The Transition From Conjugal Development to the First Vegetative Cell Division Is Dependent on \textit{RAD51} Expression in the Ciliate \textit{Tetrahymena thermophila}

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\textbf{ABSTRACT}

\textit{Rad51p}, the eukaryotic homolog of the prokaryotic \textit{recA} protein, catalyzes strand exchange between single- and double-stranded DNA and is involved in both genetic recombination and double-strand break repair in the ciliate \textit{Tetrahymena thermophila}. We have previously shown that disruption of the \textit{Tetrahymena RAD51} somatic macronuclear locus leads to defective germline micronuclear division and that conjugation of two somatic \textit{rad51} null strains results in an early meiotic arrest. We have constructed \textit{Tetrahymena} strains that are capable of \textit{RAD51} expression from their parental macronuclei and are homozygous, \textit{rad51} nulls in their germline micronuclei. These \textit{rad51} null heterokaryons complete all of the early and middle stages of conjugation, including meiosis, haploid nuclear exchange, zygotic fusion, and the programmed chromosome fragmentations, sequence eliminations, and rDNA amplification that occur during macronuclear development. However, the \textit{rad51} null progeny fail to initiate the first vegetative cell division following conjugal development. Coincident with the developmental arrest is a disproportionate amplification of rDNA, despite the maintenance of normal total DNA content in the developing macronuclei. Fusion of arrested \textit{rad51} null exconjugants to wild-type cells is sufficient to overcome the arrest. Cells rescued by cytoplasmic fusion continue to divide, eventually recapitulating the micronuclear mitotic defects described previously for \textit{rad51} somatic nulls.

\textbf{CILIATED} protozoans such as \textit{Tetrahymena thermophila} possess two structurally distinct nuclei that effectively divide the labor of germline and somatic genetic functions (reviewed in \textbf{Karrer} 2000). The micro-nucleus (MIC) serves as the germline nucleus, divides mitotically during vegetative growth, is diploid (\(2N = 10\) for \textit{Tetrahymena}), and is transcriptionally silent. In contrast, the macronucleus (MAC) serves as the somatic nucleus, divides amitotically, contains multiple copies (45C) of most genes, and is transcriptionally active.

The macronucleus is derived from a copy of the zygotic micronucleus through a series of highly controlled developmental processes that occur during sexual reproduction or conjugation (reviewed in \textbf{Orias} 1986). Development of the new MAC involves a major restructuring of the genome that includes chromosome fragmentation (\textbf{Coyne et al.} 1996), elimination of MIC-limited sequences (\textbf{Yao} 1996), high frequency intragenic recombination (\textbf{Deak} and \textbf{Doerder} 1998), and ribosomal DNA (rDNA) amplification (\textbf{Kapler} 1993). It is estimated that for \textit{T. thermophila} as many as 6000 specific deletion events account for removal of internally eliminated sequences, eliminating \(\sim 15\%\) of the MIC genome complexity to produce a mature MAC genome (\textbf{Yao et al.} 1984). These processes result in macronuclear chromosomes that range in length from 50 kb to \(> 1.5\) Mb (\textbf{Karrer} 2000). One notable exception is the ribosomal RNA locus (rDNA), which is converted to a 21-kb palindrome and amplified to 9000 copies per cell (\textbf{Kapler} 1993). Coincident with MAC development is the destruction of the old parental macronucleus. Therefore, it is only following new MAC development late in conjugation that the previously silent MIC genomes from the mated cells are brought into expression.

The highly conserved \textit{Rad51p} catalyzes strand exchange between single- and double-stranded DNA during both genetic recombination and double-strand break repair (reviewed in \textbf{Bianco} et al. 1998). It is likely that \textit{Rad51p} plays a role in the genomic restructuring that gives rise to the MAC genome, given its pattern of expression during conjugation. In addition to the response of \textit{Tetrahymena} to DNA damaging agents (\textbf{Campbell} and \textbf{Romero} 1998), \textit{RAD51} levels are elevated during conjugation at two distinct stages: early, during meiosis, and late, during exconjugant macronuclear development (\textbf{Marsh et al.} 2000). Disruption of the macronuclear \textit{RAD51} locus leads to an abnor-
TABLE 1
Genotype and phenotype of *T. thermophila* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Micronuclear genotype</th>
<th>Macronuclear genotype</th>
<th>Macronuclear phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU248.2</td>
<td>mpr1/mpr1</td>
<td>MPR1</td>
<td>mp-s, VII</td>
</tr>
<tr>
<td>CU438.1</td>
<td>pmr1/pmr1</td>
<td>PMR1</td>
<td>pm-s, IV</td>
</tr>
<tr>
<td>A*III</td>
<td>Functional amicronucleate</td>
<td>Wild type</td>
<td>III</td>
</tr>
<tr>
<td>TC104</td>
<td>mpr1/mpr1; RAD51/rad51-1::neo</td>
<td>mpr1, RAD51</td>
<td>pm-s, mp-r, V</td>
</tr>
<tr>
<td>TC105</td>
<td>mpr1/mpr1; rad51-1::neo/rad51-1::neo</td>
<td>mpr1, RAD51</td>
<td>pm-s, mp-r, V</td>
</tr>
<tr>
<td>TC106</td>
<td>mpr1/mpr1; rad51-1::neo/rad51-1::neo</td>
<td>mpr1, RAD51</td>
<td>pm-s, mp-r, III</td>
</tr>
</tbody>
</table>

Macronuclear phenotype designations: -r, resistant; -s, sensitive. Locus names are as follows: *mpr*, 6-methylpurine (mp) resistance; *rad51-1::neo*, a mutant locus disrupted by the neomycin cassette, which confers paromomycin resistance (pm-r). Mating types are designated by roman numerals. (*) Strains contain a hypodiploid micronucleus that is functionally amicronucleate. CU428.2 and CU438.1 were provided by P. Bruns (Cornell University).

Mal conjugal phenotype between *rad51* null strains, with a block occurring during meiosis (Marsh *et al.* 2000).

Unfortunately, the early conjugal block exhibited by *rad51* somatic null cells precludes an evaluation of a *rad51* null background on late events during conjugal development (Marsh *et al.* 2000). To investigate the impact that a *rad51* null background has on late conjugal processes, it was necessary to construct Tetrahymena *rad51* null heterokaryons that are capable of wild-type *RAD51* expression from their parental macronuclei during early and middle stages of conjugation. In this way a *rad51* null background is established only late in development. In this study, we demonstrate that these cells are able to complete the early stages of conjugation, including meiosis, haploid nuclear exchange, and zygotic fusion. However, the inability of the exconjugants to express *RAD51* from their macronuclear anlage during the late stages of conjugation has rather surprising consequences on rDNA amplification and initiation of the first vegetative cell division following exconjugant development.

**MATERIALS AND METHODS**

**General methods:** *T. thermophila* cultures were grown in 1–2% PPYS (proteose peptone, yeast extract, and sequestrine), as described in Yu and Blackburn (1990). All cultures were maintained at 30°C and in 1× PSF (penicillin, streptomycin, fungizone; Life Technologies). The micronuclear and macronuclear genotypes for all *T. thermophila* strains used and generated in this study are indicated in Table 1. Tetrahymena total DNA isolation by detergent lysis (Yu and Blackburn 1990) and molecular techniques were as described (Sambrook *et al.* 1989). Southern blot analysis was as previously described (Marsh *et al.* 2000).

**PCR primers and products:** PCR products were radiolabeled by the incorporation of [α-32P]dATP (specific activity 3000 Ci/ml) as described (McGormick-Graham and Romero 1996). A probe specific for the Tetrahymena *RAD51* 5′ non-transcribed sequence (NTS; Marsh 2000) was synthesized with primers P1(+/−). Portions of the Tetrahymena β-tubulin 1 (*BTU1*) 3′ NTS (Gaertig *et al.* 1993) and the rDNA 3′ NTS (Kapler 1993) were amplified with primers P2(+/−) and P3(+/−), respectively. The primers are as follows:

P1(+) GACGAATTCCGATTTGTC
P1(−) TCACCTCGTGAAGTGC
P2(+) TCGGTAGCTAAGCAGAC
P2(−) ATGGCCGTTGAGTGCA
P3(+) AAATTTAAGCGGAAAGTTC
P3(−) GTATTTATTTGATGAA

**RAD51 micronuclear gene replacement:** *T. thermophila* strains CU248.2 and A*III* (Table 1) were grown in 200-ml cultures to a density of 1.0 × 10^6 cells/ml and starved in 200 ml of 10 mm Tris HCl (pH 7.5) for 18 hr prior to mixing. Pairing efficiency (>90%) was monitored 3 hr after mixing equal numbers of cells, and the mated cells were centrifuged 0.5–1.5 hr later, washed once in 10 mm HEPES (pH 7.5), and suspended in 2 ml 10 mm HEPES (pH 7.5) at ~1 × 10^7 cells/ml. The concentrated cell suspension was transferred to filter paper in a 100-mm diameter petri dish and placed in the Bio-Rad (Hercules, CA) gene gun chamber. Au particles (0.6 μm in diameter), coated with ~1.0 μg of pTiRd51KO (Marsh *et al.* 2000) digested with *XbaI* and *KpnI*, were biolistically delivered to cells following the manufacturer’s instructions. Upon suspension in 1% PPYS (30 ml), the cells were allowed to complete round I genomic exclusion for 18 hr. The transformed cells were washed in 10 mm Tris HCl (pH 7.5), mixed with a prestarved culture of CU428.2 to complete round II genomic exclusion, and fed 12 hr later with an equal volume of 2% PPYS. Positive selection for exconjugants with 15 μg/ml 6-methylpurine (6-mp) was initiated 12 hr after feeding. Following 24 hr of 6-mp treatment, the bulk culture was distributed into 96-well plates (150 μl aliquots) and treated with 120 μg/ml paromomycin (pm) to select for transformed exconjugants. A single clonal line resistant to pm (pm-r) was identified 4 days later (TC104; Table 1) and maintained under continuous growth conditions in fresh 2% PPYS. TC104 was screened by Southern analysis for the presence of the macronuclear *rad51-1::neo* allele as previously described (Marsh *et al.* 2000) and then continually subcloned into fresh media without selection to promote both loss of pm-r through phenotypic assortment and sexual maturation. The mating type of TC104 was determined as described (Hamilton and Orías 2000), and the successful loss of the macronuclear *rad51-1::neo* allele confirmed by testing for pm-sensitive (pm-s).

Strains homozygous for the *rad51* null allele in their micronuclei (TC105 and TC106; Table 1) were generated by mating the *RAD51/rad51-1::neo* heterozygote TC104 with...
strain A*III in a single round I genomic exclusion. Mated pairs and syncytes were isolated and subsequently expanded as clonal lines for further genetic analysis. The progeny of an outcross to strain CU428.2 were monitored for pm-r to identify those parental syncytes that were homozygous rad51 nulls. Southern blot analysis of the progeny confirmed that the parental strains TC105 and TC106 were homozygous for the rad51-1:neo allele.

**Electrofusion:** Cytoplasmic fusion was as described by Gaertig and Cole (2000). Briefly, samples were washed and suspended in fusion buffer (0.1 mM CaCl2/0.5 mM sucrose solution) at a density of 5.0 × 10⁶ cells/ml. Fifty microliter were placed into a 5 mm BTX (San Diego) fusion chamber consisting of a microscope slide and two platinum wires. The cells were exposed to 18.0 V of AC current for 5 sec, followed by 80.0 V of DC current for 0.50 msec (Electro Cell Manipulator ECM 2001). The cycle of AC and DC current was repeated zero, one, or two times for three successive samples, which were pooled to ensure maximal recovery of fused cells. Following the last cycle of electrofusion, cell samples were transferred to 1 ml of 10 mM Tris-HCl (pH 7.5), diluted to ~1.0 × 10⁶ cells/ml in 1% PPS, and selected for growing cells in 100 µg/ml µm.

**Nuclear staining and visualization:** Prior to staining with 4',6-diamidino-2-phenylindole (DAPI), cells were fixed by three sequential washes of 50% MeOH, 70% MeOH, and MeOH:HOAc (70:15%) and air dried on microscope slides at 25°C. Fixed cells were stained with DAPI (0.1 µM/mL), followed by a wash in 95% EtOH. Samples were examined with an Olympus B-Max fluorescence microscope at ×320 magnification using a X40 oil-immersion objective lens, a 1.6 objective setting, and a ×5 ocular lens. Micrographs were recorded digitally using a SPOT camera and imaging software.

**Feulgen staining:** Mating pairs or exconjugant cultures (1 ml) were gently centrifuged in an IEC CL centrifuge (1000 rpm, 2 min). The supernatant was discarded, and the pellet washed in 50% methanol, 70% methanol, and 70% methanol/10% glacial acetic acid. Cell pellets were suspended in 150 µl of 70% methanol/10% glacial acetic acid and dropped onto clean microscope slides alongside drops of wild-type mating cells (CU428.2 × CU438.1), harvested, and fixed 2 hr after conjugation was initiated. The micronuclei of wild-type pairs provided a reliable diploid (2N4C) standard to quantify the macronuclear DNA content of exconjugants by cytophotometry (Doerder and de Bault 1975).

Samples were fixed for 5 min in Carnoy’s solution (EtOH:glacial acetic acid, 3:1), rinsed in dH2O, and transferred to 5 mM HCl for precisely 20 min to complete hydrolysis. Slides were rinsed and transferred to Schiff’s reagent for 2 hr at 25°C. After a rinse in dH2O, slides were treated for 10 min in dH2O:1 mM HCl:10% K2SO4 (18:1:1), rinsed twice in dH2O, and dehydrated through a series of 2-5 min washes in EtOH (35, 50, 70, 95, 100, and 100%). Air-dried slides were mounted in Euparal with coverslips.

**Cytophotometry:** A Zeiss (Thornwood, NY) Axioskop microscope with a ×63 oil immersion objective, a set of motorized filter changers, and a Zeiss MSP-21 photometer were used to measure the relative macronuclear DNA content of exconjugants. A dual wavelength cytophotometry protocol was used to mathematically transform light absorbance values of Feulgen-stained nuclei at two optimal wavelengths (480 and 560 nm), making it possible to determine the relative amount of Feulgen-stained material present in each nucleus (Mendelsohn 1958). Measured absorbance (Abs) at the two wavelengths allowed for the correction of irregularities in the brightness of the field of view (stained nuclei vs. unstained background). For each data point, 10 diploid (2N4C) micronuclei from control samples and 10 nuclei from experimental samples were measured from side-by-side drops on the same slide. The diploid control value was divided by 4 to establish an Abs value for 1C. All other nuclei were measured using this 1C value to establish ploidy.

**Biotinylated rDNA probe:** Approximately 2 µg of the Tetrahymena rDNA vector pDH18 (Yao and Yao 1991) was used to synthesize a biotinylated probe by the incorporation of 1 mM Biotin-16-dUTP (Roche) during nick translation as described (Sambrook et al. 1989). The probe was precipitated from the reaction by the addition of 1 volume of isopropanol in the presence of 20 µg microtene sperm DNA. The precipitated sample was washed once with 70% EtOH prior to dissolving in 20 µl dH2O.

**Fluorescence in situ hybridization:** Biotinylated rDNA probe hybridization to fixed Tetrahymena cells was adapted from a procedure developed by Ward et al. (1997). One-milliliter cultures (1.0 × 10⁶ cells/ml) were pelleted in an IEC CL centrifuge (1000 rpm, 2 min), washed in 10 mM Tris-HCl (pH 7.5), and the loose pellet fixed in 0.5 ml Shaudinn’s reagent [saturated HgCl2(aq)/100% EtOH/0.17 M acetic acid; 2:1:1] for 10 min at 25°C. The fixed cells were centrifuged, and the pellets washed successively in 70% EtOH and 0.1 M triethanolamine (TEA), pH 8.0. Cells were acetylated in 0.1% acetic anhydride, 0.1 M TEA for 10 min at 25°C, and then concentrated by centrifugation to washing in 2X SSC (0.3 M NaCl, 0.05 M Na citrate). The acetylated cell pellets were suspended in 0.1 ml of 70% formamide, 2X SSC, heated to 70°C for 1 min, and rapidly cooled in an ice bath for 10 min prior to dilution with 0.1 ml ice-cold dH2O. The cells were recovered as a nearly transparent pellet by centrifugation and suspended in 50 µl hybridization solution (30% formamide, 4X SSC, 0.1% BSA, 10% dextran sulfate, 5% SDS, 10 mM EDTA, pH 8.0). The cells were hybridized to ~50 ng biotinylated rDNA probe at 37°C overnight. Specific hybridization of the probe was achieved by successive 0.5-ml washes of 40% formamide, 2X SSC (37°C, 10 min), 2X SSC (25°C, 10 min), and 1X SSC (25°C, 10 min).

To detect the biotinylated rDNA probe in situ, the fixed and probed cells were incubated in a 100-µl solution with 2 µg FITC conjugated to avidin (Vector Labs, Burlingame, CA) for 30 min at 25°C [0.5 M NaCl, 0.1 M Na2CO3 (pH 8.2)]. Background binding of the fluorescent tag to the fixed cells was reduced by three sequential washes (10 min at 25°C) with 4X SSC, 4X SSC, 0.2% Triton-X-100, and 4X SSC. One drop of VectorShield w/DAPI (Vector Labs) was added to the cell pellet prior to placing 20 µl of the suspension on microscope slides. Cells were scanned under a Bio-Rad MRC1000/1024 laser confocal microscope (UMN Imaging Center, Dr. Mark Sanders, director).

**RESULTS**

**Construction of micronuclear rad51 homozygous null strains:** The *T. thermophila* RAD51 micronuclear locus was targeted for disruption with a selectable marker (pm-r) that has been described previously for disruption of the RAD51 macronuclear locus (Marsh et al. 2000). The disruption “cassette” was introduced biologically during meiosis in a “round I” genomic exclusion cross between the wild-type strain CU428.2 and the “star” strain, A*III (Table 1). Micronuclear transformation is most efficient when the transforming DNA is introduced during meiosis (Cassidy-Hanley et al. 1997).

Since progeny from a star mating, such as that described above, retain their parental macronuclei (Doerder and Shabatura 1980), it was necessary to
cross the exconjugants with a second wild-type strain for a round II genomic exclusion cross (Bruns and Cassidy-Hanley 2000). This additional mating ensured that the transformed micronuclei were brought into expression as new macronuclei during exconjugant development, making it possible to select the heterozygous transformants for their resistance to the antibiotic pm. The Rad51/rad51-1::neo heterozygote (strain TC104; Table 1) was subcloned and allowed to mature over multiple fissions in the absence of pm selection, which leads to the eventual loss of the rad51-1::neo allele from their amitotically dividing macronuclei through the process of phenotypic assortment (Orias and Flacks 1975). The resultant pm-resistant strain subsequently underwent another round I genomic exclusion cross to produce progeny, 50% of which were homozygous for the rad51-1::neo micronuclear allele. Genotypes for the resultant rad51 null heterokaryons (strains TC105 and TC106; Table 1) were confirmed by an outcross to a wild-type pm-resistant strain. One hundred percent of the progeny from the outcross exhibited both resistance to pm and a restriction digest polymorphism at the RAD51 locus (Marsh et al. 2000), indicative of disruption by the neomycin resistance cassette (data not shown). TC105 and TC106 both include wild-type RAD51 in their macronucleus, ensuring normal levels of RAD51 expression from the parental macronuclei during the early stages of conjugation.

A developmental arrest phenotype for whole genome rad51 null exconjugants: TC105 and TC106 were starved and mixed to initiate conjugation, and their progression through the various conjugal stages was monitored cytologically (Cole and Soelter 1997; Cole et al. 1997). In contrast to the meiotic prophase I arrest observed in a cross between somatic rad51 nulls (Marsh et al. 2000), conjugating TC105 and TC106 were able to successfully progress through pair separation and macronuclear development (Figure 1). Selection for pm-resistant (introduced either 12 or 24 hr after conjugation was initiated) ensured that only true exconjugants that had successfully undergone macronuclear development would propagate upon feeding. Despite normal pair separation and progression through macronuclear development, TC105 × TC106 exconjugants were incapable of division. Both anlagen persist in individual exconjugants, their cell mass increasing steadily for many days after conjugation was initiated (Figure 1). These cells survived in culture for up to 2 weeks without proliferating, in an apparent developmental arrest, prohibiting entry into the vegetative cell cycle.

Coincident with the increase in cell mass was an apparent increase in the macronuclear anlage volume for rad51 null exconjugants (Figure 1). Normal conjugants rapidly increase the DNA content of the anlage, increasing from 2C at the first postzygotic nuclear division to 16C at the time of conjugant separation, ultimately reaching a maximum of 128C in immature cells upon completion of their second vegetative cell cycle (Doerder and de Bault 1975). We conducted a cytophotometric analysis of conjugating cells to gauge whether the macronuclear DNA content of rad51 null exconjugants differs significantly from that of the wild-type cells at similar stages of macronuclear development.

Although the volume of the average rad51 null exconjugant macronucleus was greater than that of similarly immature wild-type cells a few days after pair separation (Figure 1), their DNA content rarely exceeded the normal 128C maximum (Figure 2). Both wild-type and mutant exconjugants replicate their macronuclear DNA at the same rate until shortly after pair separation, when DNA levels are ~16C for the developing anlage. Beyond this stage, macronuclear DNA accumulation is abnormally slow in the rad51 null cells, lagging 24–48 hr behind wild-type exconjugants before achieving 128C. By the time rad51 null exconjugants have achieved a macronuclear DNA content of 128C, matching their wild-type counterparts, wild-type exconjugants have passed through at least one exconjugant cell division and have entered subsequent rounds of exconjugant mitosis (Doerder and de Bault 1975).

Cytosolic “rescue” of rad51 nulls by electrofusion: It was conceivable that the inability of rad51 null exconjugants to initiate the first vegetative division is due to an irreversible block in the cell cycle, comparable to a check-point arrest. To directly address this question, we performed the following “cytosolic rescue” experiment. Wild-type pm-resistant CU428.2 cells were electrofused (Gaertig and Cole 2000) to the pm-resistant rad51 null exconjugants 2 days after conjugation was initiated. Pulses of direct current result in cell-to-cell fusion, with the subsequent mixing of cytosolic fractions between the fused wild-type and mutant cells. Following electrofusion, the mass cultures were maintained in complex media under continuous selection in 100 μg/ml paromomycin. The only electrofused cells with the potential to undergo continued cell divisions under these selective conditions are...
Southern blot analysis revealed that entry of rad51 null cells into the cell cycle was not dependent on DNA transfer during electrofusion (Figure 3A). In addition, the mitotic defect exhibited by somatic rad51 nulls (Marsh et al. 2000) was recapitulated in the electrofused rad51 nulls (Figure 3B).

**Molecular analysis of rad51 null exconjugants:** Despite an abnormally large cell size that arises as a consequence of fission block, rad51 null exconjugants retain the ability to monitor and regulate their macronuclear DNA content, rarely exceeding the G2 maximum of 128C observed for wild-type cells (Figure 2). The developmental arrest coincides precisely with the period of anlagen development, when numerous genome rearrangements occur to give rise to the macronuclear genome. To more completely characterize rad51 null exconjugants in this developmental arrest, total DNA was isolated from whole cells over a period of 7 days following conjugation and digested with restriction enzymes prior to Southern blot analysis. Somewhat surprisingly, a prominent restriction fragment pattern with an intensity that increased over time was evident in stained agarose gels (Figure 4). The pattern varied as a function of the restriction enzyme used, and the fragment sizes were indicative of the mature rDNA palindrome. Although the rDNA palindrome is normally amplified during exconjugant development (Kapler and Blackburn 1994), the extent of amplification in rad51 null cells vastly exceeds that of wild-type exconjugants at the same developmental stage. Southern blot analysis confirmed that rDNA levels were disproportionately higher than those of other macronuclear genes (Figure 4). The copy number of rDNA molecules gradually modulates down to that of normal cells if rad51 null exconjugants are cytologically rescued by electrofusion and allowed to complete numerous rounds of the vegetative cell cycle. This suggests that the continued amplification of rDNA in nonfused mutants is due to blocked entry into the first exconjugant cell division cycle and is not a direct consequence of their rad51 null background.

**Localization of rDNA in rad51 nulls:** Fluorescence in situ hybridization (FISH) has been used to localize Tetrahymena rDNA to the periphery of the macronuclear membrane in a punctate, nucleolar staining pattern (Ward et al. 1997). We have employed this methodology to determine if the highly abundant rDNA in rad51 null exconjugants is similarly organized within macronuclei. Fluorescence confocal micrographs of wild-type and rad51 null exconjugants hybridized in situ to an rDNA probe are shown in Figure 5. Both wild-type and rad51 nulls display the normal rDNA perinucleus staining pattern indicative of properly organized nucleoli (Ward et al. 1997). However, the numbers of rDNA foci are far greater for the rad51 nulls, reflective of the high levels of ongoing rDNA amplification in these cells (Figure 4).
**DISCUSSION**

**RAD51 and the cell cycle:** The macronuclear anlagen differ from macronuclei at any other stage of the Tetrahymena life cycle. In addition to palindromic rDNA amplification, which is specifically limited to this developmental period (Kapler and Blackburn 1994), all other macronuclear DNA molecules are replicated until the copy number increases 16-fold, from 2C to ~32C just prior to cytokinesis (Doerder and de Bault 1975). Even the first vegetative cell division following conjugation in Tetrahymena is unique with respect to all other cell divisions, in that two macronuclear anlagen are present in each exconjugant. Macronuclear division does not initiate under these unique circumstances, as the newly developed macronuclei simply segregate with the two daughter cells upon completion of micronuclear mitosis (Karrer 2000). We have shown that the absence of RAD51 from the macronuclear anlagen prevents initiation of the first vegetative cell division following pair separation. Consequently, both the normal replication of macronuclear DNA and the amplification of rDNA palindromes are affected (Figure 6).

We have shown previously that somatic rad51 null cells eventually become severely aneuploid over the course of multiple cell divisions, a phenomenon attributed to an anaphase delay during micronuclear mitosis (Marsh et al. 2000). The failure to complete mitosis in a timely manner does not prevent the initiation of both macronuclear division and cytokinesis, which proceed to completion despite the mitotic delay (Figure 3). Apparently, the cell cycle signals that prompt both macronuclear division and cytokinesis are dependent on the initiation of micronuclear mitosis, not on its timely completion.

In contrast, micronuclear mitosis fails to initiate in rad51 null exconjugants, preventing as-yet-undetermined downstream events that normally lead to the first vegetative division from occurring. It is not clear whether the absence of RAD51 in null exconjugants is directly or indirectly responsible for the first vegetative division cell cycle arrest. A RAD51 homolog from human...
tissues (hsREC2) has been implicated in cell cycle control via the phosphorylation of cyclinE/cdk2 (Havre et al. 2000). Furthermore, overexpression of hsREC2 causes a delay in G1 (Havre et al. 1998). It is conceivable that RAD51 fulfills a similar cell cycle control function in Tetrahymena. However, a direct effect would imply a requirement for Rad51p in initiating the first exconjugant division that is not required for initiation of subsequent vegetative cell cycles (Figure 3; Marsin et al. 2000).

It is also conceivable that RAD51’s involvement in the first cell cycle may be indirect, perhaps by ensuring the proper processing of another macronuclear locus whose function is to initiate the first cell cycle. Expression of such unknown factor(s) is not limited to the period of exconjugant development, because electrofusoin of vegetatively dividing wild-type cells to the arrested exconjugants is sufficient to prompt their division.

Although other factors may be involved, a likely candidate for mediating the cytoplasmic rescue is Rad51p itself. An experiment where somatic rad51 null cells (TC102 or TC103; Marsh et al. 2000) are electrofused to the rad51 null exconjugants would directly provide an answer to this question. Unfortunately, this experiment cannot be done with the strains at hand, as it is not possible to positively select for cytoplasmically rescued exconjugants derived from fusion of the two rad51 nulls (both TC102 and the rad51 null exconjugants share the same selectable marker, pm-r).

What is normally a narrow window for rDNA amplification (Kapler and Blackburn 1994) has been extended for an indefinite period as a consequence of the rad51 null exconjugant developmental block. It is clear that Rad51p is not directly involved in the amplification process (Figures 4 and 5), although it may be involved in rDNA maturation (see below). Despite the abnormal accumulation of rDNA, total macronuclear DNA content continues to be closely monitored by the cells, approaching but not exceeding 128C per mutant macronucleus (Figure 2). In addition, the ratio of the rDNA palindrome to a non-rDNA allele (BTU1) increases progressively over a period of days (Figure 4). This phenomenon may be indicative of the DNA replication pattern during this developmental stage, which ordinarily would not be noticeable during the normal period for rDNA amplification (2–4 hr). It is the extended rDNA amplification by virtue of the cell cycle block in rad51 null exconjugants that has accentuated the difference in rDNA vs. non-rDNA accumulation.

It is only after electrofusion of rad51 null exconjugants to wild-type cells, followed by multiple cell fissions over a period of days, that the ratio of rDNA to non-rDNA in the macronucleus approaches that of normal cells (Figure 4). This is consistent with a mechanism of rDNA copy number regulation (relative to that of the overall macronuclear DNA content) that is activated only after the first vegetative cell division.

**RAD51 and rDNA palindrome formation:** The programed genomic remodeling that occurs during development of a new macronucleus from a copy of the zygotic micronucleus in Tetrahymena is as yet poorly understood (Coyne et al. 1996). There is indirect evidence that homologous recombination may play a role in at least some of the rearrangements that occur during this process. It has been shown that intragenic recombination of the SERH1 locus within the macronuclear anlagen is responsible for wild-type expression of the SerH1 surface protein (Deak and Doerder 1998). One model for the conversion of the macronuclear rDNA monomer to the highly amplified rDNA palindrome includes homologous recombination as part of the rearrangement (Butler et al. 1995, 1996). It was hoped that Tetrahymena heterokaryons incapable of wild-type RAD51 expression from their macronuclear anlagen during exconjugant development would shed light on the involvement of RAD51 in genomic remodeling.

Despite the successful maturation of rDNA palindromes in rad51 null exconjugants (Figure 4), a role for RAD51 in the initial palindrome formation from a monomeric intermediate cannot be entirely ruled out. Northern blot analysis of RNA extracted from synchronously mated Tetrahymena reveals a continuum of RAD51 expression throughout conjugation, with two maxima occurring at prezygotic and exconjugant development (Marsh et al. 2000). It is conceivable that wild-type RAD51 expressed from the parental macronuclei in a TC105 × TC106 cross could contribute to rDNA maturation if the rearrangement occurred early enough during postzygotic development. De novo rDNA palindromes have been detected in a two-dimensional gel analysis of rDNA replication intermediates as early as 12 hr after conjugation has been initiated (Zhang et al. 1997). Maturation of even a single rDNA molecule should be sufficient for the eventual predominance of the highly amplified palindrome over the monomer.

A germline rDNA *cis* mutation that delays rDNA palindrome formation until the later stages of exconjugant development has been identified (Kapler and Blackburn 1994; Kapler et al. 1994). An altered chromosome breakage site in rmm11 (rDNA maturation and maintenance) is responsible for the processing delay, which occurs only after the normally restricted period of rDNA amplification in the developing macronucleus. The net result is a severe amplification defect caused by this rDNA excision mutation.

To address the question of RAD51 involvement in rDNA palindrome formation, we are currently constructing Tetrahymena heterokaryons that are homozygous for both rad51-1::neo and rmm11. We anticipate that during conjugation of two strains with this genotype, maturation of the rmm11 rDNA palindrome will be delayed until gene expression is entirely dependent on the developing macronuclei, which will be incapable of wild-type RAD51 expression. The cell cycle arrest at the first vegetative division (Figure 1) and continued rDNA
amplification in rad51 null exconjugants (Figures 4 and 5) will facilitate the detection of de novo rDNA molecules in the progeny from this cross. The absence of rDNA palindromes in the developing anlage would strongly suggest a role for RAD51 in palindrome formation. Conversely, detection of rDNA palindromes in exconjugants would support the conclusion that rDNA maturation is independent of RAD51.

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LITERATURE CITED


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