RAD51 Is Required for Propagation of the Germinal Nucleus in *Tetrahymena thermophila*

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ABSTRACT

RAD51, the eukaryote homolog of the *Escherichia coli recA* recombinase, participates in homologous recombination during mitosis, meiosis, and in the repair of double-stranded DNA breaks. The *Tetrahymena thermophila RAD51* gene was recently cloned, and the *in vitro* activities and induction of Rad51p following DNA damage were shown to be similar to that of *RAD51* from other species. This study describes the pattern of Tetrahymena *RAD51* expression during both the cell cycle and conjugation. Tetrahymena *RAD51* mRNA abundance is elevated during macronuclear S phase during vegetative cell growth and with both meiotic prophase and new macronuclear development during conjugation. Gene disruption of the macronuclear *RAD51* locus leads to severe abnormalities during both vegetative growth and conjugation. *rad51* nulls divide slowly and incur rapid deterioration of their micronuclear chromosomes. Conjugation of two *rad51* nulls leads to an arrest early during prezygotic development (meiosis I). We discuss the potential usefulness of the ciliates' characteristic nuclear duality for further analyses of the potentially unique roles of Tetrahymena *RAD51*.

THE exchange of information between DNA molecules fulfills two seemingly conflicting roles. Homologous recombination during meiosis generates genetic diversity within a species by mediating exchange between homologous chromosomes, whereas the same mechanism helps to maintain genetic stability by promoting exchange between sister chromatids, thereby effecting DNA repair (Thompson and Schild 1999).

It has been known for years that the *RAD51* gene from the budding yeast *Saccharomyces cerevisiae* plays an essential role in genetic recombination and DNA repair (Game 1983). Yeast clones lacking a functional *RAD51* gene are hypersensitive to DNA damaging agents, fail to sporulate, and exhibit deficiencies in mitotic homologous recombination. The *S. cerevisiae RAD51* gene shares significant sequence similarity with the bacterial *recA* gene (Basile *et al.* 1992; Shinohara *et al.* 1992). Biochemical analysis has shown that, like RecA, *S. cerevisiae* Rad51 protein is a DNA-dependent ATPase with DNA strand-exchange activity (Sung 1994). Structure analysis indicates that Rad51 protein polymerizes on double-stranded DNA to form a helical filament that is nearly identical to that formed by RecA (Ogawa *et al.* 1993).

RAD51 genes have been cloned from *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and a number of vertebrate and mammalian species, including *Homo sapiens* (Muris *et al.* 1993; Shinohara *et al.* 1993; Akaboshi *et al.* 1994; Rinaldo *et al.* 1998). In addition to primary sequence conservation, there are data that Rad51 function has also been conserved throughout evolution. First, the recombinant human Rad51 protein has strand-exchange activity similar to that of the yeast homolog (Baumann *et al.* 1996). Second, overexpression of Rad51 mRNA stimulates homologous recombination and increases the resistance of Chinese hamster ovary (CHO) cells to ionizing radiation (Vispe *et al.* 1998). Third, antisense inactivation of Rad51 renders cultured mouse cells sensitive to ionizing radiation (Taki *et al.* 1996). Fourth, Rad51 mRNA levels correlate with homologous DNA recombination activity in normal and transformed cells (Xia *et al.* 1997).

Given the apparent high degree of Rad51 conservation from yeast to mammals, it was surprising to discover that inactivation of this gene in both chicken and murine somatic cells is lethal (Tsuzuki *et al.* 1996; Sonoda *et al.* 1998), whereas the *S. cerevisiae* and *S. pombe RAD51* homologs are dispensable (Shinohara *et al.* 1992; Muris *et al.* 1993). This suggests that the vertebrate Rad51 protein may provide additional function(s) not associated with its yeast homologs. Consistent with this view, the human Rad51 protein interacts with the proteins encoded by the tumor suppressor genes p53, BRCA1, and BRCA2 (Sturzbecher *et al.* 1996; Scully *et al.* 1997; Chen *et al.* 1998). *In vitro* evidence suggests that the p53 protein may negatively regulate the activity of Rad51 (Sturzbecher *et al.* 1996). It has also been reported that c-Abl phosphorylates Rad51 following cellular irradiation and that phosphorylation of Rad51 in-

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hibits its ability to bind DNA and catalyze DNA strand transfer (Yuan *et al.* 1998). It is therefore conceivable that Rad51 may play an essential role in genome stability in higher eukaryotes.

Unfortunately, the inability to culture *rad51* null mammalian cells poses a significant barrier to gaining greater insight into this protein's function in these cells. The recent cloning of a *RAD51* from *Tetrahymena thermophila* suggests a possible means to overcome this difficulty (Campbell and Romero 1998). The ciliated protozoa possess an unusual genome organization that effectively divides the labor of germline and somatic genetic functions between two distinct nuclei (Prescott 1994). The germline micronucleus is diploid, divides mitotically, and is transcriptionally silent. In contrast, the somatic macronucleus is polyploid, divides amitotically, and is actively transcribed. During sexual reproduction (conjugation), the macronucleus is derived from a copy of the micronucleus through a developmental process that involves a series of site-specific chromosome breakage and DNA deletion events (Coyne *et al.* 1996). For Tetrahymena ($2N = 10$), 90% of the germline nuclear content is retained in the macronucleus, where the vast majority of genes are replicated and maintained at ~45 copies per cell. There are ~250 macronuclear chromosomes that average between 50 and 100 kb in length (Prescott 1994).

The nuclear dualism of *T. thermophila* provides a unique environment for the investigation of genes involved in the maintenance of genome stability. The highly regulated and sequence-specific genomic rearrangements that occur during ciliate development have prompted our investigation of *trans*-acting factors that mediate these processes. In this study, we have explored the pattern of Tetrahymena *RAD51* expression during both the cell cycle and conjugation, as well as the consequences of *RAD51* gene replacement on both mitotic division and conjugation.

MATERIALS AND METHODS

General methods: *T. thermophila* cultures were maintained in 1–2% PPYS (proteose peptone, yeast extract, and sequestrene) at 30°, as previously described (Yu and Blackburn 1990). All Tetrahymena cultures were maintained in 1× PSF (GIBCO BRL, Gaithersburg, MD) to prevent bacterial and fungal growth. Cell densities were determined with a Coulter (Hialeah, FL) particle counter. Tetrahymena total DNA was isolated by detergent lysis as described (Yu and Blackburn 1990). RNA was prepared with the MicroPoly(A)Pure kit (Ambion, Austin, TX). PCR protocols and molecular techniques were as described (Sambrook *et al.* 1989). Radiolabeled probes were generated by a PCR methodology (McCormick-Graham and Romero 1996). Hybridization was quantitated with a Molecular Dynamics (Sunnyvale, CA) phosphorImager.

Synchronization of Tetrahymena: The methodology is as described (Adl and Berger 1996), with the following modifications. A 1.5-liter logarithmically growing culture (~5×10^4 cells/ml) was delivered by peristaltic pump into a centrifugal elutriator rotor [Beckman (Fullerton, CA) JE 5.0] at a flow rate of 50 ml/min (rotor speed 850 rpm). After a 5-min equilibration and wash with 2% PPYS at 5 ml/min, the flow rate was increased to 100 ml/min and the effluent collected in 100-ml fractions. Fractions 4–7 were pooled and concentrated by brief centrifugation in an IEC clinical centrifuge. The cell density was adjusted to ~5×10^4 and incubated at 30°.

PCR primers and products: PCR primers are indicated below. P1(+/−) was designed to amplify a neomycin resistance cassette (Gaertig *et al.* 1994a). P2(+/−) and P3(+/−) were for the synthesis of radiolabeled probes specific for the Tetrahymena *RAD51* 5′ nontranscribed sequence and carboxy-terminal coding sequence, respectively (Campbell and Romero 1998). P4(+/−) was used to amplify a portion of the Tetrahymena actin coding sequence:

P1(+)CATCGATGAAACATCTCCGG
P1(−)GGAATTCTTTTGTTCCCTTT
P2(+)AGATCTTTAGTTGAATG
P2(−)ATCTAGATAACGATTTG
P3(+)GACGAATTCGGTATTGC
P3(−)TCACTCGTTGAAGTC
P4(+)GCCTGCCTTCATCGG
P4(−)GCACTTTCTGTGGAC

***RAD51* macronuclear gene replacement:** A 4.4-kb *Xba*I-*Kpn*I fragment from the *T. thermophila RAD51* genomic clone (Campbell and Romero 1998) was subcloned in a vector to create the plasmid pTtRd51XK. A portion of the Tetrahymena transformation vector p42L29B (Gaertig *et al.* 1994a) was amplified with oligonucleotides P1(+/−) for a PCR product that includes the Tetrahymena histone H4-I promoter, the coding sequence of aminoglycoside 3′ phosphotransferase-II (APH-3′-II), and the 3′ nontranslated sequence of the Tetrahymena β-tubulin 2 gene, flanked by unique *Cla*I and *Eco*RI restriction sites. This selectable marker cassette confers resistance to the antibiotics paromomycin and neomycin.

The paromomycin resistance cassette was cloned at *Cla*I (−185) and *Eco*RI (+2068) sites of pTtRd51XK to create a sequence suitable for Tetrahymena *RAD51* gene replacement (designated pTtRd51KO). Targeting to the *RAD51* locus is provided by 937 bp upstream (from −1122 to −185) and 1203 bp downstream (from +2068 to +3272) of the *RAD51* gene (Campbell and Romero 1998). Nucleotide positions indicated are relative to the Rad51 protein initiator codon.

Transformation of Tetrahymena: Tetrahymena cultures expressing different mating types were grown in 200 ml of 2% PPYS to a density of 2.5×10^5 cells/ml. The cells were washed and starved in 200 ml of 10 mm Tris HCl (pH 7.5) for 18 hr. The starved cells were mixed together in equal numbers and monitored for pairing efficiency at 3 hr (>90%). Cultures were centrifuged 10.5 hr after mixing, washed once in 10 mm HEPES (pH 7.5), and resuspended in 2 ml 10 mm HEPES (pH 7.5) to a density of ~2×10^7 cells/ml. Approximately 5×10^6 cells (250 μl) were mixed with 50 μg pTtRd51KO (digested with *Xba*I and *Kpn*I), and the cells were transformed with a BTX BCM600 electroporator (Gentronics, San Diego), as described (Gaertig *et al.* 1994a). The cells were diluted in 2% PPYS to ~2.5×10^5 cells/ml, and 150-μl aliquots distributed to 96-well plates. Paromomycin (120 μg/ml) was added 12 hr after electroporation. Clonal lines resistant to paromomycin (pm-r) 4 days after electroporation were expanded and transferred every 1–2 days into 1 ml of fresh 2% PPYS plus drug (120–960 μg/ml).

Northern blot analysis: *T. thermophila* cultures (10 ml) were lysed in guanidinium isothiocyanate, and polyadenylated RNA [poly(A) RNA] was prepared with the MicroPoly(A)Pure kit (Ambion). RNA concentrations were determined by absorbance at 260 nm, and equivalent amounts for each poly(A) RNA sample (0.7 μg) were electrophoresed in 2.2 m formalde-

hyde–1% agarose gels and transferred to Nytran filters by capillary action (Sambrook *et al.* 1989). Northern blots were equilibrated in a hybridization buffer containing 30% (v/v) formamide, 10% dextran sulfate (500,000 M_r), 5% SDS, 4× SSC (0.6 m NaCl, 60 mm sodium citrate), 1× Denhardt's solution (Sambrook *et al.* 1989), 25 mm sodium phosphate (pH 6.5), 10 mm EDTA, and 0.25 mg/ml high molecular weight RNA at 40°. Duplicate Northern blots were hybridized at 40° overnight with Tetrahymena-specific probes labeled with ^{32}P as indicated in the text. Blots were washed at 40° for 5 min in 2× SSC/0.1% SDS twice, followed by a final wash with 1× SSC/0.1% SDS at 40° for 60 min. The degree of hybridization was quantitated with a Molecular Dynamics phosphorImager.

Cytology: Cells were fixed in three sequential washes of 50% methanol, 70% methanol, and 70% methanol:15% acetic acid prior to air drying at 37° on microscope slides. Fixed cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI) and viewed with an Olympus B-Max fluorescence microscope at ×320 magnification using a ×40 oil-immersion objective lens, a 1.6 optivar setting, and a ×5 ocular lens. Micrographs were recorded either photographically with an Olympus PM-30 camera and Kodak Tech-Pan film or digitally using a SPOT camera and imaging software.

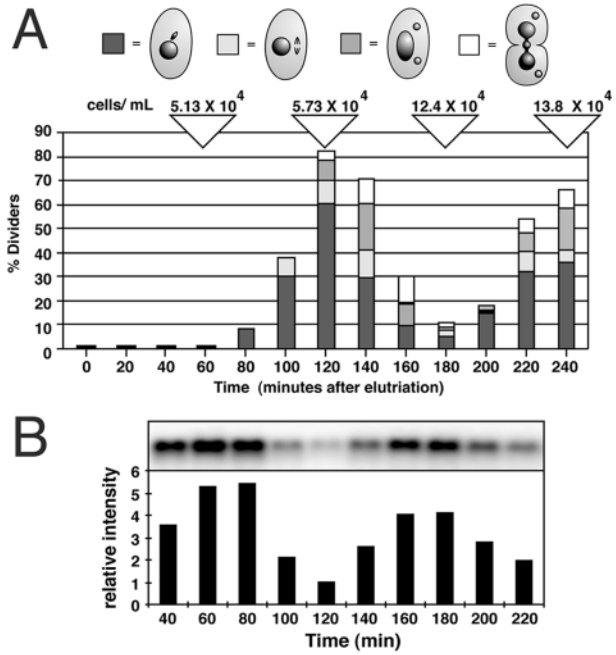

Figure 1.—*RAD51* expression during the cell cycle. (A) A schematic depiction of four characteristic stages of cell division that are detected cytologically in actively dividing Tetrahymena cultures. From left to right: the start of mitosis (elliptical micronucleus), anaphase, macronuclear elongation, and cytokinesis. The percentage of cells at each stage detected in a synchronous culture is shown graphically at 20-min intervals. Cell densities of the synchronous culture were measured every 60 min and are depicted above the histogram. (B) Northern blots of poly(A) RNA were hybridized with both *RAD51*-specific and nonspecific radiolabeled probes, as described in materials and methods. The *RAD51*-specific blot and a histogram of the relative abundance of *RAD51* mRNA from samples taken from the synchronous culture at 20-min intervals are shown.

RESULTS

***RAD51* expression during the cell cycle:** *RAD51* expression in *S. cerevisiae* is regulated through the cell cycle, with a peak occurring from late G1 to early S phase in a pattern coincident with the expression of DNA replication enzymes (Basile *et al.* 1992). Human *RAD51* is similarly regulated through the cell cycle (Scully *et al.* 1997). Because karyokinesis of the micro- and macronuclei in Tetrahymena occurs by different mechanisms, it was of interest to determine if there is a similar correlation of *RAD51* expression with the cell cycle.

To examine Tetrahymena *RAD51* expression through the cell cycle, a synchronous population was obtained by centrifugal elutriation. Daughter cells that had recently undergone cytokinesis were effectively size selected, cultured, and carefully monitored every 20 min thereafter for their progression through the cell cycle. Four characteristic cytological stages with distinct micro- and macronuclear morphologies were tabulated for the synchronous culture over 4 hr. A very high degree of synchrony (82% "dividers" at 120 min) was obtained by this method (Figure 1A).

RNA samples were prepared every 20 min and *RAD51* mRNA levels monitored by Northern blot analysis. A cell cycle dependent pattern of *RAD51* expression was observed, with a peak of expression during the 40-min interval immediately following cytokinesis (Figure 1B). Maximal levels of *RAD51* mRNA coincide with the period of DNA replication in the macronucleus (Wu *et al.* 1988). In contrast, low levels of *RAD51* mRNA were found when 70% of the cells were in micronuclear M phase. Micronuclear DNA synthesis (S phase) proceeds immediately after M phase, without a G1 interval (Doerder and DeBault 1975). As a result, the normal complement of DNA in the micronucleus is 4C during the amitotic division of the macronucleus and cytokinesis. A rapid increase in *RAD51* expression begins when the majority of the cell population has completed macronuclear division and cytokinesis (Figure 1B).

***RAD51* expression during development:** Two mature, wild-type *T. thermophila* strains expressing different mating types (CU428.2 and CU438.1; Table 1) were starved and mixed to initiate conjugation, with >95% pairing efficiency. RNA samples were prepared at 1- to 2-hr intervals from the mated cells and *RAD51* mRNA levels monitored. A bimodal pattern of *RAD51* expression is apparent, with maxima at 3–4 hr and 12–14 hr after mixing (Figure 2). The two peaks of expression coincide with both meiotic prophase and exconjugant macronuclear development (Cole and Soelter 1997).

***RAD51* gene disruption:** The *RAD51* macronuclear locus was disrupted with a selectable marker by homologous recombination as has been described for other Tetrahymena loci (Gaertig *et al.* 1994a; Hai and Gorovsky 1997; Lee *et al.* 1999; Wei *et al.* 1999). Briefly, an

TABLE 1

Genotype and phenotype of *T. thermophila* strains

Strain	Micronuclear genotype	Macronuclear genotype	Macronuclear phenotype
CU428.2	*mpr1*/*mpr1*	*MPR1*	mp-s, VII
CU438.1	*pmr1*/*pmr1*	*PMR1*	pm-s, IV
A* III	*	Wild type	III
A* V	*	Wild type	V
TC102	*mpr1*/*mpr1*; *RAD51*/*RAD51*	*mpr1*, *rad51-1*::neo	pm-r, mp-r, II
TC103	*mpr1*/*mpr1*; *RAD51*/*RAD51*	*mpr1*, *rad51-1*::neo	pm-r, mp-r, IV
TC120	*mpr1 mpr1*; *BTU1*/*BTU1*	*mpr1*, *btu1-1*::neo	pm-r, mp-r, II
TC121	*mpr1*/*mpr1*; *BTU1*/*BTU1*	*mpr1*, *btu1-1*::neo	pm-r, mp-r, IV

Macronuclear phenotype designations: -r, resistant; -s, sensitive. Locus names are as follows: (*mpr*) 6-methylpurine (mp) resistance; (*pmr1*) paromomycin (pm)-resistant *B* rDNA allele; *rad51-1* and *btu1-1* are mutant loci disrupted by the neomycin cassette, which confers paromomycin resistance. Mating types are designated by roman numerals. Star (*) strains contain a hypodiploid micronucleus that is functionally amicronucleate during conjugation. CU428.2 and CU438.1 were kindly provided by P. Bruns (Cornell University).

antibiotic resistance cassette, consisting of the aminoglycoside 3′ phospho-transferase-II (APH-3′-II) coding sequence situated between the Tetrahymena histone H4-I constitutive promoter and the β-tubulin 3′ nontranslated region, and flanked by Tetrahymena *RAD51* targeting sequence (Figure 3A), was introduced to conjugating cells by electroporation (Gaertig *et al.* 1994a,b). Transformed exconjugants expressing APH-3′-II are resistant to the antibiotic paromomycin. The nonessential β-tubulin 1 gene (Gaertig *et al.* 1994b) was similarly targeted for gene replacement with the same aminoglycoside resistance cassette as a control for these experiments [the *BTU1* targeting construct, pHAB1, was provided by J. Gaertig (University of Georgia)].

Only partial replacement of the endogenous 45 copies of the *RAD51* gene present in each developing macronucleus is achieved immediately following transformation. However, because multiple copies of macronuclear genes segregate randomly as the macronuclei divide amitotically, it is possible for one allele to be lost and the other to predominate over the course of multiple fissions (Orias and Flacks 1975). The process of phenotypic assortment can lead to the complete replacement of the targeted gene when sufficient selective pressure is applied, even if the loss of the endogenous gene leads to a deleterious but nonlethal phenotype.

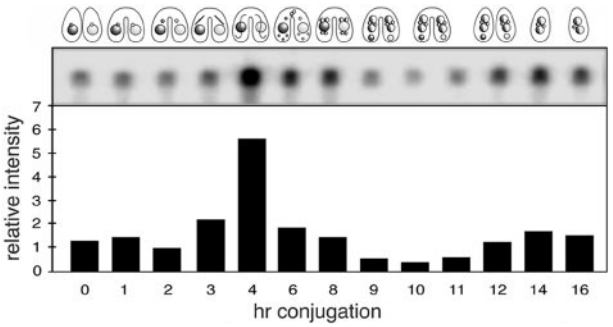

Figure 2.—*RAD51* expression during conjugation. Tetrahymena cultures expressing different mating types were starved and mixed in equal numbers to initiate conjugation. Northern blots of poly(A) RNA were hybridized with both *RAD51*-specific and nonspecific radiolabeled probes, as described in materials and methods. The *RAD51*-specific blot and a histogram of the relative abundance of *RAD51* mRNA from samples taken at 1- to 2-hr intervals after conjugation was initiated are shown. The conjugal stages typical for the various time intervals are shown schematically as previously described (Cole and Soelter 1997).

Total *RAD51* gene replacement was achieved by the incremental increase of paromomycin from 120 μg/ml to 960 μg/ml over the course of 60–80 fissions. Despite these rigorous selection conditions, a small percentage of cells under paromomycin selection tended to retain some copies of the *RAD51* allele. This phenomenon is likely due to the severe growth disadvantage for cells entirely lacking Rad51p (see below). To ensure complete *RAD51* gene replacement, single cell clonal lines were periodically isolated under increasing drug selection. In addition to Southern blot analysis (Figure 3B), putative knockout clones were evaluated by reverse transcriptase PCR of RNA isolated from candidate clones 2 hr after UV irradiation. Because *RAD51* expression is induced after exposure to UV (Campbell and Romero 1998), true knockouts, as opposed to severe knockdowns, could be confirmed by this methodology (Figure 3C).

***rad51* null vegetative phenotypes:** *rad51* nulls exhibit severe vegetative growth phenotypes (Figure 4A). The generation time for the *rad51* clones was ~25% longer than that for the *btu1* control cells (a 4-hr doubling time for the *rad51* null during logarithmic phase growth *vs.* 3.25 hr for the *btu1* null control). *rad51* cells were also more sensitive to the DNA damaging agent methylmethane sulfonate (MMS). Approximately 75% of either wild-type or *btu1* nulls survive a 30-min exposure to 20 mm MMS. In contrast, only 31% of *rad51* nulls survived under identical conditions (Figure 4B).

An examination of *rad51* nulls revealed profound defects in nuclear division, compared to wild-type and *btu1*

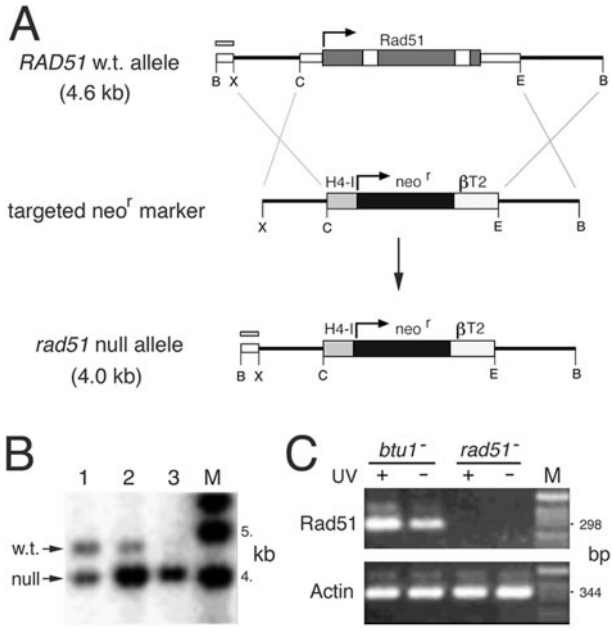

Figure 3.—(A) Wild-type and null alleles of the *RAD51* locus. The selectable marker, flanked by *RAD51* 5′ and 3′ nontranscribed sequences (thin solid lines), was introduced by electroporation. Recombination with the wild-type *RAD51* allele (4.6-kb *Bgl*II restriction fragment) results in the *rad51* null allele (4.0-*Bgl*II fragment). Also shown is a 0.2-kb radiolabeled probe derived from sequence 5′ of the *Xba*I site (shaded bar). B, *Bgl*II; C, *Cla*I; E, *Eco*RI; X, *Xba*I. Not drawn to scale. Phenotypic assortment of *rad51* null transformants. Pm-r clonal lines were expanded in increasing amounts of antibiotic as described in the text. (B) Transformant DNA, prepared from clones grown in 120, 480, and 960 μg/ml paromomycin (lanes 1, 2, and 3) digested with *Bgl*II, and hybridized to the *RAD51*-specific probe (A), reveals both wild-type and null alleles present in transformants under increasing selection. (C) Reverse transcriptase PCR analysis of transformants after *RAD51* induction by UV irradiation. Portions of both Rad51 and actin mRNAs were amplified by PCR either before (−) or 2 hr after (+) UV irradiation and analyzed in an ethidium bromide-stained agarose gel. PCR products from transformants targeted for gene replacement of the *BTU1* locus (*btu1*$^-$) or the *RAD51* loci (*rad51*$^-$) are shown.

nulls. Examples of dividing *btu1* and *rad51* cells fixed and stained with the DNA-specific dye DAPI are shown in Figure 5. There is an abnormal persistence of micronuclear mitotic spindles, even to the point where macronuclear elongation, division, and cytokinesis proceed before duplication of the micronuclei is complete. This defect in micronuclear division leads to *rad51* nulls that become hypodiploid, with an eventual subpopulation (~25%) of severely aneuploid cells (data not shown). There is also a higher-than-normal percentage of chromatin exclusion bodies (CEBs) evident in the *rad51* nulls (Figure 5). The elimination of CEBs is a mechanism to maintain the level of macronuclear ploidy (Bodenbender *et al.* 1992).

The effect of *RAD51* macronuclear gene replacement on conjugation: Assessing the effect somatic *rad51* nulls have on conjugation is problematic because a consequence of total *RAD51* gene replacement is the eventual loss of micronuclear DNA. To evaluate a *rad51* × *rad51* cross, it was necessary to first reintroduce diploid, wild-type micronuclei into these cells. This is possible in Tetrahymena due to a special type of abortive mating called round I genomic exclusion. This process occurs when wild-type cells are crossed with so-called star strains that have defective, diminutive micronuclei. Star strains can form conjugal pairs with wild-type cells but fail to contribute a migratory gametic micronucleus to the wild-type partner at the fertilization stage of conjugation. As a result, a single haploid micronucleus is contributed by the wild-type partner to the star partner, where it is endoreplicated, leading to a homozygous, diploid micronucleus in each conjugant. At this point, conjugation is aborted prematurely, and there are no postzygotic nuclear divisions. Both cells continue to express their parental phenotypes, including mating-type expression and sexual maturity, because parental macronuclei are retained by exconjugants in a star mating. Round I genomic exclusion is shown schematically in Figure 6 and described in detail elsewhere (Allen 1967; Doerder and Shabatura 1980).

We have found that hypodiploid *rad51* knockout clones behaved exactly like star strains when mated to a wild-type partner. The resultant *rad51* knockout synclones complete round I genomic exclusion and retain their old macronucleus (paromomycin resistance). Paromomycin-resistant exconjugants acquire a diploid micronucleus, which can be detected cytologically after staining with the DNA-specific dye DAPI (data not shown).

Two *rad51* null clonal lines expressing different mating types that had reacquired micronuclei through a star mating with a wild-type strain were expanded for ~20 fissions, starved, and mixed in equal numbers to initiate conjugation. As a control, two *btu1* knockout clones were mated in a parallel experiment. Whereas conjugating *btu1* knockout strains followed the nuclear developmental processes that have been well established for wild-type conjugants (Cole *et al.* 1997; Cole and Soelter 1997), the majority of *rad51* conjugants could not progress beyond the earliest micronuclear divisions (Figures 7 and 8). There was an apparent diminution of micronuclear DNA in *rad51* cells before mating was initiated (within 20 vegetative fissions), most likely due to the mitotic defect exhibited by *rad51* cells during vegetative growth (Figure 5). This loss of micronuclear DNA is somewhat variable from cell to cell, as can be seen in the relative levels of DAPI staining in mated pairs (Figure 8). Progression to developmental stages beyond prophase meiosis I was delayed and/or abortive. There were no viable progeny of the *rad51* × *rad51* cross, which is also the result when two bonafide Tetrahymena star strains are mated to each other (T. Marsh, unpublished results).

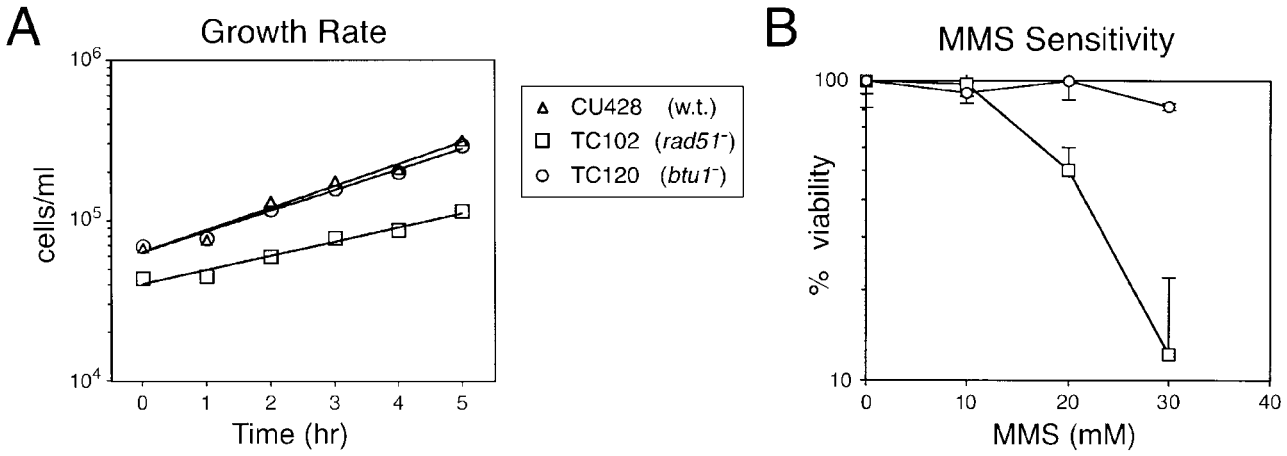

Figure 4.—Vegetative phenotypes of *rad51* null cells. (A) Cell densities for logarithmically dividing Tetrahymena cells are as indicated. Doubling times for wild-type and *btu1* null cells were ~3.25 hr, whereas that for *rad51* nulls was 4.0 hr. (B) Sensitivity to MMS. A Poisson distribution of logarithmically dividing cells was plated in 96-well plates in 2% PPYS plus MMS at the concentration indicated. Wells with proliferating cells were scored 2 days later. The percentage of wells with growing cells in the absence of MMS was set at 100% for both *btu1* and *rad51* nulls. The percentage viability shown is an average of three independent experiments.

DISCUSSION

***RAD51* expression and DNA replication:** A clear delineation of the ciliate cell cycle is complicated by the unusual nuclear dimorphism that has evolved for these protozoans. For example, it has been shown for Tetrahymena that the periods of micro- and macronuclear DNA synthesis do not overlap (Wu *et al.* 1988). Micronuclear S phase is initiated immediately following micronuclear division and migration of daughter nuclei to opposite poles of the dividing cell and is complete before the macronucleus has elongated prior to its own division (Figure 5). Approximately 10 min after the completion of micronuclear S phase, macronuclear DNA synthesis is initiated and continues through a large fraction of the interphase period between cell divisions (Wu *et al.* 1988).

We have shown that Tetrahymena *RAD51* mRNA levels peak during the cell cycle period of maximum macronuclear DNA synthesis (Figure 1). The expression of DNA replication enzymes in Tetrahymena is presumably coincident with that of *RAD51*, as has been shown for *S. cerevisiae* and humans (Basile *et al.* 1992; Scully *et al.* 1997). It is likely that damage to the micronuclear chromosomes is not recognized by DNA repair mechanisms until replication is initiated immediately following mitosis.

There is indirect support for a connection between DNA replication and homologous recombination repair in Tetrahymena, based upon the apparent subcellular localization of Rad51 protein after cells sustain DNA damage. Exposure to MMS results in a pronounced *RAD51* induction, with Rad51 protein localized primarily in macronuclei actively replicating their DNA, while it is apparently excluded from micronuclei that have already completed DNA replication (Campbell and Romero 1998). In this study, no actively dividing Tetrahymena (and therefore, no mitotic micronuclei) were detected 4 hr after treatment with 4.2 mm MMS. Localization of DNA replication and repair complexes to the germline and somatic nuclei is most likely limited to periods of DNA synthesis in a pattern reminiscent of that observed in Tetrahymena for micronuclear and macronuclear linker histones (Wu *et al.* 1988).

***RAD51* and nuclear division:** A role for *RAD51*-mediated DNA repair in the micronucleus is clearly demon-

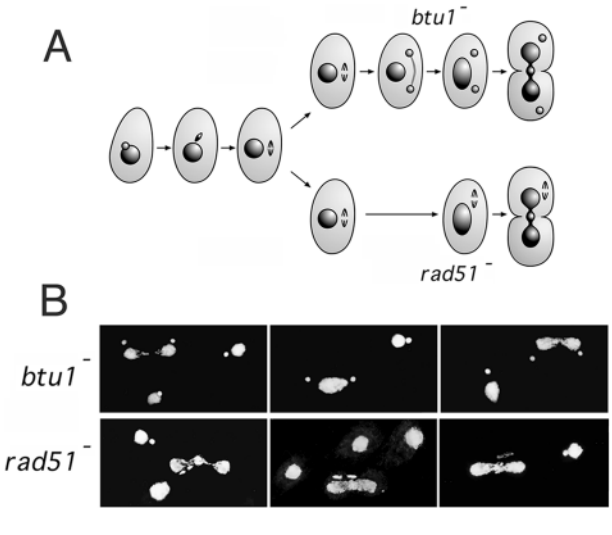

Figure 5.—Vegetative cell division in *btu1* and *rad51* nulls. (A) Schematic depiction of normal cell division by clones with targeted gene replacement of the *BTU1* locus (*btu1*), as compared to abnormal division by *rad51* nulls. (B) DAPI-stained micrographs showing the cytology of both *btu1* and *rad51* nulls. Note that macronuclear karyokinesis and cytokinesis has initiated in *rad51* nulls despite the failure to complete micronuclear mitosis.

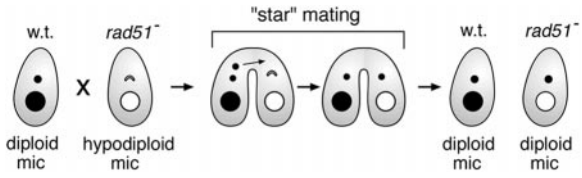

Figure 6.—Round I genomic exclusion to acquire wild-type diploid micronuclei in *rad51* null cells. The net result is the isolation of *rad51*$^-$ cells that express paromomycin resistance and have acquired a diploid micronucleus. The wild-type synclone from this mating is sensitive to paromomycin.

strated by the severe defects suffered by *rad51* knockouts during mitosis (Figure 5). Similar defects in micronuclear division have been recently reported for two other Tetrahymena mutants. Gene replacement of a cytoplasmic dynein heavy chain gene results in failure of micronuclear chromosomes to segregate during mitosis, implicating this protein in attachment of chromosomes to the kinetochore microtubules (Lee *et al.* 1999). A single amino acid substitution (S10A) that eliminates a phosphorylation site for histone H3 also results in abnormal micronuclear division, leading to a defect in mitotic chromosome segregation (Wei *et al.* 1999). The signal(s) for macronuclear division and cytokinesis in the *his3*, *dyh1*, and *rad51* mutants are unimpeded, despite the delay and/or failure of germline nuclear division. Eventually, daughter cells from *his3* and *rad51* nulls become hypodiploid and severely aneuploid, behaving as star cells in a round I genomic exclusion cross with wild-type cells.

A previously described phenotype for Tetrahymena transformants expressing a mutated telomerase RNA template is in direct contrast with those of the *his3* and *rad51* null strains. When mutant G_4T_4 repeats (instead of wild-type G_4T_2 repeats) cap the ends of micronuclear chromosomes, there is a failure of replicated chromosomes to disassociate during anaphase (Kirk *et al.* 1997). The mutant chromatids do not separate completely at the midzone, possibly due to a physical block in mutant telomere separation, and elongate up to twice their normal length. There is a concurrent block in the cell cycle that prevents the cells from initiating macronuclear division and cytokinesis. These observations are consistent with an earlier study that indicated that macronuclear karyokinesis and cell division do not initiate until replicated chromosomes are physically separated by the mitotic spindle during anaphase (Gavin 1965). These data suggest that there is a cell cycle checkpoint associated with anaphase segregation of micronuclear chromosomes. Defects in *rad51* null division can be said to occur after the successful completion of this checkpoint, given their ability to complete cytokinesis, whereas the telomere defect clearly operates upstream from this checkpoint. It is not known how the status of mitotically dividing germline chromosomes is communicated to prompt somatic nuclear division and cytokinesis or if the signal(s) are sent directly or indirectly.

***RAD51* and conjugation:** Homologous recombination factors, including Rad51p and its meiosis-specific homolog Dmc1p, play critical roles in generating genetic diversity by mediating strand exchange during meiosis in yeast (Dresser *et al.* 1997; Xu *et al.* 1997). Therefore, it is not surprising that *RAD51* expression peaks during prezygotic development in conjugating Tetrahymena (Figure 2). It is also possible that *RAD51* expression is induced during exconjugant development in order to

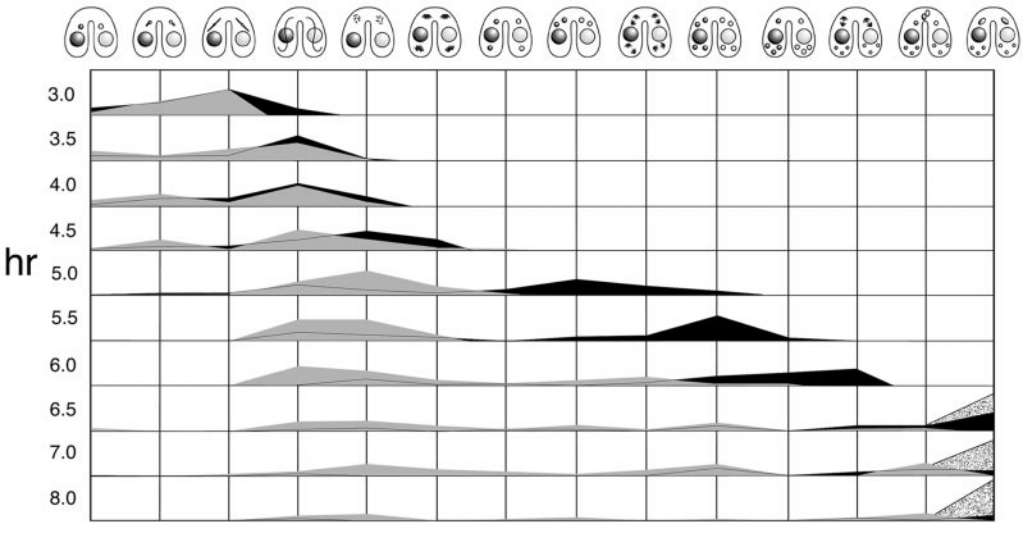

Figure 7.—Developmental profile of [*rad51* × *rad51*; TC102 × TC103] and [*btu1* × *btu1*; TC120 × TC121] matings. Samples from the two mating cultures were fixed and stained with DAPI to microscopically determine their nuclear morphologies. Mating pairs (100) were scored for each time point indicated after mating was initiated. (Top) A schematic depiction of Tetrahymena cell progression through conjugation is shown (from Cole *et al.* 1997). Shaded, *rad51* null cross; solid, *btu1* null cross. The percentage of progeny from the *btu1* cross that have progressed to postzygotic development is represented by stippling. Note the loss of synchrony between the two matings at the 4.5-hr time point when the *btu1* cross progresses normally and the majority of the *rad51* cross remains in meiotic prophase I. Approximately 86% of the *rad51* pairs showed signs of aborted development.

participate in the genomic remodeling that occurs in the macronuclear anlagen. Perhaps it is more than coincidental that DNA-mediated transformations of both germline and somatic nuclei are most efficient when Tetrahymena Rad51 levels are at their peak (Gaertig and Gorovsky 1992; Cassidy-Hanley *et al.* 1997; Hai and Gorovsky 1997).

RAD51 mRNA levels at 4 hr relative to those prior to mixing are ~5.5–1, whereas a similar comparison of *RAD51* mRNA at 14 hr is 1.5–1 (Figure 2). However, it should be noted that the macronuclear gene copy number is 45C at 4 hr for the parental macronuclei and 8C at 14 hr during development (Doerder and DeBault 1975; Allis *et al.* 1987). Each daughter cell has two macronuclear anlagen at this time, bringing each cell's macronuclear DNA content to 16C. A direct comparison of *RAD51* mRNA levels at 4 and 14 hr (5.5 to 1.5 or 3.7:1) approximates the ratio of macronuclear DNA content during these two periods of development (45C to 16C or 2.8:1). This suggests that *RAD51* expression is induced to approximately the same degree during both prezygotic and exconjugant development.

Despite their star-like behavior in completing round I genomic exclusion when crossed to wild-type cells, *rad51* nulls are not true star cells. When two star strains are mated to each other, the cells frequently complete meiosis I and II and successfully condense their chromosomes, despite severe aneuploidy (data not shown). Conversely, when two *rad51* null strains are mated, the pairs arrest at meiotic prophase I prior to chromosome condensation, rarely progressing to anaphase I (Figure 8). Furthermore, star cells express *RAD51* to wild-type levels when DNA damage is induced by UV irradiation (T. Marsh, unpublished results).

Homologous recombination plays a role in mediating some, if not all, of the extensive genomic rearrangements that occur during exconjugant development. For example, there is an intragenic recombination event between two nonsense mutations (separated by 726 bp) in the *SERH1* gene that restores wild-type expression of the SerH1 surface protein, which occurs in the macronuclear anlagen (Deak and Doerder 1998). Homologous recombination is involved in the conversion of the Tetrahymena rRNA (rDNA) gene from its micronuclear form into a highly amplified palindrome during the course of macronuclear development (Butler *et al.* 1995). It is our hope to eventually dissect the involvement of Rad51p and Rad51p-associated factors in these and other developmentally controlled genomic rearrangements. Unfortunately, the severe conjugal block during meiosis encountered in our study of a *rad51*$^{-}$ × *rad51*$^{-}$ cross prevents the evaluation of a *rad51*$^{-}$ background on macronuclear development. To characterize exconjugant development in the absence of Rad51p, it is necessary to genetically construct and mate two heterokaryons that are capable of wild-type *RAD51* expression from their parental macronuclei (to successfully complete meiosis) but are incapable of *RAD51* expression from their macronuclear anlagen during exconjugant development. Both strains must be homozygous nulls for the micronuclear *RAD51* locus. We have successfully constructed these heterokaryons and are currently evaluating the phenotypes of exconjugants from the mating experiment described above (T. C. Marsh, E. C. Cole and D. P. Romero, unpublished results).

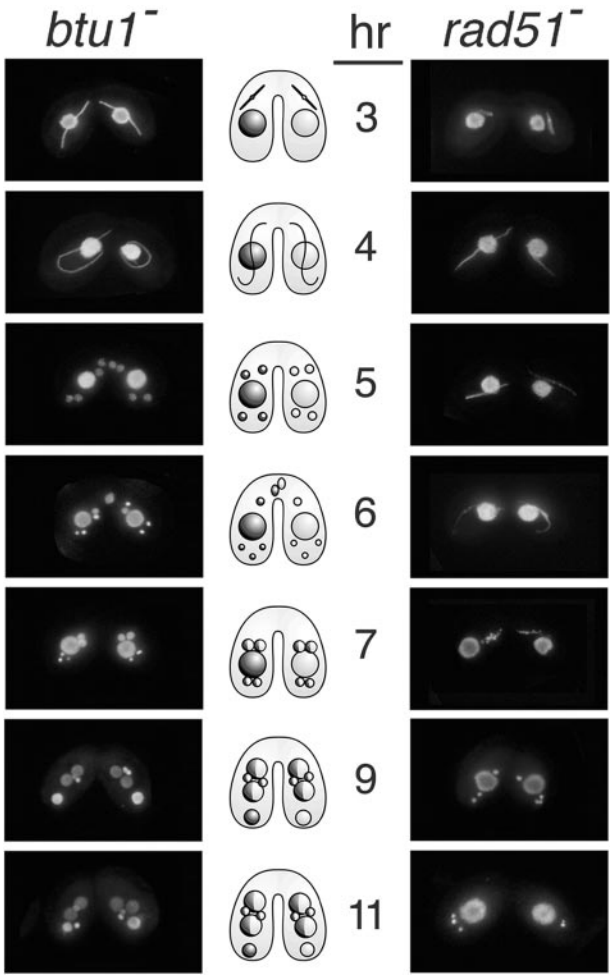

Figure 8.—The progression of *rad51* and *btu1* nulls through conjugation. Samples were fixed and stained with DAPI at various times after conjugation was initiated. A schematic of micronuclear and macronuclear morphologies as they normally occur in a wild-type cross is also shown. The various developmental stages are as follows: bipolar spindle formation (3 hr); prophase meiosis I (4 hr); completion of meiosis II (5 hr); pronuclear differentiation (6 hr); second postzygotic mitosis (7 hr); macronuclear anlagen formation (9 hr); and continued anlagen development (11 hr).

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LITERATURE CITED

Adl, S. M., and J. D. Berger, 1996 Commitment to division in ciliate cell cycles. J. Eukaryot. Microbiol. **43:** 77–86.

Akaboshi, E., Y. Inoue and H. Ryo, 1994 Cloning of the cDNA and genomic DNA that correspond to the recA-like gene of *Drosophila melanogaster*. Jpn. J. Genet. **69:** 663–670.

Allen, S. L., 1967 Cytogenetics of genomic exclusion in *Tetrahymena*. Genetics **55:** 797–822.

Allis, C. D., M. Colavito-Shepanski and M. A. Gorovsky, 1987 Scheduled and unscheduled DNA synthesis during development in conjugating *Tetrahymena*. Dev. Biol. (Orlando) **124:** 469–480.

Basile, G., M. Aker and R. K. Mortimer, 1992 Nucleotide sequence and transcriptional regulation of the yeast recombinational repair gene *RAD51*. Mol. Cell. Biol. **12:** 3235–3246.

Baumann, P., F. E. Benson and S. C. West, 1996 Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions *in vitro*. Cell **87:** 757–766.

Bodenbender, J., A. Prohaska, F. Jauker, H. Hipke and G. Cleffmann, 1992 DNA elimination and its relation to quantities in the macronucleus of *Tetrahymena*. Dev. Genet. **13:** 103–110.

Butler, D. K., L. E. Yasuda and M.-C. Yao, 1995 An intramolecular recombination mechanism for the formation of the rRNA gene palindrome of *Tetrahymena thermophila*. Mol. Cell. Biol. **15:** 7117–7126.

Campbell, C., and D. P. Romero, 1998 Identification and characterization of the *RAD51* gene from the ciliate *Tetrahymena thermophila*. Nucleic Acids Res. **26:** 3165–3172.

Cassidy-Hanley, D., J. Bowen, J. H. Lee, E. Cole, L. A. VerPlank *et al.*, 1997 Germline and somatic transformation of mating *Tetrahymena thermophila* by particle bombardment. Genetics **146:** 135–147.

Chen, J., D. P. Silver, D. Walpita, S. B. Cantor, A. F. Gazdar *et al.*, 1998 Stable interaction between the products of the *BRCA1* and *BRCA2* tumor suppressor genes in mitotic and meiotic cells. Mol. Cell **2:** 317–328.

Cole, E. S., and T. A. Soelter, 1997 A mutational analysis of conjugation in *Tetrahymena thermophila*. 2. Phenotypes affecting middle and late development: third prezygotic nuclear division, pronuclear exchange, pronuclear fusion and postzygotic development. Dev. Biol. (Orlando) **189:** 233–245.

Cole, E. S., D. Cassidy-Hanley, J. Hemish, J. Tuan and P. J. Bruns, 1997 A mutational analysis of conjugation in *Tetrahymena thermophila*. 1. Phenotypes affecting early development—meiosis to nuclear selection. Dev. Biol. (Orlando) **189:** 215–232.

Coyne, R. S., D. L. Chalker and M.-C. Yao, 1996 Genome downsizing during ciliate development—nuclear division of labor through chromosome restructuring. Annu. Rev. Genet. **30:** 557–578.

Deak, J. C., and F. P. Doerder, 1998 High frequency intragenic recombination during macronuclear development in *Tetrahymena thermophila* restores the wild-type SerH1 gene. Genetics **148:** 1109–1115.

Doerder, F. P., and L. E. DeBault, 1975 Cytofluorimetric analysis of nuclear DNA during meiosis, fertilization and macronuclear development in the ciliate *Tetrahymena pyriformis*, syngen 1. J. Cell Sci. **17:** 471–493.

Doerder, F. P., and S. K. Shabatura, 1980 Genomic exclusion in *Tetrahymena thermophila*: a cytogenetic and cytofluorometric study. Dev. Genet. **1:** 205–218.

Dresser, M. E., D. J. Ewing, M. N. Conrad, A. M. Dominguez, R. Barstead *et al.*, 1997 *DMC1* functions in a *Saccharomyces cerevisiae* meiotic pathway that is largely independent of the *RAD51* pathway. Genetics **147:** 533–544.

Gaertig, J., and M. A. Gorovsky, 1992 Efficient mass transformation of *Tetrahymena thermophila* by electroporation of conjugants. Proc. Natl. Acad. Sci. USA **89:** 9196–9200.

Gaertig, J., L. Gu, B. Hai and M. A. Gorovsky, 1994a High frequency vector-mediated transformation and gene replacement in *Tetrahymena*. Nucleic Acids Res. **22:** 5391–5398.

Gaertig, J., T. H. Thatcher, L. Gu and M. A. Gorovsky, 1994b Electroporation-mediated replacement of a positively and negatively selectable β-tubulin gene in *Tetrahymena thermophila*. Proc. Natl. Acad. Sci. USA **91:** 4549–4553.

Game, J. C., 1983 Radiation-sensitive mutants and repair in yeast, pp. 109–137 in *Yeast Genetics: Fundamental and Applied Aspects*, edited by J. F. T. Spencer, D. H. Spencer and A. R. W. Smith. Springer-Verlag, New York.

Gavin, R. H., 1965 The effects of heat and cold on cellular development in synchronized *Tetrahymena pyriformis* WH-6. J. Protozool. **12:** 307–318.

Hai, B., and M. A. Gorovsky, 1997 Germ-line knockout heterokaryons of an essential α-tubulin gene enable high-frequency gene replacement and a test of gene transfer from somatic to germline nuclei in *Tetrahymena thermophila*. Proc. Natl. Acad. Sci. USA **94:** 1310–1315.

Kirk, K. E., B. P. Harmon, I. K. Reichardt, J. W. Sedat and E. H. Blackburn, 1997 Block in anaphase chromosome separation caused by a telomerase template mutation. Science **275:** 1478–1481.

Lee, S., J. C. Wisniewski, W. L. Dentler and D. J. Asai, 1999 Gene knockouts reveal separate functions for two cytoplasmic dyneins in *Tetrahymena thermophila*. Mol. Biol. Cell **10:** 771–784.

McCormick-Graham, M., and D. P. Romero, 1996 A single telomerase RNA is sufficient for the synthesis of variable telomeric DNA repeats in ciliates of the genus *Paramecium*. Mol. Cell. Biol. **16:** 1871–1879.

Muris, D. F., K. Vreeken, A. M. Carr, B. C. Broughton, A. R. Lehmann *et al.*, 1993 Cloning the RAD51 homologue of *Schizosaccharomyces pombe*. Nucleic Acids Res. **21:** 4586–4591.

Ogawa, T., X. Yu, A. Shinohara and E. H. Egelman, 1993 Similarity of the yeast Rad51 filament to the bacterial RecA filament. Science **259:** 1896–1899.

Orias, E., and M. Flacks, 1975 Macronuclear genetics of *Tetrahymena*. I. Random distribution of macronuclear gene copies in *T. pyriformis*, syngen 1. Genetics **79:** 187–206.

Prescott, D. M., 1994 The DNA of ciliated protozoa. Microbiol. Rev. **58:** 233–267.

Rinaldo, C., S. Ederle, V. Rocco and A. La Volpe, 1998 The Caenorhabditis elegans RAD51 homolog is transcribed into two alternative mRNAs potentially encoding proteins of different sizes. Mol. Gen. Genet. **260:** 289–294.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2, Vols. 1–3, edited by C. Nolan, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scully, R., J. Chen, A. Plug, Y. Xiao, D. Weaver *et al.*, 1997 Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell **88:** 265–275.

Shinohara, A., H. Ogawa and T. Ogawa, 1992 Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. Cell **69:** 457–470.

Shinohara, A., H. Ogawa, Y. Matsuda, N. Ushio, K. Ikeo *et al.*, 1993 Cloning of human, mouse and fission yeast recombination genes homologous to *RAD51* and recA. Nat. Genet. **4:** 239–243.

Sonoda, E., M. S. Sasaki, J. M. Buerstedde, O. Bezzubova, A. Shinohara *et al.*, 1998 Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J. **17:** 598–608.

Sturzbecher, H. W., B. Donzelmann, W. Henning, U. Knippschild and S. Buchhop, 1996 p53 is linked directly to homologous recombination processes via Rad51/RecA protein interaction. EMBO J. **15:** 1992–2002.

Sung, P., 1994 Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast Rad51 protein. Science **265:** 1241–1243.

Taki, T., T. Ohnishi, A. Yamamoto, S. Hiraga, N. Arita *et al.*, 1996 Antisense inhibition of the *RAD51* enhances radiosensitivity. Biochem. Biophys. Res. Commun. **223:** 434–438.

Thompson, L. H., and D. Schild, 1999 The contribution of homologous recombination in preserving genome integrity in mammalian cells. Biochimie **81:** 87–105.

Tsuzuki, T., Y. Fujii, K. Sakumi, Y. Tominaga, K. Nakao *et al.*, 1996 Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. Proc. Natl. Acad. Sci. USA **93:** 6236–6240.

Vispe, S., C. Cazaux, C. Lesca and M. Defais, 1998 Overexpression of Rad51 protein stimulates homologous recombination and increases resistance of mammalian cells to ionizing radiation. Nucleic Acids Res. **26:** 2859–2864.

Wei, Y., L. L. Yu, J. Bowen, M. A. Gorovsky and C. D. Allis, 1999 Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. Cell **97:** 99–109.

Wu, M., C. D. Allis and M. A. Gorovsky, 1988 Cell-cycle regulation as a mechanism for targeting proteins to specific DNA sequences

in *Tetrahymena thermophila*. Proc. Natl. Acad. Sci. USA **85:** 2205–2209.

Xia, S. J., M. A. Shammas and R. J. Shmookler Reis, 1997 Elevated recombination in immortal human cells is mediated by HsRAD51 recombinase. Mol. Cell. Biol. **17:** 7151–7158.

Xu, L., B. M. Weiner and N. Kleckner, 1997 Meiotic cells monitor the status of the interhomolog recombination complex. Genes Dev. **11:** 106–118.

Yu, G.-L., and E. H. Blackburn, 1990 Amplification of tandemly repeated origin control sequences confers a replication advantage on rDNA replicons in *Tetrahymena thermophila*. Mol. Cell. Biol. **10:** 2070–2080.

Yuan, Z. M., Y. Huang, T. Ishiko, S. Nakada, T. Utsugisawa *et al.*, 1998 Regulation of Rad51 function by c-Abl in response to DNA damage. J. Biol. Chem. **273:** 3799–3802.

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