MOLECULAR AND CELLULAR BIOLOGY, Dec. 2006, p. 8731–8742 Vol. 26, No. 23
0270-7306/06/$08.00+0 doi:10.1128/MCB.01430-06

Induction of Gene Silencing by Hairpin RNA Expression in *Tetrahymena thermophila* Reveals a Second Small RNA Pathway▽

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Received 2 August 2006/Returned for modification 24 August 2006/Accepted 18 September 2006

Unlike in other eukaryotes, in which it causes gene silencing, RNA interference (RNAi) has been linked to programmed DNA deletion in the ciliate *Tetrahymena thermophila*. Here we have developed an efficient method to inducibly express double-stranded RNA hairpins and demonstrated that they cause gene silencing through targeted mRNA degradation in all phases of the life cycle, including growth, starvation, and mating. This technique offers a new tool for gene silencing in this model organism. Induction of RNA hairpins causes dramatic upregulation of Dicer and Argonaute family genes, revealing a system capable of rapidly responding to double-stranded RNA. These hairpins are processed into 23- to 24-nucleotide (nt) small RNAs, which are distinctly different from the 28- to 30-nt small RNAs known to be associated with DNA deletion. Thus, two different small RNA pathways appear to be responsible for gene silencing and DNA deletion. Surprisingly, expression of the RNA hairpin also causes targeted DNA deletion during conjugation, although at low efficiencies, which suggests a possible crossover of these two molecular paths.

Small RNAs are major components of gene regulation in eukaryotic cells. This regulation occurs by several different mechanisms, including mRNA degradation, translational repression, and chromatin remodeling (reviewed in reference 61). Factors determining the action and specificity of small RNAs vary among organisms. In some cases, double-stranded RNAs (dsRNAs) from different origins (microRNA [miRNA] genes, viruses, transposons, transgenes, or direct introduction) are processed and interact with different orthologs of RNA interference (RNAi) machinery proteins, which determine the mode of action of the resulting small RNA. For example, *Arabidopsis thaliana* has 4 Dicer-like genes and 10 Argonaute family genes. Specific functions have been assigned to many of these genes, although some functional redundancy exists (3). The different Dicer-like proteins produce small RNAs of different lengths and functions: DCL1 produces 21-nt endogenous miRNAs involved in developmental gene regulation, DCL2 produces some virus-derived RNAs, DCL3 produces 24-nt RNAs involved in heterochromatin formation, and DCL4 produces the 21-nt RNAs that mediate RNAi (16, 55). AGO1 in *Arabidopsis thaliana* is responsible for miRNA function and experimentally induced posttranscriptional gene silencing, both of which result in mRNA degradation (4, 17, 51). AGO4, however, is involved in heterochromatin formation and transcriptional gene silencing (62). Other organisms, such as fission yeast, have only one copy each of key genes necessary for small RNA functions and yet still are able to specify multiple pathways of small RNA action. *Schizosaccharomyces pombe* Ago1 functions both in the RNA-induced silencing complex, to mediate mRNA degradation, and in the RNA-induced initiation of transcriptional gene silencing complex, to promote formation of heterochromatin (52, 53). Studies of these multiple small RNA pathways have resulted in new understanding of developmental processes, centromere maintenance, virus neutralization, and transposon suppression (1, 26, 31, 53, 55).

Tetrahymena thermophila is a single-cell model organism known for RNA-guided DNA deletion (58). Small RNAs have been identified as part of the *T. thermophila* sexual reproductive cycle of conjugation (35, 38, 39). *T. thermophila*, like other ciliates, is binucleate. The micronucleus (MIC), or germ line nucleus, contains five pairs of chromosomes that are silent during normal cellular growth. The transcriptionally active macronucleus (MAC) is derived from a copy of the zygotic MIC during sexual reproduction. During this process, the chromosomes are fragmented, and approximately 15% of the genome is deleted (12, 56, 57). Much is still unknown about the mechanism of DNA deletion, but it is thought that that the process is mediated by 28- to 30-nucleotide (nt) small RNAs. These small RNAs begin to accumulate shortly after mating begins and are likely derived from double-stranded RNAs produced by bidirectional transcription of sequences in the MIC that are destined for elimination in the MAC (10, 38). The Dicer-like protein Dcl1p and the PAZ/PIWI domain protein Twi1p are required for accumulation and stabilization of the small RNAs, and all three of these factors are necessary to promote the required methylation of histone H3 tail lysine 9 (H3K9) at the loci to be deleted (33, 35, 38, 39, 48). The chromatin modifications that occur in the deletion elements of *T. thermophila* are very similar to those that occur in small RNA-guided transcriptional gene silencing in fission yeast, fruit flies, and plants

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▽ Published ahead of print on 25 September 2006.

(48, 53, 62). The outcome (deletion of the targeted DNA), however, is unique to ciliates.

Another ciliate species, *Paramecium tetraurelia*, also undergoes developmental DNA deletion. The role of small RNAs in this process has been less well characterized than in *T. thermophila*. However, the introduction of dsRNA into *Paramecium* by bacterial feeding or high-copy-number transgenes leads to homology-dependent silencing which is linked with the accumulation of 22- to 23-nt small RNAs (21–23).

To date, transcriptional or posttranscriptional gene silencing by RNAi has not been reported for *Tetrahymena*. A class of small RNAs of 23 to 24 nt in length has recently been identified in *T. thermophila* cells during normal vegetative growth (29). These RNAs map to the antisense strand of several predicted gene clusters. The function of these 23- to 24-nt RNAs is unknown. Twenty-three- to 24-nt RNAs are also observed in mating cells lacking both Dcl1p and the 27- to 28-nt class of small RNAs (35, 39). The presence of this second class of small RNAs suggests that *T. thermophila* may have a second RNAi pathway capable of posttranscriptional gene silencing, but there is currently no evidence of this function.

In support of the presence of a second small RNA pathway, *T. thermophila* expresses other homologs of RNAi machinery genes in addition to those active during mating. Dicer homologs *DCR1* and *DCR2* are both expressed throughout the cell's life cycle (35, 39). *DCR1* is nonessential, and *DCR1* knockout cells still produce the 23- to 24-nt small RNAs (29). *DCR2* is an essential gene with a domain architecture very similar to other Dicer genes, including a conserved helicase domain and two RNase III domains, suggesting it could be responsible for processing long dsRNA into the 23- to 24-nt RNAs (29). At least 10 other Argonaute-like PAZ/PIWI domain (PPD) genes have been identified in the *T. thermophila* genome; the cDNA sequence has been reported for one of these, designated *TWI2*, but this gene has not yet been characterized (http://db.ciliate.org/cgi-bin/search/textSearch?query=twi2&type=homolog).

Here we present an efficient method for RNA-induced gene silencing (RNAi) in *Tetrahymena thermophila* that will provide a valuable tool for reverse genetics in *T. thermophila* and allow rapid functional analysis of genes. Using this method, we have demonstrated the presence of a second small RNA pathway in this model organism. We also showed that the mRNA degradation pathway can interact with and affect the DNA deletion small RNA pathway and lead to DNA deletion at low rates.

MATERIALS AND METHODS

Construction of RNA hairpin expression vectors. The plasmid pIBF-1, a gift from Douglas Chalker, was used as the backbone of all hairpin constructs used in this study. This plasmid contains the *MTT1* metallothionine promoter, the blue fluorescent protein gene, and the 3′ portion of the rpL29 gene as a transcriptional terminator cloned into the NotI site of the ribosomal DNA (rDNA) vector pD5H8 (44, 59). A 90-bp fragment containing a small intron was amplified from predicted gene 117.m00123, CH 445424, by use of a forward primer containing PmeI and XmaI restriction sites and a reverse primer containing XhoI and ApaI restriction sites. This PCR product was cloned into the pCRII-TOPO vector to create pCRII-I3. Target sequences used in hairpin constructs were amplified using two different sets of primers: a forward primer containing the PmeI site paired with a reverse primer with an XmaI site, and a forward primer containing the ApaI site paired with a reverse primer with an XhoI site (see Table 1 for primer sequences). Amplified target fragments were cloned into the PmeI/XmaI and XhoI/ApaI sites, respectively, of the pCRII-I3 vector to create

TABLE 1. Sequences of oligonucleotides used for hairpin vector construction, deletion assays, and probe amplification

Primer and purpose	Sequence
Hairpin vector construction	
DCR2	
apa5239-Dcr2for	GCGGGCCCGTGCAATCAAAGATTAGAATTTTAG
xho5650-Dcr2rev	CGCTCGAGCGCTCCAACTAAACTTTCAATTAC
Pme5239-Dcr2for	CGTTTAAACGTGCAATCAAAGATTAGAATTTTAG
Sma5650-Dcr2rev	CGCCCGGGCGCTCCAACTAAACTTTCAATTAC
apa4176-Dcr2for	GCGGGCCCCACCAACTAATGGAGGGATAC
xho4568-Dcr2rev	CGCTCGAGCCTATGGGAGAAAGCGTTTC
Pme4176-Dcr2for	CGTTTAAACCACCAACTAATGGAGGGATAC
Sma4568-Dcr2rev	CGCCCGGGCCTATGGGAGAAAGCGTTTC
TWI1	
Pme-Twi1-2711	CGTTTAAACCAGAGACGGTGTTGGTGAAG
Apa-Twi1-2711	CGGGCCCCAGAGACGGTGTTGGTGAAG
Sma-Twi-3240r	GCCCGGGCTCCAGTCCAGTTATAGTAC
Xho-Twi1-3240r	GCTCGAGCTCCAGTCCAGTTATAGTAC
ATU1	
PmeAtub2541	CGTTTAAACGTCAAGGTGGTATCCAAGTC
ApaAtub2541	CGGGCCCGTCAAGGTGGTATCCAAGTC
SmaAtub3029r	GCCCGGGGATAGATGGTGAAGCCCAAC
XhoAtub3029r	GCTCGAGGATAGATGGTGAAGCCCAAC
SERH3	
PmeSerH32782	CGTTTAAACGTTCTGCTTCATGCACAGC
ApaSerH32782	CGGGCCCGTTCTGCTTCATGCACAGC
SmaSerH33205r	GCCCGGGGAAGCACTTGATGCACAAGC
XhoSerH33205r	GCTCGAGGAAGCACTTGATGCACAAGC
RPL21	
1027RPL21-Pme	CGTTTAAACGGTCTTGAAGGATTTATCCGG
1487RPL21-Sma-r	GCCCGGGATAGCTTACGTAGAGGGGTC
1027RPL21-Pme-Apa	CGTTTAAACATAGGGCCCGGTCTTGAAGGATTTATCCGG
1487RPL21-Sma-Xho-r	GCCCGGGATCTCGAGATAGCTTACGTAGAGGGGTC
NDC1	
1399NDC1-Pme	CGTTTAAACCGGCTTCGCCGCCTCAACCC
1819NDC1-Sma-r	GCCCGGGCAAGATGGGGTAAGCACC
1399NDC1PmeApa	CGTTTAAACATAGGGCCCCGGCTTCGCCGCCTCAACCC
1819NDC1SmaXhor	GCCCGGGATCTCGAGCAAGATGGGGTAAGCACC
TTN2-I3 linker	
TTN2I3-PmeSma	CGTTTAAACAACCCGGGGTAAGTAACGGATATGCAAAAG
TTN2I3-ApaXho-r	CTGGGCCCATACTCGAGCCTTTGAATTCTTCTTTTATCTA
Probe amplification	
Atub-3221	CTTTGACTGCCTCCCTCAG,
Atub-3720r	GCTCTCTTGGCGTACATAAG
Twi-1988	GGAACTGTTGATACTAAGACTG
Twi1-1564r	CTTGGCATAGTGAAGGAATC
SerH3-3229	CTAATACCGCTGGTTCTGC
SerH3-3698r	GCTAACAAAGATCTAACCACTG
2001-NDC1for	CCAAGCCATAAATGGCAG
NDC1rev	TAAGCGAGGGCATCAGAGTT
RPL21for	CCTTGAAAGACCCGGTACAA
RPL21rev	CCTTCGAATTCGGTCTTGAA
Dcl1for	GATACCCTTGAATCAGTTGTTG
Dcl1rev	CTTGATGCGACACTGACTC
Dcr1for	GTAACTTTGGCTCCTTGCTTG
Dcr1rev	GGATTGACTGTATCTCGTG
Dcr2for	GAAATAGCAGTAGACGATGATG
Dcr2rev	CATTAAGTTGATTAAACAGAGGG
112-Twi2f	CCCTAGTCTTCTGATTCTGATG
1030-Twi2r	CTGTCATTCCTGTCATATTGC
DNA deletion analysis	
1012-NDC1	GTTAATGAATGGGGTTAACCG
NDC1-rev	TAAGCGAGGGCATCAGAGTT
Atub-3720r	GCTCTCTTGGCGTACATAAG
Atub-1801	GGTTGACTCTGATGGGAG

the hairpin cassette. Hairpin cassettes were then removed from the pCRII-TOPO backbone by digestion with PmeI and ApaI and ligated into the pIBF backbone digested with PmeI and ApaI to remove the blue fluorescent protein gene fragment.

Transformation and selection. Wild-type (WT) strains B2086 II, CU428, and CU427 were obtained from Peter Bruns (Cornell University, Ithaca, NY). *DCR1* knockout strains 3-7-1 and 2-12-1 were obtained from Martin Gorovsky (University of Rochester, Rochester, NY). Cells were grown in SPP medium and starved in 10 mM Tris (pH 7.5) to induce mating (41). Matings of B2086 II and CU428 and of CU427 and CU428 were transformed by electroporation using 10 μg of hairpin vector DNA (20). Transformants were selected in 120 μg/ml paromomycin in SPP medium.

Northern blotting. RNA samples were prepared using an RNeasy mini kit with Qiashredder (QIAGEN). Samples were combined 1:3 with NorthernMax formaldehyde load dye (Ambion) and electrophoresed on a 1.2% agarose formaldehyde MOPS (morpholinepropanesulfonic acid) gel. Gels were transferred to Hybond XL nylon membranes (Amersham Biosciences), cross-linked, and hybridized with probes overnight at 65°C in Church's hybridization buffer (1% [wt/vol] bovine serum albumin, 1.0 mM EDTA, 0.5 M phosphate buffer, 7% [wt/vol] sodium dodecyl sulfate [SDS]). Probes were made by random prime labeling PCR products amplified from genomic DNA (see Table 1 for primer sequences) (43). After hybridization, blots were washed three times for 15 min in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS and exposed to film. Bands were quantified using ImageQuaNT software (Molecular Dynamics, Inc).

Evaluation of *TWI1* RNAi phenotype. Two different cell lines transformed with the *TWI1* hairpin construct (1. 2H3 and 2. 3H12) were starved and incubated with 0.05 μg/ml $CdCl_2$ for 2 hours. Pretreated cells were washed to remove Cd and then resuspended in 10 mM Tris. Pretreated and untreated cells were mated with each other or with WT strain CU427. To determine if mating cells produced viable progeny, 88 individual pairs were isolated in drops of growth medium and incubated for 48 h. Drops containing more than 1,000 cells were scored as viable, and drops containing fewer than 10 cells were counted as inviable. Viability is expressed as the percentage of viable drops out of the total. Progeny production was tested by assaying drug resistance phenotypes of cells in drops scored as viable. *TWI1*hp progeny were tested for resistance to the antibiotic paromomycin. *TWI1*hp transformants are resistant to paromomycin due to a mutation in the rDNA vector carrying the hairpin expression cassette. Progeny of these cells are sensitive to paromomycin, as their new macronuclei will no longer carry the rDNA vector. Progeny of CU427 and CU428 cells were tested for cycloheximide resistance, as CU427 cells carry a micronuclear marker that confers cycloheximide resistance to their progeny. Progeny production is expressed as the percentage of drops containing cells with the appropriate drug phenotypes out of the total of 88.

To determine at what stage of mating *TWI1*hp-expressing cells arrested, the nuclear morphology of mating cells was examined 24 h after the initiation of mating. Cells were fixed in 70% ethanol and stained with the fluorescent DNA stain DAPI (4′,6′-diamidino-2-phenylindole) to visualize the nuclei.

Small RNA Northern blotting. RNA samples were prepared using TRIzol reagent (Invitrogen). Total RNA (10 to 20 μg) was combined 1:1 with Gel Loading Buffer II (Ambion) and run on a 20% polyacrylamide (19:1 acrylamide-bisacrylamide) Tris-borate-EDTA (TBE) gel containing 8.0 M urea (43). A denatured 10-bp DNA ladder or the RNA Decade marker system (Ambion) was used as the size marker. Gels were run in a Bio-Rad mini-protean apparatus for approximately 6 hours, until bromophenol blue loading dye reached the bottom of the gel. Gels were then either stained with ethidium bromide or transferred to Hybond XL nylon membranes (Amersham Biosciences) by use of a Panther semidry electroblotter (OWL Separation Systems). Small RNA blots were hybridized with oligonucleotides end labeled with high-specific-activity (7,000 Ci/mmol) [γ-^{32}P]ATP by use of polynucleotide kinase (43). Blots were hybridized overnight at 37°C in ULTRAhyb Oligo hybridization buffer (Ambion) and then washed at room temperature in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.5% SDS three times for 15 min and exposed to film.

Deletion PCR assays. DNA samples were prepared from mating cells 24 h after mixing or from cultures grown from isolated pairs of mating cells using the DNAzol reagent from Molecular Research Center, Inc. PCR across the targeted loci was performed using primers annealing 400 to 500 bp upstream and downstream from the region homologous to the hairpin (Table 1). When products smaller than the expected full-length size were observed, these products were isolated from an agarose gel, cloned into the pCRII vector (Invitrogen), and sequenced.

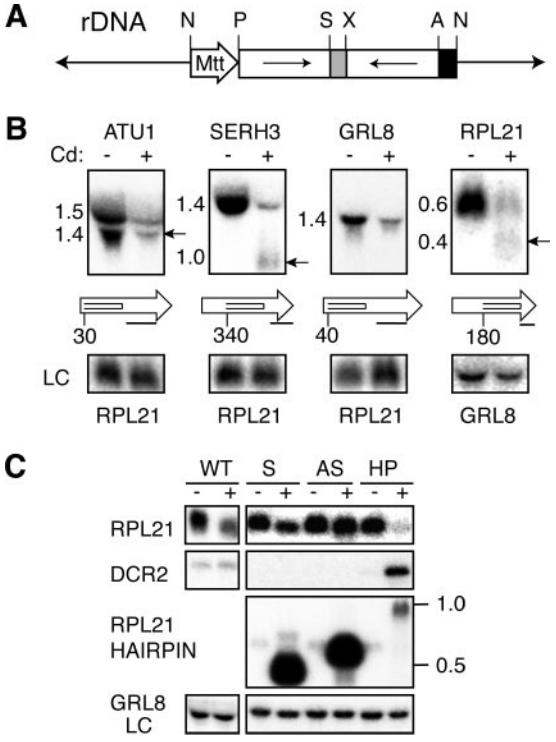

FIG. 1. Expression of hairpin RNA in *T. thermophila* leads to reduction in target mRNA. (A) Schematic map of hairpin RNA constructs (not to scale). Hairpin expression cassettes were cloned into the *T. thermophila* rDNA vector (outer black arrows). Open arrow, *MTT1* cadmium-inducible promoter; gray box, 90-bp intron linker region; white boxes, 400- to 500-bp segments of target gene, with small arrows indicating orientation; black box, transcription terminator. Restriction sites are marked with the following letters: N, NotI; P, PmeI; S, SmaI; X, XhoI; A, ApaI. (B) Northern blots of RNA samples from starved *T. thermophila* cells transformed with hairpin RNA constructs that were uninduced (−) or induced for 2 hours with 0.1 μg/ml $CdCl_2$ in 10 mM Tris (+). The top panels show hybridizations against targeted mRNAs; arrows indicate likely mRNA degradation products. Approximate sizes (kilobases) of RNAs are indicated to the left of each panel. Open arrows represent targeted mRNAs, with double lines inside indicating the regions targeted by the hairpin RNA and lines below showing the regions covered by the probe used for mRNA detection. The distances (number of nucleotides) from the beginning of the coding sequence to the beginning of the hairpin homology are indicated by the numbers beneath open arrows. The bottom panels show hybridizations of loading controls (LC), as indicated underneath. (C) Double-stranded RNA expression is required for silencing of RPL21 as well as the induction of *DCR2*. WT cells, cells expressing the sense (S) or antisense (AS) strands of the *RPL21* target, and *RPL21* hairpin-expressing cells (HP) were treated with 0.05 μg/ml $CdCl_2$ for 2 hours and then harvested. Northern blots were hybridized with *RPL21* mRNA probe to show silencing, with *DCR2* to show RNAi activation, with *RPL21*hp sequence to show expression of induced RNA, and with *GRL8* as a loading control. Approximate sizes (in kilobases) of induced RNAs are indicated to the right of the *RPL21* hairpin panel. Silencing of *RPL21* and induction of *DCR2* occurs only in the sample expressing hairpin RNA.

RESULTS

Expression of hairpin RNA in *Tetrahymena thermophila* leads to silencing of the targeted gene. We developed an inducible system for RNA-induced gene silencing in *T. thermophila* by use of a highly expressed double-stranded RNA hairpin construct similar to that used for plants and worms (45,

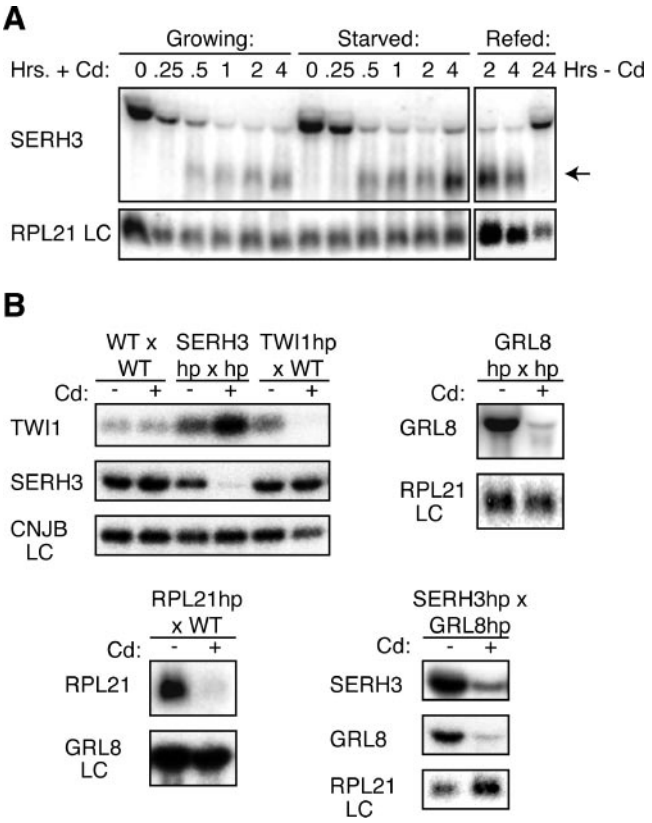

FIG. 2. RNAi is effective in growing, starved, and mating cells. (A) Induction and persistence of RNAi in *SERH3* hairpin transformants. RNA was taken from *SERH3*hp cells at indicated times after the addition of $CdCl_2$ (1.0 μg/ml in growing cells, 0.1 μg/ml in starved cells). Starved cells induced for 4 hours were washed to remove Cd and resuspended in growth medium; samples were then harvested at 2, 4, and 24 h after refeeding. Northern blots were hybridized with *SERH3* probes to determine degree of mRNA degradation, and *RPL21* was used as a loading control. The arrow indicates the *SERH3* mRNA degradation product. (B) Constitutively expressed and mating-specific genes can be silenced during mating, and mating of two different hairpin-expressing strains results in silencing of both targeted genes in the paired cells. Mating cells of the indicated genotypes were left untreated (−) or were treated with 0.05 μg/ml $CdCl_2$ for 2 hours prior to mating (+), and RNA was prepared from cells at 4 h after the initiation of mating. Northern blots were hybridized with the probes indicated to the left of each panel, *CNJB* was used as loading control specific to mating cells, and other loading controls (LC) are indicated (36).

49). A 400- to 500-base pair length of target sequence was cloned in an inverted orientation around a 90-bp linker region containing a 50-bp intron from an unrelated gene (Fig. 1A). This hairpin was then inserted into the polylinker of a *T. thermophila* rDNA vector containing the Cd-inducible metallothionine promoter *MTT* (44). When introduced into mating *T. thermophila*, this plasmid was processed and maintained as a high-copy-number autonomously replicating chromosome (59). The combination of using a strong promoter and placing the construct in a highly amplified chromosome was chosen to ensure robust expression of the hairpin RNA.

A variety of essential, nonessential, and mating-specific genes were chosen as targets to test the effectiveness and versatility of the system. *GRL8*, a dense core granule protein gene (11), and *SERH3*, a cell surface antigen gene (50), were chosen as nonessential genes; ribosomal protein gene *RPL21* (42) and alpha tubulin gene *ATU1* (6) were chosen as essential gene targets, and *TWI1*, the PAZ-PIWI protein gene involved in developmentally regulated DNA deletion, was chosen as a mating-specific gene target (38).

Cells transformed with vectors targeting the constitutively expressed genes were starved briefly and then treated with Cd to induce hairpin expression. Northern blots of RNA samples harvested after 2 hours of induction were hybridized with probes for the target mRNAs to determine whether silencing occurred. In all cases, Cd induction of hairpin RNA expression led to a large reduction of target message (Fig. 1B). Induction of hairpins targeting *ATU1*, *SERH3*, *GRL8*, and *RPL21* led to reductions of message to 5%, 15%, 18%, and 10% of uninduced levels, respectively. In Cd-treated *SERH3* hairpin (*SERH3*hp), *RPL21*hp, and *ATU1*hp samples, a distinct degradation band was present. The size of this band is consistent with mRNA cleavage occurring near the region complementary to the 5′ end of the hairpin (Fig. 1B and 2A). The *ATU1* degradation band is also visible in untreated cells, suggesting that the hairpin construct may be slightly leaky and cause a low level of silencing even in the absence of Cd. In cell lines expressing the *RPL21* hairpin or just the sense or antisense halves of the hairpin, only cells expressing the full hairpin showed reduction of *RPL21* mRNA (Fig. 1C). This indicates that double-stranded RNA is necessary for initiating a silencing response.

Growth curves and viability assays were used to demonstrate the functional consequences of targeting essential genes. In 24 h of growth, cells expressing hairpins targeting *RPL21* or *ATU1* went through no more than two doublings, whereas cells expressing the *GRL8*- or *SERH3*-targeting hairpins doubled four or five times, similar to wild-type cells (data not shown). Viability assays were conducted by isolating single cells of wild-type or hairpin transformant lines in drops of media with and without Cd to induce hairpin expression. After 2 days of growth, the approximate number of cells in each drop was determined, and drops containing greater than 1,000 cells were counted as viable, while drops containing 10 cells or fewer were regarded as inviable. Wild-type cells, *SERH3*hp cells, and *GRL8*hp cells showed 99 to 100% viability either with or without Cd, whereas *RPL21*hp and *ATU1*hp cells gave 0% viable clones in medium with Cd versus 100% and 96.5% viable without Cd. *ATU1*hp-expressing cells did not divide in medium with Cd, and only 18% of *RPL21*hp cells were able to undergo limited division (one to four rounds) (Table 2).

TABLE 2. Viability assays of hairpin-transformed cell lines

Cell line	% with indicated viability in assay with:				
	No $CdCl_2$		0.5 μg/ml $CdCl_2$		
	>1,000 Cells	0–1 Cells	>1,000 Cells	0–1 Cells	2–10 Cells
WT B2086 II	100	0	99	1	0
*SERH3*hp	100	0	100	0	0
*GRL8*hp	100	0	100	0	0
*RPl21*hp	100	0	0	82	18
*ATU1*hp	96.5	3.5	0	100	0

TABLE 3. Matings of *TWI1*hp-expressing cells show reduced viability and progeny production and often arrest before completion of conjugation

Mating	Pair isolation[a]		Nuclear morphology, % showing:			Total counted
	Viability	Progeny production	2 MAC, 1 MIC	2 MAC, 2 MIC	Earlier mating stage	
*TWI1*hp-1 × WT CU427	62.5	45.4	99	1	0	72
*TWI1*hp-1 × WT CU427 + Cd	45.5	3.4	13	72	15	87
*TWI1*hp-2 × WT CU427	79.5	55.6	99	1	0	105
*TWI1*hp-2 × WT CU427 + Cd	28.4	19.3	76	24	0	95
*TWI1*hp-1 × *TWI1*hp-2	82.9	69.3	96	3	1	170
*TWI1*hp-1 × *TWI1*hp-2 + Cd	5.6	2.3	59	38	3	218
*GRL8*hp-1 × *GRL8*hp-2	96.0	42.0	86	5	9	222
*GRL8*hp-1 × *GRL8*hp-2 + Cd	91.7	48.5	82	9	9	113
WT CU427 × CU428	86.9	72.2	86	5	9	256
WT CU427 × CU428 + Cd	87.5	73.3	82	9	9	245

[a] Viability and progeny production expressed as percentage of 88 isolated pairs that grow and complete mating.

Silencing is effective in all stages of the *Tetrahymena thermophila* life cycle. To explore the biology of this phenomenon and demonstrate the versatility of the technique, hairpin expression was performed under a variety of conditions. *SERH3* hairpin transformants were used to determine the time course of message reduction in both starved and growing cells. In starved cells treated with 0.1 μg/ml Cd, or in growing cells treated with 1.0 μg/ml Cd, maximum reduction of message was achieved within 1 h of induction (Fig. 2A). Silencing can be reversed by washing out Cd to stop hairpin induction. Starved *SERH3* hairpin-expressing cells induced for 4 hours were washed to remove Cd and resuspended in growth medium. mRNA levels remained reduced for at least 4 hours but returned to near normal by 24 h after Cd removal (Fig. 2A).

We also tested whether silencing was effective during cell mating, when programmed DNA deletion and other interesting developmental processes occur. Because cells do not initiate mating efficiently while Cd is present, hairpin expression was induced during starvation prior to mating. Experiments using *GRL8*hp-expressing cells where both mating partners expressed the hairpin showed a clear reduction in target mRNA (Fig. 2B). This was also the case in matings of two *SERH3*hp-expressing strains (Fig. 2B). Silencing was also effective in matings where only one partner expressed the hairpin RNA, as demonstrated by matings of wild-type CU428 cells with *TWI1*hp-expressing cells or *RPL21*hp-expressing cells (Fig. 2B). In cases where only one of the mating partners expressed a hairpin, the reduction of message in the mating population was much greater than 50%, indicating that RNAi is a cytoplasmically dominant effect between mating partners. Paired cells undergo substantial cytoplasmic mixing, and silencing complexes are likely to pass from one cell to the other, which could explain this dominant effect (37). This facilitates simultaneous silencing of two gene targets during mating. This point is directly demonstrated by mating *SERH3*hp cells with *GRL8*hp cells (Fig. 2B). Both the *SERH3* and *GRL8* messages were greatly reduced in Cd-treated matings of these cells: the *SERH3* message was reduced to 7.1% and the *GRL8* message was reduced to 6% of that of the uninduced cells, indicating effective silencing of both targets in the paired cells. The ability to easily silence two genes simultaneously in paired cells will allow investigation of the relationships of gene functions important in meiosis and nuclear development.

Silencing of *TWI1* by RNAi recapitulates the phenotype of *TWI1* knockout strains, further supporting the effectiveness of this gene silencing effect in mating cells. When *TWI1* macronuclear knockout cells are mated to each other they are unable to produce progeny, and the mating cells arrest before the completion of macronuclear development (38). Arrested cells are characterized by the presence of two micronuclei and two new macronuclei, while normal cells resorb one of the two micronuclei at the final stage of conjugation. This arrest phenotype is commonly caused by mutations in genes necessary for macronuclear development (13, 35, 38, 40). Matings either between two *TWI1*hp strains or between a *TWI1*hp strain and a wild-type strain preinduced with Cd showed viability and progeny production much lower than that of uninduced cells (Table 3). Matings of wild-type cells or cells expressing the *GRL8* hairpin showed similar levels of viability and progeny production between untreated and Cd-pretreated matings, indicating that the effect is specific to the *TWI1* hairpin induction. When the nuclear morphology of the matings was examined using DAPI staining, we found that in matings with cells expressing the *TWI1* hairpin, a large fraction of cells had failed to resorb one micronucleus at the final stage of mating (Table 3). *TWI1*hp cells that complete mating and produce progeny are likely the result of incomplete silencing in that particular pair. These results show that it is possible to use RNAi to silence genes expressed only during mating and to produce a loss-of-function phenotype consistent with previous knockout results.

RNAi genes are upregulated in response to dsRNA. Of the Dicer and Argonaute homologs in *T. thermophila*, only *DCL1* and *TWI1* have been well characterized. We hypothesized that if the other homologs were involved in RNAi, mRNA levels of these genes may change in response to high levels of experimentally induced dsRNA. Therefore, we examined the mRNA levels of all three Dicer genes for changes in response to expression of the double-stranded RNA hairpin. RNA samples from cells transformed with the *SERH3* hairpin construct were probed for *DCR1*, *DCR2*, and *DCL1* transcripts (Fig. 3A). *DCR1* and *DCR2* both normally have low levels of expression in growing, starved, and mating cells, while *DCL1* expression is restricted primarily to mating cells (29, 35, 39). In starved, uninduced *SERH3*hp transformants, no transcript was detected for any of the three Dicer genes, which is consistent with previous reports. However, in starved cells in which hairpin expression was induced by Cd addition, *DCR1* transcript was

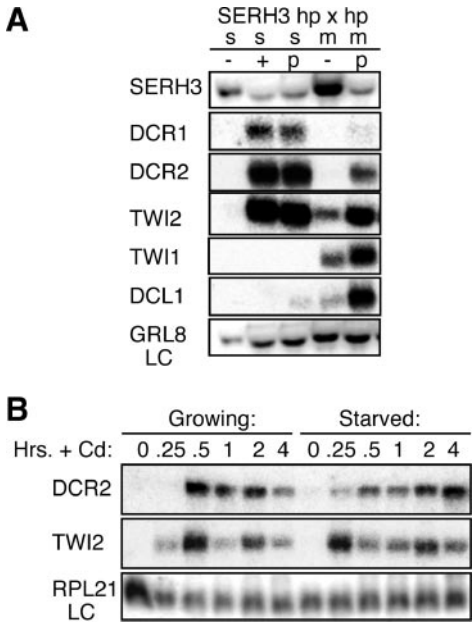

FIG. 3. Cells respond to dsRNA by upregulating RNAi genes. (A) Starved *SERH3*hp cells were left untreated (s −), were treated with 0.05 μg/ml $CdCl_2$ (s +), or were pretreated with 0.05 μg/ml $CdCl_2$ and then washed (s p). Pretreated or untreated cells were then mated for 4 hours (m p, m −), and RNA was harvested. Northern blots were hybridized with the probes indicated to the left of the gels. *DCR1*, *DCR2*, and *TWI2* all show increased mRNA levels after induction of hairpin RNA. Mating-specific genes *TWI1* and *DCL1* also show increased expression in mating cells when hairpin RNA is induced prior to mating. (B) Induction time course of *DCR2* and *TWI2* mRNAs in growing and starved *SERH3*hp cells. The Northern blots shown in Fig. 2A were hybridized with probes to *DCR2* and *TWI2*. Upregulation of both genes occurs within 15 to 30 min of hairpin induction.

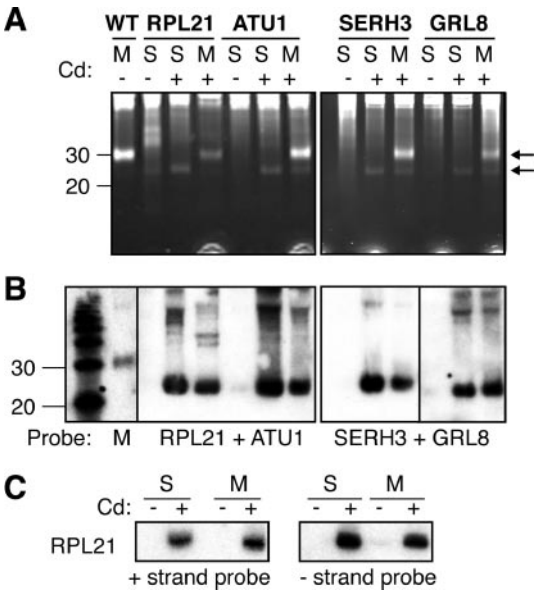

FIG. 4. Small RNAs (23 to 24 nt) are detected in cells after the induction of hairpin RNAs. These RNAs are of a size distinct from that of small RNAs produced from mating cells. (A) RNA samples were prepared from untreated (−) or Cd-treated (+) starved (S) or mating (M) cells transformed with the indicated hairpin constructs. Small RNAs were separated by electrophoresis in 20% polyacrylamide gels and visualized after staining with ethidium bromide. Arrows indicate the two species of small RNAs in the gel. (B) Small RNA Northern analysis. Duplicates of the small RNA gels shown in panel A were blotted and hybridized with ^{32}P-labeled oligonucleotides complementary to the indicated hairpin sequences. The lane containing RNA from wild-type mating cells was hybridized with oligonucleotides complementary to the M deletion element to emphasize the difference in size between mating-specific small RNAs and silencing small RNAs. (C) RNAs corresponding to both strands of the hairpin can be detected by hybridizing *RPL21*hp samples with oligonucleotides complementary to the plus or minus strand of the hairpin.

readily detectable, and *DCR2* expression was extremely robust (Fig. 3A). Dicer genes' upregulation was less dramatic in mating cells; however, it was still clear for *DCR2*. The same pattern of regulation was detected in cells expressing the *GRL8* and *RPL21* hairpins (data not shown). In the time course of *SERH3* hairpin induction, the reduction of *SERH3* message corresponded closely with the increase of *DCR2* message (Fig. 3B). This effect of increasing mRNA levels of the Dicer genes in response to hairpin expression suggests that the cell has a robust system to detect the presence of double-stranded RNA and responds by upregulating genes involved in RNAi. *DCR2* showed the most dramatic response to hairpin RNA induction, which suggests that it could be the Dicer gene responsible for dsRNA processing in *T. thermophila*. Expression of only the sense or antisense strand of RNA did not cause increased expression of *DCR2*, indicating that this response is specific to dsRNA (Fig. 1C).

Because the Dicer genes showed interesting changes in expression in response to dsRNA, we asked if other RNAi pathway genes showed similar changes. The Argonaute protein is a member of the RNA-induced silencing complex and is responsible for binding the small interfering RNA (siRNA) and specifically cleaving the target mRNA (25, 32). *T. thermophila* has many genes containing PPDs, similar to Argonaute genes in other organisms, but only two PPD genes (*TWI1* and *TWI2*) have been annotated in the *T. thermophila* genome. *TWI1* has been implicated in the RNA-guided process of DNA deletion and macronuclear development in mating cells (38). The *TWI2* gene has not been characterized; however, the cDNA sequence has been reported to GenBank (GI:34555686) (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=34555686).

Northern blots of starved and mating *SERH3* and *GRL8* hairpin transformants were hybridized with probes for *TWI1* and *TWI2* (Fig. 3A and B and data not shown). *TWI2* showed an expression pattern similar to that of *DCR2*, with very low levels of expression in uninduced starved cells and high levels in cells treated with Cd to induce hairpin expression. Uninduced mating cells showed higher levels of *TWI2* expression than starved cells, but there was still a marked increase of *TWI2* message in Cd-treated mating cells. *TWI2* expression increased dramatically within 15 to 30 min of hairpin induction but decreased slightly after prolonged induction (Fig. 3B). *TWI2* gene expression shows the same increase in response to induction of the hairpin RNA as *DCR2* gene expression does, suggesting that the two genes may act in the same RNAi pathway. The expression pattern of *TWI1* is similar to that of *DCL1*, showing somewhat increased levels in mating cells treated with Cd prior to mixing. *TWI1* and *DCL1* are both

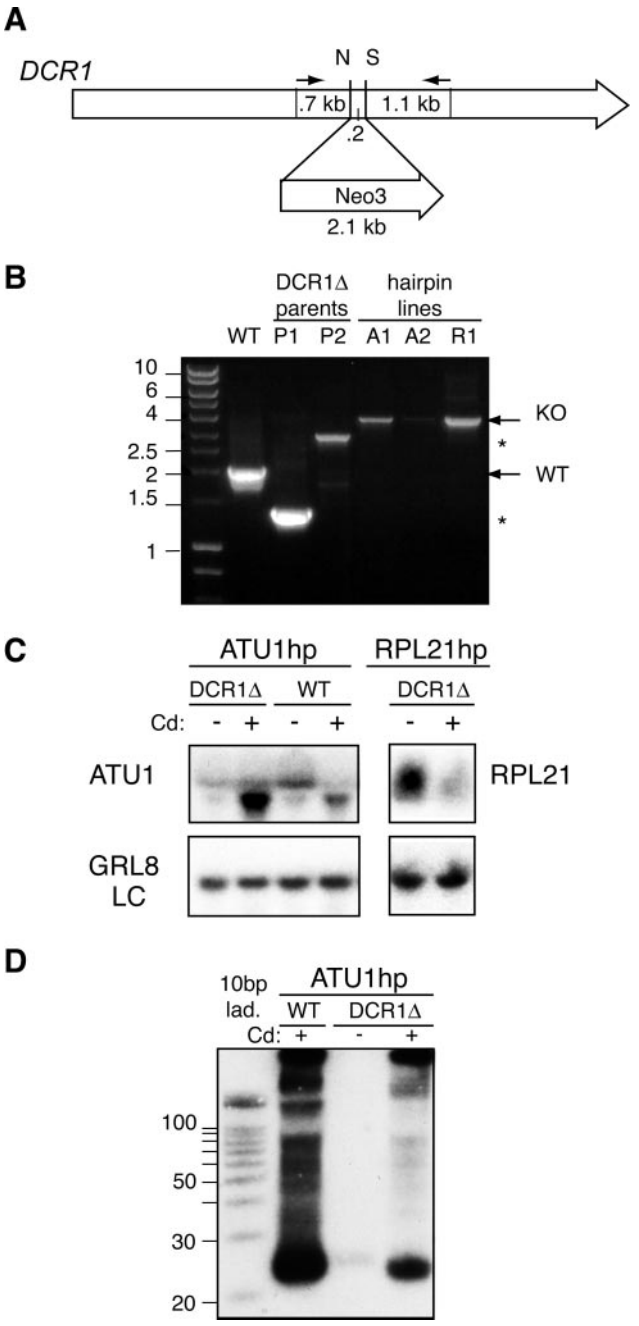

FIG. 5. RNAi occurs normally in *DCR1* knockout cells. (A) Diagram of the *DCR1* locus indicating the insertion of the Neo cassette to disrupt gene expression. The letters N and S indicate NcoI and SpeI restriction sites. The Neo3 cassette eliminates a portion of the helicase domain of *DCR1*. Arrows indicate primers used to amplify the sequence used in the knockout construct and in PCR assays to confirm knockout. (B) PCR assay to confirm *DCR1* knockout in parental and hairpin-transformed lines. The primers indicated in panel A were used to amplify genomic DNA from *DCR1* knockout cells (DCR1Δ) and their progeny, which were transformed with *ATU1* and *RPL21* hairpin constructs. The product from wild-type cells (WT) is 2 kb, while the knockout product (KO) is 4 kb. Parental lines contain deletions of the Neo cassette and therefore show products of different sizes (*). PCR on transformed *DCR1* knockout progeny lines (A1 and A2, *ATU1*hp; R1, *RPL21*hp) produced only the 4-kb knockout product. (C) RNAi occurs normally in *DCR1* knockout cells. Wild-type and *DCR1* knockout cells transformed with *ATU1* and *RPL21* hairpin constructs were starved briefly, induced with 0.05 μg/ml Cd for 2 h, and then harvested. Northern blots were hybridized with probes for *ATU1* and *RPL21* to show mRNA degradation and with a probe for *GRL8* as a loading control. *ATU1* and *RPL21* messages are reduced in both *DCR* knockout and wild-type transformants treated with Cd, and prominent degradation bands can be seen in the *ATU1* samples. (D) *DCR1* knockout cells produce small RNAs normally. Wild-type and *DCR1* knockout cells transformed with the *ATU1* hairpin construct were induced as in panel C, and RNA was harvested for small RNA Northern blotting. Hybridization with oligonucleotide probes complementary to the *ATU1* hairpin detected the 23- to 24-nt RNAs in both wild-type and *DCR1* knockout samples, indicating that *DCR1* is not required for processing the hairpin RNA into small RNAs. 10bp lad., 10-bp ladder.

genes involved in the 28- to 30-nt small RNA pathway that guides DNA deletion, which could explain why these two genes show similar responses to the introduction of large amounts of exogenous dsRNA during mating.

Hairpin RNAs are processed into 23- to 24-nt RNAs. RNA-induced silencing in other organisms is mediated by 18- to 22-nucleotide small RNAs that are created by cleavage of longer double-stranded RNA by the RNase III enzyme Dicer (5). During mating in *Tetrahymena thermophila*, double-stranded RNAs produced by bidirectional transcription of micronucleus-limited sequences are processed into 28- to 30-nucleotide RNAs (10, 38). In vegetatively growing cells, small amounts of 23- to 24-nt RNAs have been identified, but their function is unclear (29). We determined that the long double-stranded RNAs expressed from the hairpin constructs were processed into 23- to 24-nt small RNAs, distinct from the 28- to 30-nt mating RNAs. Total RNA from uninduced and induced transformed strains was isolated and analyzed by electrophoresis in a 20% denaturing polyacrylamide gel. In the wild-type mating cells, the mating-specific 28- to 30-nt small RNAs were clearly visible in the ethidium bromide-stained gels, migrating slightly slower than the 30-nt DNA marker (Fig. 4A). In cells expressing a hairpin RNA, a faint faster-migrating band was visible, suggesting that the long hairpin RNA is processed into small RNAs of approximately 23 to 24 nt in size. In samples from mating cells expressing hairpin RNAs, both sizes of small RNAs could be detected, suggesting that at least two different Dicer proteins are active at this time. Oligonucleotides complementary to the expressed hairpins strongly hybridize to the 23- to 24-nt RNAs in all Cd-treated hairpin samples (Fig. 4B). As a control, the wild-type mating RNA sample was hybridized with oligonucleotides complementary to the M deletion element. This showed weaker hybridization to the 30-nt small RNAs, which is expected given the high sequence complexity of this class of small RNAs. Oligonucleotides complementary to either the sense or the antisense strand of the RPL21 hairpin both hybridized well, indicating that both strands of the hairpin RNA are present in the small RNA population (Fig. 4C).

The roles of *DCR1* and *DCR2* in RNAi. *DCR1* and *DCR2* mRNAs are both upregulated after hairpin induction. Therefore, it seems likely that one or both of these genes are involved in processing the hairpin RNA into the 23- to 24-nt RNAs. To determine if *DCR1* is necessary for RNAi in *T. thermophila*, we transformed homozygous germ line *DCR1*

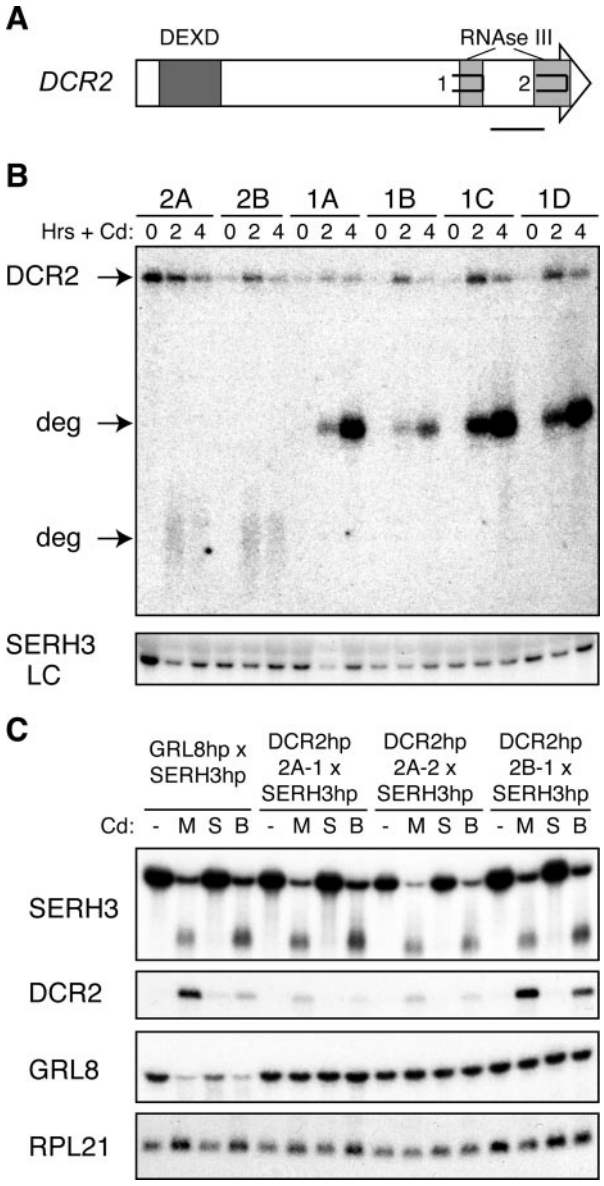

FIG. 6. Knockdown of *DCR2* by RNAi. (A) Diagram of the *DCR2* gene. DEXD helicase and RNase III domains are represented by gray boxes. The regions used for hairpin constructs 1 and 2 are represented by joined parallel lines. The region used to probe for *DCR2* mRNA is indicated by the dark line under the gene diagram. (B) Expression of *DCR2* hairpins 1 and 2 leads to both induction and degradation of *DCR2* mRNA. Cells transformed with *DCR2*hp1 (1A to 1D) and *DCR2*hp2 (2A and 2B) were starved briefly and then induced with 0.05 μg/ml Cd for 2 or 4 h. Northern blots were hybridized with a probe for *DCR2* to determine mRNA levels and with a probe for *SERH3* as a loading control. Degradation products (deg) are visible as smears in the *DCR2*hp2 samples and as distinct bands in the *DCR2*hp1 samples. (C) Preinitiation of RNAi of *DCR2* in mating cells does not affect subsequent RNAi of a second target. Starved *DCR2*hp lines (2A-1, 2A-2, 2B-1) or control *GRL8*hp cells were either left untreated or induced for 2 h and then were washed to remove Cd. These cells were then mixed with *SERH3*hp cells and allowed to mate for 2 h. Then, the mating cultures were divided, and half were induced with Cd again to initiate RNAi of *SERH3*, while the other half continued mating untreated. −, matings of untreated cells; M, cells treated only after mating had begun; S (for "starved"), matings of preinduced cells that were not induced a second time; B (for "both"), matings of preinduced cells that received the second induction. Cells were harvested 2 h after the second induction. Northern blots were hybridized with probes to *SERH3* and *DCR2* to determine message degradation and with probes to *GRL8* to show control RNAi was functioning and to *RPL21* as a loading control.

knockout cells with hairpin RNA constructs targeting *RPL21* and *ATU1*. Induction of hairpin expression in *DCR1* knockout cells caused the same lethality as in WT cells (data not shown), and Northern blotting indicated that mRNA degradation occurred normally, as did the production of 23-nt RNAs (Fig. 5C, D). These results indicate that *DCR1* is not necessary for the production of siRNAs in *T. thermophila*.

Because knockout strains of *DCR2* are not viable, we chose to examine the effect of the reduction of *DCR2* expression by use of RNAi. Wild-type cells were transformed with two different hairpin constructs targeting *DCR2* (Fig. 6A). Some strains expressing the DCR2 hairpins showed slightly slower growth than uninduced cells, but in general, RNAi against *DCR2* was not lethal (data not shown). This is likely due to incomplete silencing, as *DCR2* transcript was still detectable after 4 hours of induction, although large amounts of degradation products were also visible, indicating that the hairpin constructs were effective (Fig. 6B). To test whether RNAi against *DCR2* affected silencing of a second target, we mated *DCR2*hp-expressing cells with *SERH3*hp or *GRL8*hp strains and looked at silencing of the second target, but we saw no change in silencing (Fig. 6C and data not shown). It is likely that expression of the *DCR2* hairpin leads to increased mRNA levels while simultaneously causing degradation of the now more abundant mRNA, which results in a steady state of *DCR2* mRNA that is no lower than that seen when the cell is in its normal "resting" state. For this reason, it is unclear whether *DCR2* is necessary for double-stranded RNA processing and initiation of silencing. However, these results again clearly show that *DCR2* is strongly induced by dsRNA. As indicated by the analysis of *DCR1*, *DCR2*, and *TWI2* expression in response to hairpin induction, it is likely that many genes involved in the RNAi pathway are subject to this inductive effect, which may prevent the analysis of these genes by RNA-induced silencing.

Hairpin expression can lead to deletion of DNA. Previous experiments have shown that injection of double-stranded RNA into *T. thermophila* during the time of macronuclear development can cause deletion of the corresponding DNA sequence in the macronuclear chromosomes, although it is not clear which small RNA is involved (58). To determine whether the expression of the hairpin RNA during mating can also cause DNA deletion, the progeny of hairpin-expressing cells were examined using a PCR assay. Primers were designed to amplify a 1.5- to 2-kb region of DNA spanning the portion of the gene used to create the hairpin construct. These primers were used to amplify DNA from progeny of mating *GRL8*, *SERH3*, *ATU1*, and *RPL21* hairpin-expressing cells. PCR products smaller than expected were indicative of a deletion event and were cloned and sequenced to determine the boundaries of deletion. Deletions were detected in progeny of *GRL8* and *ATU1* hairpin-expressing cells but not in those of *RPL21* or *SERH3* hairpin cells. Deletions were detected in individual progeny lines from isolated pairs or from populations of mat-

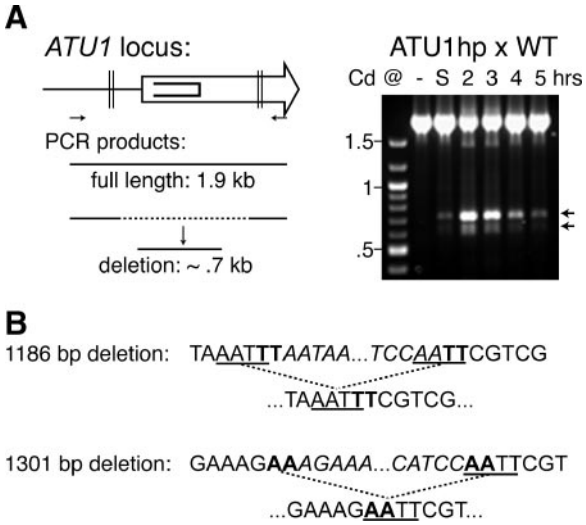

FIG. 7. DNA deletion is caused by hairpin RNAs. (A) PCR analysis of DNA from pools of *ATU1*hp cells mated with WT strain CU428. Mating cells were untreated (−), treated with Cd during starvation prior to mating (s), or treated with Cd at the indicated hour after initiating mating. DNA was prepared from cells 24 h after mixing. In the *ATU1* locus diagram, the expressed gene is represented by an open arrow, with the region corresponding to the hairpin indicated by the joined horizontal lines; annealing locations of PCR primers are indicated by arrows under the gene diagram. Approximate borders of DNA deletion are represented by vertical parallel lines. Next to the PCR assay gel, arrows indicate PCR products consistent with DNA deletion, which are present in all Cd-treated samples. (B) Sequence analysis of deletion products. Two representative deletion boundaries are diagrammed, with the top line showing the WT sequence with deleted bases in italics, microhomologies in bold, and AT sequences underlined. The bottom line shows the sequence after deletion has occurred.

ing cells. In mating populations, *ATU1*hp cells were treated with Cd prior to mating or at 2, 3, 4, or 5 h after the initiation of mating. Cells were allowed to complete mating, and then DNA was harvested. PCR analysis of the *ATU1* locus showed smaller-than-expected products in all mating cells treated with Cd, with the highest amount of deletion product occurring in samples treated at 2 or 3 h after beginning mating (Fig. 7A). Results were similar for *GRL8*hp matings (data not shown). The frequency of deletion events was assayed using cells treated at 2 h after the initiation of mating. Individual mating pairs were isolated 4 h after treatment, and their progeny were analyzed for deletion events. Of the 30 to 40% of isolated pairs from hairpin-expressing cells that produced progeny, only 3 to 6% had a detectable deletion in the locus corresponding to the expressed hairpin. PCR products amplified from this shorter DNA were cloned and sequenced to determine deletion boundaries. Sequence analysis showed the extent of deletion to be greater than the region defined by the hairpin RNA, with deletions spanning 800 to 900 bp in the *GRL8* locus (data not shown), and 1,100 to 1,300 bp in the *ATU1* locus (Fig. 7A). These sizes are similar to those seen for deletions induced by injecting dsRNA, which were often larger than the injected RNA (58). The boundaries of deletion were AT rich and contained microhomologies at the deletion boundaries (Fig. 7B).

DISCUSSION

We have shown that in *Tetrahymena thermophila*, expression of a double-stranded RNA hairpin homologous to a gene leads to a significant reduction of the corresponding mRNA levels. This is most likely due to targeted mRNA degradation by an RNA-induced silencing complex incorporating small RNAs derived from the dsRNA hairpin. Specific mRNA degradation products are observed in Northern blots of targeted transcripts, indicating that silencing is posttranscriptional, although it is possible that transcriptional silencing may occur as well. Small RNA Northern blotting in cells expressing hairpin RNAs reveals the presence of 23- to 24-nt RNAs homologous to the expressed hairpin. These siRNAs can be produced during any phase of the *T. thermophila* life cycle and are distinct from mating-specific small RNAs, which are 28 to 30 nt in length. This evidence shows that in addition to the RNAi-like deletion pathway active during mating, *T. thermophila* has a second small RNA pathway that is responsible for posttranscriptional gene silencing.

The discovery that the introduction of dsRNAs into cells can lead to small RNA-guided mRNA degradation, or RNAi, has led to the development of a powerful genetic tool (18). In many model organisms, methods are available for the introduction of dsRNAs that are then incorporated into the RNAi pathway, leading to specific gene silencing. This technique is not only useful for studying the function of specific genes of interest but is readily adaptable to high-throughput genome-wide screens (19, 27). We have now developed an effective method for delivering double-stranded RNA in *Tetrahymena thermophila* for the purpose of gene silencing, or RNAi. This technique will facilitate the study of gene functions as an alternative to gene knockout by homologous recombination and will be especially useful in the study of essential genes. Currently, the only method available to study essential genes in *T. thermophila* is to produce a heterokaryon in which the gene of interest is disrupted in the micronucleus but remains intact in the macronucleus (24). When heterokaryon knockout cells are mated, the resulting progeny will have null alleles in both nuclei and can be studied for only a few divisions before death (7). Conditional alleles can be created by transforming heterokaryon knockouts with a construct expressing the essential gene from the inducible *MTT1* promoter (44). However, these methods are laborious and have some limitations. The inducible RNAi technique we describe here allows for the silencing of any gene of interest at any point in a cell's life cycle. This is effective for constitutively expressed genes such as *SERH3* and *GRL8*, for developmentally expressed genes such as *TWI1*, and for essential genes such as *RPL21* and *ATU1*. As demonstrated in this study, silencing initiated during starvation is carried over into mating, allowing one to study the effect of the loss of function of a gene during mating but maintain gene function during normal cell growth. The RNAi effect is also cytoplasmically dominant, which enables the simultaneous silencing of two different genes in mating cells. RNAi strategies will also be useful as genetic screening methods for *T. thermophila*. Previous methods of screening using antisense ribosome technology have been effective, and RNAi is likely to expand on these results because it can also be used against essential genes (11, 46).

Some evidence suggests that DNA deletion in *T. thermophila* serves as a form of genome defense against invasive DNA elements (34, 58). It is possible that RNAi serves as another form of genome defense, as in other organisms. Transposons and viruses often produce RNAs that are incorporated into small RNA pathways, and many RNAi genes are necessary for transposon suppression in *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Chlamydomonas reinhardtii* (2, 28, 47, 54). In *Arabidopsis*, DCL2, DCL4, and DCL3 have been shown to have specific functions in virus and transposon silencing (14, 31, 55). In all of these organisms, repetitive elements are a common trigger of silencing mechanisms. This could be the case for *T. thermophila* as well. Small RNAs cloned from vegetatively growing cells mapped to 12 clusters of highly related genes of unknown function in the macronuclear genome. Distinct transcripts from these genes are not detectable by Northern blotting. The function of the small RNAs is not clear, but one explanation is that they could be part of a mechanism for silencing these "repetitive" genes (29). The discovery of an RNA-guided gene silencing mechanism in *T. thermophila* suggests that this organism, too, could regulate the activity of repetitive or invasive genetic elements. Between the action of DNA deletion and RNAi, *T. thermophila* may have evolved a very effective double surveillance system to protect itself from genome invasion.

This study has also offered some insight into the cellular response to an "invasive" RNA species. *DCR1*, *DCR2*, and *TWI2* are present at relatively low levels throughout the life cycle of *T. thermophila*. Expression of the dsRNA hairpin in the cell leads to a dramatic increase in mRNA levels of these genes. This suggests that the cell is able to mount a very specific and rapid response to the presence of dsRNA and alludes to the presence of a signal transduction pathway capable of detecting dsRNA and activating the transcription of RNAi genes. Rapid upregulation of Dicer and Argonaute genes has not been reported to take place in other organisms, although specific RNA-dependent RNA polymerases have been shown to be induced by viral infection in plants (60). This immediate and robust response may be particularly critical to protozoans, which ingest foreign biological materials, including genetic elements, into the cell as food. In *Tetrahymena*, the immediate action of RNAi complements the stable long-term effect offered by DNA deletion.

Evidence from this study hints at how two RNAi pathways can function simultaneously in *T. thermophila* during conjugation. Because the small RNAs that accumulate after hairpin induction are of a size distinctly different from that of the mating-specific small RNAs (23 to 24 nt versus 28 to 30 nt), it is likely that different Dicer proteins produce them. Dcl1p is required for the production of the 28- to 30-nt small RNAs and localizes primarily to the meiotic micronucleus (35, 39). It is clear that one of the other Dicer proteins, likely Dcr2p, is responsible for processing the hairpin RNA into the 23- to 24-nt siRNA. Although the subcellular localization of Dcr2p is not known, it could be in the cytoplasm, as Dicer proteins involved in gene silencing in other eukaryotes are (30). We suggest that spatial restriction of the proteins and RNA molecules in each small RNA pathway allows the separate functions of gene silencing and DNA deletion to remain distinct. Thus, only those dsRNAs produced in the micronucleus (or the newly developed macronucleus) are processed into 28- to 30-nt small RNAs and cause DNA deletion; those produced from the macronucleus, such as the hairpin RNA studied here, are processed into the 23- to 24-nt siRNA and lead to mRNA degradation. This hypothesis is supported by analysis of progeny of hairpin RNA-expressing cells, which shows that deletion of the DNA sequence homologous to the expressed hairpin occurs at a very low frequency, and for some genes, was never detected at all. This suggests that the mechanisms for keeping the two pathways separate in *T. thermophila* are quite effective, even when faced with the production of high levels of dsRNA. This is in contrast to dsRNA-induced deletion in *Paramecium tetraurelia*, which is quite robust and correlates directly with the levels of 22- to 23-nt RNAs (23). The efficiency of deletion caused by the hairpin RNA could also be subject to epigenetic regulation by the old macronucleus, as the presence of a sequence in the macronucleus can reduce or prevent deletion of the homologous sequence in the micronucleus (9). In *Tetrahymena*, however, this inhibition of deletion is rarely complete, even when the macronucleus is loaded with high copy numbers of the normally deleted sequences.

The small degree of DNA deletion caused by the RNA hairpin observed in this study is clear and points to an interesting crossover of the two pathways. Although we have failed to detect any 28- to 30-nt small RNA with the hairpin sequence in cells induced during conjugation, it is technically possible that a very small amount of the hairpin RNA is processed into the 28- to 30-nt form. In this case, the crossover would be due to leakage of dsRNA from one compartment into another. A perhaps more interesting possibility is that some 23- to 24-nt siRNA is used in the DNA deletion pathway, thus implying that molecules from one pathway might interact with or even interfere with those in the other. Deletion in the dsRNA injection study reported earlier can be examined under this new light (58). In that study, dsRNA was injected into the cytoplasm of mating cells and DNA deletion was observed at high rates, sometimes in greater than 50% of the progeny. Since a massive amount of RNA was injected and the cell was highly stressed, both nuclear leakage and mistargeting of small RNAs are likely to occur. Although not a certainty, it is probable that both classes of small RNA were produced from the injected dsRNA.

Ciliates display one of the most unusual epigenetic inheritance phenomena. A genetic element in a cell's somatic nucleus can determine or influence the passage of the same element from the germ line to the next somatic genome (9, 15, 23). Recent studies have revealed the role of DNA deletion in this process, and models that use small RNA to communicate between the somatic nucleus and the germ line nucleus have been proposed (8, 10, 23, 38, 58). The realization that dsRNA produced in these two nuclei follow different small RNA pathways provides an important basis for further understanding of this phenomenon. Clearly, putting a genetic element in the wrong compartment (e.g., micronucleus-specific sequences in the macronucleus) could alter the small RNA it may produce. This alteration could potentially interfere with the activity of the normal form in a sequence-specific manner. The binucleate nature of *Tetrahymena thermophila* perhaps has facilitated the evolution of distinctive RNAi effects using different compart-

ments, and it provides a special platform for understanding their interactions.

ACKNOWLEDGMENTS

We are grateful to Nina Salama, Sue Biggins, and Nels Elde for critical reading of the manuscript. We thank members of the Yao lab for helpful discussions and Aida Flor de la Cruz for help with experiments.

National Institutes of Health grant GM26210 supported this work.

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