Molecular Genetic Analysis of an SNF2/brahma-Related Gene in Tetrahymena thermophila Suggests Roles in Growth and Nuclear Development§

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We used a reverse genetic approach to identify three members of the SNF2 superfamily of chromatin remodeling genes in the ciliated protozoan Tetrahymena thermophila in order to investigate possible functions of ATP-dependent chromatin remodeling factors in growth and nuclear development. Comparative sequence analysis of the gene product of the Tetrahymena brahma-related gene (TtBRG1) indicates it is a member of the SNF2/BRM subgroup of the SNF2 superfamily. Northern analysis suggests that TtBRG1 has roles in growth and nuclear development in Tetrahymena. Indirect immunofluorescence analysis during nuclear development indicates that TtBrp1p localizes to both the parental and developing macronucleus of Tetrahymena during the time period corresponding to genome rearrangements. We generated germ line knockout heterokaryons for TtBRG1 and demonstrated that expression of the gene is required to complete nuclear development of Tetrahymena. In addition, the formation of distinct Pdd1p-containing structures is disturbed during the late stages of conjugation in TtBRG1 germ line knockout heterokaryons. We discuss these results in light of possible roles of SNF2-related proteins in growth and nuclear development of Tetrahymena.

DNA transactions, such as recombination, replication, and transcription, occur within a chromatin medium. The basic modular unit of chromatin is the nucleosome, which is composed of 147 bp of DNA wrapped 1.7 times around a histone octamer (33). Histones contain a core globular domain and a flexible, amino-terminal tail that projects from the nucleosome. Chromatin remodeling is a general term used to refer to the fact that DNA must be completely or partially unraveled from the histone octamer for DNA transactions, such as transcription, recombination, and replication, to occur (1). There are three methods utilized to remodel chromatin. The first involves various covalent modifications of specific residues in the histone N-terminal tail, such as acetylation, methylation, phosphorylation, and ubiquitination (19). The second method occurs through the ATP-dependent physical disruption or the movement of the nucleosome (61), and the third involves the insertion of histone variants into chromatin (29). The latter two examples of chromatin remodeling appear to be performed by multisubunit protein complexes that are nucleated by a variety of DNA-dependent ATPases. These ATPase subunits are members of the SNF2 superfamily of nucleic acid-stimulated ATPases (13). This superfamily has three major groups based upon the presence or absence of other conserved protein motifs flanking the core ATPase. The CHD class (chromodomain helicase DNA-binding domains) contains two copies of a chromodomain, the SNF2 class, a C-terminal bromodomain, and the ISWI class, neither.

The ciliated protozoan Tetrahymena thermophila exhibits nuclear dimorphism with a mostly transcriptionally silent diploid germ line nucleus (micronucleus [MIC]) and a polyploid and transcriptionally active somatic nucleus (macronucleus [MAC]) contained within the same cell. When two cells of complementary mating types undergo sexual development (conjugation), the micronucleus in each divides meiotically and mitotically to generate a haploid gametic nucleus that is reciprocally exchanged and fuses with that of its partner to form a zygotic nucleus. This zygotic nucleus divides and from one of the products develops a new macronucleus. Macronuclear development involves extensive programmed DNA rearrangements, including chromosome fragmentation, DNA amplification, and the site-specific interstitial DNA deletion of internal eliminated sequences (IESs) (9).

We performed a PCR screen as a first approach to studying the function of SNF2 proteins in growth and development in Tetrahymena. We adopted a reverse-genetics approach and identified several SNF2-related genes, and in this study we report the beginning of their molecular analysis. We have obtained and analyzed the complete cDNA sequence of the SNF2-related gene TtBRG1 (Tetrahymena brahma-related gene). We demonstrate by indirect immunofluorescence analysis that TtBrp1p localizes to the parental macronucleus as well as the developing macronucleus during conjugation. We show that the TtBRG1 gene is expressed throughout growth and development and is essential for growth and development. Using the recently completed Tetrahymena genome database (www.ciliate.org), we have identified potential members of a Tetrahymena SWI/SNF complex, as well as the predicted full complement of SNF2-related genes in the organism. We discuss the usefulness of Tetrahymena as a model organism for the molec-
ular analysis of the function of ATP-dependent chromatin remodeling in growth and nuclear development.

**MATERIALS AND METHODS**

**Cell strains.** *T. thermophila* strains Cu428 (Mpr/Mpr [VII, mp-s]) and B286 (Mpr'/Mpr' [II, mp-s]) of inbreeding line B were provided by J. Gaertig, University of Georgia, Athens. Cells were cultured axenically in 1× SPP at 30°C as described previously (45).

**DNA manipulations.** Whole-cell DNA was isolated from *Tetrahymena* strains as described by Gaertig et al. (20) (modified in reference 17). Molecular biology techniques were carried out using standard protocols (50) or by following a supplier’s instructions. Double-stranded DNA probes for Northern and Southern analysis were labeled by random priming with [α-32P]dATP (Amersham). Oligonucleotides used as probes in Northern analysis were end labeled using T4-Phosphatase (Amersham). DNA-modifying enzymes were obtained from New England Biolabs. Northern and Southern blots were imaged and quantified with a Canberra Packard Instant Imager.

**DNA sequencing and PCR.** Sequencing was performed using automated cycle sequencing with dye-labeled dideoxy terminators and a PEABI 373A or 377 sequencer at the Core Molecular Biology Facility, York University, Toronto, Ont., Canada. PCR was performed using conditions as specified by the enzyme supplier (Biobasic; Toronto). Long PCR was performed using the Expand Long Template PCR system (Roche).

**Isolation of TtBRG cDNA and genomic DNA.** *Tetrahymena* spNF2-related genes were identified by amplification of whole-cell DNA using the degenerate primers TSNF2F and TSNF2R (Table 1). We cloned and sequenced the 550-bp PCR products obtained using 3′ rapid amplification of cDNA ends (RACE) of mRNA extracted using the mRNA capture kit (Roche) from 8 h conjugating *Tetrahymena* with primers Inv2 and QT (Table 1) using Titan one-step reverse transcriptase PCR (Roche). We used a combination of inverse PCR (43) and chromosome walking to clone and sequence the entire genomic locus of *TtBRG1* (see Fig. 1B) and amplified 5′ cDNA using a vegetative cDNA library (16) using the Inv1 primer (Table 1) and a universal oligo(dT) primer. We verified the identity of the 5′ end of the cDNA using 5′-RACE with the primer 5′ RBRGACE3-3 in combination with a poly(dG) and an anchor primer (Table 1). First-strand cDNA was obtained from mRNA of 14-h conjugating cells, which was then tailcd with dCTP using terminal deoxynucleotidyl transferase.

**RNA isolation and Northern analysis.** Total RNA was isolated and analyzed by Northern analysis as described previously (18). Nylon filters were stripped of the hybridized probe by boiling in 1× Tris-EDTA–1% sodium dodecyl sulfate (SDS) for 5 min, cooling to room temperature over 10 min, and then washing in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min. The stripped filters were immediately covered with Saran wrap before being imaged for verification and then stored at 4°C.

**Mycronuclear gene replacement.** Plasmid pTBRGKO was constructed by amplifying a 4,365-bp fragment of *TtBRG1* genomic DNA using the primers GBRGF and GBGRK (Table 1). The PCR product digested with EcoRV was cloned into the Smal site of pcDNA3.1(+) (Invitrogen). The plasmid was transformed into competent *E. coli* using the primers GBRGKOF and GBRGKOR (Table 1) digested with EcoRV and ligated to the 1.4-kb Smal/EcoRV fragment of pUT2-1 (20) to yield pTBRGKO.

**Mycronuclear gene replacement.** To construct the germ line knockout, we made pTBRGNEO3 (a 0.6-kb fragment of the *TtBRG1* 5′ flanking sequence and 1.0-kb fragment of the 3′ flanking sequence), which was PCR amplified from genomic DNA using primers which introduced restriction sites at the ends of the products. The amplified products were inserted into the pMNBL vector, flanking the neo3 cassette (52). The 5′ flanking sequence was cloned from Apal to XhoI, while the 3′ flanking sequence was cloned from BamHI to SalI.

**Tetrahymena transformation.** One-micrometer gold particles (60 mg/ml; Bio-Rad) were coated with 5 μg of EcoRI/BamHI-digested pTBRGKO and introduced into the *Tetrahymena* macronucleus using biolistic transformation using a PDS-1000/He Biolistic particle delivery system (Bio-Rad) (7). Transformants were identified by growth to saturation in a paromomycin concentration of 60 μg/ml. Transformants were grown in increasing concentrations of paromomycin to a final concentration of 1 mg/ml.

The *TtBRG1* gene was disrupted using biolistic particle bombardment (7). We transformed conjugating B286 and CU426 cells from 2.5 h to 4 h after mixing with 5×-Apal-Sacl fragment released from plasmid pTBRGNEO3. After bombardment, the cells were resuspended in starvation medium and permitted to complete conjugation, after which they were transferred to SPP medium supplemented with 1 μg/ml CuCl2 and incubated with gentle shaking for 4 h at 30°C before being divided into aliquots in 96-well microtiter plates in the presence of 100 μg/ml paromomycin. Knockout heterokaryon TtBRG1 strains (BRG4 and BRG5) with different mating types were created as described by Hai et al. (22). These strains contain two copies of the disrupted *TtBRG1* gene in their micronuclei and the wild-type gene in their macronuclei. When these two strains conjugate, the old, paromomycin-sensitive macronuclei are replaced by new, paromomycin-resistant macronuclei.

**Western analysis.** Whole-cell extracts were prepared by washing and resuspending 106 cells in 100 μl of 10 mM Tris, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (Sigma). Cells were lysed by addition of 100 μl of 2× SDS-loading buffer and boiling for 5 min. Proteins from 0.25 × 106 cells (5 μl) were separated by electrophoresis through an 8% SDS-polyacrylamide gel (PAGE), transferred to a nitrocellulose filter (Amersham), and blocked in 5% skim milk (Bio-Rad) in phosphate-buffered saline (PBS). The blots were incubated in primary polyclonal antisera at a 1:2,500 dilution overnight at 4°C. Blots were washed extensively in PBS, incubated in blocking buffer with 1:5,000-diluted alkaline phosphatase-conjugated secondary goat antirabbit antibody (Sigma), and then visualized using the AP-conjugate substrate kit (Bio-Rad) according to the supplier’s instructions. Rabbit polyclonal antibody to Pdd1p was kindly provided by David Alles, Rockefeller University, New York, N.Y.

**Generation of a polyclonal antibody against an internal peptide of TtBRG.** Because the ciliated protozoa use a nonstandard genetic code (26), we were not able to express full-length TtBRG1 in bacteria. To generate a polyclonal antibody recognizing TtBRG1p, we generated an internal sequence from amino acid 931 to 1010 that did not contain UAA or UAG codons and fused it to a six-His epitope tag. The sequence was amplified from genomic DNA using the primers CBRGF and CBRGR (Table 1) and cloned as an EcoRI/XhoI fragment into pET28a (Novagen), expressed with a six-His tag in Escherichia coli BL21, and purified on a nickel-nitrilotriacetic acid resin. We used SDS-PAGE to identify the purest fractions, which were mixed with appropriate adjuvant and injected into rabbits.

**Indirect immunofluorescence.** Cells were harvested and fixed for indirect immunofluorescence using the method of Wenkert and Allis (63). Incubation of fixed cells on coverslips with primary rabbit anti-TtBRG1p antiserum or its preimmune serum was at a 1:1200 dilution in 5% Bovine serum, 0.1% PBS, at 4°C overnight. Anti-Pdd1p was used at a 1:20,000 dilution as described previously (34). Secondary antibody was fluorescein isothiocyanate-conjugated goat antirabbit (Pierce) diluted at 1:2000 in 5% Bovito, 0.1% goat serum, 1× PBS at 1 h at room temperature. Nuclear counterstaining was with 4,6-diamidino-2-phenylindole dihydrochloride for fluorescein.

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**TABLE 1. Sequences of oligonucleotides used in this study**

| Oligonucleotide | Sequence                  |
|-----------------|---------------------------|
| TISNF2F         | 5′-GCNGAYGARATGGGNYNT     |
| TISNF2R         | 5′-CATHCKRTGNCCYCTTCT     |
| TISWRHR         | 5′-ATAGA                   |
| INV2            | 5′-TCACAAATTTGCAGTGGGAA   |
| INV1            | 5′-GAGAGGAGGACGAGCATAAA   |
| QT              | 5′-GGTGTGGGCGTTGTTTGTGTTT |
| 5′RACEBRG1      | 5′-GAATCTGACGATTTAGCTGTT |
| 5′BRGACE2       | 5′-AACTACGTGTTATACGTTTCT |
| 5′RACE ANCHOR   | 5′-ATGAGAGAGAGAGAGAGAGAG |
| PADER           | 5′-CCGCCCTGAGGTTAAATATAAAAAT |
| TUTR1           | 5′-AGATGCGAACGCTGATGTGTTAA |
| TUTR2           | 5′-CCGCGATCCGAGATTGTTTCTT |
| CBRGF           | 5′-CCGCGATCCGAGATTGTTTCTT |
| CBRGR           | 5′-CCGCGATCCGAGATTGTTTCTT |
| PDF             | 5′-ATGAGAGAGAGAGAGAGAGAG |
| PDDR            | 5′-CTTCTACGTGAGATTGTTTCTT |
| 17S rNA         | 5′-GGAAATACCTTCTTGCGGAC |

**EUKARYOT. CELL**

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cence microscopy and propidium iodide for confocal microscopy (11). The anti-
methyl H3K9 antibody (Upstate) was used at a dilution of 1:500.

Nucleotide sequence accession numbers.

Sequence data from this article have
been deposited with the EMBL/GenBank data libraries under accession no.

AAP31846 and AY268943.

RESULTS

A PCR screen reveals

Tetrahymena encodes several ATP-dependent chromatin-remodeling enzymes. To analyze the
role of ATP-dependent chromatin remodeling proteins in both
growth and development in

Tetrahymena thermophila,
we used

a PCR-based approach to clone members of the SNF2 super-
family. We designed degenerate PCR primers with a bias to-
wards

Tetrahymena
codon use (64) based upon the highly con-
served amino acid sequence corresponding to amino acids 776
to 781 and 879 to 884 of hBRG1 (Fig. 1A) of the DNA-
dependent ATPase domain. We designed an additional for-
ward primer that changes the DEGH motif to DEAH (Fig.
1A). We amplified several DNA fragments whose predicted
encoded proteins were found by BLASTX analysis to have
similarity to members of the SNF2 superfamily. One of the
sequences encoded a protein that was most similar to the
founding member of the SNF2 superfamily SWI2/SNF2, as
well as hBRG1 (Fig. 1A). The other two sequences obtained
from our PCR screen are members of the

SNF2
-like super-
family (SNF2L1 and SNF2L2) (Fig. 1A). We isolated the en-
tire cDNA of

TtBRG1
using colony screening of a full-length
cDNA library constructed from vegetative cells and verified its
5\textsuperscript{\prime}H11032
terminus using 5\textsuperscript{\prime}H11032
-RACE.

Gene structure of

TtBRG1
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Alignment of the 4,304-bp

TtBRG1
cDNA with corresponding amplified genomic se-
quence revealed that 13 introns interrupt the

TtBRG1
coding sequence (Fig. 1B). The

TtBRG1
gene is relatively intron rich,
containing the second-most introns described to date within a

Tetrahymena

gene (5, 59). The size of the introns ranges from
61 bp (intron 7) to >800 bp (intron 3) (Fig. 1B). The 5\textsuperscript{\prime}
untranscribed region and the 3\textsuperscript{\prime} untranscribed region are 275
bp and 260 bp, respectively, and are highly AT rich. The G+C
content for the spliced open reading frame is 33.2%, typical for

Tetrahymena
protein coding sequences (64). The

TtBRG1
cDNA contains a predicted open reading frame of 1,228 amino
acids encoding a putative protein of molecular mass \sim 145

FIG. 1. Tetrahymena encodes several SNF2-related genes. (A) Multiple sequence alignment of partial sequence of three Tetrahymena SNF2-related proteins obtained in this study with corresponding sequence of Saccharomyces cerevisiae SNF2 (accession no. AAA35059), Drosophila Brahma (accession no. P25439), and human BRG1 (accession no. AAB40977). In all multiple sequence alignments presented in this study, black and gray backgrounds indicate invariant and similar amino acid residues, respectively. (B) Genomic and cDNA structure of

TtBRG1
compared to the primary amino acid structure of

TtBRG1.p.
In the genomic locus of

TtBRG1, clear boxes represent the exons and solid boxes introns as well as upstream and downstream genomic sequence. The stippled box represents sequence remaining uncloned. The initiator methionine of

TtBRG1.p
is represented with an M. The striped box of

TtBRG1.p
represents the conserved ATPase domain. (C) Southern analysis of

Tetrahymena
whole-cell DNA digested with SacI (S) and HindIII (H). The SacI restriction fragment of the cDNA (probe A) was utilized as the probe for Southern analysis and the Northern analysis of Fig. 4A. E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SacI; X, XbaI.
kDa. In agreement with this predicted size, crude sera from a rabbit immunized with a six-His-tagged TtBrg1p internal peptide recognizes a polypeptide of approximately this size in *Tetrahymena* whole-cell extracts (data not shown but see Fig. 4A). Southern analysis of *Tetrahymena* whole-cell DNA suggests that *TtBRG1* is a single-copy gene (Fig. 1C).

Analysis of the predicted TtBrg1 protein reveals that it shares several conserved domains with the BRM/SWI2/SNF2 group (Fig. 2A). High similarity (48% identity, 66% similarity over 629 and 657 amino acids in TtBrg1p and hBrg1p, respectively) is observed in the DNA-dependent ATPase domain (Fig. 2B), which contains the seven well-conserved consensus motifs of the helicase superfamily. The first of the seven motifs contains the conserved A-box GKT tripeptide of the ATP-binding motif (Fig. 1A). The lysine residue of the GKT motif has been shown to be required for ATPase activity and transcriptional activation by *SNF2/SWI2* (31). The N-terminal region of TtBrg1p has several regions that are conserved with BRM, hBRG1, and Snf2 (Fig. 2B; also see Fig. 5A in the supplemental material) but does not contain a region corresponding to the proline/glutamine-rich region of domain I. Domain II is a highly charged region well conserved between hBrg1 and Brm and to a lesser extent Snf2 proteins (60). The domain II multiple sequence alignment shows extensive conservation between Brm, hBrg1, and TtBrg and for a shorter distance with Snf2 (see Fig. 5A in the supplemental material). The regions C-terminal to the ATPase domains of Brm, hBrg1, and Snf2 contain a bromodomain (65). This protein module was initially identified as a conserved sequence motif shared between several proteins, including Snf2 and Brm, from human, *Drosophila*, and yeast, all implicated in transcriptional activation (24). Structural studies have shown that the bromodomains of several histone acetyltransferases function as acetyl-lysine-binding modules (12, 28) with specificity for the N-terminal tails of histones. Our analysis of the C-terminal domain of TtBrg1p indicates that it does not encode a canonical bromodomain. In addition, our molecular analysis of the C-terminal region of TtBrg1p indicates that this region is dispensable for growth and nuclear development (see Fig. 5B in the supplemental material).

**Expression studies of TtBRG1 suggest roles in growth and development.** We asked whether TtBRG1 is expressed during conjugation, the developmental cycle that includes meiosis and large-scale genome rearrangements that are associated with macronuclear development in *Tetrahymena*. Northern analysis was performed on whole-cell RNA extracted from growing or starved cells or cells harvested at different times in conjugation using a DNA probe from the 5′/H11032 region of the TtBRG1 cDNA (Fig. 1B). We found that TtBRG1 is expressed during vegetative growth (Fig. 3A), to a lesser extent in starved cells, and strongly and consistently throughout conjugation (Fig. 3A and 5). The relevant nuclear events of the first 14 h of conjugation include meiosis (−2 to 4 h), prezygotic nuclear division (−4.5 h), postzygotic nuclear divisions (−6 to 7 h), and macronuclear development (−8 to 14 h) (37). The programmed genome rearrangements characteristic of macronuclear development occur in the time period 10 to 14 h after the initiation of conjugation (2, 51). TtBRG1 is strongly expressed during the period of genomic rearrangements (Fig. 3A and 5G). The blot was stripped and reprobed to examine the expression pattern of a gene expressed only in conjugation, *PDD1* (34) (Fig. 3B), and a gene that is highly expressed during growth, *ARP1* (25) (Fig. 3D). As expected, no expression was observed for *PDD1* in growing or starved cells, but strong induction for this devel-
Development-specific gene was observed throughout conjugation, consistent with previous studies (34). *ARP1* is expressed strongly in growing cells and in early conjugation during the time corresponding to meiosis, but then the expression decreases significantly, not increasing again until late in macro-nuclear development (Fig. 3D). This expression pattern is similar to that of genes expressed strongly in growth, such as *ACT1* (8). We also stripped and reprobed the Northern blot with the
partial sequence we obtained of the two putative *Tetrahymena* SNF2L chromatin remodeling proteins (Fig. 1A). Both genes are expressed at a low level during growth and are induced during conjugation with nonoverlapping peaks of expression (Fig. 3E and F). The Snf2l1 probe appears to hybridize to two different-size transcripts (Fig. 3E).

**The TtBRG1 gene product localizes to the macronucleus during the time of conjugation that corresponds to programmed genomic rearrangements.** The mRNA expression profile of TtBRG1 suggests that it has a function during macronuclear development. We therefore examined the expression of TtBrg1p during conjugation. In agreement with the Northern analysis, the amount of TtBrg1p throughout conjugation appears constant (Fig. 4A). We then examined the cellular localization of TtBrg1p using indirect immunofluorescence analysis on cells fixed at several time points during conjugation. The late crescent stage corresponds to late prophase of meiosis I (37), a time where the micronucleus is
briefly transcriptionally active (38) and when several proteins implicated in transcription localize to the meiotic micronucleus (54, 55). Unlike these proteins, the localization of TtBrg1p during meiotic prophase (∼3 h into conjugation) is exclusively macronuclear (Fig. 4B). After the fusion of haploid gametic nuclei, the zygotic nucleus divides twice to produce four nuclei, two of each moving to the anterior and posterior of the cell. The anterior nuclei develop as macronuclei, while the two posterior nuclei develop into new micronuclei. At this stage, ∼6 h into conjugation and just prior to the onset of macronuclear development, TtBrg1p localizes to the parental macronucleus (Fig. 4C) but not to any of the zygotic nuclei. The localization of TtBrg1p in the parental macronucleus is lost at the onset of macronuclear development, a stage where the two anterior nuclei (the anlagen) have become visibly larger than the posterior nuclei (Fig. 4D and E). We did not see any cells that contained TtBrg1p in both the parental macronucleus and the anlagen at the same time. Anlagen-specific localization of TtBrg1p is seen in late macronuclear development (∼12 to 14 h into conjugation), where the old macronucleus has been degraded. This is the time of macronuclear development corresponding to programmed genomic rearrangements (Fig. 4F).

**TtBRG1 expression is essential for viability.** The expression and immunolocalization experiments indicate that TtBrg1p likely functions in both growth and development. To determine if TtBRG1 has an essential function during growth of *Tetrahymena*, we attempted to replace all the endogenous macronuclear copies of TtBRG1 with a disrupted version (the NEO-based TtBRG1 knockout construct) (Fig. 5A). The initial transformation event confers resistance to a low level of paromomycin (∼12 to 14 h into conjugation), where the old macronucleus has been degraded. This is the time of macronuclear development corresponding to programmed genomic rearrangements (Fig. 4F).
wild-type allele (21). Southern analysis (Fig. 5B) demonstrates that it was not possible to replace all of the wild-type alleles in the transformants. Since we were not able to replace all of the endogenous copies, we concluded that expression of \( \text{TtBRG1} \) is essential for growth of \( \text{Tetrahymena} \).

In order to further analyze the role of \( \text{TtBRG1} \) during nuclear development, we used the NEO3-based \( \text{TtBRG1} \) knockout construct (Fig. 5A) to generate and conjugate germ line \( \text{TtBRG1} \) knockout heterokaryons (23). About 90 single pairs from the mating between two \( \text{TtBRG1} \) knockout heterokaryon strains and the same number of single pairs from a wild-type control mating were cloned into drops of growth medium. Importantly, 0/90 of the mating pairs produced cells that could complete conjugation and produce viable progeny on return to growth medium that when screened showed a paromomycin-resistant phenotype. By contrast, 82/90 pairs from the wild-type mating divided and grew upon return to nutrient conditions. Therefore, if we observe the \( \text{TtBrg1p} \) signal in the anlagen, it would originate from the parental macronucleus.

We did not observe \( \text{TtBrg1p} \) in the developing macronucleus (Fig. 5C), indicating that it is not subject to \( \text{trans}-\text{nuclear transport} \) during the development of the anlagen. At the end of a wild-type conjugation, cells undergo pair separation, degeneration of old macronuclei, and resorption of one micronucleus. The \( \text{TtBRG1} \) knockout heterokaryon conjugation resulted in cells that delayed in pair separation, and the majority of the knockout cells did not resorb one of their two micronuclei. As a result, these cells remained arrested at the 2MAC-2MIC stage, even after 58 h.
The fact that we were not able to generate viable offspring of the conjugation of knockout heterokaryons for *TtBRG1* that appear to be arrested in the 2MAC-2MIC stage implies that zygotic expression of the gene is essential for completion of conjugation in *Tetrahymena*.

The arrest of the conjugating knockout heterokaryons in the 2MAC-2MIC stage is reminiscent of the result of conjugating cells deleted for their macronuclear copies of the development-specific *PDD1* gene (10). These cells also exhibit defects in the ability to undergo the large-scale genome rearrangement characteristic of macronuclear development. We were therefore interested in whether the absence of TtBrg1p in the developing macronucleus would similarly have an effect on large-scale genome rearrangements. During the late stages of a wild-type conjugation, indirect immunofluorescence analysis of Pdd1p shows that it goes from a diffuse distribution in the developing macronucleus to form distinct punctuate and peripheral foci that have been referred to as “dumposomes” due to the fact that they contain micronucleus-limited DNA destined for deletion from the developing macronucleus (34). As a first approach to addressing this question, we analyzed Pdd1p localization during late-stage conjugation of the *TtBRG1* germ line knockout heterokaryons. In our wild-type conjugation, Pdd1p staining in the anlagen becomes punctuate 10 h into conjugation and subsequently disappears by 18 h postmixing as expected (Fig. 6A to C). However, in the mating of the knockout heterokaryons, Pdd1p staining remains uniform through and at the periphery of the anlagen and does not organize into punctuate foci (Fig. 6D to F). Thus, the formation of the distinct Pdd1p-containing structures appears to be disrupted, suggesting that TtBrg1p has a direct role in programmed genome rearrangements.

Since the formation of these distinct Pdd1p-containing structures likely occurs downstream of the generation of methylated lysine 9 of histone H3 (methyl-H3K9) in the *Tetrahymena* anlagen (58), we asked whether there was any change in this...
histone modification in the heterokaryon knockout. As with Pdd1p, methyl-H3K9 has been demonstrated to be enriched in anlagen but not detectable in MICs or old MACs (58). We also saw this pattern of methyl-H3K9 in indirect immunofluorescence analysis of a wild-type conjugation. At 12 h and 15 h postmixing, the appearance and pattern of the methyl-H3K9 epitope in the anlagen appears normal at 12 h and 15 h postmixing (Fig. 7D and E), but as described above for Pdd1p, the epitope persists and is still present at 18 h postmixing (Fig. 7E).

**Genomic analysis of putative Tetrahymena SWI/SNF complex members and SNF2-related proteins.** To address whether a canonical SWI/SNF complex exists in Tetrahymena, we identified putative members by querying the Tetrahymena genome database with sequences encoding subunits of the yeast SWI/SNF complex that have human homologues. The results, summarized in Table 2, indicate that Tetrahymena has homologues of SWI/SNF proteins conserved from yeast to human. We did not obtain homologues when we queried the database with proteins that were specific to Saccharomyces cerevisiae, such as SNF11 and SNF6.

| Yeast query | Tt ID | E value (Sc-Tt) | Conserved domain | E value (Tt-Sc) | E value (Tt-Hs) |
|-------------|-------|----------------|------------------|----------------|----------------|
| Swi1p (BAF-250b) | 21.m00216 | 4.70E–08 | ARID (3E–20) | 1.00E–05 | 4.00E–07 |
| Swi3p (BAF-170) | 80.m00137 | 6.20E–34 | SANT (2E–04) | 3.00E–36 | 2.00E–46 |
| Snf5p (IN11) | 29.m00182 | 3.30E–11 | SWIRM (5E–23) | 4.00E–17 | 3.00E–24 |
| Snf12p (BAF-60) | 165.m00081 | 4.00E–05 | SWIB (1E–12) | 0.021 | 1.00E–35 |

Other PCR screen for SNF2-related genes (Fig. 1A) combined with Southern blotting (Fig. 1C) had indicated that it was unlikely that there were two closely related SNF2/brachma-related genes in Tetrahymena, unlike the case for yeast (SNF2 and STH1) or human (BRG1 and brahma). To confirm this observation, we queried the Tetrahymena genome database with the amino acid sequence of the DNA-dependent ATPase region of the yeast Snf2 protein (amino acids 281 to 1400). The results are summarized in Table 3 and confirm that although there are a variety of SNF2-related genes encoded by Tetrahymena, there is only one bona fide SNF2 gene, TtBRG1, in agreement with results obtained by Southern blotting (Fig. 1C).

**DISCUSSION**

**Comparative sequence analysis of TtBRG1.** The conservation of several domains aside from the well-conserved ATPase domain argues that TtBrG1 is a bona fide member of the BRM/SNF2 group of chromatin remodeling proteins. Accord-

**TABLE 2. Putative members of a Tetrahymena SWI/SNF complex**

| Yeast query | Tt ID | E value | Conserved domain | E value | E value |
|-------------|-------|---------|------------------|---------|---------|
| Swi1p (BAF-250b) | 21.m00216 | 4.70E–08 | ARID (3E–20) | 1.00E–05 | 4.00E–07 |
| Swi3p (BAF-170) | 80.m00137 | 6.20E–34 | SANT (2E–04) | 3.00E–36 | 2.00E–46 |
| Snf5p (IN11) | 29.m00182 | 3.30E–11 | SWIRM (5E–23) | 4.00E–17 | 3.00E–24 |
| Snf12p (BAF-60) | 165.m00081 | 4.00E–05 | SWIB (1E–12) | 0.021 | 1.00E–35 |

**TABLE 3. Genes identified in Tetrahymena genome that give significant matches to S. cerevisiae SNF2**

| Tt ID | E value | Top match, Sc (Hs) | E value, Sc (Hs) | Domain(s) |
|-------|---------|-------------------|-----------------|-----------|
| 288.m00027 (TtBRG1) | 4.30E–173 | SNF2 (HBRM) | 0.0 (0.0) | 2 C-terminal SANT domains (3.2E+0, 2.1E+01) |
| 10.m00436 | 4.30E–118 | ISW2 (hSNF2H) | E–151 (4E–179) | |
| 438.m00086 | 2.30E–104 | SWR1 (SRCAP) | E–180 (1E–121) | |
| 35.m001105 | 9.60E–99 | INO80 (INO80-like protein) | 0.0 (0.0) | |
| 41.m00227 | 1.50E–96 | ISW1 (hSNF2H) | E–136 (2E–147) | 1 C-terminal SANT domain (1.6E+0) |
| 16.m00319 (TtSNF2L1) | 2.40E–90 | CHD1 (CHD-7) | E–107 (2E–148) | 2 N-terminal chromodomains (5.1E–17, 5.1E–17) |
| 3.m01803 (SNF2L2) | 1.90E–61 | CHD1 (CHD3) | E–102 (9E–129) | 3 C-terminal PHD domains (2.9E+0, 5.7E–01, 5.4E+0) |
| 20.m00257 | 2.40E–53 | RAD54 (RAD54) | E–102 (2E–101) | 1 GATA Zn finger (8E–02) |
| 66.m00231 | 1.20E–49 | MOT1 (BTAF1) | E–99 (2E–106) | 2 N-terminal PHD domains (4.7E–11, 4.7E+0) |
| 88.m00108 | 4.50E–33 | SNF2 (SMARCAL1) | 3E–61 (8E–58) | 2 N-terminal chromodomains (7.4E+0, 4.5E–01) |
| 46.m00228 | 1.20E–31 | RAD5 (SMARCAL3) | E–38 (3E–56) | |
| 30.m00324 | 5.50E–25 | RAD5 (lodestar) | E–100 (2E–45) | RING (3.5E–05) |
| 2.m02413 | 3.40E–24 | RAD5 (SMARCAL3) | E–20 (3E–21) | EFhand (4.1E–03) |
| 80.m00204 | 2.10E–18 | MOT1 (SMARCAL1) | 2E–34 (4E–74) | |
| 222.m00048 | 7.30E–11 | ISW1 (SMARCAL1) | 3E–21 (6E–57) | |
| 28.m00182 | 5.20E–10 | RAD5 (SMARCAL3) | 4E–20 (3E–21) | RING (1.2E–02) |

a Tetrahymena (Tt) gene identification indicator of match from BLASTP analysis (www.ciliate.org) using the amino acid sequence of Snf2 of S. cerevisiae.

b E value of the match.

c Top match for S. cerevisiae (Sc) from the query of the Tetrahymena protein against the GenBank NR database (www.ncbi.nlm.nih.gov).

d Top match for human (Hs) from the query of the Tetrahymena protein against the GENBANK NR database (www.ncbi.nlm.nih.gov).

e Conserved domains with E values in the SMART analysis (http://smart.embl-heidelberg.de/).
ingly, TtBrg1p may nucleate a large multipolypeptide complex similar to the SWI/SNF (48) and BRG1-associated factor (62) complexes. The more-extensive similarity throughout domain II between TtBrg1p, Brm, and hBrg1 suggests that TtBrg1p may share a function with these proteins that it does not yet with the yeast protein Snf2p. Domain II of hBrg1 has been implicated in a variety of protein-protein interactions, such as with β-catenin (3), the Fanconi anemia protein, FANCA (46), and Hp1α (41). Our genomic analysis suggests that, similar to the case with Drosophila, there is only one BRM/SW1/SNF2-group member in Tetrahymena, raising the question of whether TtBrg1p nucleates multiple SWI/SNF-like complexes, such as in yeast and humans (32). Affinity purification of TtBrg1p-containing complexes from isolated macronuclei of epitope-tagged TtBrg1p strains should provide answers to this question, and these experiments are in progress (J. Garg, J. S. Fillingham, and R. E. Pearlman, unpublished).

**TtBRG has a role during vegetative growth of Tetrahymena.**

The strong level of TtBRG1 transcription during vegetative growth, combined with the fact that it is an essential gene, argues that it has a significant role in growth of Tetrahymena. Genetic analysis of SNF2 superfamily genes in different organisms has revealed a variety of growth-related phenotypes (6, 14, 49, 57) related to defects in transcription activation. The fact that TtBrg1p does not have a canonical bromodomain or a distinct P/Q-rich region, both regions related to transcriptional activation, hints that its essential role may not be related to defects in transcriptional activation. It will be interesting to determine if TtBrg1p associates with histone deactetylases like its human counterpart (53) or if, like Snf2p, TtBrg1p is involved in the repression of gene expression during growth (36).

**Expression of TtBRG1 suggests role in nuclear development.**

The expression profile of TtBRG1 during conjugation suggests a role in nuclear development. Northern analysis of TtBRG1 showed transcription at a relatively constant rate throughout conjugation, and this expression pattern is observed at the protein level. The expression of a gene at a consistent level throughout conjugation is significant, since there is a demonstrated decrease in global transcription that occurs after meiosis and does not increase again until early macronuclear development (38) (Fig. 3D).

The localization of TtBrg1p to the parental macronucleus in conjugating cells with no micronuclear staining suggests that TtBrg1p is not likely to be involved in germline-specific transcription in the meiotic prophase macronucleus. Several proteins with a demonstrated role in transcription have been immunolocalized to the germ line macronucleus during a time period when it is transcriptionally active (55, 63). Indeed, transcriptional differences between the macronucleus and the micronucleus have been shown to be associated with the selective loss of chromatin components from developing micronuclei more than their appearance in developing macronuclei (54). Since TtBrg1p does not apparently have a role in this process, it is possible that its function in development is not related to transcriptional activation.

The macronuclear localization of TtBrg1p has a striking property in that its appearance in the parental macronucleus and the anlagen appears to be mutually exclusive. One other Tetrahymena polypeptide that shares this property is the development-specific Twi1p. The function of Twi1p is to organize chromatin regions in the developing macronucleus for programmed elimination (39). The developmentally specific polypeptides Pdd1p and Pdd2p also function in this pathway and exhibit localization to both the parental macronucleus and the early-stage anlagen (34, 42). Thus, the localization pattern of TtBrg1p is correlated both temporally and spatially with the extensive programmed genomic rearrangements that occur during nuclear development in Tetrahymena.

It has been suggested that functional differences between the polyploid macronucleus, specialized for transcriptional activity, and the transcriptionally silent micronucleus may be related to the lack of classic heterochromatic regions in the macronucleus (9, 27). Molecular support for this comes from recent studies that show that in vegetatively growing Tetrahymena, the macronucleus and micronucleus lack the methyl-H3K9 modification (4, 58). However during nuclear development, Tetrahymena uses the methyl-H3K9 modification to target IESs for programmed DNA deletion (58). The methyl-H3K9 modification is considered to be a marker of constitutive and facultative heterochromatin (35, 47). The chemistry of IES excision suggests that its mechanism shows some similarity to other examples of developmentally programmed DNA deletion, such as V(D)J recombination. The recent description of specific histone modifications correlated with V(D)J recombination (56) and class switch recombination (44) suggests that an underlying common mechanism could exist from ciliates to humans controlling programmed DNA deletion. Several studies have implicated hSWI/SNF in V(D)J recombination. Kwon et al. (30) demonstrated that hSWI/SNF combined with histone acetylation to greatly stimulate V(D)J recombination (30). Using chromatin immunoprecipitation, Ng et al. (40) found that large amounts of hBrg1, the catalytic subunit of hSWI/SNF, acetylated histone H3, and the methyl-H3K9 modification are correlated with V(D)J recombinationally active loci. This combined with the localization of TtBrg1p during conjugation suggests the possibility that, by analogy, TtBrg1p may directly remodel chromatin to activate it for IES excision. Support for this idea is given by the altered distribution of chromodomain-containing Pdd1p in the developing macronucleus of TtBRG1 germ line knockouts. The fact that the generation of the methyl-H3K9 epigenetic mark appears normal in the mating of knockout heterokaryons suggests that if there is a direct role for TtBrg1p in IES excision, it functions downstream of the generation of methyl-H3K9 and upstream of the methyl-H3K9-Pdd1p interaction. One possibility is a possible physical interaction between Pdd1p and TtBrg1p. A physical interaction has been demonstrated to occur between human Brg1p and the chromodomain-containing HP1-alpha (41).

It will be interesting to analyze the components of the putative TtSWI/SNF complex isolated during this time period to determine if it contains subunits that are related to human lymphoid-specific genes. The ability to generate large populations of Tetrahymena synchronously undergoing programmed genomic rearrangements should allow putative developmentally specific TtSWI/SNF complexes to be isolated and analyzed.

**SNF2 genes in Tetrahymena.**

Here we report the cloning and initial molecular characterization of ATP-dependent SNF2 chromatin-remodeling genes in Tetrahymena, the first de-
scribed to date in the ciliated protozoa. In addition to TbBRG1, Tetrahymena encodes at least two other members of the SNF2 superfamily with expression profiles that, similar to the case with TbBRG1, suggest roles in growth as well as nonoverlapping roles in nuclear development. The two SNF2L genes do not have related genes other than themselves (data not shown).

The expression pattern of TsSNF2L indicates it may encode splice variants, a phenomenon not yet described for Tetrahymena. Our analysis of the Tetrahymena genome database indicates the two SNF2L genes are of the CHD class (SNF2L1, CHD5; SNF2L2, CHD3), both of which contain two chromodomains. Significantly, the gene represented by SNF2L1 (CHD5) contains a bromodomain situated between the chromodomains. This highly unique sequence organization combined with its development-specific expression pattern suggests its molecular analysis (Vythilingum, Fillingham, Garg, and Pearlman, unpublished observation) will be of particular interest in deciphering the histone code of programmed genome rearrangements in Tetrahymena.

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