The SUMO Pathway Is Developmentally Regulated and Required for Programmed DNA Elimination in *Paramecium tetraurelia*†‡

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Extensive genome-wide remodeling occurs during the formation of the somatic macronuclei from the germ line micronuclei in ciliated protozoa. This process is limited to sexual reproduction and includes DNA amplification, chromosome fragmentation, and the elimination of internal segments of DNA. Our efforts to define the pathways regulating these events revealed a gene encoding a homologue of ubiquitin activating enzyme 2 (*UBA2*) that is upregulated at the onset of macronuclear development in *Paramecium tetraurelia*. *Uba2* enzymes are known to activate the protein called small ubiquitin-related modifier (SUMO) that is covalently attached to target proteins. Consistent with this relationship, Northern analysis showed increased abundance of SUMO transcripts during sexual reproduction in *Paramecium*. RNA interference (RNAi) against *UBA2* or SUMO during vegetative growth had little effect on cell survival or fission rates. In contrast, RNAi of mating cells resulted in failure to form a functional macronucleus. Despite normal amplification of the genome, excision of internal eliminated sequences was completely blocked. Additional experiments showed that the homologous *UBA2* and SUMO genes in *Tetrahymena thermophila* are also upregulated during conjugation. These results provide evidence for the developmental regulation of the SUMO pathway in ciliates and suggest a key role for the pathway in controlling genome remodeling.

Small ubiquitin-related modifier (SUMO) is the most intensively studied member of the ubiquitin-like protein family and is conserved in all eukaryotes. Modification of target proteins by SUMO requires E1 (ubiquitin activating), E2 (ubiquitin conjugating), and, in many cases, E3 (ubiquitin ligating) enzymes (13, 14, 22). In multicellular organisms, SUMO is expressed in all tissues and developmental stages (13). It is required for survival of all eukaryotic organisms tested so far due to its involvement in the regulation of protein localization, protection from ubiquitin-mediated degradation, cell cycle progression, DNA repair, chromatin cohesion, stress response, heterochromatin formation, and apoptosis (8, 13, 14, 22, 23, 31). We report here that the SUMO pathway is developmentally regulated in ciliates, and it is essential for formation of the somatic macronucleus.

Ciliates, including *Paramecium tetraurelia* and *Tetrahymena thermophila*, have two distinct nuclei in a single cell. The germ line micronucleus contains transcriptionally silent, diploid chromosomes which are transmitted to the next sexual generation. The somatic macronucleus contains acentric polygenomic chromosomes that are actively transcribed. The old macronucleus is destroyed, and the new macronucleus develops from the fertilized zygotic nucleus after sexual reproduction. The newly formed macronucleus undergoes DNA amplification and developmentally programmed, genome-wide DNA rearrangements (reviewed in references 1, 12, and 41). Chromosomes are fragmented into smaller pieces, and telomeres are added de novo to these ends (7, 16). In *Paramecium tetraurelia* the DNA is amplified 500-fold, and more than 50,000 unique germ line-specific sequences called internal eliminated sequences (IESs) are excised at a specific developmental stage (2, 6). *Paramecium* IESs are short (26 to 883 bp) and AT rich (about 80%), and they contain no significant open reading frames (34). All known *Paramecium* IESs are flanked by an 8-bp terminal inverted repeat consensus sequence (5'-TAYAGYNR-3') that has similarity to the consensus found at the termini of the *mariner/Tc1* superfamly of transposable elements (15). IES elimination is precise, leaving a single copy of the 5'-TA-3' dinucleotide in the macronuclear destined sequences.

In this paper, we show that SUMO and *UBA2* (SUMO-activating E1 enzyme) are upregulated during sexual reproduction in *Paramecium* and *Tetrahymena*. *Uba2* is one of two subunits of E1 enzyme and establishes a thioester bond with the SUMO peptide. RNAi knockdown of *UBA2* and SUMO in *Paramecium* prevented IES excision but did not prevent developmental DNA amplification or normal vegetative growth. Thus, in ciliates, sumoylation is a developmentally regulated pathway required for genome remodeling. MATERIALS AND METHODS

**Cell lines and culture.** *Paramecium tetraurelia* stock d4-110 (hr-k/hr-k) was used for synchronized conjugation for RNA isolation and whole-cell PCR analysis. For genetic analysis, strain d4-502 homozygous for *pwc-A502* and *nldb-1* and strain a3093 homozygous for *pdb-b9* and *nld-b* were used (kindly supplied by Mihoko Takahashi, University of Tsukuba). Otherwise, *nldb (nldb-1/nldb-1)* was used as the *Paramecium* wild-type strain. Paramecia were cultured in a pea medium (1.25 to 2.5 g Austrian winter pea in 800 ml double-distilled H2O prepared in the autoclave for 20 min [38]) 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; modified from Dryl's original solution [5]) supplemented with 1.25 mg/liter stigmasterol. The peas were purchased from...
Outsidepride.com. The medium was inoculated with *Klebsiella pneumoniae* 1 to 2 days prior to use. *Paramecium* cell lines were cultured at 27°C as described by Sonneborn (33). *Tetrahymena thermophila* stocks B2086 and CU428.1 were cultured in NEFF medium (0.5% dextrose, 0.25% yeast extract, 0.25% protease peptone, 3.3 mM FeCl₃) at 30°C with shaking at 85 rpm.

**Concentration of conjugating cells.** The mating efficiency of *Paramecium tetraurelia* is relatively modest, therefore we used a procedure by Yang and Takahashi to enrich for conjugating cells (40). Mating-reactive cells at a density of >2,000 cells/ml were mixed and incubated at 27°C. After 15 min, the upper part of the medium (containing mostly single cells) was removed with a pipette. Two hours after mixing, a few drops of iron dextran particles prepared according to Vosskübler and Tiedtke (39) were added, and the culture was incubated for 5 min. Single cells that ingested the iron particles were pulled down by strong neodymium magnets, and the supernatant containing free-swimming conjugating cells was collected. This was repeated twice for the supernatants, and cells were washed thereafter with K-DS. The procedure resulted in a culture 80 to 99% pure for conjugating *Paramecium*. Conjugating *Tetrahymena* cultures with mating efficiencies between 70 to 90% were obtained as previously described (18).

**Total RNA isolation.** Total RNA samples from 50 to 100 ml of *Paramecium* cell culture (100 to 1,000 cells/ml) or 20 ml of *Tetrahymena* cell culture (3 x 10⁴ cells/ml) were isolated as previously described (18), using the RNeasy Mini kit (QIAGEN) supplemented by a QIA shredder (QIAGEN) for homogenization and the RNase-free DNase set (QIAGEN) for genomic DNA elimination according to the manufacturer’s instructions.

**Differential display.** Differential display and subsequent cloning of the cDNAs were carried out using the RNA image kit 1 (GenHunter) according to the procedure supplied by the company. cDNAs were amplified by PCR using an oligonucleotide primer of a 5-mer arbitrary primer and a specific primer. Two sets of independently amplified PCR products were examined to verify reproducibility of the bands on 6% denaturing polyacrylamide gel electrophoresis. Purified DNA fragments from the gels were cloned into pGEM-Teasy (Promega) and were subsequently used to produce probes for Northern blots and sequencing of the insert.

**Microinjection and observation of GFP fluorescence.** Plasmids containing the green fluorescent protein (GFP) fusion genes were derived from pZC/H11032/H9004 (a gift from J. R. Preer, Jr., Indiana University (12), accession number X99490). Plasmid pRXIU/ba2 contains the entire *UBA2* open reading frame (ORF) between the *GFP* region and the full-length ORF of *SUMOI*. GFP-156 (C-terminal fusion, and the *SUMOI*) was derived from pZC/H11011 (see probe B in Fig. 3A). This reaction amplifies the circularized IES4578 of the *UBAX* gene with 5'-TGTTGTTAC CCCTCGG-3' as forward primer and 5'-ACATCTCTAGTTGAGG-3' as reverse primer. The sequences for the microinjection and *SUMO-I* alleles were taken from the GenBank database (accession numbers L26124 and M65163, respectively). The PCR cycles for whole-cell PCR consisted of 2 min of 94°C, followed by 25 cycles of 92°C for 30 s, 50°C or 53°C for 1 min, 72°C for 1 min, and then 5 min at 74°C.

**Whole-cell PCR amplification.** Twenty cells in approximately 5 μl of double-distilled H₂O were mixed with 2 μl of 50 mM EDTA (pH 8.0) and immediately placed at 94°C for 25 min. After incubation, the solution was adjusted to 20 μl with distilled water. A 25 μl PCR was set up with 2 μl of the above cell solution, 1× commercial buffer, 0.2 mM each deoxynucleoside triphosphate, 0.5 μM each primer, 1.5 mM MgCl₂ (after adjusting for the concentration of EDTA), and 0.625 U ExTaq DNA polymerase (Takara Inc., Japan). Micronuclear amplification products surrounding *IES4040* in the *A-I* allele (pp1 and pp2 in Fig. 3A) were amplified using primer F4386 (5'-CACTACCTCAGTTGATG-3') and a primer inside *IES4578* (5'-CTTTCTTAAATATCAGCT-3') and IES6435 (pp3 and pp4 in Fig. 3A) were obtained using primer F6411 (5'-TGTTGTTAC TCCCTCGCC-3') plus a primer inside IES6469 (5'-ATATCACAAGGGCT CTC-3').

**Total DNA isolation from exotomalous cells.** Cells were fed with 50 ml of culture fluid containing double-stranded RNA producing *E. coli* and grown to the maximum cell density for the purpose of culturing. Cells were scored for 100% autogamy by staining with Carbol fuchsin solution to examine macronuclear fragments (3). The entire 50-ml *UBA2* or *SUMO* RNAi culture or 5 to 10 ml of vector control culture was fed with fresh 100-mI culture fluid with *Klebsiella pneumoniae* and incubated for approximately 12 h. Cells from control and experimental treatments were collected when about two-thirds of control cells contained one new macronuclear and one-third still contained two macronuclei. Total genomic DNA samples from the cell cultures (~3,000 cells/ml) were isolated as previously described (18). Cells were resuspended in 1.0 ml of culture fluid, squirted into 2 ml of low-EDTA lysing solution (10 mM Tris-HCl, 50 mM EDTA, 1% sodium dodecyl sulfate [SDS], pH 9.5), and incubated at 65°C for 8 min. The DNA was purified by phenol-chloroform extraction and ethanol precipitation.

**Northern and Southern blot analyses.** RNA samples (20 μg) were separated on 2.5% (1:10) agarose gel. Southern blots were performed according to the method of Sambrook and Russell (30). Genomic DNA samples (10 μg) were separated on a 0.7 to 0.8% agarose gel. PCR products were separated on 3% agarose or 4% NuSieve 3:1 agarose gel. The resulting fragment was transferred to a GenBank insert was taken from the GenBank database (accession numbers L26124 and M65163, respectively). The PCR cycles for whole-cell PCR consisted of 2 min of 94°C, followed by 25 cycles of 92°C for 30 s, 50°C or 53°C for 1 min, 72°C for 1 min, and then 5 min at 74°C.
RESULTS

**UBA2 and SUMO genes are upregulated in mated Paramecium.** Macronuclear differentiation begins around 8 h after mixing mating reactive cells. IES excision in the new macronucleus then follows, with a peak of excision activity around 14 h after mixing. We used differential displays to compare RNA samples from mated (8 and 13 h after mixing), log-phase, and starved *Paramecium tetraurelia* organisms. One of the differentially expressed cDNAs encoded a homologue of *UBA2* that was upregulated in mated cells. Northern analysis of total RNA using *UBA2* as a probe showed low levels of transcripts in log-phase and starved cells but a dramatic increase 8 to 10 h after mixing mating reactive cells (Fig. 1A).

The *Paramecium* Uba2 sequence was approximately 29% identical over the entire predicted amino acid sequence to Uba2 orthologs from other organisms and 33% across the region of the Uba2 conserved domain (cd01489) (Fig. S1A and B in the supplemental material).

We searched the *Paramecium* genome (http://www.genoscope.cns.fr/externe/paramecii/) to identify other possible *UBA2* isoforms. Among the *UBA* genes found in the *Paramecium* genome, some had clear identity to *UBA1* or *UBA3*. However, one gene showed significant similarity to *UBA2* orthologs, ~24% identical to the orthologs across the entire amino acid sequence, and 35% identical across the region of the Uba2 conserved domain (cd01489) (see Fig. S1A and B). We will refer to our original gene as *UBA2* and the second as *UBAX*. Amino acid sequences of *UBA2* and *UBAX* share only 23% identity, therefore these genes are distantly related. Although we cannot eliminate the possibility that *UBAX* is a *UBA2* isoform, the Uba C-terminal domain (Pfam no. PF02134) is typically well conserved among other *UBA2* genes but contains a 15-amino-acid insertion in *UBAX* (Fig. S1B). We present evidence later in this report that *UBAX* has a different biological role in the *Paramecium* genome than *UBA2*. That is, SUMO may not be the substrate for UbaXp.

To evaluate the cellular localization of Uba2p, Uba2-GFP was constructed in a plasmid driven by the *UBA2* upstream region (see Materials and Methods). Cells transformed with a *UBA2-GFP* fusion construct were mated and examined using fluorescence microscopy. The results showed that Uba2-GFP localized to the developing macronuclei but not to old macronuclear fragments (Fig. 2A and C). The results from an N-terminal fusion showed the same localization (data not shown). The GFP fluorescence was found uniformly throughout the new macronuclei, except a few spots (~3-macronuclei) of <1.5 μm in diameter close to the nuclear membrane, where the GFP signal was very intense (Fig. 2A). Protein abundance was estimated from the fluorescence of Uba2-GFP in mated cells. Consistent with the Northern analysis (Fig. 1A), the GFP signal increased during macronuclear development (10 and 13 h in Fig. 1B and 2C). The combination of increased Uba2p levels and localization to the new macronucleus are consistent with a role for *UBA2* in *Paramecium* macronuclear development.

Since *UBA2* was upregulated in mated cells, we asked whether the gene encoding its substrate, i.e., SUMO, also was upregulated during sexual reproduction in *Paramecium*. We identified at least three loci encoding SUMO homologues in the *Paramecium* genome (*SUMOI*, *SUMOII*, and *SUMOIII*). Genomic Southern blots and reverse transcription-PCR confirmed the presence of each gene (data not shown). The three genes were almost identical (81 to 91% identical in the coding nucleotide sequences and 98 to 100% identical in the deduced amino acid sequences across the region containing the mature protein), and they showed 30 to 55% identity to known orthologous SUMO proteins (Fig. S1C). A Northern blot probed with a mixture of *SUMOI*, *SUMOII*, and *SUMOIII* ORFs revealed that global SUMO mRNA levels are also developmentally regulated with a peak early in conjugation (Fig. 1A).

GFP-SUMOI fusion proteins, expressed using the *SUMOII* promoter, localized to new macronuclei in autogamous cells (Fig. 2B). Unlike Uba2-GFP, ~1.5-μm spots were not apparent in the new macronuclei, although many small (~0.5-μm) dots were found instead (Fig. 2B). Estimation of protein abundance with GFP-SUMO showed accumulation of GFP fluorescence early in conjugation consistent with the Northern analysis (Fig. 1B), yet the protein abundance was still high during macronuclear development (10 to 13 h in Fig. 1B and 2D). Figure 2D shows intense GFP signal in old macronuclear fragments in exconjugants, which was not always observed in autogamous cells (Fig. 2B). GFP-SUMO protein was accumulated in the (old) macronuclei early in conjugation and stayed there for hours (Fig. 2D), unlike Uba2-GFP, which was not abundant before new macronuclei appeared.

![Graph and Figure](image_url)

**FIG. 1.** Expression of *UBA2* and SUMO in *Paramecium*. (A) Northern blot of total RNA (20 μg per lane) from *Paramecium* probed sequentially, first with *Paramecium* *UBA2* and then with a mixture of *SUMOI*, *SUMOII*, and *SUMOIII* genes. Etidium bromide staining of rRNA was used as a loading control. (B) Protein levels of Uba2-GFP (filled circle) and GFP-SUMOI (open circle) were estimated by measuring fluorescence intensity (in arbitrary units) in vegetative and mated cells. Expression of each GFP fusion gene was driven by its own 1.5-amino-acid insertion in *UBAX* may not be the substrate for UbaXp.

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FIG. 2. Cellular localization of GFP fusion proteins. (A and B) Fusion protein of GFP to the C terminus of Uba2p (A) or N terminus of SUMO I (B) in autogamous cells expressed by their own promoters. The nuclear events in these cells correspond to approximately 12 to 20 h in conjugating cells.
these data demonstrate that UBA2 and SUMO in Paramecium are developmentally regulated.

**UBA2 and SUMO in Paramecium are required for macro-nuclear development but not for meiotic processes and developmental DNA endoreplication.** To investigate the role of UBA2 and SUMO during conjugation, gene expression was knocked down using RNA interference (RNAi) by feeding double-stranded RNA-producing bacteria (9). Northern analysis showed that full-length UBA2 transcripts were reduced to <30% in autogamous cells treated with UBA2 RNAi compared to control RNAi (empty vector) (Fig. 3A). The autogamous cells used here (Fig. 3A) contained two new macronuclei which were easily recognized at low magnification (×100); this should correspond to stages of mated cells after 12 h. Unlike control RNAi, UBA2 and SUMO RNAi treatments reduced survival of exconjugants and prevented marker gene transmission (Table 1). Each parental cell line used for conjugation was marked with a different homozygous recessive allele (see Materials and Methods). Since successful Paramecium conjugation results in heterozygous loci, F₁ progeny should have the wild-type phenotype. Some F₁ progeny in the control cross (6 of 46; 13%) did not show the wild-type phenotype. This is most likely the result of a failure in nuclear exchange that is associated with a small fraction of cell pairs in Paramecium genetic crosses and perhaps amplified by the effects of the mutant alleles used as genetic markers. Nevertheless, this cannot account for the lack of wild-type phenotype in 100% of surviving progeny from the UBA2 RNAi cross (25 of 25). The mutant phenotype of the marker genes in the exconjugant cells suggests that development of the new macronucleus failed, and the alternative developmental pathway, called macronuclear regeneration (MR), was followed. Normally, in the wild type old macronuclear fragments remain in the exconjugants and are transcriptionally active for several cell divisions, but DNA replication no longer occurs. Fragments disappear 8 to 10 cell divisions after sexual reproduction (either actively or by dilution). When the new macronucleus is incapable of division after sexual reproduction, one or more parental macronuclear fragments regenerate into a single macronucleus which is capable of DNA replication, amitotic division, and transcription (33). MR results in a heterozygous micronuclear genotype from cross-fertilization, while the macronuclear genotype remains parental. Several selected progeny that survived were followed into autogamy (self fertilization) to reveal the micronuclear genotypes in the F₁ progeny. The resulting F₂ lines from UBA2 RNAi produced wild-type (as well as mutant) progeny, indicating that surviving F₁ progeny were heterozygous in the micronucleus and thus resulted from MR. Therefore, the nuclear events of conjugation were successful, but the formation of the mature macronucleus was not successful when UBA2 was inhibited with RNAi.

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Fluorescent images are projections of optical sections obtained by confocal microscopy. The GFP fluorescence localized to the new macronuclei, which show weaker propidium iodide staining (DNA) than the old macronuclear fragments. Pictures on the right are ×3 magnifications of a new macronucleus. The bar is 15 μm for the left pictures and 5 μm for ×3 magnification on the right. (C and D) GFP fluorescence from Uba2-GFP (C) and GFP-SUMO (D) expressed by their own upstream regions in mated cells. Pictures for GFP fluorescence were taken at the same exposure under a conventional fluorescent microscope. The bar in panel C corresponds to 20 μm for both panels C and D.
In the case of SUMO RNAi, we could not produce enough F₂ progeny to directly test for MR, therefore it is possible that the surviving progeny (only 14%) with mutant phenotypes resulted from failure of conjugation. This result is still consistent with a defect in macronuclear development.

Cytological observations of RNAi-treated cells were consistent with the genetic analysis. Normal exconjugants produce two macronuclear anlagen, which assort into the daughter cells after the first cell division, approximately 18 h after induction of conjugation. RNAi treatment of UBA2 or SUMO resulted in a significant proportion (14 to 30%) of cells with two macronuclear anlagen at 24 to 36 h after induction of conjugation (Fig. 3B and C). This means that exconjugants most likely never went through the first cell division after conjugation. Thus, cell cycle progression after conjugation seems to be affected by RNAi of these genes (though vegetative cell division is not affected, as shown later in Results). In addition, we observed cells undergoing unequal assortment of a nondividing new macronucleus into a daughter cell, which is consistent with the absence of the new macronucleus in a small fraction of cells (Fig. 3C). Many cells (more than 50%), however, showed a normal cytological phenotype from time points 24 to 54 h after mixing (Fig. 3C and data not shown). The genetic analysis (Table 1) indicates that the apparent normal cells either die late or complete MR, since none of the surviving progeny expressed wild-type genetic makers.

DNA content of the developing macronucleus in exconjugants treated with UBA2 RNAi was indistinguishable from that of control RNAi in the timing of development examined (Fig. 3D). The results showed that UBA2 affects macronuclear formation without affecting developmental DNA endoreplication. **UBA2 and SUMO are required for IES excision**. We decided to investigate whether the defect in macronuclear development observed in RNAi-treated cells (Table 1 and Fig. 3) included the failure to remove internal eliminated sequences (IESs) from the new macronucleus. Unfortunately, analyzing IES excision is complicated by the presence of DNA from the old macronuclear fragments, the developing macronucleus, and the micronucleus in one cell. To detect IES excision, PCR primers were designed with one primer in the macronuclear-destined DNA and one primer inside an IES to amplify micronuclear sequences. Such PCR products (named pp1 to pp4 in Fig. 4A) should increase as DNA is amplified and decrease.

**TABLE 1. Phenotype of Paramecium exconjugants treated with RNA interference (RNAi)**

| Gene for RNAi | No. of exconjugants examined | Survival (%) | No. of exconjugants with the marker gene phenotype |
|--------------|-------------------------------|--------------|------------------------------------------|
|              |                               |              | Wild type | Mutant |
| Vector       | 46                            | 100          | 40        | 6      |
| UBA2         | 54                            | 46           | 0         | 25     |
| SUMO         | 36                            | 14           | 0         | 5      |

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**FIG. 4. Inhibition of excision of six IESs in the A-51 allele by RNAi of UBA2 and SUMO.** (A) A partial map of the micronuclear version of the A-51 allele showing locations of IESs (boxes), SspI recognition sites (arrow heads), and sizes of fragments generated by SspI digestion. Positions of expected PCR products (pp1 to pp4) 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 (conj.). (A) The predicted PCR products correspond to pp1 to pp4 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 either with 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 contains 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, 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.
Effects of RNAi against \( \text{UBA2} \) and \( \text{SUMO} \) were measured (Fig. 5A). When GFP-Uba2p was expressed using a constitutive promoter without an RNAi signal, GFP signal was effectively reduced (solid circles; bars on the circles show 95% confidence), while cell division rates in both vector and \( \text{UBA2} \) RNAi-treated cells were unaffected (vertical bars show standard deviation). (B) Daily isolation lines (12 to 24 lines) of wild-type (GFP-nontransformed) cells were treated with RNAi for three continuous days, and survival of the cell line and fissions per day were counted.

As IESs are excised (Fig. 4B, vector lanes). Small IESs included in PCR products serve as another indicator for IES excision, producing faster-migrating bands in the gels when it is excised prior to the IES containing the primer site (pp2 and pp4 in Fig. 4A and B). RNAi of \( \text{UBA2} \) and \( \text{SUMO} \) showed a gradual increase in the amount of full-length (IES-containing) PCR product over the developmental time course and no evidence of the shorter product resulting from excision of the neighboring IES (Fig. 4B). These results demonstrate that RNAi of \( \text{UBA2} \) and \( \text{SUMO} \) inhibits IES excision but does not inhibit developmental DNA amplification.

Since endoreplication in the new macronucleus proceeded normally in RNAi-treated cells (Fig. 3D), Southern hybridization of total DNA should detect DNA in the new macronucleus. Normally, the micronuclear version of DNA is undetectable in a Southern blot due to its lower copy number compared to that of macronuclear DNA. DNA for Southern analysis was isolated from large cultures induced to enter autogamy (self fertilization). Total DNA was isolated from a well-fed exautogamous cell culture when about 50% of control cells (empty vector RNAi) had undergone the first cell division. The DNA was digested with SaPl, which has frequent recognition sites in IESs but only one in the coding region of the \( A-51 \) allele. A probe containing macronuclear DNA from the \( A \) gene (HincII-PstI in Fig. 4A) detected the 1.3- and 2.0-kb fragments in \( \text{UBA2} \) and \( \text{SUMO} \) RNAi-treated cells expected for amplification of the unprocessed \( A-51 \) gene (Fig. 4C). Such DNA was not found in control RNAi experiments using empty vector (Fig. 4C). Probe IES4578 (Fig. 4A) contained only the sequence from IES4578 of the \( A \) gene, and it detected the IES-containing bands migrating as a single 500-bp species in \( \text{UBA2} \) and \( \text{SUMO} \) RNAi-treated DNA (Fig. 4C), as expected. Together, these results show that RNAi against \( \text{UBA2} \) or \( \text{SUMO} \) specifically inhibits IES excision in developing macronuclei.

RNAi against \( \text{UBA2} \) and \( \text{SUMO} \) has little or no effect on vegetative growth. Expression patterns of \( \text{UBA2} \) and \( \text{SUMO} \) transcripts suggested that the major role of the \( \text{SUMO} \) pathway is in sexual reproduction, not in vegetative growth. To test this prediction, RNAi treatments were performed on vegetative cells expressing GFP-Uba2p, and the cell division rate and GFP signal were measured (Fig. 5A). When GFP-Uba2p was expressed using a constitutive promoter without an RNAi treatment, strong signals were detected at the periphery of the micronucleus and diffusely in the macronucleus (data not shown). After RNAi treatment of \( \text{UBA2} \), most GFP-Uba2p signal disappeared by day 2; however, no decrease in cell division rate was observed in cells treated with \( \text{UBA2} \) RNAi or those treated with empty vector RNAi (Fig. 5A).

Effects of \( \text{SUMO} \) and \( \text{UBA2} \) RNAi on vegetative cell division were minor (Fig. 5B). RNAi treatment of the \( \text{UBAX} \) gene greatly affected cell survival and cell division (Fig. 5B), suggesting that this gene is involved in a different genetic pathway from \( \text{SUMO} \). Although these data do not exclude a role for \( \text{SUMO} \) in vegetative growth, it is clear that more dramatic effects occur during conjugation.

**UBA2 and SUMO also are upregulated in conjugating Tetrahymena.** Our results provide clear evidence for developmental regulation of the \( \text{SUMO} \) pathway in Paramecium. If the function of this pathway is fundamental to ciliate macronuclear development, we expect it to be conserved in other ciliates. \( \text{UBA2} \) and \( \text{SUMO} \) homologues from Tetrahymena thermophila were also examined for developmental regulation of expression. The deduced Tetrahymena Uba2 amino acid sequence is 30% identical and Tetrahymena SUMO is 66% identical to the respective Paramecium homologues. Northern blot analysis showed that they are also developmentally regulated (Fig. 6). The expression pattern of one gene parallels considerably that of the other between the two species (Fig. 1 and 6). These
results suggest that SUMO plays a major role during conjugation and may be required for programmed DNA rearrangements in all ciliates.

**DISCUSSION**

**Developmental regulation of the SUMO pathway in* Paramecium.** Our analysis of UBA2 and SUMO in *Paramecium tetraurelia* showed that their mRNA levels and estimated protein levels significantly increased in mated cells (Fig. 1). The same developmental regulation of UBA2 and SUMO mRNA expression were obtained from another ciliate, *Tetrahymena* (Fig. 6). The developmental regulation of Uba2p activity is unusual, since sumoylation in other organisms is cell cycle regulated or induced by external cues, such as hormone or cytokine treatment (reviewed in reference 11). To our knowledge, ciliates are the first organisms that show developmental regulation of the SUMO pathway genes. Furthermore, RNAi silencing of either UBA2 or SUMO showed no detectable phenotype in vegetative *Paramecium*, unlike most other organisms, which require constitutive SUMO functions (Fig. 5). In fission yeast, a SUMO gene deletion is not immediately lethal, but it causes defective growth and increased genome instability (35). Although we cannot claim that UBA2 and SUMO are completely dispensable for vegetative survival in *Paramecium*, we showed that RNAi against a recombinant GFP-UBA2 gene is effective in vegetative cells, which suggests that only minor levels (<5%) of wild-type Uba2p could be present (Fig. 5A). The decreased fission rate and poor survival of vegetative cells treated with RNAi against the UBA2 gene also argues that the RNAi treatment is effective and that Uba2p activity is conjugation specific. Together, our results from *Paramecium* and *Tetrahymena* suggest that the SUMO pathway is upregulated during sexual reproduction in diverse ciliates.

The SUMO pathway is required for *Paramecium* DNA elimination. Our results provide clear evidence for the role of the SUMO modification pathway in *Paramecium* macronuclear development and DNA elimination. RNAi treatment against either gene during sexual reproduction results in the failure of macronuclear development and inhibition of DNA elimination. In contrast, RNAi against UBA2 or SUMO during vegetative culture had little effect on the rate of cell division or viability.

Other factors required for DNA elimination are also known. The importance of heterochromatin and chromodomain proteins (Pdd proteins) in DNA elimination has been well established in *Tetrahymena thermophila* (4, 28, 36). In addition, there is now considerable evidence that an RNAi-related pathway is essential for DNA elimination in *Tetrahymena* (reviewed in reference 27). This includes a requirement for a piwi-related protein during conjugation (25), functional evidence for the role of double-stranded RNA (42), and a requirement for a dicer-like protein (26). Evidence for the involvement of an RNA-mediated pathway in *Paramecium* DNA elimination has also been discovered (10), and putative RNA binding proteins called NOWA1p and NOWA2p have been analyzed (29). The work presented in this study shows that the SUMO pathway is another critical biochemical activity for DNA elimination. Interestingly, UBA2 and SUMO inhibition in *Paramecium* has no effect on DNA amplification in the new macronucleus, even though it completely inhibits DNA elimination. This is different from the loss of Pdd proteins in *Tetrahymena*, which affects DNA endoreplication as well as IES excision (4, 28).

The precise biochemical role of the SUMO pathway in *Paramecium* macronuclear development is not clear. In other organisms, SUMO participates in nuclear functions such as transcriptional repression, chromatin cohesion, DNA repair, protein transport, and so on (15, 25, 35). Studies in yeast demonstrate a role for SUMO in genome integrity and chromosomal cohesion, but this seems an unlikely explanation in *Paramecium*, since the initial meiotic and mitotic events of conjugation appear normal and formation of the macronucleus does not require mitosis. A possible connection between heterochromatin formation through the epigenetic pathway mentioned above and transcriptional repression through sumoylation is intriguing. Transcriptional repression is known to be associated with sumoylation in mammals (15, 31). However, most *Paramecium* IESs are smaller (90% are less than 78 bp) than a single nucleosome (145 bp), and thus histone modification through the RNAi-like pathway may not be sufficient for precise recognition of *Paramecium* IESs. Furthermore, RNAi of NOWA1 and NOWA2 does not inhibit excision of all IESs (33), while RNAi of SUMO inhibited all IES excision so far examined, suggesting that SUMO has different or additional roles for IES excision that is required for excision of all IESs. We believe the SUMO pathway in *Paramecium* most likely functions during conjugation to control import (or export) of proteins into the developing macronucleus. In other organisms, the protein components of the SUMO pathway localize to the nuclear pore complex (reviewed in reference 13). In addition, substrates for SUMO include RanGAP1, which plays an important role in regulating the transport of ribonucleoproteins across the nuclear pore complex (17, 21). If our nuclear traffic hypothesis is true, then the transport is selective for only a subset of proteins, since DNA amplification appears normal in the developing macronucleus. Abundant GFP-SUMO I in the old macronucleus prior to formation of the new macronucleus seems to be carried over into the old macronuclear fragments hours after macronuclear development (Fig. 2B and D). This fluorescence was reduced in exatogamous cells (Fig. 2B), which undergo longer times before the first cell division under starved conditions compared to well-fed exconjugant cells (Fig. 2D), through either transport toward the new macronucleus or protein degradation. However, a small amount of SUMO in the old macronuclear fragments may imply some minor role in this nucleus as well. No substrates of SUMO have been identified in *Paramecium*, and our results suggest that a systematic search for conjugation-specific targets of SUMO could reveal proteins directly involved in IES excision.

**SUMO variants in* Paramecium.** We have experimentally confirmed the presence of at least three closely related SUMO genes in the *Paramecium* genome. There are two or three additional hits of almost identical SUMO genes in the *Paramecium* genome (data not shown). However, we were unable to confirm these loci by Southern blots probed with a mixture of SUMO1, SUMO2, and SUMO3 cDNA fragments. Although the presence of these additional genes is still possible, they may be present at an extremely low copy number in the macronucleus or were produced by errors in the sequence assembly process.
In mammals, SUMO variants (SUMO-1, SUMO-2,3, and SUMO-4) are selectively expressed and ligated to distinct target proteins and, thus, exert different biological roles. Since our Northern blot and RNAi analyses on the Paramecium SUMO genes were carried out using a mixture of SUMO1, SUMOII, and SUMOIII cDNA fragments, this report analyzed the global role of SUMO genes in Paramecium. Predicted amino acid sequences of SUMO genes are, however, very similar in the mature protein products (the amino-terminal part from the first methionine to glycine at the 80th position). SUMO1p and SUMO1Ip are identical, and there is only one amino acid difference between SUMO1p or SUMOIIp and SUMOIIIp (at the 29th position). Furthermore, the genome of Tetrahymena, like other single-celled organisms such as budding yeast, does not seem to have multiple SUMO variants. These observations support a simple interpretation that multiple SUMO genes in Paramecium are paralogous and biochemically equivalent.

The expression of individual SUMO genes was not examined in this report, except for SUMOI, which showed developmental upregulation when GFP-SUMOI was used as a reporter with the SUMOI promoter. We do not deny the possibility that SUMOII and SUMOIII could be expressed in the vegetative stage but not in macronuclear development. The lack of vegetative phenotype does not eliminate this possibility. For example, if vegetative SUMO was required for DNA repair, then the phenotype might not be detected after a small number of cell divisions. Even if this is the case, it is unlikely that UbaXp is responsible for activation of these SUMO variants, since the phenotypes of SUMO and UBA2 are clearly different (Fig. 5B). This suggests that the substrate of UbaXp is a different ubiquitin-like protein from SUMO. The effects of RNAi against SUMO were slightly more severe than those against UBA2 after conjugation (Table 1). We are not sure whether this is due to the efficiency of RNAi silencing or a separate role of SUMO independent from UBA2.

Macronuclear regeneration and postconjugation cell cycle defects in RNAi cells. Although RNAi treatment of SUMO and UBA2 prevented the formation of a new macronucleus in conjugating cells, a significant fraction of the progeny survived (46% for UBA2 and 14% for SUMO). Our genetic and cytological analyses showed that this was the result of macronuclear regeneration (MR), a process discovered decades ago but normally occurring in a small fraction of exconjugant progeny. Previously, in P. tetraurelia, MR was observed primarily in mutants defective in amitosis of the vegetative macronucleus (3, 32). In another species of Paramecium, P. caudatum, MR is a default pathway unless the new macronucleus fully develops before the first amitotic division (the third postzygotic cell division) (24). In P. caudatum, MR can be inhibited if exconjugants are kept in a nutrient-free medium that allows the new macronucleus to develop but suppresses macronuclear fragments from regenerating. The results presented here suggest that MR is an efficient default pathway in P. tetraurelia if the new macronucleus fails to form.

Our results showed that UBA2 and SUMO RNAi treatment caused inhibition of cell division after sexual reproduction (Fig. 2). It is unclear whether this results directly from a loss of SUMO function and its effect on cell cycle or an indirect effect from the disruption of IES excision in the new macronucleus. Direct involvement of SUMO in the postconjugation cell cycle may be supported by the observation that GFP-Uba2p expressed vegetatively localized to the periphery of the micronuclei (data not shown) but not during macronuclear development (Fig. 1). Thus, localization of Uba2p from the developing macronucleus to the micronuclei may be necessary to enter the first postconjugation mitotic cycle. In fact, in Tetrahymena, exit from conjugation and entry into the first postconjugation cell cycle is distinct from progression of normal vegetative cell cycles (19). Alternatively, an indirect effect could result from disruption of many ORFs by IESs in the new macronuclear genome, causing dominant-negative effects from truncated gene products. The study of additional genes required for IES excision in Paramecium will be required to differentiate between these two possibilities.

Our results demonstrate that the SUMO pathway plays a critical role in ciliate macronuclear development. Additional investigations of SUMO targets and their role in genome remodeling may provide unique insights into the role of sumoylation in a variety of organisms.

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