be completed in wild-type cells. PCR using primers able to detect both the rearranged (micronuclear form of the locus) and rearranged (macronuclear form) IESs allowed assessment of the level of excision. Whereas DNA from wild-type mating populations showed predominantly the rearranged locus for each IES, DRB2 mic knockout or control DCL1 knockout matings exhibited accumulation of the unrearranged form of both IES B and the M IES (Fig. 4 and data not shown). IES B is a 327-bp IES found within the *LA2* gene, and the M IES is a well-studied intergenic IES that undergoes alternative rearrangement that removes either 0.6 kb or the complete 0.9-kb
IES (2, 22a). PCR analysis of IES B clearly showed that the 597-bp product indicative of the micronuclear locus was over-represented in the \textit{DCL1} and \textit{DRB2} mic knockout matings relative to wild type (Fig. 5A). It is important to note that the cell populations monitored included some percentage of unmated cells, whose DNA likely contributed much of the template for the 270-bp product representing the rearranged form in the mutant cell lines. The PCR analysis of the M IES utilized three primers for PCR, which we have found provides a more quantitative assessment of its rearrangement. Two bands at 1,192 bp and 386 bp resulted from amplification of micronuclear DNA containing the IES, while two other bands at 592 bp and 292 bp were the products of removal of either 0.6 kb or 0.9 kb of the M IES locus. As observed for IES B, the unarranged form of the M IES was overrepresented in the \textit{DCL1} and \textit{DRB2} mic knockout mating populations relative to wild-type matings (Fig. 5B). This difference was less apparent in \textit{DRB2} mic knockout matings than in the \textit{DCL1} mutant, which may have been due to persistence of parental Drb2p. Analysis of other IESs further demonstrated that these mutants exhibit...
substantial failure of RNA-directed DNA elimination (data not shown).

Assessment of chromosome breakage near the LIA1 locus also showed that DRB2 mic knockout progeny fail to properly fragment chromosomes (Fig. 5C). Before the completion of conjugation, the chromosomes in the zygotic macronuclei, which contain 5 chromosomes amplified to between 4 and 8 copies, are fragmented at approximately 180 chromosome breakage sites (CBSs) to produce the shortened macronuclear chromosomes. In knockouts of genes essential for genome rearrangement, including DCL1 and TWI1, chromosome breakage fails, as does IES elimination (42, 47). In a Southern blot assay of wild-type progeny, chromosome breakage at the LIA1 locus resulted in a band of approximately 2.5 kb in the zygotic macronuclei. The copies of this chromosome from the parental macronucleus were visible as a 2.6-kb band, as they have longer telomeres relative to newly fragmented ends. Unbroken micronuclear chromosomes were detected as a 10.5-kb band. The probe also detected a 7.8-kb fragment present in all nuclei. Due to the increased copy number of the locus in the macronucleus in the progeny of wild-type crosses, the 2.5-kb and 2.6-kb fragments are more intense than the larger 10.5-kb micronucleus-specific fragment. As in the control matings of DCL1 knockout cells, the postconjugation populations of DRB2 mic knockout crosses have increased levels of the 10.5-kb unarranged fragment and lack the 2.5-kb fragment indicative of de novo chromosome breakage (Fig. 4C). A previous report on chromosome breakage in a somatic knockout of PDD1 showed that chromosome fragmentation was able to occur (15). Here we report that crosses of homozygous PDD1 knockout strains showed failure of chromosome breakage, as observed with DCL1 and DRB2 mic knockouts, emphasizing the importance of zygotic expression of PDD1 and DRB2 in chromosome breakage (Fig. 5C).

**DRB2 colocalizes with Pdd1p in DNA elimination bodies.** Failure of DNA elimination and chromosome breakage in DRB2 mic knockout strain matings indicated that the conjugation arrest phenotype described earlier was a result of failure to complete RNA-directed DNA elimination. The localization of Drb2p-CFP into large foci 14 h into conjugation, which is when DNA elimination normally occurs, prompted us to ascertain whether Drb2p-CFP was localized into DNA elimination bodies. These nuclear structures are enriched for the essential DNA elimination, chromodomain-containing protein Pdd1p and are the putative sites of IES removal. Strains expressing Drb2p-CFP or Pdd1p-YFP were mated, and localization of both proteins was monitored at 8 h into conjugation, very early in zygotic macronuclear differentiation, and later at 14 h into conjugation, when DNA elimination occurs (Fig. 6). As was previously reported, Pdd1p-YFP was diffusely localized in the zygotic macronuclei at 8 h, and as conjugation proceeded toward DNA elimination around 14 h, Pdd1p-YFP localization gradually became unevenly dispersed, forming first small foci and then finally large foci (Fig. 6) (40, 41). Localization of Drb2p-CFP in the zygotic macronuclei at 8 h into conjugation was not markedly different from Pdd1p-YFP localization, with small Drb2p-CFP foci throughout the nucleus (Fig. 5). However, at 14 h into conjugation Drb2p-CFP foci aggregated into larger foci, which colocalized with the Pdd1p-

**TABLE 2. Progeny production of DRB1 knockouts in wild-type and knockout matings**

<table>
<thead>
<tr>
<th>Cross</th>
<th>% pair survival (S/N)*</th>
<th>% progeny production (P/N)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU427 × DRB1 KO 5.1.3</td>
<td>97.2 (171/176)</td>
<td>96.5 (165/171)</td>
</tr>
<tr>
<td>CU427 × DRB1 KO 6.1.6</td>
<td>98.8 (87/88)</td>
<td>98.9 (86/87)</td>
</tr>
<tr>
<td>CU427 × DRB1 KO 6.1.12</td>
<td>99.0 (169/176)</td>
<td>98.2 (166/169)</td>
</tr>
<tr>
<td>CU427 × DRB1 KO 6.1.122</td>
<td>98.9 (174/176)</td>
<td>97.1 (169/174)</td>
</tr>
<tr>
<td>CU427 × DRB1 KO 7.1</td>
<td>97.7 (129/132)</td>
<td>98.4 (127/129)</td>
</tr>
<tr>
<td>CU427 × DRB1 KO 7.7.2</td>
<td>99.2 (131/132)</td>
<td>94.7 (124/131)</td>
</tr>
<tr>
<td>DRB1 KO 5.1.3 × 6.1.12</td>
<td>93.5 (247/264)</td>
<td>33.3 (6/18)</td>
</tr>
<tr>
<td>DRB1 KO 5.1.3 × 6.1.122</td>
<td>94.7 (250/264)</td>
<td>51.5 (35/68)</td>
</tr>
</tbody>
</table>

* The pair survival is the percentage of pairs alive (S) of the total pairs (N) isolated.
* Progeny production is the percentage of surviving pairs (S) that successfully completed conjugation and made new macronuclei (P).

**FIG. 4. Zygotic expression of DRB2 is necessary for completion of conjugation.** (Top) Terminal arrest phenotype of wild-type (WT), ΔDCL1, ΔDRB1, and ΔDRB2 mic cells 30 h into conjugation. WT, ΔDCL1, ΔDRB1, and ΔDRB2 mic cells were mated and harvested after 30 h into conjugation. Cells were then DAPI stained, and DIC (left) and DAPI (right) images were obtained. White arrowheads, micronuclei; white arrows, zygotic macronuclei. (Bottom) Cells with the indicated terminal arrest phenotype of WT, ΔDCL1, ΔDRB1, and ΔDRB2 mic 30 h into conjugation.
TABLE 3. Progeny production of DRB2 mic knockouts in wild-type and knockout matings

<table>
<thead>
<tr>
<th>Cross</th>
<th>% pair survival (S/N)a</th>
<th>% progeny production (P/S)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>B<em>VII427 × B</em>VIID2DA2D2</td>
<td>99.6 (263/264)</td>
<td>98.8 (260/263)</td>
</tr>
<tr>
<td>B<em>VII427 × B</em>VIID2DA2D2</td>
<td>99.2 (262/264)</td>
<td>99.2 (260/262)</td>
</tr>
<tr>
<td>CU427 × B*VIID2DA2D2</td>
<td>95.1 (251/264)</td>
<td>99.6 (250/253)</td>
</tr>
<tr>
<td>CU427 × B*VIID2DA2D2</td>
<td>97.3 (257/264)</td>
<td>100 (257/257)</td>
</tr>
</tbody>
</table>

a Pair survival is the percentage of pairs alive (S) of the total pairs (N) isolated. 
b Progeny production is the percentage of surviving pairs (S) that successfully completed conjugation and made new macronuclei (P).

YFP-containing DNA elimination bodies, indicating a possible interaction with each other in zygotic macronuclei.

Localization of Pdd1-YFP and Drb2p-CFP is not exclusive to the zygotic macronuclei. Residual localization of both proteins was seen in the parental macronucleus as well. At 8 h into conjugation, both proteins formed strong, distinct foci in the nuclear periphery and Drb2p-CFP foci found in the nuclear interior. During DNA elimination at 14 h into conjugation, remaining Pdd1p-YFP was found throughout the parental macronucleus but away from the interior, while Drb2p-CFP was still seen only in the interior. The significance of this late parental macronuclear localization remains to be explored.

**Pdd1p fails to form DNA elimination bodies in DRB2 mic knockouts.** To understand if Pdd1p and Drb2p colocalization is relevant to the conjugation arrest phenotype and failure of DNA elimination in DRB2 mic knockouts, we sought to determine how Pdd1p localization was affected in DRB2 mic knockout strain matings. DRB2 mic knockout strains were transformed with an inducible Pdd1p-YFP expression construct, and the resulting transformants were mated and their Pdd1p-YFP localization was examined. At 10 h into conjugation during zygotic macronuclear differentiation, Pdd1p-YFP localization in both DRB2 mic knockouts crossed to wild-type strains, which rescues loss of DRB2 from the mating partner, and DRB2 mic knockout matings appeared mottled throughout the developing zygotic macronucleus without obvious defects (Fig. 7A). However, late in conjugation (14 h), Pdd1p-YFP failed to form DNA elimination bodies in zygotic macronuclei in DRB2 mic knockout matings (Fig. 7B). Thus, Pdd1p-YFP foci fail to mature into DNA elimination bodies without zygotic DRB2 expression. These data indicate that DRB2 participates in the maturation of DNA elimination bodies and implicates a possible role for uncharacterized dsRNAs in genome reorganization.

**DISCUSSION**

Our analyses of DRB1 and DRB2 have revealed that each has unique and important functions. While both are predominantly nuclear proteins, they localize into distinct subnuclear foci. Furthermore, disruption of the each gene showed that Drb2p has essential functions during both growth and development, while Drb1p appears to be important for prezygotic development. The similarities of these two proteins outside their predicted DSRMs suggest that they may have arisen from an ancestral gene duplication. If that is the case, they have significantly diverged in function since the duplication event.

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![DNA rearrangements of IESs and chromosome breakage](http://ec.asm.org/)
Upon initial recognition that the *T. thermophila* genome encodes two DSRM-containing proteins, we looked for evidence that would connect them as protein partners for the Dicer homologues encoded by *DCL1* and *DCR2* (42, 49). Tandem DSRM-containing partner proteins for Dicer and Drosha family proteins, including R2D2, Loqs, and Pasha in *D. melanogaster*, RDE-4 in *C. elegans*, and TRBP2 and DGCR8 in *H. sapiens* and other mammals, play vital roles in RNAi by ensuring proper sRNA delivery and in many cases cleavage of sRNA precursors (12, 17, 23, 27, 37, 57, 65). Our analyses provided little support that Drb1p or Drb2p serve as major Dicer partners. Neither protein showed abundant localization in meiotic micronuclei, where Dcl1p acts (Fig. 2A and B, top rows) (42, 49). We also did not find defects in scnRNA accumulation in complete DRB1 knockouts (data not shown). As Drb2p is essential for growth, we were unable to generate full knockouts with which to examine scnRNA accumulation upon its loss. The *T. thermophila* Dicer protein, Dcr2p, is also essential for growth, but a previously published characterization of Dcr2p complexes did not find Drb2p to be an interacting protein (34, 35).

While we did not find evidence that these proteins act with Dcl1p, we uncovered a critical role for Drb2p in the RNAi-directed DNA elimination pathway. Loss of zygotic expression was sufficient to block DNA rearrangement; thus, Drb2p is needed well downstream of scnRNA biogenesis by Dcl1p (Fig. 2A and B). Colocalization of Drb2p with Pdd1p-containing DNA elimination bodies and loss of these DNA elimination bodies in *DRB2* mic knockouts implicate zygotically expressed Drb2p in promoting development or stabilizing these large nucleoprotein structures (Fig. 6 and 7). This may indicate that Drb2p/RNA complexes mediate the formation of mature DNA elimination bodies through facilitating protein-RNA or protein-protein interactions within these structures. Although the exact mechanism of Drb2p action remains to be discovered, its importance in late stages of genome reorganization suggests an unrecognized role for dsRNA in RNAi-directed DNA elimination.

Drb2p is also required for vegetative growth, as we were unable to replace all wild-type *DRB2* gene copies with the disrupted allele. We tried extensively to assort *DRB2* out of the macronucleus without success (data not shown). Furthermore, when *DRB2* partial knockout strains were grown in nonselective medium (without paromomycin), the remaining wild-type *DRB2* copies rapidly replaced the *DRB2* knockout allele (data not shown). As both Drb2p and Dcr2p are essential for growth, it remains possible that they act in the same pathway (34, 35). We cannot rule out the possibility that these proteins transiently interact, as do RDE-4 and DCR-1 in *C. elegans* (65).

Further investigation of the function of Drb2p during growth may provide key insights into the role of this protein during both growth and genome reorganization.

While Drb1p is predominantly a macronuclear protein, it also localizes to one or both ends of the crescent micronucleus during the prophase of meiosis I (Fig. 2 and data not shown). Further investigation of this micronuclear point localization indicated that colocalization of Drb1p with cenH3, the centromeric histone H3 (unpublished data) (9, 16, 39). Knockouts of *DRB1* were able to complete conjugation, yet a significant percentage of pairs aborted mating without forming new macronuclei. Together, the localization of Drb1p near centromeres and possibly with telomeres and the reduction in knockout
addition to tandem DSRMs, are putative nuclear, nucleotide transferases that participate in DNA repair and RNA transport (32, 59, 60, 73; reviewed in reference 31). Further study of DRB1 and DRB2 in *T. thermophila* may reveal new roles for tandem DSRM-containing proteins. The great evolutionary distance between ciliates and other eukaryotes could also facilitate understanding of how DSRM-containing proteins evolved within the eukaryotic lineage (54). Much remains to be gleaned about the roles of DSRM-containing proteins in eukaryotes, and we expect further investigation of Drb1p and Drb2p functions will provide greater understanding of RNAi-directed DNA elimination and roles for dsRNA in regulating chromosome structure.

**ACKNOWLEDGMENT**

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