

The Conjugation-Specific Die5 Protein Is Required for Development of the Somatic Nucleus in both *Paramecium* and *Tetrahymena*[∇]

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Development in ciliated protozoa involves extensive genome reorganization within differentiating macronuclei, which shapes the somatic genome of the next vegetative generation. Major events of macronuclear differentiation include excision of internal eliminated sequences (IESs), chromosome fragmentation, and genome amplification. Proteins required for these events include those with homology throughout eukaryotes as well as proteins apparently unique to ciliates. In this study, we identified the ciliate-specific *Defective in IES Excision 5 (DIE5)* genes of *Paramecium tetraurelia* (*PtDIE5*) and *Tetrahymena thermophila* (*TtDIE5*) as orthologs that encode nuclear proteins expressed exclusively during development. Abrogation of PtDie5 protein (PtDie5p) function by RNA interference (RNAi)-mediated silencing or TtDie5p by gene disruption resulted in the failure of developing macronuclei to differentiate into new somatic nuclei. *Tetrahymena* Δ *DIE5* cells arrested late in development and failed to complete genome amplification, whereas RNAi-treated *Paramecium* cells highly amplified new macronuclear DNA before the failure in differentiation, findings that highlight clear differences in the biology of these distantly related species. Nevertheless, IES excision and chromosome fragmentation failed to occur in either ciliate, which strongly supports that Die5p is a critical player in these processes. In *Tetrahymena*, loss of zygotic expression during development was sufficient to block nuclear differentiation. This observation, together with the finding that knockdown of Die5p in *Paramecium* still allows genome amplification, indicates that this protein acts late in macronuclear development. Even though DNA rearrangements in these two ciliates look to be quite distinct, analysis of *DIE5* establishes the action of a conserved mechanism within the genome reorganization pathway.

The biology of ciliates offers an extreme case of differential regulation of separate copies of the genome (see reference 34). These protists possess two morphologically and functionally distinct types of nuclei coexisting within a common cell (reviewed in references 20 and 43). The somatic macronucleus is polygenomic and transcriptionally active, whereas the germinal micronucleus is diploid and transcriptionally inert throughout the vegetative growth cycle.

When ciliates undergo development during the sexual phase of the life cycle, the existing somatic macronuclei disappear and new micro- and macronuclei arise from germ line-derived precursors. These genetically identical precursor nuclei are formed upon the conjugation of two mating-compatible partners, which induces meiosis of germ line micronuclei. The products of meiosis and a postmeiotic division are two haploid gametic nuclei in each conjugate, one of which is exchanged between partners. The migratory pronucleus fuses with the stationary copy to form a diploid zygotic nucleus that subsequently divides and differentiates. The new micronuclei are maintained in a silent state, while the differentiating macronuclei acquire extensive chromatin modifications that mediate

regulated gene expression. In some ciliate species, nuclear differentiation can alternatively occur through a self-fertilization process called autogamy, which occurs without cell pairing and exchange of genetic material, but nonetheless starts with meiosis and leads to the production of new micro- and macronuclei.

Macronuclear differentiation includes genome-wide DNA rearrangements that extensively remodel the developing somatic chromosomes. The processes of chromosome fragmentation and DNA elimination dramatically alter the genome found in the mature somatic macronucleus after conjugation. Different ciliate species eliminate anywhere from 15% to 90% of the germ line (micronucleus-limited) DNA during this process (reviewed in references 43 and 57). In addition to these physical alterations, the genome is endoreplicated to tens or even thousands of copies per macronucleus, a ploidy level that varies between species.

The DNA segments eliminated from the developing macronucleus are called internal eliminated sequences (IESs), which can vary in structure both within a species and between different species. In *Paramecium tetraurelia*, IESs are generally short (26 to 883 bp) and have a modestly conserved 8-bp inverted repeat that has some similarity to the termini of the mariner/Tc1 superfamily of transposable elements (22). Excision of these IESs is precise, leaving a single copy of the 5'-TA-3' dinucleotide in the macronucleus-retained sequences. The mechanism generates double-stranded breaks at both ends, followed by joining of the macronucleus-destined DNA (see references 5 and 19). On the other hand, IESs in another

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ciliate, *Tetrahymena thermophila*, are relatively large (0.6 to >20 kb) and show substantial heterogeneity in their excision boundaries. The elimination of the *Tetrahymena* IESs shows some similarity to the imprecise elimination of transposon-like sequences from the developing genome of *Paramecium* (24). Unlike the *Paramecium* IESs, those in *Tetrahymena* show no obviously conserved sequence characteristics at their excision boundaries. The model proposed for their excision also differs from that of *Paramecium*. Evidence from the characterization of rearrangement intermediates suggests that a double-stranded break at one end of the IES is followed by transesterification of the freed end to the other boundary, generating the macronuclear junction (45, 46).

Despite substantial variability in the size, structure, and excision mechanisms of IESs in different ciliate species, there is a growing body of molecular evidence that IES excision in both *Tetrahymena* and *Paramecium* involves an RNA interference (RNAi)-like pathway (17, 34, 36, 38, 56). This pathway is more extensively characterized for *Tetrahymena*. A large pool of germ line-derived small (27- to 30-nucleotide [nt]) RNAs is generated by cleavage of RNA produced by extensive, bidirectional transcription within meiotic micronuclei (11, 36). These "scan RNAs" (scnRNAs), generated by the Dicer-like protein Dcl1p (30, 37), direct methylation of histone H3 on lysine 9 (K9) and/or lysine 27 (K27), marking the IESs at the beginning of nuclear differentiation for later removal from the genome (26, 50). A role for small RNAs has also been identified in *Paramecium* and possibly in *Stylonychia*, but it is less clear if these act by directing specific chromatin modifications on or near IESs (17, 21, 25). Further studies and comparative analyses should reveal additional biochemical pathways that mediate these remarkable genome reorganization processes. For example, a recent study discovered a domesticated piggyBac transposase in *Paramecium* that is required to remove the IESs from the developing macronuclei and reported that a similar, developmentally expressed transposase gene is contained within the *Tetrahymena* genome (4).

In this report, we show that a gene named *Defective IES Excision 5* (*DIE5*) is expressed during sexual reproduction and is required for removal of germ line-specific sequences from the *Paramecium* genome. Although significant sequence identity with *DIE5* could not be found in most other eukaryotes, we identified a candidate homologue in *Tetrahymena thermophila* (*TiDIE5*) that is also developmentally regulated. Disruption of *TiDIE5* revealed that it is also required for IES excision and chromosome breakage. Further investigations demonstrated that loss of *TiDIE5p* does not appear to disturb well-characterized steps in macronuclear development, such as small-RNA accumulation and formation of DNA rearrangement foci. Comparison of our results for both species revealed a conserved role for *Die5p* in the formation of the ciliate macronucleus yet also uncovered significant differences in the action of *Die5p* between these organisms. We believe this type of comparative study has the power to discern key conserved features of ciliate genome reorganization.

MATERIALS AND METHODS

Cell lines and culture. *Paramecium tetraurelia* stock d4-110 (*hr-b/hr-b*) was used to generate cultures undergoing synchronized conjugation for RNA isolation and whole-cell PCR analysis. Strains d4-502 (*pwA-502/pwA-502*; *nd6-1/*

nd6-1) and a3093 (*pwB-96/pwB-96*; *nd9-c/nd9-c1*) (from Mihoko Takahashi, University of Tsukuba) were used for genetic analysis. Elsewhere, *nd6* (*nd6-1/nd6-1*) was used as a wild-type control. *Paramecia* were cultured at 27°C as described by Sonneborn (49) in 1.25 to 2.5 g Austrian winter pea (Outsidepride) in 800 ml double-distilled water (ddH₂O) buffered with K-DS (4 mM sodium citrate, 2.8 mM sodium phosphate dibasic, 1.2 mM potassium phosphate monobasic, 1.5 mM calcium chloride) supplemented with 1.25 mg/liter stigmasterol and inoculated with *Klebsiella pneumoniae* 1 to 2 days prior to use (53). *Tetrahymena thermophila* stocks B2086, CU427, and CU428.1 were cultured axenically in Neff's (0.5% dextrose, 0.25% yeast extract, 0.25% proteose peptone, 3.3 mM FeCl₃) or SPP (0.2% dextrose, 0.1% yeast extract, 1% proteose peptone, 0.003% Sequestrene) medium at 30°C as described previously (18, 42).

For conjugation, mating-reactive *Paramecium* cells in starvation medium were mixed at a density of >2,000 cells/ml and incubated at 27°C. Conjugating cells were enriched for by the methods described by Yang and Takahashi (55) and Vosskuhler and Tiedtke (54), which resulted in cultures 80 to 99% pure for conjugating *Paramecium*. Conjugating *Tetrahymena* cells were prepared for mating by washing out growth medium and culturing them in 10 mM Tris-HCl (pH 7.4) for >6 h. Mixing cultures of complementary mating types at equal cell density cultures with mating efficiencies of 80 to 95% (29).

Total RNA isolation. Total RNA was isolated from 50 to 100 ml of *Paramecium* cell culture (100 to 1,000 cells/ml) with an RNeasy minikit (Qiagen) supplemented by a QIAshredder for cell homogenization and an RNase-free DNase set (Qiagen) for elimination of genomic DNA. All products were used according to the manufacturer's directions. RNA was extracted from 10 to 20 ml *Tetrahymena* cell culture (2 × 10⁵ cells/ml), using RNAzol extraction (14). Total RNA was used in reverse transcription (RT)-PCR to monitor expression in wild-type and *ΔDIE5* lines, using *TiDIE5* oligos 5'-GTTTATGTTTTCTAATTGAGCTT T-3' and 5'-CTGGTATAATCATTAAATGCTCG-3' or HHP1 (*Tetrahymena* HP1 gene) oligonucleotides 5'-GGAGCTTCAACTCATTAACACG-3' and 5'-TCGGGAGAAGCATACTTAGCA-3', which amplify 252-bp or 371-bp cDNA products, respectively.

Microinjection and observation of GFP fluorescence. *Paramecium* plasmids containing the green fluorescent protein (GFP)-*DIE5* fusions were derived from pZC'ΔRI (kindly provided by Eric Meyer, CNRS, Paris, and Jean Cohen, CNRS, Gif-sur-Yvette). Plasmid p5AGN5At contains a 652-bp upstream region, the GFP gene, and the full-length open reading frame (ORF) of *DIE5a* (we designated the gene identified by differential display as *DIE5a* and its paralog as *DIE5b*) followed by the 330-bp *DIE5a* downstream region. Plasmid sequences are available on request. Approximately 2 μl of plasmid solution (~5 μg/μl) in distilled water was injected into the macronuclei of *Paramecium* cells as previously described (32). GFP-expressing cells were fixed and stained with propidium iodide (Vector Laboratories, Burlingame, CA) as described previously (32). Confocal microscopy was performed with a Bio-Rad MRC 1024 UV/Vis system.

To examine *Die5p* localization in *Tetrahymena*, the *TiDIE5* coding region (genome coordinates CH445530:323,516 to 324,279) was amplified and cloned into the pENTR-D plasmid to create pENTR-*DIE5*, which is compatible with Gateway recombination cloning (Invitrogen). Subsequently, LR Clonase II was used to recombine the *DIE5* coding sequence into a destination vector containing an MTT1-inducible GFP expression cassette cloned upstream of a cycloheximide-resistant *rpl29* allele. This construct was linearized with HindIII in the flanking *rpl29* sequences and introduced into starved *Tetrahymena* cells by biolistic transformation. Transformants were selected in SPP medium containing 12.5 μg/ml cycloheximide. To induce GFP-*DIE5* expression, 0.08 μg/ml CdCl₂ was added to mating cells 3.5 h postmixing. Cells at 6 h to 14 h postmixing were fixed in 2% paraformaldehyde, counter stained with DAPI (4',6-diamidino-2-phenylindole), and visualized, using a Nikon model Eclipse E600 microscope outfitted with a QImaging Retiga EX CCD camera driven by Openlab image acquisition software (Improvision).

RNAi and phenotypic observation. A cDNA fragment corresponding to the region of *DIE5a* between a HincII site and the polyA addition site was cloned into pL4440 (52). (Note that a 23-bp segment in this region perfectly matches *DIE5b* and therefore likely silences both paralogs). RNAi experiments were performed by feeding *Paramecium* *Escherichia coli* producing double-stranded RNA as previously described (<http://Paramecium.cgm.cnrs-gif.fr/RNAi/>). Conjugation or autogamy of RNAi-treated cells was induced within 48 h of this feeding. Conjugating pairs were isolated in fresh culture medium for phenotypic analysis. For genetic studies, exconjugants were isolated and grown separately for ~10 cell divisions prior to observation of phenotypes. To observe the phenotypes of the F₂ generation, ~10 starved F₁ cells were transferred to fresh culture fluid and allowed to grow for additional cell divisions, and their phenotype was scored after autogamy (self-fertilization). Nuclear DNA was stained with propidium

iodide in Vectashield after cells were fixed with 4% paraformaldehyde as described previously (32).

Micronuclear and macronuclear Δ DIE5 strains. A *Tetrahymena DIE5* knock-out construct was generated, using a MultiSite Gateway cloning kit (Invitrogen). *DIE5* upstream (814 bps; nts 322753 to 323567 of contig CH445530) and downstream (761 bps; nts 324295 to 325056 of contig CH445530) flanking sequences were amplified by PCR and cloned into the Gateway donor vectors, pDONR-P4-P1R and pDONR-P2R-P3, respectively, using BP recombinase. The *MTTI-NEO* (2,079 bps) selection cassette derived from pMNBL (47) was amplified and cloned into donor plasmid pENTR-D by a topoisomerase-mediated reaction. These three donor plasmids were mixed in equal molar ratios with the destination vector pDEST-R4-R3 and LR Clonase Plus, and the resulting recombination created the gene disruption vector pKO-*TIDIE5*.

Plasmid pKO-*TIDIE5* was digested with StuI and introduced by biolistic transformation into either starved CU427 and CU428 populations (macronuclear transformation) or mating B2086 and CU428 populations (germ line transformation) 2.5 to 3.5 h postmixing as described previously (7, 8). Putative transformants with *DIE5* disrupted (Δ DIE5) within either their macronuclei or both their macro- and micronuclei were selected by growth in the presence of paromomycin. Micronuclear knockouts were verified by crossing original transformants to CU427 to test for segregation of the *MTTI-NEO* cassette among the cycloheximide-resistant progeny. The heterozygous micronuclear Δ DIE5 transformants were crossed to the star strains B*VI and B*VII to generate homozygous micronuclear Δ DIE5 and Δ DIE5 micronuclei/wild-type macronuclei heterokaryons. For both somatic and germ line transformants, which initially contained a mixture of wild-type and Δ DIE5 alleles in their macronuclei, cells were subcloned and cultured in growth medium containing increasing concentrations of paromomycin until only mutant alleles remained, thus producing complete macronuclear-knockout strains. The elimination of the *DIE5* gene in the macronucleus and micronucleus was confirmed by PCR and Southern blot hybridization analysis as described previously (30).

Whole-cell PCR amplification. PCR amplifications were performed on whole-cell *Paramecium* as described previously (32) (see Fig. 3).

Northern and Southern blot analyses. DNA from *Paramecium* cultures was isolated as previously described (23). *Tetrahymena* genomic DNA was isolated from 1×10^6 to 2×10^6 cells, using a Wizard genomic DNA isolation kit (Promega), followed by resuspension in 10 mM Tris-HCl by incubation at 65°C for 1 h or at 4°C overnight. Northern and Southern blots were performed as described previously (30). Probes for Northern hybridization were derived from cloned cDNA fragments containing whole *Paramecium* or *Tetrahymena DIE5* ORFs. Plasmids used to generate Southern probes were pSA2.1HP for the 2.1-kb HincII and PstI or 1.5-kb BglII-PstI fragments of the macronuclear *A-51* allele or p4578c containing a 787-bp fragment of the *A-51* allele generated by PCR, using a forward primer (5'-GGATCTGTGGATCAACTAG-3') and a reverse primer (5'-CTGATAGCGTATTTGGATTAG-3') with total genomic DNA from ex-conjugant cells (see probes in Fig. 3A). This reaction amplifies the circularized IES4578 of the *A-51* allele that is present transiently in the genomic DNA of cells during sexual reproduction. To examine the *Tetrahymena DIE5* locus in the knockout lines, isolated genomic DNA was digested with BstBI and separated on 0.9% agarose gel at 40 V overnight. The probe used for analysis of the knockout lines was isolated from pDONR-Die5, created for generating the pKO-TIDie5 plasmid. To assess failure of chromosome breakage, total genomic DNA isolated from wild-type or Δ DIE5 cells after 16 h of mating was digested with EcoRI, fractionated, and probed with a 0.8-kbp fragment that spans the EcoRI site at position 335013 of chromosomal scaffold CH445662.

Fluorescence microscopy. For examining the nuclear morphology of knockout strains and for cellular localization of GFP-Die5p, cells were fixed in 2% paraformaldehyde and stained with DAPI (1 μ g/ml) for 10 to 30 min. Cells were then immobilized under 22- by 22-mm coverslips in 5 μ l of 2% methylcellulose. For the visualization of DNA elimination structures, an integrative PDD1-YFP fusion construct was introduced into *Tetrahymena* cells by biolistic transformation. Conjugating transformants induced with 0.05 μ g/ml CdCl₂ were fixed with 2% paraformaldehyde at 14 h postmixing and were counter-stained with DAPI. For histone modification analysis, 9-h conjugating cells were fixed with Schaudinn's fixative (2 parts saturated mercuric chloride to 1 part 95% ethanol) and dehydrated with methanol. The cells were then rehydrated with Tris-buffered saline (TBS) and blocked in 1% bovine serum albumin (BSA) plus 0.01% Tween 20. Anti-H3K9me2 rabbit polyclonal (Upstate Biotechnology) and anti-H3K27me3 mouse monoclonal (Abcam) antibodies were used at 1:500 dilution for immunostaining. Secondary antibodies used were Alexa Fluor 488-conjugated anti-rabbit and anti-mouse antibodies (1:1,000; Invitrogen).

Nucleotide sequence accession numbers. The nucleotide sequences of the *Paramecium DIE5a* and *DIE5b* genes are present in the GenBank database

under accession numbers 124427424 and 124429605, respectively. The *Tetrahymena DIE5* gene is found on genomic scaffold scf_8254365 under GenBank accession number CH445530. The *Tetrahymena DIE5* gene is designated TOTHERM_00686240, and the protein ID in GenBank is EAS04981.1. Preliminary *Paramecium* and *Tetrahymena* genome sequence data were obtained from Genoscope (<http://www.genoscope.cns.fr/>) and the J. Craig Venter Institute (formerly the Institute for Genomic Research; <http://www.jvri.org/>), respectively (2, 13).

RESULTS

***Paramecium DIE5* encodes a novel, developmentally expressed nuclear protein.** As the genome remodeling that creates the somatic macronucleus is a major event in *Paramecium* development, we used differential display to identify proteins expressed exclusively during conjugation, as these are candidates that promise to be important for this nuclear differentiation (32). Upon further examination of the expression of individual candidate genes by Northern blot analysis, one in particular exhibited a dramatic increase in its mRNA abundance at 13 h into conjugation (Fig. 1A), and overall its expression closely corresponded with the known timing of IES excision (10 to 22 h, with a peak at 14 h) (6, 23). We also detected a low level of expression in starved cell populations, but this is likely derived from developing cells spontaneously undergoing autogamy (self-fertilization).

We named this promising candidate Defective IES Excision 5 (*DIE5*), due to its gene knockdown phenotype described below. *DIE5* is predicted to encode a 199-amino-acid (aa) protein with a molecular mass of 24 kDa. A nearly identical protein is encoded by a second locus in the *Paramecium* genome (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Paramecium/>). This copy is clearly a *DIE5* paralog derived from the recent whole-genome duplication in the *Paramecium* lineage (2), as the two genes are remarkably similar (86% nucleotide identity; 98% amino acid identity) and both loci contain homologous copies of the neighboring gene, *NMD3*, present in the same orientation. Therefore, following the nomenclature convention for *Paramecium* genes (1), we designated the gene identified by differential display as *DIE5a* and its paralog as *DIE5b*. (We refer to both genes below simply as *DIE5*, given that 196 of 199 aa are conserved between them, and we used the *DIE5a* sequence in the design of all functional experiments described.) Analysis of the *Paramecium tetraurelia* Die5p amino acid sequence revealed no conserved protein domains or motifs except for two classical nuclear localization signals (NLSs); however, an identifiable homologue was found in the genome of the ciliate, *Tetrahymena thermophila* (described below). (When necessary to distinguish the *Paramecium* and *Tetrahymena* genes, we add the prefix *Pt* or *Tt* before *DIE5*). Thus, *DIE5* appears to be a novel gene that is conserved within the oligohymenophora lineage.

The two putative NLSs, along with the timing of expression, suggested to us that *DIE5* may encode a nuclear protein that participates in macronuclear differentiation. To further investigate this possibility, we examined Die5p localization by fusing GFP to its N terminus in a transgene expressed from the *DIE5* promoter. *Paramecium* cells containing this transgene showed no detectable GFP fluorescence during logarithmic growth, starvation, or the early stages of conjugation. However, consistent with *DIE5* mRNA expression (Fig. 1A), GFP-Die5p

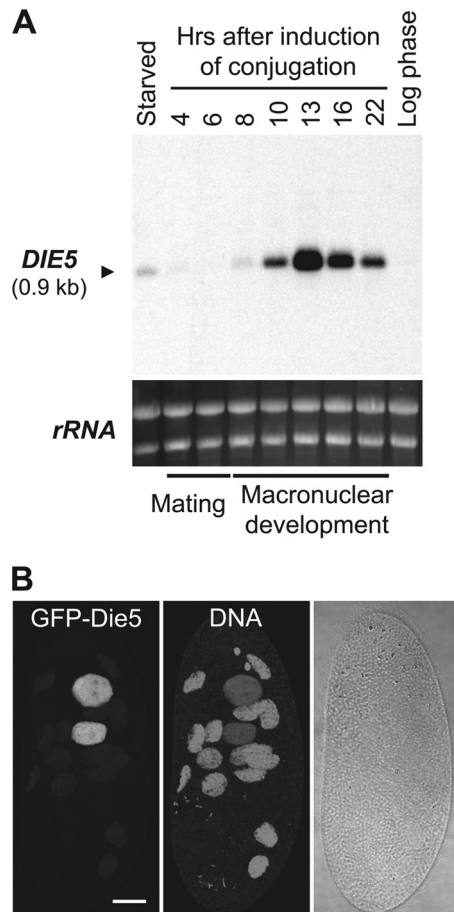


FIG. 1. *DIE5* is a developmentally regulated gene encoding a nuclear protein in *Paramecium*. (A) Northern blot of total RNA (20 μ g per lane) from *Paramecium* probed with *Paramecium DIE5a*. Ethidium bromide staining of rRNA was used as a loading control. (B) Cellular localization of GFP-Die5a expressed from an extrachromosomal DNA driven by the *DIE5a* promoter. Fluorescent images are projections of optical sections obtained by confocal microscopy. The GFP fluorescence exclusively localized to the new macronuclei, which show weaker propidium iodide staining (DNA) than the old macronuclear fragments. The image corresponds to a cell approximately 14 h after the start of mating. The bar corresponds to 20 μ m.

was observed in cells by 7 h after the initiation of mating. The fusion protein was found to localize exclusively within the developing macronuclei but not in the fragments of old macronuclei of conjugating or autogamous cells (Fig. 1B and data not shown). Thus, *PtDIE5* encodes a developmentally expressed protein that appears to act in differentiating macronuclei.

Silencing of *PtDIE5* inhibits formation of a functional macronucleus. To investigate whether the expression timing and localization of Die5p is indicative of a function in macronuclear development, we knocked down *DIE5* expression, using RNAi-mediated gene silencing, by feeding *Paramecium* cells *E. coli* expressing double-stranded RNA corresponding to a fragment of *DIE5* (16). To assess the level of knockdown achieved by this approach, we monitored protein expression of GFP-Die5p in transformed cells, using GFP-specific antibodies, which provided a proxy for endogenous expression. West-

ern blot analysis showed as substantial reduction in GFP-Die5p levels, to $\sim 35\%$ of that observed upon feeding cells *E. coli* transformed with the empty RNAi vector (data not shown).

To determine whether *DIE5* is essential to complete development, we knocked down expression in mating cells that were genetically marked to allow true progeny to be distinguished from the parental lines. The two parental cell lines used in this experiment were each homozygous for a recessive allele at different loci (*pwA* or *pwB*; see Materials and Methods). Since successful conjugation of these cells generates F_1 progeny that are heterozygous at all loci, these F_1 exhibit a wild-type phenotype (genotype *PWA/pwA*, *PWB/pwB*). Both *DIE5* RNAi-treated and control cultures (i.e., cells fed bacteria containing the empty RNAi vector) produced high percentages of viable cells (78 and 97%, respectively), but only the control cells gave rise to true progeny that were phenotypically wild-type, whereas all viable cells of *DIE5* RNAi-treated cells exhibited the parental mutant phenotypes (Fig. 2A). Thus, loss of Die5p resulted in failure to form new macronuclei.

We visually followed the *DIE5* RNAi-treated conjugants throughout development to ascertain whether the lack of sexual progeny was caused by a failure in prezygotic events (e.g., meiosis, nuclear exchange, or karyogamy) or in events associated with postzygotic differentiation of the new macronucleus. Control matings that produced wild-type cells generated exconjugants with two macronuclear anlagen that eventually segregated to the daughter cells at the first postmating cell division (~ 18 h after induction of conjugation). In contrast, cytological observations of *DIE5* RNAi-treated cells (examined 30 and 36 h after induction of conjugation) showed a dramatic reduction in cells with differentiating macronuclei, as only 2 to 8% of exconjugants had two new macronuclei, while 12 to 23% had no macronuclear anlagen at all (Fig. 2B and C). The no-macronucleus cells are expected to include those that did not survive conjugation (lethal). The increase in no-macronucleus cells at 54 h may also include cells that entered autogamy shortly after conjugation. This is not possible in a normal mating but could occur in cells that have undergone macronuclear regeneration. Despite these nuclear abnormalities, most *DIE5* RNAi-treated conjugants proceeded through the first postconjugative cell division. These results are substantially different from those for silencing *UBA2* (which encodes a SUMO-activating enzyme), where most cells were arrested with two micronuclei and two macronuclei (32). Together with the genetic analysis of exconjugants, these results reveal that knockdown of *DIE5* expression blocks the completion of macronuclear development.

Despite the defects observed upon *DIE5* knockdown, these cells still exhibited a high level of viability upon exit from conjugation. This observation suggests that *DIE5* RNAi treatment likely induced the alternative developmental pathway of parental macronuclear regeneration (MR), which can occur in *Paramecium* when new macronuclei fail to form. This pathway has been observed upon silencing of other genes during conjugation (32, 41). Normally in wild-type cells, old macronuclear fragments remain in the exconjugants and are transcriptionally active for several postconjugative cell divisions. DNA replication no longer occurs, though, and these fragments are typically lost within 8 to 10 cell divisions (either actively or by

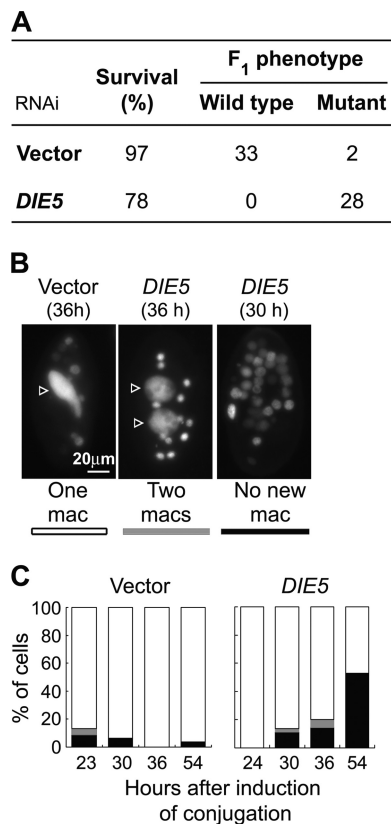


FIG. 2. Silencing of *DIE5* disrupts nuclear events during conjugation. (A) Progeny from conjugation treated with RNAi. Survival (%) and phenotype of the marker genes are shown. Both parental strains for conjugation were homozygous for Mendelian recessive mutant alleles of different marker genes (*pwA* and *pwB*; required for ciliary reversal); thus, successful conjugation should produce the wild-type phenotype of backward swimming, while failure in either nuclear exchange, fertilization, or formation of the new macronucleus should result in the mutant phenotype, i.e., no backward swimming upon stimulation. (B) Representative DAPI-stained *Paramecium* exconjugants with normal and defective cytological phenotypes observed after RNAi treatments. Each picture contains a single cell with or without the new macronucleus (arrowheads) and old macronuclear fragments. Phenotypic classes were assigned to white, gray, or black, as indicated below the pictures and plotted in the graphs in panel C. mac, macronucleus.

dilution). When the new macronucleus is incapable of division after sexual reproduction, one or more of the parental macronuclear fragments regenerate into a single macronucleus that is again capable of DNA replication, amitotic division, and transcription. After MR, the macronuclear genotype is the same as that of the parental lines (e.g., a mutant for *pwA* or *pwB*), while the micronuclear genotype is heterozygous, as nuclear exchange occurs between conjugates. To determine whether MR had occurred, several cells that survived *DIE5* knockdown during conjugation were followed into autogamy to reveal their micronuclear genotypes. The resulting F₂ lines of the *DIE5* RNAi F₁ survivors produced wild-type (as well as mutant) progeny, which demonstrates that the F₁ cell lines contained heterozygous micronuclei. Thus, the prezygotic events of conjugation (i.e., meiosis and nuclear exchange) must

have occurred normally in the *DIE5* RNAi-treated cells, and the surviving F₁ were the result of MR.

DIE5 is required for *Paramecium* IES excision. The failure to form mature macronuclei after *DIE5* RNAi treatment despite successful formation of the zygotic nucleus raised the possibility that this protein is important for the removal of IESs. To examine the effect of *DIE5* knockdown on IES excision, we first employed a PCR-based approach to specifically amplify micronucleus-derived sequences. By locating one PCR primer for each amplicon within an IES and its partner in the flanking macronucleus-destined DNA, we could distinguish DNA in the macronuclear anlagen (and new micronuclei) from the abundant, rearranged DNA of the old macronuclear fragments. The abundance of the resulting PCR products (named “pp1,” “pp2,” and “pp3” in Fig. 3A) should increase as anlagen DNA is amplified and decrease as the IESs containing the primers are excised (Fig. 3B, vector lanes). In addition, as small IESs are contained within the PCR amplicons, we could follow their fates, as their excision prior to removal of the IESs containing the primer sites generated smaller products observed as faster-migrating bands in the gels (pp1s, pp2s, and pp3s in Fig. 3A and B). RNAi of *DIE5* showed a gradual increase in the amount of full-length (IES-containing) PCR product over the developmental time course, indicating that loss of *DIE5* does not inhibit developmental DNA amplification (Fig. 3B). Nevertheless, we saw no evidence of shorter products, which are readily detectable in control cells, that would indicate IES excision had occurred.

To further demonstrate that loss of *DIE5* blocked IES excision, we used Southern blot hybridization to analyze the rearrangement status of the new macronuclei. For this study, total DNA was isolated from large cultures of postautogamous cells at a point when about 50% of well-fed control cells (treated with the empty RNAi vector) had undergone the first cell division. The DNA was digested with *SspI*, which has frequent recognition sites in IESs but only one in the coding region of the *A-51* allele. The probe containing macronuclear DNA from the A gene (*HincII*-*PstI* in Fig. 3A) detected only the macronuclear form of this region (indicated by the 5.7-kbp band in Fig. 3C) in DNA from control cells (Fig. 3C, labeled vector). In contrast, two additional bands of 1.3 and 2.0 kb were observed in DNA from *DIE5* RNAi-treated cells. These are the sizes expected for amplification of the unprocessed *A-51* gene (Fig. 3C). Furthermore, using a probe for IES4578 (Fig. 3A) that contained only micronucleus-limited DNA, we detected a single 500-bp DNA fragment, which corresponds to the IES-containing locus in DNA from *DIE5* RNAi-treated cells but not from control cells (Fig. 3C). The unprocessed DNA can be observed in these experiments because amplification of DNA continues in the developing macronuclei despite the absence of IES excision. Together the results show that knockdown of *DIE5* expression inhibits IES excision in the developing macronuclei of *Paramecium*, yet the DNA is amplified to levels comparable to that for normal developing macronuclei.

Tetrahymena DIE5 is required for macronuclear differentiation. The PtDie5p sequence contained no conserved domains that offered clues to its biochemical function; however, we did find a putative *DIE5* homologue (*TtDIE5*) encoded within the *Tetrahymena thermophila* genome. These two ciliate proteins are similar in size (199 and 207 aa, respectively) and share 21%

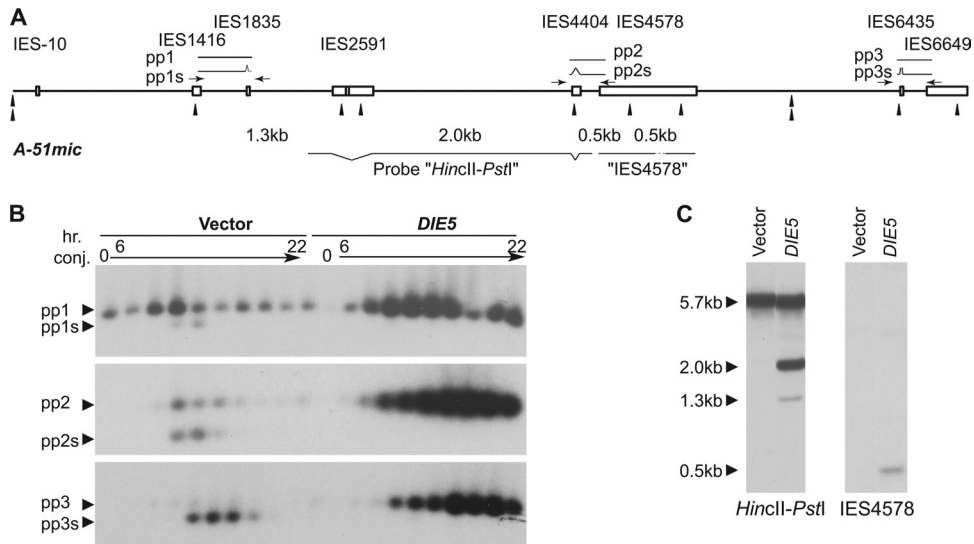


FIG. 3. Silencing of *DIE5* inhibits excision of IESs. (A) A partial map of the micronuclear version of the *A-51* allele showing locations of IESs (boxes), SspI recognition sites (arrowheads), and sizes of fragments generated by SspI digestion. Positions of expected PCR products (pp1 to pp3) and probes for Southern hybridization are also indicated. Arrows show positions of the primers relative to IESs not drawn to scale. (B) Whole-cell semiquantitative PCRs of RNAi-treated exconjugants, using one primer in the macronuclear sequence and the other primer inside the IESs. Each lane represents whole-cell PCR products taken at 2-h intervals from 6 to 22 h after induction of conjugation. The predicted PCR products correspond to pp1 to pp3 in panel A. Due to excision of smaller IESs during rearrangement, two bands are expected for each primer set. (C) SspI-digested total genomic Southern blots of RNAi-treated exautogamous cells probed with either a HincII-PstI fragment or IES4578, as indicated in panel A. Total DNA (~10 μg) was isolated from an exautogamous cell culture when about 50% of control cells (RNAi using empty vector) had undergone the first cell division. Most IESs in the micronuclear version of the *A-51* allele contain SspI sites (single arrow heads in panel A), while only one site is present in the macronuclear-destined sequence of the *A-51* allele (the double arrow head in panel A). Thus, for probe HincII-PstI, 1.3- and 2.0-kb fragments are expected for unprocessed DNA, while a 5.7-kb fragment is expected for the processed DNA, including abundant old macronuclear DNA in exconjugants. Probe IES4578 contains only the IES sequence and detects 0.5-kb fragments if the IES is present at high levels in exautogamous cells.

amino acid identity throughout their coding regions (Fig. 4A). In addition, when the sequences were analyzed for predicted secondary structures using PSIPRED (33), a common domain structure that predicted beta sheets and alpha helices in the form β1α1α2β3β4 was revealed within the first 110 aa of both proteins (data not shown). Intriguingly, our Northern blot analysis revealed that *TtDIE5* is expressed exclusively during conjugation, providing data to support that this *Tetrahymena*

gene may have a function similar to that of its *Paramecium* counterpart. *TtDIE5* expression was first detected 4 h into conjugation, which corresponds to the end of meiosis, and peaked at 6 h, when new macronuclei first emerge. Expression levels declined slightly at 8 h but continued at a low level until the end of macronuclear development (about 15 h after cells first paired) (Fig. 4B). The expression pattern we observed is nearly identical to recently published microarray data (35).

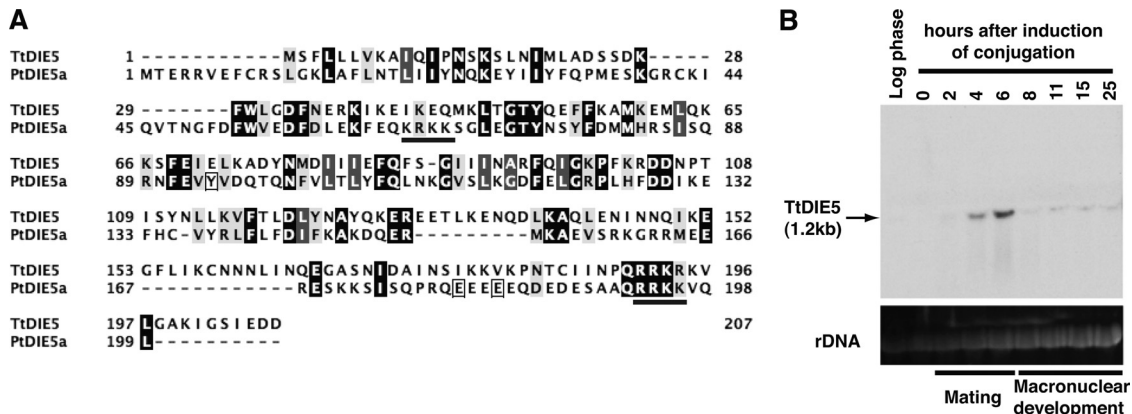


FIG. 4. *Tetrahymena* homologue (*TtDIE5*) is developmentally expressed. (A) Pairwise alignment of *Paramecium* Die5 protein from paralog a (*PtDIE5a*) and *Tetrahymena* Die5p sequences. The boxes around three amino acids indicate differences between the two *Paramecium* sequences (the differences are C, D, D, respectively). The underlined regions indicate potential nuclear localization signals in the *Paramecium* sequence. (B) Northern blot analysis of total RNA (20 μg per lane) extracted from growing (log), starved, or conjugating cells (between 2 and 25 h after mixing populations of compatible mating types). Ethidium bromide staining of rRNA was used as a loading control.

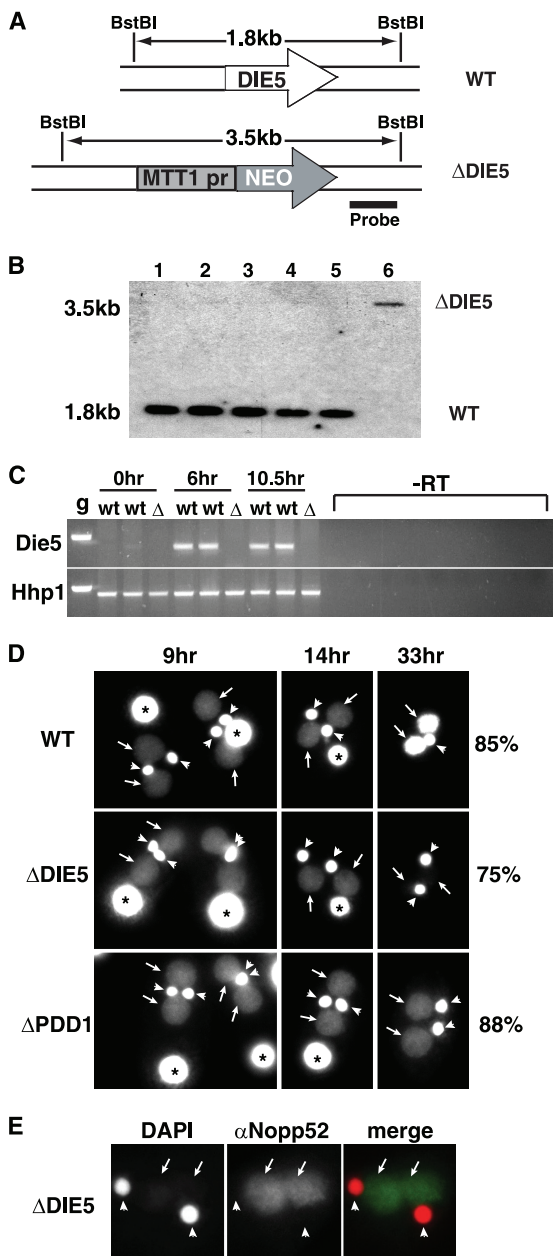


FIG. 5. *TiDIE5* zygotic expression is essential to complete conjugation. (A) Diagram of the gene disruption construct showing replacement of the coding sequence with the *neo3* selectable cassette (47). Restriction enzyme sites (*Bst*BI) and the region corresponding to the radiolabeled probe fragment used for Southern blot analysis are indicated. WT, wild type; MTT1pr, metallothionein gene 1 promoter. (B) Southern blot hybridization of DNA isolated from the wild type (lane 1), *DIE5* micromuclear-knockout lines (lanes 2 to 5), and the *DIE5* complete knockout line (lane 6) using the probe shown in panel A. Longer exposure of the blot reveals the 3.5-kb band in *DIE5* micromuclear-knockout samples corresponding to the two copies of *DIE5* disrupted by the *neo3* cassette in their micromuclei. (C) RT-PCR for expression of *DIE5* transcripts in conjugating wild-type (wt) and *DIE5* complete knockout (Δ) cells at indicated time points. The bottom panel shows control RT-PCR with *HHP1* primers. Both *DIE5* and *HHP1* primers span an intron of their respective genes. g, *Tetrahymena* genomic DNA used as a control for amplification. (D) Fluorescent images of representative DAPI-stained wild-type (WT), micromuclear-knockout (Δ *DIE5*), and Δ *PDD1* strains at 9 h, 14 h, and 33 h postmixing. For each mating strain, the percentage of cells exhibiting their

Our RNAi knockdown experiments showed that Die5p is essential for macronuclear differentiation in *Paramecium*. If *TiDIE5* is a homologue of the *Paramecium* protein, it is likely to have an essential role in *Tetrahymena* development as well. To investigate this possibility and learn more about Die5p function, we used homologous gene replacement to disrupt *TiDIE5*. A knockout construct (Fig. 5A) consisting of the *neo3* selectable cassette (45) flanked on each side by DNA sequences from immediately upstream and downstream of the *TiDIE5* locus was introduced by biolistic transformation into starved or conjugating *Tetrahymena* cells to disrupt the macromuclear or micromuclear copies, respectively. Paromomycin-resistant transformants were obtained by both strategies and were subsequently cultured to generate full macromuclear knockouts or homozygous micromuclear-knockout lines (see below and Materials and Methods). Genetic crosses of strains lacking all macromuclear *DIE5* copies produced viable progeny (data not shown), indicating that *DIE5* expression prior to transcriptional activation of developing macromuclei is not required to complete conjugation. In contrast, attempts to generate complete knockout strains (lacking *DIE5* in both micro- and macromuclei) by crossing heterozygous germ line (micromuclear) knockout strains were unsuccessful. One-quarter of the progeny resulting from these crosses should have been homozygous knockouts (in both nuclei), yet only homozygous wild type or heterozygous knockout strains were found among the >30 viable progeny screened (data not shown). This finding provided the first indication that *TiDie5p* is critical for development, as is the *Paramecium* protein.

As strains lacking all macromuclear *DIE5* copies grew normally and produced viable progeny when mated, the inability of heterozygous *DIE5* micromuclear knockouts to produce homozygous knockout progeny must result from the loss of zygotic *DIE5* expression during macromuclear differentiation. To allow us to further investigate Die5p's role during *Tetrahymena* development, we generated homozygous micromuclear-knockout heterokaryon strains of different mating types by performing genomic exclusion crosses between heterozygous micromuclear knockouts and two different star strains, B*VI and B*VII, which have defective micromuclei. These abortive matings resulted in the transfer of a haploid micromucleus from the *DIE5* knockout to its star strain partner without inducing new macromuclear development, such that after pair separation and micromuclear endoreplication, both exconjugants were homozygous in their germ line. The resulting paromomycin-sensitive exconjugants (the star strain partner) with homozygous knockout micromuclei and wild-type macromuclei were identified by genomic locus PCR (data not shown) and Southern blot analysis (Fig. 5B) and then verified by genetic crosses (data not shown). Herein we refer to these cell lines as *DIE5* micro-

respective arrest phenotype at 33 h is indicated on the right. Asterisks, arrows, and arrowheads indicate old/parental macromuclei, new macromuclei, and the micromuclei, respectively. (E) The nuclear envelope remains intact in arrested Δ *DIE5* micromuclear-knockout cells. Postconjugative Δ *DIE5*mic cells were fixed with 2% paraformaldehyde and stained with α Nopp52 antibody and DAPI. New macromuclei and micromuclei are indicated as described for panel D.

nuclear knockouts or $\Delta DIE5mic$ (these cells were used for most experiments; therefore, all figures labeled $\Delta DIE5$ refer to micronuclear knockouts). Phenotypic assortment of the paromomycin-resistant, homozygous, micronuclear-knockout exconjugants allowed us to generate complete (micro-/macronuclear) knockout strains (referred to herein as *DIE5* complete knockouts). We confirmed the loss of all *DIE5* copies and expression during conjugation by Southern blot analyses and RT-PCR (Fig. 5B and C), respectively. The ability to generate *DIE5* complete knockouts confirms that Die5p is dispensable for vegetative growth.

$\Delta DIE5mic$ strains are paromomycin sensitive but are homozygous for the *DIE5::NEO3* allele in their silent micronuclei, so that when mated, their progeny, if viable, would be paromomycin-resistant. To measure the ability of these germ line knockout strains to produce $\Delta DIE5$ progeny, $>10^6$ post-conjugative cells were cultured in growth medium containing paromomycin (plus $CdCl_2$). In most trials, all cells died upon the addition of the drug, providing further support that zygotic expression of *DIE5* is essential for the completion of conjugation. We did obtain paromomycin-resistant cells for some matings (up to two survivors per 10^6 mating pairs). We interpret this to mean that some cells express sufficient Die5p from their wild-type parental macronuclei to provide for the essential functions of this protein late in macronuclear differentiation. To further test this possibility, we created an N-terminal GFP-*TtDie5p* transgene expressed from the strong, cadmium-inducible *MTT1* promoter, integrating the construct upstream of the macronuclear *rpL29* genomic locus in the $\Delta DIE5mic$ strains. Expression of this transgene by the addition of cadmium during conjugation increased progeny survival by 3 to 4 orders of magnitude. Thus, inducing additional Die5p from the parental macronucleus partially rescued the loss of *DIE5* zygotic expression from macronuclear anlagen, which provided clear evidence that loss of *DIE5* expression is the cause of lethality upon mating $\Delta DIE5mic$ cells and that the GFP-*TtDie5p* fusion is functional. We were also able to increase survival of $\Delta DIE5mic$ conjugates by introducing an rDNA-based expression vector by electroporation that carries the GFP-*TtDIE5* transgene at ~ 9 h into conjugation, an observation which further supports that this protein is required relatively late in macronuclear development.

Tetrahymena conjugation can be readily staged by the configuration of nuclei within mating pairs or exconjugants (31). To begin to ascertain why mating $\Delta DIE5mic$ cells fail to produce viable progeny, we fixed and DAPI-stained cells to monitor their development. For most of conjugation, the progression of $\Delta DIE5mic$ conjugates was indistinguishable from that of wild-type mating cells as they completed meiosis, karyogamy of gametic nuclei, and the subsequent nuclear divisions to generate macronuclear anlagen (Fig. 5D). Mating pairs separated, and their nuclear morphology appeared normal until ~ 14 h of conjugation, after which it became evident that most knockout cells arrested their development. The last visible event to be triggered during conjugation is the elimination of one of the two micronuclei in each conjugant. Wild-type cells remain with the characteristic nuclear configuration of one micronucleus and two new macronuclei (Fig. 5D, 33 h) until they are fed, at which point they divide their one remaining micronucleus and undergo a specialized postconjugative cyto-

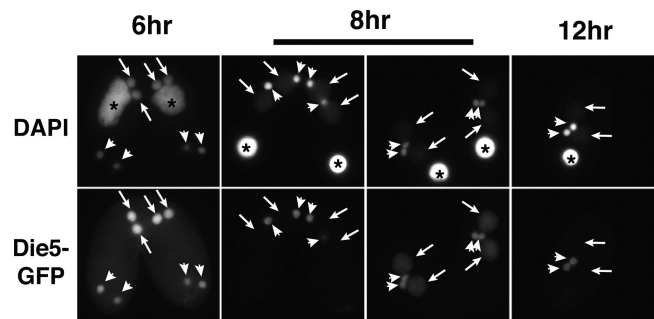


FIG. 6. Cellular localization of GFP-Die5p in conjugating *Tetrahymena* cells at 6 h, 8 h, and 12 h. The cells were fixed with 2% paraformaldehyde and counterstained with DAPI. Asterisks, arrows, and arrowheads indicate old/parental macronuclei, new macronuclei, and the micronuclei, respectively.

kinesis to partition one micro- and one macronucleus to each daughter. In contrast, the $\Delta DIE5mic$ conjugates failed to trigger micronuclear resorption, arresting with two micro- and macronuclei, and were unable to divide when returned to growth medium.

The two micro-/two macronuclei arrest phenotype has been described for knockouts of several genes required for programmed DNA rearrangement, including $\Delta PDD1$ strains (Fig. 5D, bottom panels). Such mutant strains not only fail to eliminate one micronucleus, but unlike wild-type cells, stop amplification of the DNA in the new macronuclei (Fig. 5D, compare the DAPI-staining intensity of $\Delta PDD1$ cells to that of the wild type at 33 h). Intriguingly, not only did $\Delta DIE5mic$ exconjugates stop anlagen DNA amplification, but the majority of cells (75%) actually lost most of the DNA content of the anlagen, as indicated by the loss of DAPI staining between 14 and 33 h postmixing (Fig. 5D). The macronuclear structure appeared to remain intact, as immunofluorescence to detect a nucleolar protein, NOPP52, revealed the integrity of the nuclear compartment (Fig. 5E). We are unsure of the mechanism of DNA loss, as attempts to detect chromosome degradation by the presence of unprotected ends revealed that unlike the degrading old macronucleus, these new macronuclei do not label in terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays (data not shown). Nevertheless, the loss of macronuclei in the $\Delta DIE5mic$ exconjugates is consistent with the *Paramecium DIE5* RNAi phenotype in which anlagen form but fail to become the macronuclei of the surviving exconjugants.

As the *DIE5* expression profile and knockdown/knockout phenotypes were very similar in both *Paramecium* and *Tetrahymena*, we expected that *TtDIE5* would also encode a protein localized primarily to macronuclear anlagen. However, we were surprised when we examined the localization of the GFP-*TtDie5p* fusion used in the rescue experiments described above. Although GFP-*TtDie5p* was detected in the developing macronuclei during early stages of macronuclear development (Fig. 6, 6-h to 8-h arrows), this localization was rapidly lost as conjugation progressed, before the period when the essential zygotic expression would occur. In contrast, we observed the GFP fluorescence within micronuclei through all stages of conjugation (Fig. 6, arrowheads). Therefore, while Die5p is a

developmentally expressed nuclear protein in both ciliates, their localization patterns suggest some differentiation in their action.

Zygotic expression of *DIE5* is required for programmed DNA rearrangement in *Tetrahymena*. Transcription of *TiDIE5* was induced by 4 h into conjugation (Fig. 4A) and peaked at 6 h; thus, it was somewhat unexpected that we found that the macronuclear copies were dispensable. Additionally, complete-knockout cells lacking *DIE5* from both macro- and micronuclei exhibit the same phenotype as the germ line knockouts, which further confirms that the critical *TiDIE5* expression occurs from macronuclear anlagen. This suggests that *DIE5* functions at late stages of *Tetrahymena* conjugation when DNA rearrangement occurs. To determine whether zygotic *Die5p* is required for *Tetrahymena* DNA rearrangements, we monitored IES excision and chromosome breakage in $\Delta DIE5$ mic exconjugants. To assess the process of IES excision, we examined the elimination of the M IES, which is a >1-kbp sequence located on micronuclear chromosome 4 (3, 9). This IES has two equally used left deletion boundaries that are 300-bp apart, such that successful IES excision generates two alternative products through the elimination (Δ) of either 0.6 kbp or 0.9 kbp from the locus (Fig. 7A). These two forms can be easily distinguished by a PCR-based IES excision assay, using primers designed to amplify across the IES. By crossing $\Delta DIE5$ mic lines together or with wild-type strain B2086, each of which contains only the M Δ 0.9-kbp rearranged form in their macronuclei, we could test for appearance of the M Δ 0.6-kbp deletion as an indicator of new rearrangement in the anlagen. Whereas the slower-migrating PCR product indicative of the M Δ 0.6-kbp deletion was detected when each $\Delta DIE5$ mic line was crossed to B2086, we saw no evidence of M-element rearrangement in crosses of the two $\Delta DIE5$ mic strains (Fig. 7B).

Since RNAi knockdown of *Paramecium DIE5* blocked chromosome fragmentation (data not shown) as well as IES excision (Fig. 3), we tested whether $\Delta TiDIE5$ mic knockouts also fail to fragment chromosomes. We isolated DNA from post-conjugative $\Delta DIE5$ mic cells and used Southern blot analysis to examine chromosome fragmentation at a site which lies 2.2 kbp downstream of the *LIA1* gene (Fig. 7C) (30). During conjugation, breakage occurs at this sequence, followed by the addition of 300 bp to 400 bp of telomeric DNA during growth. Restriction digestion of genomic DNA with *EcoRI* allows simultaneous detection of the unprocessed micronuclear form of this locus at 10.5 kbp, the fragmented chromosome of parental macronuclei with fully elongated telomeres that migrates at 2.5 to 2.6 kbp, and the 2.2-kbp form that results from *de novo* breakage and minimal telomere addition (Fig. 7C). Whereas this 2.2-kbp fragment was easily observed in genomic DNA samples from wild-type matings, the DNA from $\Delta DIE5$ mic conjugants showed no evidence of chromosome fragmentation, as the predominant band observed was ~2.5 kbp, which represents DNA from parental macronuclei of unmated cells in the population (Fig. 7D). These results together with experiments with *Paramecium* (data not shown) indicate that *DIE5* is required for IES excision and chromosome fragmentation in both ciliates.

Critical events leading to DNA rearrangement are unaffected in $\Delta DIE5$. IES excision is guided by small RNAs. In *Tetrahymena*, it is known that these small RNAs target H3K9

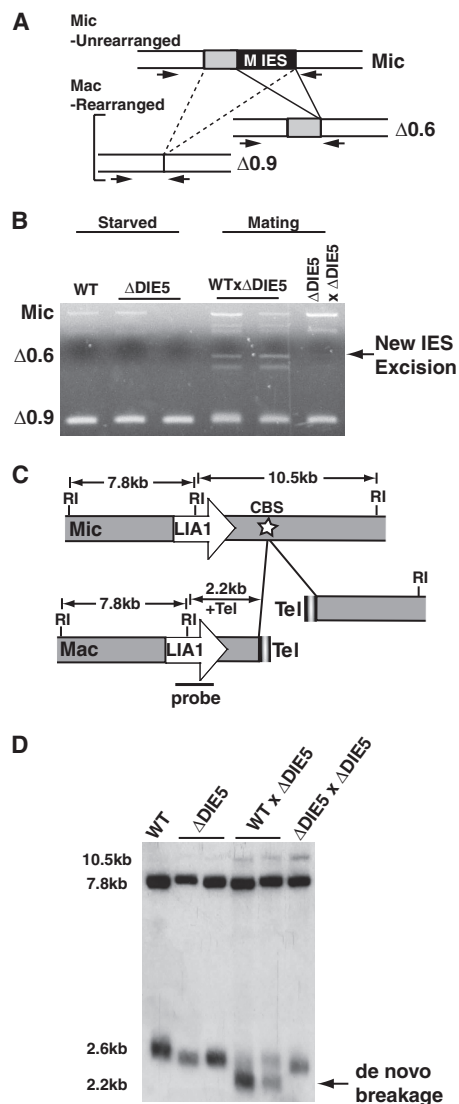


FIG. 7. Germ line *TiDIE5* is required for *Tetrahymena*-programmed DNA rearrangement. Total genomic DNA was isolated from starved wild-type (WT) and *DIE5* micronuclear-knockout strains ($\Delta DIE5$), as well as from cells 16 h after crosses of *DIE5* micronuclear knockouts to the wild type (WT \times $\Delta DIE5$) and two *DIE5* micronuclear knockouts ($\Delta DIE5 \times \Delta DIE5$). The DNA was used for PCR-based IES excision assays (A and B) or Southern blot analyses for chromosome breakage (C and D). (A) Schematic of PCR-based IES excision assay strategy. Arrows denote forward and reverse primers used to amplify across the M element. Alternative rearrangement products resulting from deletion of 0.6-kbp (Δ 0.6) or 0.9-kbp (Δ 0.9) are shown. (B) M-element excision PCR. Arrow indicates new IES excision. (C) Diagram shows the macronuclear chromosomal scaffold surrounding the *LIA1* gene, which lies within 2.2 kbp of a chromosomal-breakage sequence (CBS) (white star). Relevant *EcoRI* (RI) restriction sites used for the Southern blot analysis are shown. The probe spans the central *EcoRI* site and detects a 7.8-kbp fragment common to both nuclei as well as to either the 10.5-kbp micronucleus-specific fragment or a 2.5- to 2.6-kbp macronucleus-specific fragment (2.2 kbp of unique sequence plus 300 to 400 bp of telomeric DNA). Tel, telomere. (D) Southern blot analysis to assess chromosome breakage. Arrow indicates the product of *de novo* breakage.

and K27 methylation to IES chromatin, which is bound by the Pdd1 and Pdd3 chromodomain-containing proteins that are reorganized into distinct foci that are hypothesized to be the site of IES excision (reviewed in reference 10). The timing of

these events has been well described, and proteins required for many of these steps have been identified (11, 26, 36, 50). To investigate the role of *DIE5* in *Tetrahymena* DNA rearrangement, we examined these events to determine whether they are perturbed by the loss of *DIE5*.

Production of developmental-specific small RNAs occurs during meiosis, before the emergence of zygotic expression. As zygotic, not somatic, expression of *DIE5* is essential for conjugation in *Tetrahymena*, we expected that *DIE5* would not be required for their biogenesis or accumulation. We isolated small RNAs from conjugating complete-*DIE5*-knockout cells, and as predicted, we found that the levels of total small RNAs and those homologous to the M IES observed were comparable in wild-type and Δ *DIE5*mic conjugating cells (data not shown).

We also examined whether H3K9 and H3K27 methylation was perturbed by the loss of *DIE5*. Although establishment of these marks occurs during the time that we observed GFP-TiDie5p to localize to developing macronuclei, deposition of these modifications in conjugating *DIE5* knockout cells appeared to be unaffected (Fig. 8A). Therefore, Die5p is not required for the establishment of these heterochromatic modifications in *Tetrahymena*, further suggesting that the essential function of *DIE5* occurs at later stages of conjugation.

The chromodomain-containing protein Pdd1p is an essential component of the DNA rearrangement machinery and a major constituent of DNA rearrangement foci (12, 27). Disruption of genes required for IES excision can block the formation of these foci (26, 44). To investigate chromatin reorganization upon loss of Die5p, we examined the localization of a Pdd1p-YFP fusion expressed in Δ *DIE5*mic cells. Even though these mutant lines fail to excise IESs, Pdd1p foci in the developing macronuclei appear to form, as we observed in wild-type conjugants, albeit with a slight delay in their maturation (Fig. 8B). Given that all events prior to IES excision described to date appear normal in Δ *DIE5* knockouts, we suspect that Die5p acts after these known events.

DISCUSSION

Orthologous *DIE5* genes in *Paramecium* and *Tetrahymena* are essential for macronuclear development. Research over the past 10 years has revealed that some key components of the genome reorganization pathway in ciliates are well-known proteins in other eukaryotic organisms. Examples include proteins of the RNA interference pathway, histone-modifying enzymes, and transposases (4, 26, 30, 36). Other studies have identified proteins with recognizable domains, and yet clear orthologs cannot be identified, even in other ciliate genomes (58). *DIE5* is a member of a third group, proteins that are both conserved among ciliates (*Paramecium* and *Tetrahymena*) but novel to this class of organisms. This group may contain core components that account for the unusual precision and efficiency of ciliate genome reorganization. Our results provide strong evidence that *Paramecium* and *Tetrahymena* encode orthologous Die5 proteins. Although the amino acid identity between the proteins is modest (21%), each is the top reciprocal BLAST hit in comparisons of their respective genomes. Additionally, the

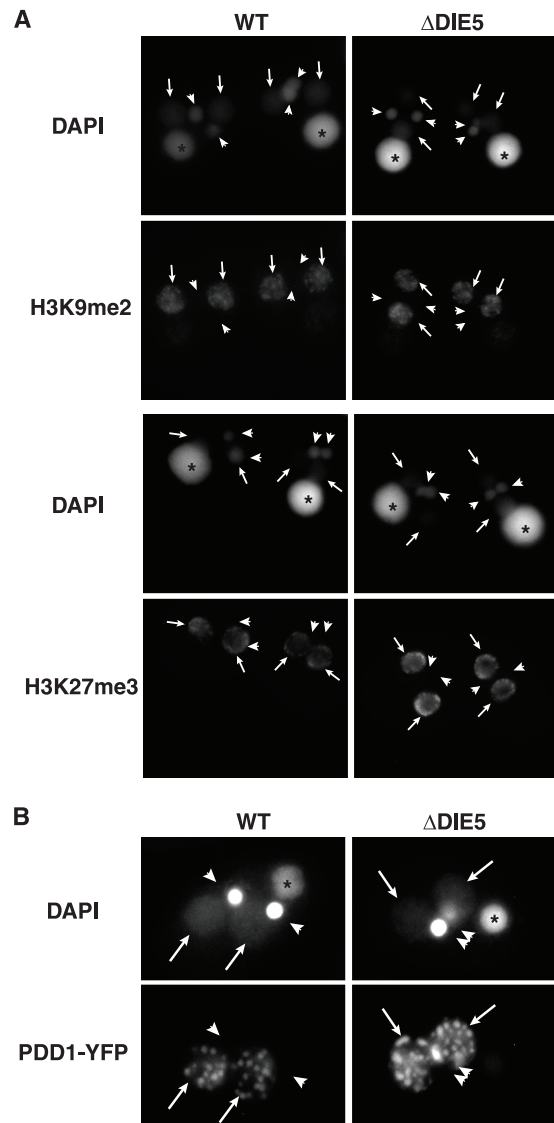


FIG. 8. Germ line knockout of *TiDIE5* does not inhibit critical events leading to IES excision. (A) H3K27me3 and H3K9me2 histone mark deposition is unaffected in *TiDIE5* germ line knockouts. Nine-hour-conjugating wild type (WT) and micronuclear-knockout (Δ *DIE5*) cells were fixed with Schaudinn's fixative and stained with either H3K27me3 or H3K9me2 antibodies at 9 h into conjugation. The cells were counter stained with DAPI. (B) Formation of PDD1 foci is not disrupted in Δ *DIE5*mic cells. Conjugating wild-type and *DIE5* micronuclear-knockout cells (Δ *DIE5*) expressing an inducible *PDD1*-YFP transgene were fixed at 14 h with 2% paraformaldehyde and counter stained with DAPI. Asterisks, arrows, and arrowheads indicate old/parental macronuclei, new macronuclei, and the micronuclei, respectively.

amino acid identity is not centered in a single region, as expected for a shared domain; rather, it is spread across the entire coding region. Both are small, nuclear proteins (199 aa and 207 aa in *Paramecium* and *Tetrahymena*, respectively) expressed exclusively during conjugation. Finally, RNAi knockdown (*Paramecium*) and gene disruption (*Tetrahymena*) demonstrate that these Die5 proteins are essential for genome rearrangements in their respective species. To-

gether the data demonstrate that *DIE5* encodes a conserved component of the macronuclear development pathway in ciliates.

Die5 is one of a few conserved ciliate proteins known to be required for DNA rearrangements. Previous studies have identified several developmentally regulated proteins that are required for genome rearrangements in *Paramecium* or *Tetrahymena*. These include chromatin-associated proteins (27, 28, 39, 40, 48, 58), a transposase (4), components of the RNAi pathway (30, 36, 37) and SUMO pathways (32), and a putative RNA binding protein (41). Of these identified components, only the RNAi-associated proteins, piggyMac transposase, and SUMO components show clear homologues between these different ciliates. In contrast, the Pdd and Lia (localized in macronuclear anlagen) DNA rearrangement proteins of *Tetrahymena* do not have obvious homologues in *Paramecium* (58). This search for homologues is complicated by the significant sequence diversity between *Paramecium* and *Tetrahymena*, a point that was evident even before complete-genome sequences were available (reviewed in reference 15). Thus, failure to detect homologues based on primary sequence data is not definitive, and one must consider the possibility that protein three-dimensional structure is maintained with minimal sequence identity. Functional homology is the most relevant criteria, but few of the proteins involved in these rearrangements have known biochemical roles. For those that do, such as the *Tetrahymena* chromodomain-containing Pdd1 and Pdd3 proteins that have been shown to associate with IES chromatin-containing histone H3 methylated on lysine 27 and/or lysine 9, respectively, evidence for an analogous role in *Paramecium* DNA rearrangements is lacking (26, 50). The Nowa proteins in *Paramecium* are required for elimination of germ line transposons and a subset of IES that are controlled by maternal effects (41). The N-terminal domains contain repeated elements with similarity to RNA binding motifs in other species, and evidence of nucleic acid binding activity was obtained. While there are *Tetrahymena* proteins, including CnjBp (41, 51), that share similar glycine-rich repeats, none are clearly identifiable as homologues. Whereas the Pdd, Lia, and Nowa proteins reinforce the divergence between the DNA rearrangement machinery in the two species, Die5p reveals a novel connection between the two systems.

***DIE5* is required late in macronuclear development.** Although the novel sequence of Die5p limits speculation on its biochemical function, the molecular events that are disrupted (or not disrupted) by inhibiting *DIE5* expression in two species argue for late action in macronuclear development. First of all, prezygotic events of meiosis and pronuclear exchange occur normally upon *DIE5* RNAi silencing in *Paramecium*. The survivors of conjugation upon *DIE5* knockdown are heterokaryons with heterozygous micronuclei (with alleles from both parents), but they have macronuclei regenerated from fragments of their parental macronuclei. This phenotype has been observed upon knockdown of other genes involved in macronuclear development (32, 41). Likewise, early-stage events of scnRNA biogenesis (data not shown) and accumulation of specific chromatin modifications (Fig. 8) associated with DNA elimination appear unaltered upon disruption of *DIE5* in *Tetrahymena*. This is in contrast to disruption of RNAi components (e.g., *DCL1* or *TWIL*) that are defective in these events.

Even relatively late-stage events prior to IES excision were not disrupted upon loss of *DIE5* expression. Macronuclear anlagen form and substantial DNA amplification occurs in RNAi-treated *Paramecium* even though IES excision is inhibited. In *Tetrahymena* Δ *DIE5*mic conjugants, Pdd1p foci formed as expected for wild-type cells (Fig. 8B). These observations, together with the fact that we saw no phenotype when *DIE5* was disrupted only from the parental macronucleus, suggest that its essential role occurs after activation of zygotic expression of the macronuclear anlagen. Most striking is our observation that *Tetrahymena* heterokaryons with wild-type macronuclei but homozygous for the Δ *DIE5* allele in their micronuclei fail to excise IESs and arrest prior to the elimination of one of the two micronuclei. These results clearly demonstrate a late role for Die5p in macronuclear differentiation.

While loss of *DIE5* expression blocked IES excision in both ciliates studied here, we did observe some differences in terminal phenotypes, in particular the degree of anlagen DNA amplification. *DIE5* RNAi-treated *Paramecium* appeared to extensively amplify their anlagen DNA, whereas Δ *DIE5*mic *Tetrahymena* cells arrested their anlagen differentiation at a low amplification level. We favor the explanation that this most likely represents distinct differences in nuclear differentiation events (e.g., macronuclear regeneration occurs in *Paramecium* but not in *Tetrahymena*) in these evolutionarily diverse organisms. Nevertheless, we cannot rule out that such phenotypic differences may result from low levels of PtDie5p remaining after RNAi silencing. What is clear is that RNAi silencing that was robust enough to prevent IES excision in *Paramecium* to the same extent as was observed in *Tetrahymena* Δ *DIE5*mic conjugants elicited differential effects on anlagen DNA amplification.

The surprising observation that anlagen DNA is degraded in *Tetrahymena* Δ *DIE5* conjugants has not been observed in other DNA rearrangement mutants (12, 30, 36, 40). The mechanism of degradation is not understood, and we were unable to demonstrate labeling in TUNEL assays. It is interesting to note that anlagen DNA degradation appears to be the outcome in *Paramecium* *DIE5* RNAi silencing, but as mentioned previously, these cells survive by regenerating a functional macronucleus from an old macronuclear fragment. This is possible in *Paramecium* because macronuclear fragments persist throughout conjugation and continue transcription for multiple cell divisions after conjugation. In contrast, degradation of the old macronucleus in *Tetrahymena* is complete prior to formation of the new genome and leaves no recovery pathway if development is defective. Further analysis will be required to establish whether the degradation of *Tetrahymena* anlagen DNA has a similar molecular basis as the fate of *Paramecium* anlagen in *DIE5*-silenced cells.

The significance of TtDie5p localization to the micronuclei but not anlagen at late time points is unclear. This may reflect the limitation of using a macronuclear GFP-*DIE5* transgene that cannot be expressed after degradation of the old macronucleus. Alternatively, the localization could be evidence of an unexpected role of Die5p from the newly formed micronucleus. Unusual observations such as these underscore the importance of investigating the role of conserved proteins such as Die5p in the DNA rearrangement process to elucidate the evolution and regulation of this massive genome-remodeling

process and its connections to large-scale genome reorganizations in other species.

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