A Developmentally Regulated Gene, ASI2, Is Required for Endocycling in the Macronuclear Anlagen of *Tetrahymena*[^7]

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Ciliated protozoa contain two types of nuclei, germ line micronuclei (Mic) and transcriptionally active macronuclei (Mac). During sexual reproduction, the parental Mac degenerates and a new Mac develops from a mitotic product of the zygotic Mic. Macronuclear development involves extensive endoreplication of the genome. The present study shows that endoreplication of macronuclear DNA in *Tetrahymena* is an example of endocycling, a variant of the mitotic cycle with alternating S and G phases in the absence of cell division. Thus, endocycling is conserved from ciliates to multicellular organisms. The gene ASI2 in *Tetrahymena thermophila* encodes a putative signal transduction receptor. ASI2 is nonessential for vegetative growth, but it is upregulated during development of the new Mac. Cells that lack ASI2 in the developing Mac anlagen are arrested in endoreplication of the DNA and die. This study shows that ASI2 is also transcribed in the parental Mac early in conjugation and that transcription of ASI2 in the parental Mac supports endoreplication of the DNA during early stages of development of the Mac anlagen. Other molecular events in Mac anlage development, including developmentally regulated DNA rearrangement, occur normally in matings between ASI2 knockouts, suggesting that ASI2 specifically regulates endocycling in *Tetrahymena*.

In order to maintain the appropriate DNA content in dividing cells, the DNA must be replicated once, and only once, per mitotic cell cycle. Stringent controls exist to ensure this is the case. However, some specialized cells in multicellular organisms exit the mitotic cycle and enter a variant cell cycle, the endocycle. In endocycling cells, alternating S and G phases in the absence of cell division produce cells with multiple copies of the genome. This occurs in specific tissues of evolutionarily diverse organisms, including the egg chambers of *Drosophila melanogaster*, the leaves of plants, and the placenta of mammals (reviewed in reference 11).

*Tetrahymena thermophila* is a unicellular eukaryote with two structurally and functionally different nuclei, the micronucleus (Mic) and the macronucleus (Mac) (reviewed in reference 14). The Mic is the diploid germ line nucleus. It determines the genotype of the progeny and is transcriptionally inactive during vegetative growth. The polyploid (~50C) Mac is the transcriptionally active somatic nucleus and determines the phenotype of the cell. During sexual reproduction, the parental Mac is degraded and a new Mac develops from a mitotic product of the zygotic Mic. Macronuclear development involves extensive reorganization of the genome. The events include developmentally regulated chromosome breakage (36), elimination of specific elements constituting 10 to 20% of the germ line genome (IES) (38), and *de novo* methylation of about 0.8% of the adenine residues to [\(^{3}\)H]methyladenine (12).

Another feature of Mac development is a dramatic increase in DNA content during conjugation and the early vegetative fissions. *Tetrahymena* is induced to mate by starvation (5). Under conditions of continuous starvation, the DNA content of the Mac reaches 8 to 14C by the time the cells of a mating pair have separated (9, 17). Upon refeeding, the DNA content increases to 128C (9, 22), but it returns to the 50C characteristic of vegetatively growing cells within about 50 vegetative fissions (10, 34).

The question we address here is whether the endoreplication of DNA in the Mac anlagen of *Tetrahymena* is an example of endocycling, as characterized by alternating S and G phases. In all known cases in multicellular organisms, endoreplication occurs in alternating S and G phases. However, there are some significant differences between the ciliate anlagen and endocycling cells in the higher eukaryotes. In multicellular organisms, the cells make the transition from the mitotic cycle to the endocycle. With only rare exceptions, endocycling produces terminally differentiated cells that will never divide again. In the ciliates, the Mac anlage is a transition state between the mitotically dividing Mic and the new Mac, which will divide amitotically during the vegetative growth of the cell.

In the mitotic division of the Mic in vegetatively growing cells, there is virtually no G1 phase; the DNA is replicated immediately after the separation of the sister chromatids (23, 34). Similarly, DNA replication occurs immediately after the prezygotic mitosis and each of the two postzygotic mitotic divisions that produce the progenitors of the new micro- and macronuclei in the progeny cells (Fig. 1) (1, 9). Consequently, the early anlagen quickly attain a DNA content of 4C. For the next few hours (about 7 to 11 h postmixing), there is little incorporation of [\(^{3}\)H]thymidine in the developing Macs, suggesting that DNA synthesis is arrested at 4C until the macro-

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nuclear anlagen begin endoreplication at about 12 h of conju-
gation (1, 29).

Flow cytometry of nuclei from starved, late-stage conjugat-
ing cells revealed two distinct populations of nuclei with dif-
ferent DNA contents that approximate a doubling of the ge-
nome (2, 17). These were thought to represent the 4C early ma-
cronuclear anlagen and the nuclei in early endoreplication.
The DNA content of the latter class increased slightly with
time, which indicated that at least some nuclei attain DNA
contents higher than 8C (9, 17). However, under conditions of
continuous starvation, the developing Macs do not undergo
multiple rounds of DNA replication. Thus, in order to deter-
mine whether endocycling occurs in the developing Mac an-
lagen, it is necessary to examine reed cells.

Studies of endocycling in higher eukaryotes often use flow
cytometry to demonstrate the presence of discrete populations
of nuclei, usually with a characteristic doubling of the DNA
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In the present study, the DNA content in the developing
Macs of mating cells was measured in Feulgen stain prepara-
tions. This method allowed the unambiguous identification of
Mac anlagen. Since mating cells are never in perfect developmen-
tal synchrony, there was the added advantage that the nuclear
morphology of individual cells could be monitored to determine
whether endoreplication was correlated with a particular cytolog-
ical stage. The DNA content of mating cells under conditions of
continuous starvation was compared to that in reed cells to iden-
tify possible artifacts due to extended starvation.

Endoreplication in the developing Mac of *Tetrahymena* is
dependent upon *ASI2* (anlage stage-induced gene 2), a gene
that encodes a putative signal transduction receptor. *ASI2* is
nonessential for vegetative growth, but it is upregulated during
the development of the macronuclear anlagen, and *ASI2* is
required in the vegetative Mac to make viable progeny. DNA
replication arrest in progeny of matings between *ASI2* germ
line knockouts (lacking *ASI2* in the developing Mac), and
the cells die shortly after separation of the mating pairs (17).

The early stages of conjugation in *Tetrahymena* are driven by
transcription in the parental Mac. The experiments described
here were designed to determine whether *ASI2* was transcribed
in the parental Mac, whether parental *ASI2* transcription
was required for viability of the progeny, and which molec-
ular events were dependent on transcription of *ASI2* in the
parental Mac.

**MATERIALS AND METHODS**

*Strains and culture conditions.* The genotypes and phenotypes of strains used in this study are listed in Table 1. Cells were grown in SP medium (1% proteose peptone, 2% glucose, 1% yeast extract, and 0.03% sequestrene) at 30°C with shaking at 90 to 100 rpm. To induce mating, *Tetrahymena* cells were starved in 10 mM Tris-HCl (pH 7.4) for at least 10 h before cells of two different mating types were mixed (5).

**Construction of *Tetrahymena ASI2-GFP* cell lines.** *Tetrahymena ASI2-GFP* strains were obtained by biolistic transformation with the plasmid pST2-ASI2-GFP. The plasmid consisted of 2.3 kb of the *ASI2* coding region fused to the complete green fluorescent protein (GFP) coding sequence (amplified from MTT-NRK2-GFP, provided by J. Gaertig, University of Georgia, Athens, GA), the RPL29 transcription termination sequence (excised from ncuB+GRL8eGFP, provided by A. P. Turkewitz), the neod cassette (25), and approximately 1 kb of *Tetrahymena* genomic DNA 3′ to *ASI2* (17). The plasmid was purified, digested with SacI, and introduced into starved cells (CU428 or CU427) by biolistic bombardment (Bio-Rad particle delivery system) (6). Transformants were selected on the basis of paromomycin resistance.

*Southern blots.* *Tetrahymena* Mac DNA (5 to 6 μg) was digested with restric-
tion enzymes, and the fragments were separated by electrophoresis on 0.7% agarose gels. DNA fragments were transferred to GeneScreen Plus nylon mem-
brane (NEN) with 0.4 N NaOH overnight. The DNA was cross-linked to the
membrane in a UV Stratallinker cross-linker (Stratagene). The membranes were
prehybridized and hybridized as described previously (35).

*Western blots.* Cells were pelleted from an aliquot of a mating culture, and
total protein was solubilized in cell lysis buffer with loading dye (50 mM Tris-
HCl, pH 6.8, 100 mM dithiothreitol [DTT], 2% SDS, 0.1% bromophenol blue,
and 10% glycerol). Proteins were separated by 6% SDS-PAGE gel electrophore-
sis and transferred to a nitrocellulose membrane (Protran, Whatman). Blots
were incubated with rabbit anti-Pdd1p antibody (from C. D. Allis) at a 1:10,000

### TABLE 1. Genotypes and phenotypes of *T. thermophila* strains

| Strain | Micronuclear phenotype | Macronuclear phenotype | Phenotype |
|--------|------------------------|------------------------|-----------|
| CU427  | chx1-1/chx1-1          | CHX1                   | cy-s, VI  |
| CU428  | mpr1-1/mpr1-1          | MPR1                   | mp-s, VII |
| MU111  | ASI2/ASI2 mpr1-1/mpr1-1| ASI2/neod              | pm-r, mp-s, VII |
| MU119  | asi2/neod/asi2/neod    | asi2/neod              | pm-r, mp-s, VII |
| MU127  | ASI2/ASI2 chx1-1/chx1-1| asi2/GFP-neo4 CHX1    | cy-s, VI  |
| MU128  | ASI2/ASI2 mpr1-1/mpr1-1| asi2/GFP-neo4 MPR1    | pm-r, mp-s, VII |

* Macronuclear phenotype designations. Phenotypes for mutant genes are as follows: mp-r, 6-methylpurine resistant; cy-s, cycloheximide resistant; pm-s, paro-

mocycin resistant; "-" indicates susceptibility, asi2/neod is the knockout of the *ASI2* allele, disrupted by the neod cassette, which confers paromomycin resis-
tance. Mating types are designated by Roman numerals.
dilution or mouse anti β-tubulin (Sigma) at a 1:20,000 dilution, followed by incubation with goat horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibody (Bio-Rad) at a 1:5,000 dilution. 

**Cytology.** Fifty-microliter aliquots of cells were removed from a mating culture, air-dried on a slide, and fixed with 95% ethanol at room temperature for 30 s. The fixed cells were incubated with 100 ng/ml DNA-specific dye, 4',6-diamidino-2-phenylindole (DAPI) (Roche) in 70% ethanol–300 mM NaCl for 1 min, rinsed in 70% ethanol for 15 s and 35% ethanol for 15 s,53, and air dried. 

**RNA extraction.** Total RNA was extracted from *Tetrahymena* by TRIzol (Invitrogen) extraction (26) and dissolved in formaldehyde for small scan RNA (scRNA) gels or in RNase-free water for reverse transcription.

**Reverse transcription.** After DNase (RNase free; Roche) treatment, 2 μg of total RNA was mixed with first-strand primer, and the first-strand DNA was synthesized with Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (Promega) according to the manufacturer’s instructions.

**Immunohistochemistry.** For PddIp detection, cells were fixed in Lavidowsky’s fixative (ethanol-formal-acetic acid-water, 50:10:1:39) overnight at 4°C and immobilized on a cover glass (19). Fixed cells were incubated in blocking solution (10% normal goat serum, 3% bovine serum albumin [BSA], 0.1% Tween 20, PBS). The samples were incubated with rabbit anti-PddIp (1:5,000 dilution) (C. D. Allis, Rockefeller University) followed by Cy3-conjugant goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories). The cells were incubated with 1 μg/ml DAPI in PBS for 5 min, rinsed three times for 5 min with shaking, and observed by fluorescence microscopy.

**RNA gels.** Total RNA (15 μg/lane) was separated on 15% polyacrylamide-urea-1x TBE (Tris-borate-EDTA) gels (21).

**Feulgen staining.** A 100-ml mating culture of CU427 and CU428 was prepared, and 5-ml aliquots were removed every 2 h from 8 to 24 h after mixing. The DNA content of the developing Macs in mating was measured to determine whether endocycling in the developing Mac anlagen occurs primarily in two distinct periods between 8 and 12 and 16 to 18 h postmixing.

The abrupt increase in the average DNA content between 16 and 18 h of mating suggested that the bulk of DNA replication occurred relatively late in Mac anlage development. In order to determine whether this was the case, developing Mac anlagen were divided into four cytological stages. The analysis of DNA content in cells at each developmental stage revealed that the bulk of the DNA replication occurs after the degradation of one of the Mics, around the time that the mating pair separates (Fig. 3). This was in accord with the observation that substantial incorporation of tritiated thymidine occurred in exconjugants (1).

Because mating cells are not in perfect synchrony, the average DNA content at any particular time reflects contributions from nuclei with different amounts of DNA. In order to determine whether nuclei accumulate at discrete ploidyic, indicative of gaps between S phases, the DNA contents of nuclei from individual mating cells were plotted. Developmentally regulated DNA elimination occurs between 10 and 14 h postmixing (3, 4). Complications imposed by the simultaneous events of DNA elimination and DNA replication were avoided by limiting the analysis to cells at 14 h of conjugation and later.

Analysis of the DNA content of individual nuclei (Fig. 4) showed that the Mac anlagen from starved cells fell into three classes, with DNA contents of about 3C, 5C, and 10C. This supported the hypothesis that successive rounds of DNA replication in the *Tetrahymena* Mac were separated by gap phases. 

The analysis of DNA content was done in cells mating under conditions of continuous starvation. The molecular events of IES elimination, chromosome breakage, and *de novo* methylation all occur in the anlagen under conditions of continuous starvation (4, 12, 37). However, the availability of nutrients might reasonably be expected to limit DNA synthesis, particularly since *Tetrahymena* is auxotrophic for both purines and pyrimidines (15). Since the DNA content of the developing Mac anlagen increased to ~10C under starvation conditions, at least some DNA replication occurred without added nutrients, but it was necessary to determine whether the plateau at 10C is part of the developmental program or an artifact of starvation.

**RESULTS**

**DNA endoreplication in *Tetrahymena* occurs in alternating DNA synthesis and gap phases.** The endocyte is an alternative to the mitotic cycle in which DNA replication is uncoupled from cell division. In endocycling tissues, nuclei accumulate with DNA contents representative of each genome doubling (18, 32). The DNA content of the developing Macs in mating *Tetrahymena* cells was measured to determine whether endoreplication in the *Tetrahymena* Mac was an example of endocycling or whether the 181 macronuclear chromosomes were replicated in a less regular fashion. If S phases were alternating with gap phases, nuclei were expected to accumulate with doublings of DNA content resulting from each successive round of DNA replication.

Most studies of Mac development in *Tetrahymena* are done under conditions of continuous starvation. Under these conditions, the average DNA content of developing Mac anlagen increased slightly between 8 and 12 h postmixing from about 2C to 4C (Fig. 2 and Table 2). From 12 to 16 h, the DNA content remained fairly constant, and then it increased abruptly between 16 and 18 h to about 10 to 12C, where it remained for the duration of the experiment until the mating cells separated. The data showed that under conditions of continuous starvation, DNA replication in the developing Mac anlagen occurs primarily in two distinct periods between 8 and 12 and 16 to 18 h postmixing.

FIG. 2. Average DNA content of the developing macronuclear anlagen during conjugation of *Tetrahymena*. Gray, conditions of continuous starvation; black, cells refed at 8 h postmixing; bars, standard error. The DNA content of at least 50 cells was measured at each time point.
A mating culture of CU427 and CU428 was refed to a final concentration of 0.5% SPP at 8 h postmixing. Samples were taken and slides were prepared as in the previous experiment. Refed cells do not arrest at stage 4 but divide and return to vegetative growth. Once progeny have completed the first post-conjugation cell division, they can no longer be distinguished from nonmating cells. For this reason, cells were analyzed only through the exconjugant stage prior to the first cell division.

The DNA contents in the developing Macronuclei of starved and refed cells were similar until 16 h past mixing, when the average DNA content in the refed cells increased to about twice that in the starved cells (Fig. 2). By 18 h, Macronuclei in both starved and refed cells attained a DNA content of 10 to 12C. In starved cells, there was no further increase in DNA content. In refed cells, a 10C to 12C plateau was evident from

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\text{TABLE 2. Average DNA content of developing macronuclei}
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| Condition            | DNA content at postmating time (h) | DNA content (C) ± standard error (number of nuclei) |
|----------------------|-----------------------------------|-------------------------------------------------|
| Continuous starvation| 8                                 | 2.32 ± 0.06 (93)                                  |
| Refeed at 8 h        | 2.01 ± 0.07 (75)                 | 2.96 ± 0.08 (175)                                |
|                      | 12                                | 2.72 ± 0.08 (128)                                |
|                      | 14                                | 4.03 ± 0.09 (112)                                |
|                      | 16                                | 3.15 ± 0.13 (285)                                |
|                      | 18                                | 11.07 ± 0.66 (80)                                |
|                      | 20                                | 12.87 ± 0.52 (83)                                |
|                      | 22                                | 15.60 ± 0.60 (55)                                |
|                      | 24                                | 16.69 ± 0.96 (59)                                |
|                      |                                    | 20.71 ± 1.08 (56)                                |
|                      |                                    | 32.55 ± 1.77 (62)                                |

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\text{FIG. 3. Average DNA content of Macronuclei at different cytological stages. Stage 1, paired cells in which the anterior postzygotic nuclei have started to swell and develop as anlagen, with the parental Macronucleus in the center of the cell; stage 2, paired cells in which the Micronuclei and the Macronucleus have moved to a central location in the center of the cell and the parental Macronucleus has migrated to the posterior of the cell, where it is undergoing degradation; stage 3, paired cells or exconjugants with two Micronuclei and two Macronuclei in which the parental Macronucleus was completely degraded; stage 4, paired cells or exconjugants containing two Macronuclei and one Micronucleus. Gray, continuous starvation; black, cells refed at 8 h postmixing. Error bars indicate the standard errors.}
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\text{FIG. 4. DNA content of individual Macronuclei in starved and refed cells. DNA contents were rounded to the nearest whole integer. All nuclei in samples fixed from 14 to 24 h of mating were included. Dotted line, nuclei from starved cells; solid line, nuclei from refed cells.}
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In order for the strategy to be effective, it was necessary to determine that the cassette was inserted at the endogenous ASI2 site. This was confirmed by PCR analysis of DNA from the transformants. The expected 1.15-kb PCR products were obtained from somatic ASI2-GFP cell lines but not wild-type cells (Fig. 5B).

Somatic ASI2-GFP transformants were grown in increasing concentrations of paromomycin until most or all the wild-type ASI2 alleles were replaced by the ASI2-GFP allele. Substantial replacement of endogenous ASI2 genes was confirmed by Southern blot analysis of DNA from purified macronuclei (Fig. 5C). Transformant 1 in Fig. 5C was designated MU127, and transformant 2 was designated MU128.

To determine whether ASI2-GFP had any effect on sexual reproduction, the somatic ASI2-GFP lines MU127 and MU128 were mated under conditions of continuous starvation. The mating was cytologically normal (17). Developing Mac anlagen were first seen at 9 h of mating, and by 12 h, approximately 83% of the cells were at some stage of Mac anlage development. By 24 h, more than 90% of cells were in the exconjugant stage, with two Macs and one Mic in each cell. Thus, the substitution of ASI2-GFP in the parental Macs had no effect on the cytological progress of mating.

Because a cytologically normal mating can produce nonviable pairs or viable clones that have aborted the mating, the fertility of somatic ASI2-GFP tagged cell lines was confirmed genetically. Single pairs from a mating were cloned and tested for growth. True progeny were identified on the basis of 6-methylpurine resistance and paromomycin sensitivity. Ninety-eight percent (93/96) of isolated single pairs grew and formed viable clones, and 99% (92/93) of the clones were sexual progeny, based on their drug resistance characteristics. Thus, somatic ASI2-GFP is sufficient to support the development of viable progeny.

ASI2-GFP heterokaryons MU127 and MU128 were mated; total RNA was isolated from an aliquot of the culture every 3 h, and RT-PCR was carried out with allele-specific primer pairs (Fig. 6A and Table 3). The level of mRNA for H3.3, a histone variant that is transcribed constitutively during conjugation (24, 40), was followed as a control for RNA concentration. ASI2-GFP mRNA transcribed from the parental Macs was present in mating cells from 3 to 9 h postmixing. By 12 h, this signal was no longer detectable (Fig. 6B). Zygotic ASI2 mRNA transcribed in the developing Mac anlagen was prominent from 9 h postmixing (when the Mac anlagen are just beginning to form) through 24 h. Thus, the parental Mac is the source of ASI2 transcripts early in conjugation, and the Mac anlagen are the source of transcripts in later stages.

Viability is reduced in matings between somatic ASI2 knockouts. The data shown in Fig. 6 demonstrate that ASI2 is transcribed in the parental Mac in the early stages of mating, from meiosis to early Mac anlage development. To determine whether parental ASI2 is required for sexual reproduction, somatic ASI2 knockout cells with a wild-type Mic (strain MU111) were mated with an ASI2 knockout homokaryon, MU119, lacking the ASI2 gene in both the Mic and the Mac (17). In this mating, germ line ASI2, which is known to be required to produce progeny, is supplied by the MU111 Mic.

In the absence of parental ASI2, the viability of progeny was lower than that in matings between wild-type cells. Only 48%
of pairs isolated from a mating between ASI2 somatic knockouts grew in nutrient medium (Table 4). In contrast, 85% of the pairs isolated from matings between ASI2 somatic knockout strains and the ASI2 wild-type strain CU427 were viable. Thus, there was reduced viability of pairs isolated from matings between somatic ASI2 knockouts, and the phenotype was recessive in matings of the ASI2 somatic knockouts with ASI2 wild-type cells.

Infertile Tetrahymena cells will often form mating pairs that abort the mating and return to vegetative growth. In order to determine whether matings between somatic ASI2 knockouts were fertile, the phenotype of clones established from individual mating pairs (synclones) was examined. Because there were no drug resistance markers in this cross that could be used to identify sexual progeny, this was done by testing for sexual immaturity. Sexual progeny should be unable to form mating pairs with the tester strain, CU427, until they have undergone at least 65 vegetative fissions (28). Clones from pairs that aborted mating, on the other hand, were expected to mate with CU427 and form progeny, which would be cycloheximide-resistant due to transmission of the chx1-1 allele from the CU427 Mic to the Macs of the progeny (Table 1). Ten μl of a dense culture of each individual surviving clone from pairings between ASI2 somatic knockouts was mixed with 100 μl starved CU427 cells at 2 × 10⁵ cells/ml, and pairing was monitored over a period of 48 h. Five of the 46 synclones (11%) formed large numbers of mating pairs with CU427, and all of these 5 matings produced cycloheximide-resistant progeny, indicating that these synclones were parental ASI2 knockout cells from aborted mating pairs. In contrast, the other 41 synclones formed no pairs at all with CU427 and were therefore judged to be immature sexual progeny from the mating between ASI2 somatic knockout cells. No cycloheximide-resistant cells were recovered in these wells, confirming that the cells were sexually immature cells and unable to mate with CU427. In summary, although the viability of pairs from matings between ASI2 somatic knockouts was low, 89% of the surviving synclones were identified as true progeny, and thus, the fertility of the viable clones was comparable to that of the wild type (Table 4).

To determine whether matings between ASI2 somatic knockouts were arresting at an early stage of development, an aliquot of cells was removed from a mating between the somatic knockout lines MU111 and MU119 every 3 h, and the cells were fixed and stained with DAPI (Fig. 7). At the cytological level, nuclear differentiation and progression of mating between somatic ASI2 knockouts were similar to those previously described for wild-type cells (17). Developing Mac anla-
gen were first detected at about 9 h of mating, and by 12 h, about 83% of the cells were in some stage of Mac anlage development. By 24 h of mating, 78% of the cells were in the exconjugant stage. That is, although only 48% of the pairings between ASI somatic knockouts produce viable progeny, the mating pairs proceed through the mating as far as the exconjugant stage.

**DNA endoreplication is delayed in somatic ASI2 knockouts.**

It was previously shown that matings between germ line ASI2 knockouts are infertile because DNA endoreplication is arrested in the developing macronuclear anlagen (17). The DNA content in the developing Mac of progeny from matings between the ASI2 somatic knockout MU111 and the ASI2 knockout homokaryon MU119 was monitored in order to determine whether the absence of somatic ASI2 synthesized early in mating had any effect on the DNA replication in the Mac anlagen.

At 10 h postmixing, very early in Mac anlage development, the DNA content in the stage 1 anlagen in progeny of ASI2 somatic knockouts was slightly less than that of the progeny of wild-type cells. In addition, the DNA content did not increase in the stage 2 nuclei as it did in wild-type cells (Fig. 8).

Between 10 and 14 h postmixing, the time during which IES elimination occurs, the DNA content of the developing Mac anlagen of ASI2 somatic knockout progeny decreased to 2.3C in nuclei at stage 2 and to 1.8C at stage 3. In contrast, the DNA content in Mac anlagen of wild-type cells remained fairly constant. This is presumably because the first round of DNA endoreplication in wild-type cells masks the reduction of DNA content due to DNA elimination. By 18 h postmixing, the C value began to increase in the Mac anlagen of ASI2 somatic knockout progeny. Thus, replication of the genome resumed after transcription of ASI2 began in the Mac anlagen.

In summary, the somatic ASI2 knockout phenotype is consistent with the germ line knockout phenotype. Transcription of ASI2 from the parental Mac supports endoreplication in the early stages of Mac anlage development, and transcription in the developing Mac is required to maintain endoreplication in the later stages.

**Dynamic distribution of Pdd1p is independent of somatic ASI2.**

In order to determine whether ASI2 was specifically required for endocycling or whether it was a general regulator of the developmental program, two molecular events that occur early in conjugation were examined in matings between ASI2 somatic knockouts. One of the well-characterized events of early conjugation is the accumulation and dynamic distribution of Pdd1p, a chromodomain protein. Pdd1p mRNA is transcribed in the parental Mac (7). Pdd1p, for programmed DNA degradation, is initially concentrated in the parental Mac and then transferred to the Mac anlagen, where it is required for the stability of scRNA and for elimination of IES (7, 26).

The absence of the PDD1 gene in the parental Mac is lethal to progeny (7).

Figure 9A shows a Western blot of proteins isolated from mating cells at 6, 8, and 10 h after mixing of the cells. Relative to the β-tubulin control, the amount of Pdd1p present in the progeny of ASI2 somatic knockouts is indistinguishable from that in progeny of wild-type cells. In particular, there is substantial expression of Pdd1p at 8 h postmixing, even though transcription of zygotic ASI2 mRNA is not upregulated until the stage 1 anlagen are present at 9 h postmixing (Fig. 6). The data suggest that Pdd1p synthesis is independent of parental ASI2.

The subcellular distribution of Pdd1p in matings between ASI2 somatic knockouts is also similar to that previously described for wild-type cells (7, 17, 20) (Fig. 9B). The Mac and Mic of unpaired cells showed no staining for Pdd1p. The protein was first detectable in the cytoplasm and then began to accumulate in the parental Mac early in meiosis I. The staining of the parental Mac increased in intensity as the cells progressed through prezygotic mitosis. During the postzygotic mitosis, strong staining was shown in the conjusome, an organelle of unknown function that is present specifically during conjugation (13). At stage 1 of Mac development, when the anlagen are just beginning to swell, staining was detectable in both the parental Mac and the Mac anlagen. By stage 2 of anlage development, staining with anti-Pdd1p was reduced in the parental Mac, and the majority of the Pdd1p protein was located in the new Mac anlagen. Pdd1p staining in the anlagen took on a

![FIG. 8. Average DNA content of Mac anlagen in progeny of matings between ASI2 somatic knockout strains MU111 and MU119 and wild-type control strains CU427 and CU428. Gray, wild type; black, ASI2 somatic knockouts. Error bars indicate the standard errors. Statistically significant differences from results for wild-type progeny are shown at the following confidence levels: *, P < 0.05; **, P < 0.01.](image-url)
punctuate appearance in the exconjugants after the degeneration of the parental Mac. Of particular note was the presence of Pdd1p at the early stages of conjugation between the somatic knockouts. Since the parental Macs with the knockout allele were not producing $ASI2$ mRNA and transcription in the Mac anlagen does not begin until the stage 1 Mac anlagen are present, production of Pdd1p at the early stages of conjugation is independent of $ASI2$.

**DISCUSSION**

Endoreplication in the developing Mac anlagen of *Tetrahymena* is an example of endocycling. Development of the *Tetrahymena* Mac involves the developmentally regulated, site-specific breakage of the 5 germ line chromosomes into 181 smaller Mac chromosomes, which undergo endoreplication until the Macs reach a DNA content of 128C (9, 22). The data
described here show that endoreplicating *Tetrahymena* cells accumulate at Mac anlage ploidies characteristic of a doubling of the genome, suggesting that endoreplication of the processed chromosomes occurs in alternating S and G phases, a hallmark of endocycling.

An early analysis of the *Tetrahymena* Mac anlagen combined cyphotometry with autoradiography to study the kinetics of DNA replication in the Mac anlagen. In that study, it was assumed that the genome was undergoing successive doublings. Nuclei were assigned to stages of development based on their DNA content rather than by cytological stage or hours of development, and nuclei that were actively incorporating [³H]thymidine were excluded from the analysis. Although the mean DNA content of the classes approximated genome doublings, the distributions of DNA content in each stage were quite broad, with significant overlap in DNA content between the stages (29). These observations raised the possibility that endoreplication might occur in an irregular fashion rather than as discrete doublings. In the early study, some of the variability in DNA content for each stage might be ascribed to the fact that data from starved and refed cells were combined. The experiments described here show that starved and refed cells accumulate at very different distributions in DNA content and the data from cells maintained under the different conditions must be considered separately. Another source of the variability in DNA content in the early study arises from the fact that elimination of DNA sequences occurs between the first and second rounds of endoreplication (29). The analysis in Fig. 4 of the present study includes only cells after 14 h of conjugation, by which time most of the DNA elimination is complete. Thus, the present study confirms the conclusion of Roth and Cleffmann (29) regarding the alternating S and G phases while clarifying some of the ambiguities in their analysis.

The data described here are largely in accord with those of an earlier analysis using flow cytometry (2). In that study, a population of isolated nuclei from starved cells that was enriched in Mac anlagen showed two classes of nuclei, consistent with our Feulgen analysis of Mac anlagen in conjugating starved cells. In contrast to our finding that the Mac anlagen of refed cells accumulate with DNA contents representing genomic doublings, flow cytometry of total nuclei from refed cells revealed a broad distribution of nuclei without any clearly distinguishable classes (29). The total nuclei in this experiment included Mics, degenerating parental Macs, and nuclei from nonmaters. The background from all these different nuclei probably obscured the data from the developing Mac anlagen. The DNA content of nuclei in developing Macs undergoes approximate doublings whether the cells are maintained under conditions of continuous starvation or refeed. However, the DNA contents at which nuclei accumulate are different, suggesting that the nutritional state may affect the fraction of the genome that is replicated. In endocycling cells in multicellular organisms, late-replicating DNA is often underreplicated (11). Late-replicating DNA is generally equated with heterochromatin, which is largely repeated DNA. By 14 h of mating in *Tetrahymena*, most of the repeated elements have been eliminated from the macronuclear genome (39). Whether or not there is a late-replicating component of the *Tetrahymena* Mac genome will be an interesting topic for future study.

**Role of parental Asi2p in sexual reproduction.** Transcription of *ASI2* from the parental Mac early in mating supports DNA replication early in Mac anlage development, and transcription from the Mac anlagen is required to sustain endocycling at later stages. A variety of molecular events that occur during conjugation in *Tetrahymena* appear to be normal in matings between *ASI2* knockouts. The data described here show that the early events in sexual reproduction are independent of parental *ASI2* transcription. The amount and deposition of Pdd1p are normal (Fig. 9). Since progeny of *ASI2* somatic knockouts accumulate scRNA (Fig. 10), *ASI2* is not required in the parental Mac to activate any of the genes required for the production or stability of scRNAs, including *DCL1* (27, 21) and *TWI* (26).

Similarly, DNA replication in mid- to late Mac anlage development requires transcription of zygotic *ASI2* in the developing Mac. Other events in the later stages of anlage development occur normally in germ line *ASI2* knockouts, including Pdd1p deposition, IES elimination (17), and *de novo* DNA methylation (L. Yin and K. M. Karrer, unpublished data). Thus, *ASI2* seems to have a specific role in the support of endoreplication of the DNA during Mac anlage development.

In vegetatively dividing *Tetrahymena*, the Mic divides mitotically and the Mac divides amitotically. Both Mics and Macs have distinctive S phases, albeit at different points in the cell cycle (23). Somatic *ASI2* knockouts replicate DNA in both the Mic and the Mac, and vegetative fission occurs at a rate indistinguishable from that of wild-type cells (17). Thus, *ASI2* is not required for DNA replication per se. With respect to the requirement for *ASI2*, endocycling in *Tetrahymena* is different from both the mitotic and amitotic S phases in vegetatively dividing cells. This is similar to the situation in multicellular organisms, where mitotically dividing and endocycling cells have different requirements to drive the cell cycle (reviewed in reference 18).

**Incomplete penetrance of the *ASI2* somatic knockout phenotype.** Previous studies showed that an absence of germ line *ASI2* is lethal to progeny due to the arrest of endoreplication in the developing Mac (17). In this study, the *ASI2* somatic knockout cells were shown to have a similar phenotype but with incomplete penetrance. *ASI2* knockout homokaryons were mated with *ASI2* somatic knockouts. In these mating pairs, the only source of *Asi2p* was from the developing Macs, which have a mixture of wild-type and *ASI2* null alleles. The reduced viability of pairs isolated from matings between *ASI2* somatic knockouts suggests that parental *ASI2* transcripts contribute to fertility. Why is it that some pairs are viable and others are not? One possibility is that without parental *ASI2*, *ASI2* transcription in the heterozygous Mac anlagen is marginally sufficient to trigger downstream events. In some pairs, the signal level is above the threshold and exconjugants survive, while in other pairs, the signal level is below the threshold and the exconjugants are unable to undergo sufficient DNA replication for survival. A second possibility is that endoreplication of the two alleles in the developing Mac anlagen is unequal. This might lead to variation in the progeny cells with respect to the level of *Asi2p*. According to this hypothesis, cells with a sufficient number of copies of the wild-type allele produce viable progeny, and cells with a preponderance of the *ASI2* knockout allele are unable to complete Mac development.
Transcription of ASI2 in the parental Mic and early Mac anlagen of mating *Tetrahymena*. Although we did not detect any wild-type alleles in the Mac DNA of ASI2 somatic knockouts by Southern blot analysis (Fig. 5C), we cannot exclude the possibility that a few copies may remain in the parental Mac. Indeed, faint RT-PCR signals were detected at 3 and 6 h postmixing. These may be transcripts of wild-type alleles in the parental Mac. Alternatively, these products may be generated from whole-genome transcription of the micronucleus, which has been postulated to be the source of the scRNAs involved in IES elimination (26).

In progeny of *ASI2-GFP* heterokaryons, *ASI2* mRNA transcribed from the developing Mac anlagen was upregulated by 9 h postmixing (Fig. 6). This suggests that the Mac anlage is transcriptionally active very early in its development, as soon as it can be distinguished cytologically (Fig. 6 and 7).

Although transcription of *ASI2* in the Mac anlagen was detected at 9 h of mating (Fig. 6), DNA replication in progeny of *ASI2* somatic knockouts (Fig. 8) was still significantly delayed at 14 h. This suggests that synthesis and processing of *Asi2p* and transmission of the signal require several hours.

**Control of endocycling in multicellular organisms and ciliates.** Little is known regarding the control of endocycling in ciliates. In *Drosophila* follicle cells, the signal transduction receptor Notch controls the mitosis-to-endocycle switch by up- or downregulating genes required for the G1/S, G2/M, and M/G1 cell cycle transitions (31, 30). It is not known whether the changes in transcriptional activity of these genes are a direct or indirect effect of Notch signaling. Interestingly, the endocycle is regulated by different signals in *Drosophila* nurse cells, which do not require Notch for endocycling (18), and in *Arabidopsis* and *Tetrahymena*, which lack Notch homologs. *ASI2*, which encodes a putative signal transduction receptor, is required to sustain endocycling in *Tetrahymena*. Surprisingly, no homolog of *ASI2* was found in the *Paramecium* genome database. However, *Tetrahymena* and *Paramecium* are separated by more than 100 million years of evolution (8). There is a predicted gene in the genome of the more closely related ciliate, *Ichthyophthirius* *multifilis*, whose product bears 26% identity and 47% similarity to that of *ASI2* over 522 amino acids (expect = 5E−34) (L. Hannick and R. Coyne, personal communication). It is not known whether the gene regulates endocycling in *Ichthyophthirius*.

The data described here suggest that endocycling as a variant of the cell cycle is conserved from ciliates to multicellular organisms and the gene *ASI2* is specifically required for endocycling in *Tetrahymena*.

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**REFERENCES**

1. Allis, C. D., M. Colavito-Shepanski, and M. A. Gorovsky. 1987. Scheduled and unscheduled DNA synthesis during development in conjugating *Tetrahymena*. Dev. Biol. 124:469–480.
2. Allis, C. D., and D. K. Dennison. 1982. Identification and purification of young macronuclear anlagen from conjugating cells of *Tetrahymena* thermophila. Dev. Biol. 92:519–533.
3. Austerberry, C. F., C. D. Allis, and M. C. Yao. 1984. Specific DNA rearrangements in synchronously developing nuclei of *Tetrahymena*. Proc. Natl. Acad. Sci. U. S. A. 81:7352–7357.
4. Brunk, C. F., and R. K. Conover. 1985. Elimination of micronuclear specific DNA sequences early in anlagen development. Mol. Cell. Biol. 5:93–98.
5. Bruns, P. J., and T. B. Brussard. 1974. Pair formation in *Tetrahymena* pyriformis, an inducible developmental system. J. Exp. Zool. 188:337–344.
6. Cassidy-Hanley, D., J. Bowen, J. H. Lee, E. Cole, L. A. VerPlank, J. Gaertig, M. A. Gorovsky, and P. J. Bruns. 1997. Germline and somatic transformation of mating *Tetrahymena* thermophila by particle bombardment. Genetics 146:135–147.
7. Coyne, R. S., M. A. Nikiforov, J. F. Smothers, C. D. Allis, and M. C. Yao. 1999. Parental expression of the chromodomain protein Pdd1p is required for completion of programmed DNA elimination and nuclear differentiation. Mol. Cell. 4:665–682.
8. Dessen, P., M. Zagulski, R. Gronadka, H. Plattner, R. Kissmehl, E. Meyer, M. Betermier, J. E. Schultz, J. U. Linder, R. E. Pearlman, C. Kung, J. Forney, B. H. Satir, J. L. Van Houten, A. M. Keller, M. Freindl, S. Sperling, and J. Cohen. 2001. Paramecium genome survey: a pilot project. Trends Genet. 17:306–308.
9. Doerder, F. P., and L. E. DeBault. 1975. Cytolournometric analysis of nuclear DNA during meiosis, fertilization and macronuclear development in the ciliate *Tetrahymena pyriformis*, syngen 1. J. Cell Sci. 17:471–493.
10. Doerder, F. P., and L. E. DeBault. 1978. Life cycle variation and regulation of macronuclear DNA content in *Tetrahymena thermophila*. Chromosoma (Berl.) 91:1–19.
11. Edgar, B. A., and T. L. Orr-Weaver. 2001. Endoreplication cell cycles: more for less. Cell 105:297–306.
12. Gorovsky, M. A., S. Hattman, and G. L. Pleger. 1973. "[N]Methyl adenine in the nuclear DNA of a eucaryote, *Tetrahymena* pyriformis. J. Cell Biol. 56:697–701.
12a. Hardie, D. C., T. R. Gregory, and P. D. N. Hebert. 2002. From pixels to picograms: a beginners’ guide to genome quantification by Feulgen image analysis densitometry. J. histochem. Cytochem. 50:735–749.
13. Janetopoulos, C., E. S. Cole, J. F. Smothers, C. D. Allis, and K. J. Auferheide. 1999. The conjusome: a novel structure in *Tetrahymena* found only during sexual reorganization. J. Cell Sci. 112:1003–1011.
14. Karrer, K. M. 2000. *Tetrahymena* genetics: two nuclei are better than one. Methods Cell Biol. 62:127–186.
15. Kidder, G. W., V. C. Dewey, R. E. Parks, Jr., and M. R. Heinrich. 1950. Further studies on the purine and pyrimidine metabolism of *Tetrahymena*. Proc. Natl. Acad. Sci. U. S. A. 36:451–439.
16. Kiersnowska, M., A. Kaczanowski, and G. de Haller. 1993. Inhibition of oral organophagy during conjugation of *Tetrahymena thermophila* and its resumption after cell separation. Eur. J. Protistol. 29:359–369.
17. Li, S., L. Yin, E. S. Cole, R. A. Udani, and K. M. Karrer. 2006. Progeny of germ line knockouts of *ASI2*, a gene encoding a putative signal transduction receptor, is required to sustain endocycling in *Tetrahymena*. Surprisingly, no homolog of *ASI2* was found in the *Paramecium* genome database. However, *Tetrahymena* and *Paramecium* are separated by more than 100 million years of evolution (8). There is a predicted gene in the genome of the more closely related ciliate, *Ichthyophthirius multifilis*, whose product bears 26% identity and 47% similarity to that of *ASI2* over 522 amino acids (expect = 5E−34) (L. Hannick and R. Coyne, personal communication). It is not known whether the gene regulates endocycling in *Ichthyophthirius*.

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1352 YIN ET AL. EUKARYOT. CELL
28. Rogers, M. B., and K. M. Karrer. 1985. Adolescence in Tetrahymena thermophila. Proc. Natl. Acad. Sci. U. S. A. 82:436–439.
29. Roth, J., and G. Cleffmann. 1986. Pattern of DNA increase in macronuclear anlagen of Tetrahymena. J. Cell Sci. 83:155–164.
30. Scharf, V., C. Althauser, H. R. Scherbata, W. M. Deng, and H. Ruohola-Baker. 2004. Notch-dependent Fizzy-related/Hec1/Cdh1 expression is required for the mitotic-to-endocycle transition in Drosophila follicle cells. Curr. Biol. 14:630–636.
31. Scherbata, H. R., C. Althauser, S. D. Findley, and H. Ruohola-Baker. 2004. The mitotic-to-endocycle switch in Drosophila follicle cells is executed by Notch-dependent regulation of G1/S, G2/M and M/G1 cell-cycle transitions. Development 131:3169–3181.
32. Smith, A. V., and T. L. Orr-Weaver. 1991. The regulation of the cell cycle during Drosophila embryogenesis: the transition to polyteny. Development 112:997–1008.
33. Stuart, K. R., and E. S. Cole. 2000. Nuclear and cytoskeletal fluorescence microscopy techniques, p. 291–311. In D. J. Asai and J. D. Forney (ed.), Methods in cell biology, vol. 62. Academic Press, San Diego, CA.
34. Woodard, J., E. Kaneshiro, and M. A. Gorovsky. 1972. Cytochemical studies on the problem of macronuclear subnuclei in Tetrahymena. Genetics 70:251–260.
35. Wuitschick, J. D., J. A. Gershon, A. J. Lochowicz, S. Li, and K. M. Karrer. 2002. A novel family of mobile genetic elements is limited to the germline genome in Tetrahymena thermophila. Nucleic Acids Res. 30:2524–2537.
36. Yao, M.-C., C.-H. Yao, and B. Monks. 1990. The controlling sequence for site-specific chromosome breakage in Tetrahymena. Cell 63:763–772.
37. Yao, M.-C., J. Choi, S. Yokoyama, C. Austerberry, and C.-H. Yao. 1984. DNA elimination in Tetrahymena: a developmental process involving extensive breakage and rejoining of DNA at defined sites. Cell 36:433–440.
38. Yao, M.-C., S. Duharcourt, and D. L. Chalker. 2002. Genome-wide rearrangement of DNA in ciliates, p. 730–758. In N. L. Craig, R. Craigie, M. Gellert, and A. M. Lambowitz (ed.), Mobile DNA II. ASM Press, Washington, DC.
39. Yao, M.-C., and M. Gorovsky. 1974. Comparison of the sequences of macronuclear and micronuclear DNA of Tetrahymena pyriformis. Chromosoma 48:1–18.
40. Yu, L., and M. A. Gorovsky. 1997. Constitutive expression, not a particular primary sequence, is the important feature of the H3 replacement variant hv2 in Tetrahymena thermophila. Mol. Cell. Biol. 17:6303–6310.