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RESEARCH ARTICLE

DRH1, a p68-related RNA helicase gene, is required for chromosome breakage in *Tetrahymena*

Stephen L. McDaniel¹, Erica Zweifel², Peter K. W. Harris¹, Meng-Chao Yao³, Eric S. Cole² and Douglas L. Chalker¹,*

ABSTRACT

The p68 DEAD box helicases comprise a widely conserved protein family involved in a large range of biological processes including transcription, splicing and translation. The genome of the ciliate *Tetrahymena thermophila* encodes two p68-like helicases, Drh1p and Lia2p. We show that *DRH1* is essential for growth and completion of development. In growing cells, Drh1p is excluded from the nucleus and accumulates near cortical basal bodies. In contrast, during sexual reproduction, this protein localizes to meiotic micronuclei, initially in punctate foci in regions where centromeres and telomeres are known to reside and later in post-zygotic differentiating somatic macronuclei. Differentiation of the macronuclear genome involves extensive DNA rearrangements including fragmentation of the five pairs of germline-derived chromosomes into 180 chromosomal sub-fragments that are stabilized by *de novo* telomere deletion. In addition, thousands of internal eliminated sequences (IESs) are excised from loci dispersed throughout the genome. Strains with *DRH1* deleted from the germline nuclei, which do not express the protein during post-zygotic development, fail to fragment the developing macronuclear chromosomes. IES excision still occurs in the absence of *DRH1* zygotic expression; thus, Drh1p is the first protein found to be specifically required for chromosome breakage but not DNA elimination.

KEY WORDS: Ciliate, Genome rearrangements, DEAD-box RNA Helicase, p68, Chromosome, Meiosis

INTRODUCTION

Like all ciliated protozoans, *Tetrahymena thermophila* contains two functionally distinct types of nuclei: a somatic macronucleus and a germline micronucleus. When *Tetrahymena* cells mate, they undergo a predictable genetic program (reviewed by Cole and Sugai, 2012) of meiosis, cross-fertilization (exchange of gametic nuclei), karyogamy (fusion of gametic nuclei), DNA replication, nuclear division, and new macronuclear genome differentiation. Nuclear differentiation involves extensive genome remodeling encompassing two processes: massive DNA elimination and chromosome fragmentation. DNA elimination removes nearly one third of the germline-derived genome from the newly forming somatic genome. The thousands of DNA segments removed, termed internal eliminated sequences (IESs), are composed largely of A+T-rich non-coding sequences, transposable elements, and other repetitive DNA (Chalker and Yao, 2011). The resulting chromosomal breaks are repaired by non-homologous end joining (Lin et al., 2012). This pathway generates a gene-enriched genome specialized for its somatic role in growing progeny.

Chromosome fragmentation occurs when the five pairs of germline-derived chromosomes are processed into ~180 macronuclear chromosomes (Hamilton et al., 2005); this reduction in chromosome size is thought to be important to facilitate the amitotic division of the macronucleus in growing cells. The loci at which genome fragmentation occurs contain a highly conserved 15 bp chromosomal breakage sequence (CBS), which is necessary and sufficient for this processing (Fan and Yao, 1996, 2000). This sequence is entirely removed from the somatic chromosomes, and new telomeres are added within 25 bp of the former position of the CBS.

The process and many of the proteins involved in IES elimination are well characterized. First, non-genic transcripts produced during meiosis are processed by Dicer-like 1 (*Dcl1p*) into ~30 nt scanRNAs (scanRNAs). These scanRNAs bind the *Tetrahymena* Piwi1 (*Twil1*)-interacting protein and mark the sequences to be eliminated in the differentiating macronucleus (Mochizuki et al., 2002; Chalker and Yao, 2001; Malone et al., 2005; Mochizuki and Gorovsky, 2005). Next, Twil1p-bound scanRNAs target histone H3 lysine 9 (K9) and K27 methylation to homologous sequences in the developing macronucleus. Finally, the IESs are excised by the domesticated transposase Tpb2p (Taverna et al., 2002; Liu et al., 2007; Cheng et al., 2010). The RNA helicase Ema1p is also essential for the histone methylation that leads to IES elimination. Ema1p is thought to accomplish this by stimulating base-pairing between Twil1p-bound scanRNAs and noncoding transcripts in both the parental and developing somatic nuclei.

Unlike the DNA elimination pathway, the proteins that recognize and cleave at the CBS and eventually recruit telomerase remain to be discovered. Deletion of genes encoding components (e.g. *DCL1*) of the DNA elimination machinery block chromosome fragmentation, possibly indicating overlap in these pathways. Alternatively, the developmental arrest caused by these mutations may occur before chromosomal fragmentation initiates (Malone et al., 2005). The developmental timing and interdependence between these two types of rearrangements remains to be determined. To identify the molecular machinery that performs IES elimination and chromosome breakage, we have focused on proteins that have induced expression during development, localize to nuclei where these processes occur, and/or share homology to proteins previously linked to these events (Yao et al., 2007; Matsuda et al., 2010). The importance of homologous RNAs in DNA rearrangements, the
known role for Ema1p in DNA rearrangements (Aronica et al., 2008), and the abundance of RNA helicases encoded within the *Tetrahymena* genome led us to further investigate members of this family of proteins for possible roles in genome reorganization.

RNA helicases participate in the majority of biological processes involving RNA, including transcription, processing, and degradation. In these processes, RNA helicases unwind secondary structures and participate in assembly/disassembly of ribonucleoprotein complexes and other mechanisms requiring RNA structural manipulation (Fuller-Pace, 2006; Linder, 2006; Cordin et al., 2006). Ema1p belongs to the DExD/H box family of helicases, which includes the well-known DEAD box proteins. Some DExD/H proteins are thought to act in single pathways, whereas others participate in multiple biological processes (Linder and Jankowsky, 2011). The human, yeast, and *Tetrahymena* genomes encode 37, 26, and 45 (DRH1-45) DExD/H box family members, respectively (Fairman-Williams et al., 2010). All evidence indicates that these proteins have undergone extensive diversification during their evolution.

Although called helicases, how, or even whether, specific DExD/H box proteins unwind RNAs is uncertain (Jankowsky, 2011). They clearly can alter RNA structure in ways that lead to RNA unwinding or assist in RNA annealing. However, some family members have been implicated in processes for which their action may not be dependent on RNA. For example, the p68 (DDX5) and related p72 (DDX17) proteins have roles in transcriptional regulation that appear to be independent from their helicase activity (Endoh et al., 1999; Watanabe et al., 2001). Nevertheless, these same proteins participate in pre-mRNA, rRNA, and microRNA processing (Fuller-Pace and Moore, 2011). How these proteins can participate in such a diverse array of cellular events is not well understood.

In previous work, we identified the DEAD box helicase gene *LIA2* in a screen for developmentally expressed proteins that localize within developing somatic nuclei (Yao et al., 2007). The function of this protein is unknown, but preliminary data indicated that this protein is not essential for growth (Yao et al., 2007; Fass et al., 2011). Here, we have investigated the *Tetrahymena* protein most closely related to the protein encoded by *LIA2*, the p68-like DEAD box RNA helicase Drh1p. We report that its zygotic expression is essential for chromosome fragmentation, but not DNA elimination. This finding makes Drh1p the first protein known to be required specifically for chromosome breakage.

**RESULTS**

*Tetrahymena* encodes two putative p68-like RNA helicases

We previously identified *LIA2* (*DRH3*) in a screen for proteins that localize within developing macronuclei. We disrupted the gene by recombining the neo3 (*MTT1-neo*) selectable marker (which confers paromomycin resistance to *Tetrahymena* cells) (Shang et al., 2002) into the middle of the coding region (Fig. S1A) but did not detect any obvious phenotypes (Yao et al., 2007; Fass et al., 2011). During subsequent work, we observed that mutant cells divided more slowly than wild-type cells when grown in stationary cultures (data not shown), but this growth difference was not apparent when cells were grown in flasks with shaking (Fig. S1B). Thus, any growth impairment proved too weak to use to help uncover processes in which the Lia2 protein (Lia2p) might act.

*LIA2* is one of 45 *Tetrahymena* genes (*DRH1-DRH45*) encoding putative DExD/H box RNA helicases and is highly similar to a second gene, *DRH1* (*TThERM_00190830*). Both genes encode putative homologs in the p68/DDX5 family of helicases, although the similarity is confined to the ~430 amino acid helicase domains (DEADc and HELICc in Fig. 1A; see Fig. S2). *LIA2* and *DRH1* are apparent paralogs as they are more similar to each other than to any other sequence in the Genbank non-redundant database (Fig. 1B). Reciprocal BLAST searches and multi-sequence alignments showed that both genes cluster with p68 proteins, whereas the *Tetrahymena* protein (encoded by *DRH2*, Ttherm_00420420) possessing the next greatest similarity to p68 proteins clusters with the DDX46 family of helicases (Fig. 1B).

Both *LIA2* and *DRH1* are expressed throughout the life cycle. Both genes are expressed at moderately high levels during growth and starvation (a condition of nutrient deprivation that readies cells for conjugation when cells of complementary mating types are mixed) and are upregulated during conjugation. *LIA2* mRNA accumulation increases steadily at the beginning of post-zygotic development (6 h into conjugation) (Yao et al., 2007), whereas *DRH1* is upregulated starting in pre-zygotic development (4 h; Fig. 1C), when meiosis occurs. *DRH1* mRNA levels decrease during the early post-zygotic stages of development (6 to 8 h post-pairing) and rise again by 10.5 h, which corresponds to the onset of DNA rearrangements that remodel the somatic genome within differentiating macronuclei. These differences in sequence and expression suggest that these two paralogs have diverged somewhat in function.

**Drh1p shows dynamic organization in nuclei during development**

The p68 RNA helicases, including Lia2p, are primarily nuclear-localized proteins that have been implicated in a variety of cellular processes (Fuller-Pace and Moore, 2011). To gain insight into...
functions of Drh1p, we examined the sub-cellular localization of a green fluorescent protein (GFP)-DRH1 fusion protein expressed throughout the Tetrahymena life cycle from the cadmium-inducible MTT1 promoter. In vegetatively growing cells, GFP-Drh1p accumulated in the cytoplasm but was largely undetectable in nuclei, which is not what we expected. The protein was somewhat enriched at the cell cortex, possibly near basal bodies (Fig. 2A; Fig. S3), but the significance of this cortical localization is not known.

Shortly after mixing pre-starved populations of complementary mating types, GFP-Drh1p strongly accumulated within germline micronuclei (Fig. 2A). Protein import occurred rapidly, even prior to cell pairing, during the period of co-stimulation. The GFP fluorescence within micronuclei was initially observed in about five punctate spots (inset, Fig. 2A). Tetrahymena micronuclei contain five pairs of germline chromosomes that undergo recombination during late meiotic prophase. It is possible that centromeres of homologous chromosomes align even at this earlier stage. If so, the ten centromeric regions would appear as five independent foci such as we observed here. We speculate that the five GFP-decorated foci are the centromeric regions of the paired micronuclear chromosomes.

Shortly after cell pairing, micronuclei elongate to create the ‘crescent’ micronucleus, which reaches its maximum extended length at prophase stage IV (Fig. 2B). During stage III, the elongating micronuclei have a narrow ‘head’ and a bulb-shaped trunk. Chromosomes occupy a non-random configuration within this structure, with the telomeres concentrated at the tip of the head and centromeres positioned near the base of the trunk (Loidl and Scherthan, 2004; Cervantes et al., 2006). At this stage, GFP-Drh1p was enriched at both ends of crescent micronuclei, concentrated at the tip of the head (the telomere end) and exhibiting a more dispersed granular appearance near the apical end of the trunk (centromeres). Approximately ten GFP-labeled aggregates could be counted in the trunk region of each crescent micronucleus, which is consistent with the number of centromeres at this stage. As the crescent micronucleus begins to condense (the transition between prophase stages IV and V), the GFP fluorescence at both ends became more dispersed throughout the crescent (Fig. S4). Other cells at this stage showed diffuse Drh1p localization at both ends and a single large aggregate near the centromeric end. This dynamic localization near telomeres and centromeres is consistent with a role of Drh1p in meiotic chromosome function.

Entering metaphase of meiosis I (note: Tetrahymena undergoes closed meiosis without nuclear envelope breakdown), GFP-Drh1p was localized over both fibers and distinct foci within the spindle apparatus (Fig. 2C). We frequently observed a single dense focus located mid-spindle that later appeared as two foci that each appeared to migrate to opposite poles at anaphase (Fig. 2C). Drh1p showed localization to both similar fibers and in puncta during Meiosis II as we described for Meiosis I. Overall, this highly organized and dynamic localization of GFP-Drh1p throughout meiosis may reflect association with and/or regulation by the meiotic spindle.

GFP-Drh1p exhibited additional, notable changes in its localization during early conjugation. We did not detect it in the nucleus during the third pre-meiotic division (a mitotic division) (Fig. S4), but did detect it in the nucleus after nuclear exchange and formation of the zygotic nucleus (Fig. 2D, left panel; Fig. S4). During synkaryon formation (the nuclear fusion that produces the zygotic genome), multiple GFP-Drh1p foci appeared to coalesce and segregate during mitotic anaphase. In these zygotic nuclei, we observed GFP-Drh1p as a single large aggregate is some cells, but in others the protein was divided into two or four nuclear foci (Fig. 2D, left panel). This difference may reflect varying degrees of spindle integration that must occur upon nuclear fusion, as individual spindles form prior to karyogamy (Cole and Sugai, 2012). After karyogamy, the newly formed zygotic nucleus undergoes two rounds of mitosis to produce precursors of the new micro- and macronuclei. Following the second post-meiotic mitosis, the two anterior division products enlarge and begin differentiation into...
macronuclei. At this stage of development, GFP-Drh1p was in these developing macronuclei and was excluded from the micronuclei. We also observed some transient localization in the parental macronucleus just before its degeneration. It is quite intriguing that Drh1p switches its localization rapidly from micronuclei throughout pre-zygotic development to macronuclei in post-zygotic development (Fig. 2D; Fig. S4). The localization in the differentiating macronuclei was diffuse and did not exhibit the highly organized patterns similar to what we observed in dividing micronuclei. Whether the changing localization reflects different functions for Drh1p in these different nuclear compartments is yet to be determined.

**DRH1 is essential for growth and development**

The localization of Drh1p suggested that this protein has important roles in critical nuclear events throughout development. To identify processes in which this protein might act, we disrupted the gene and assessed cells for loss-of-function phenotypes. We generated a gene disruption construct composed of ∼1 kilobase pairs (kb) DNA fragments corresponding to genomic DNA flanking the *DRH1* coding sequence, cloned on each side of the *neo3* selectable marker. We introduced this *DRH1* knockout cassette into conjugating *Tetrahymena* strains (strains CU428×B2086) by particle bombardment to disrupt gene copies in both the micro- and macronuclei. *DRH1* knockout strains were identified by selection for paromomycin-resistant progeny. Homologous gene replacement was confirmed by PCR (data not shown) and Southern blot analysis (Fig. 3A). The transformed progeny that we initially generated were heterozygous for the knockout allele in their micronuclei (Δ*DRH1*mic/+ and had a significant portion of their *DRH1* macronuclear gene copies disrupted. We also obtained transformants of non-mated cells in the population with only the macronuclear copies disrupted (Δ*DRH1*mac).

*Tetrahymena* macronuclear division is amitotic, allowing random segregation (called assortment) of alleles during growth. Repeated attempts to obtain transformed cells with all macronuclear copies replaced with the knockout allele by sub-cloning transformants into paromomycin-containing growth medium were unsuccessful, indicating that *DRH1* is likely essential for growth. We next generated cells lacking all *DRH1* copies by mating two strains each homozygous in their micronuclei for the knockout allele (Δ*DRH1*mic strains 1B and 3A). This genetic cross offered the possibility of characterizing phenotypes of cells lacking all *DRH1* expression. No viable progeny resulted from this cross. The majority of the 176 mating pairs cloned into growth medium died, and the only viable cells had aborted conjugation without making new macronuclei (Table 1); thus, *DRH1* has essential functions. Crossing either Δ*DRH1*mic strain to wild-type cells resulted in the loss of *DRH1* expression from the knockout partner, resulting in a high percentage (64-91%) of viable progeny (Table 1).

Because the progeny of homozygous Δ*DRH1*mic strains lack all wild-type *DRH1* copies, their failure to survive could have resulted from an inability to (1) grow once all maternally expressed protein was depleted or (2) even complete conjugation without zygotic *DRH1* expression. To first determine whether mated pairs could return to vegetative growth, we isolated individual pairs into growth medium (Table 1). In contrast, the mated Δ*DRH1*mic cells arrested prior to the elimination of one micronucleus and, based on the lower intensity of DAPI fluorescence, exhibited some degree of micronuclear degeneration. It is quite intriguing that Drh1p switches its localization rapidly from micronuclei throughout pre-zygotic development to macronuclei in post-zygotic development (Fig. 2D; Fig. S4). The localization in the differentiating macronuclei was diffuse and did not exhibit the highly organized patterns similar to what we observed in dividing micronuclei. Whether the changing localization reflects different functions for Drh1p in these different nuclear compartments is yet to be determined.

Table 1. *DRH1* zygotic expression is required for conjugation

<table>
<thead>
<tr>
<th>Cross</th>
<th>Total</th>
<th>% Viable (%)</th>
<th>% Progeny (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU427×CU428</td>
<td>88</td>
<td>98% (86)</td>
<td>87% (75)</td>
</tr>
<tr>
<td>Δ<em>DRH1</em>mic-1B×427</td>
<td>176</td>
<td>97% (170)</td>
<td>91% (155)</td>
</tr>
<tr>
<td>Δ<em>DRH1</em>mic-3A×428</td>
<td>176</td>
<td>83% (146)</td>
<td>64% (94)</td>
</tr>
<tr>
<td>Δ<em>DRH1</em>mic-1B×3A</td>
<td>176</td>
<td>45% (80)</td>
<td>0% (0)</td>
</tr>
</tbody>
</table>
DAPI fluorescence, without fully amplifying the DNA in developing macronuclei (Fig. 3B). We conclude that DRH1 zygotic expression is required for cells to complete late stages of conjugation, and that DRH1 is essential for both growth and development.

As the progeny of the ΔDRH1mic strains arrested in development, this genetic cross did not allow us to examine the phenotype of loss of DRH1 function in vegetative cells. In an attempt to overcome this limitation, we generated cells lacking all endogenous DRH1 copies in macronuclei by expressing an inducible allele of the gene from an ectopic locus. We started with two partially assorted (pa) ΔDRH1mac strains into which we introduced a construct consisting of a GFP-DRH1 fusion expressed from the cadmium inducible MTTL promoter and integrated upstream of the rpl29 locus. By expressing the GFP-DRH1 fusion allele in these cells, the knockout allele was able to completely replace the endogenous DRH1 gene, which we demonstrated by using reverse-transcription (RT) PCR in which only mRNA from the GFP-DRH1 allele was detectable (Fig. 3C). In the absence of all endogenous protein, GFP-Drh1p expressed during vegetative growth localized primarily in the cytoplasm or near basal bodies, supporting the idea that the localization of the GFP-Drh1p expressed during vegetative growth localized primarily in the cytoplasm or near basal bodies, supporting the idea that the localization of the GFP-Drh1p expressed from the rDNA vector described above was biologically relevant.

To examine the effect of loss-of-function phenotypes in growing cells, we placed the fully assorted DRH1expressed from the rDNA vector described above was biologically relevant. To examine the effect of loss-of-function phenotypes in growing cells, we placed the fully assorted ΔDRH1mac:GFP-DRH1 strains into medium lacking cadmium. We found that the cells continued to grow; however, we could detect low levels of GFP-DRH1 expression by fluorescence microscopy (data not shown) and RT-PCR (Fig. 3C), suggesting that the MTTL promoter was too leaky to observe loss-of-function phenotypes in growing cells.

We utilized these ΔDRH1mac:GFP-DRH1 cells to examine loss of DRH1 expression during early conjugation. These mutant cells, when starved and mated without addition of cadmium, accumulated limited amounts of Drh1p as judged by the low intensity of GFP fluorescence. When these mated pairs were isolated into growth medium, most died and produced no viable progeny. In contrast, crossing these strains to wild-type cells or crossing them together in medium, most died and produced no viable progeny. In contrast, crossing these strains to wild-type cells or crossing them together in the presence of cadmium produced viable progeny. Examination of the mated cell population showed that about 60% of the exconjugants arrested with two micro- and two macronuclei, and the remainder appeared to complete conjugation and amplify their new macronuclear genomes, even though no viable progeny resulted (Fig. 3B). This partially penetrant phenotype is consistent with a knockdown, rather than a knockout, phenotype. Nevertheless, the inability of ΔDRH1mac:GFP-DRH1 strains to produce viable progeny demonstrates that expression of DRH1 from parental macronuclei is required for Tetrahymena development.

DRH1 zygotic expression is required for chromosome breakage

The two micro- and two macronuclei developmental arrest point, which we observed upon mating ΔDRH1mic strains, is a common phenotype described for several strains lacking genes required for developmentally programmed DNA rearrangements (Shieh and Chalker, 2013; Rexer and Chalker, 2007; Motl and Chalker, 2011; Malone et al., 2005; Horrell and Chalker, 2014; Noto et al., 2010; Mochizuki et al., 2002; Bednenko et al., 2009). To determine whether the developmental arrest observed upon loss of DRH1 expression was associated with defects in genome rearrangements, we assessed IES excision and chromosome breakage efficiency in ΔDRH1mic strains post-conjugation. We used PCR of single exconjugants to monitor the rearrangement of the well-characterized ΔDRH1mic strains tested (Table 2). This is in striking contrast to control mating of DNA rearrangement mutants ΔDCL1 or ΔPDD1, whose exconjugants fail to eliminate the M IES as indicated by a larger PCR amplification product (Fig. 4A).

To further characterize DNA rearrangement efficiency in ΔDRH1mic exconjugants, we isolated total DNA from populations of mated cells and used PCR (Fig. 4B) or Southern blot analysis (data not shown) to examine the fate of four other IES-containing loci. For each IES examined, the relative abundances of PCR products or DNA fragments that corresponded to unrearranged or rearranged forms in the macronuclei of progeny. We detected exclusively the rearranged locus in the 18 exconjugants of ΔDRH1mic tested (Table 2). This was consistent with our hypothesis that ΔDRH1mic strains have a common genetic background.

Table 2. IES rearrangement and chromosome breakage in single exconjugants

<table>
<thead>
<tr>
<th>Cross</th>
<th>Total</th>
<th>% Rearranged (n)</th>
<th>% Unrearranged (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M IES Rearrangement:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔDRH1mic</td>
<td>18</td>
<td>100% (18)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>WT</td>
<td>20</td>
<td>95% (19)</td>
<td>5% (1)</td>
</tr>
<tr>
<td>ΔDCL1</td>
<td>13</td>
<td>46% (6)</td>
<td>53% (7)</td>
</tr>
<tr>
<td>ΔPDD1</td>
<td>19</td>
<td>58% (11)</td>
<td>42% (8)</td>
</tr>
<tr>
<td>Chromosome breakage at the Tt819 locus:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔDRH1mic</td>
<td>26</td>
<td>8% (2)</td>
<td>92% (24)</td>
</tr>
<tr>
<td>WT</td>
<td>25</td>
<td>84% (21)</td>
<td>16% (4)</td>
</tr>
<tr>
<td>ΔDCL1</td>
<td>23</td>
<td>4% (1)</td>
<td>96% (22)</td>
</tr>
<tr>
<td>ΔPDD1</td>
<td>31</td>
<td>10% (3)</td>
<td>90% (28)</td>
</tr>
</tbody>
</table>
unable to excise IESs, when compared to wild type. It is important to note that because we analyzed IES rearrangement in populations of cells, which include unmated cells, we detected the rearranged IESs in all samples. Overall, IES excision was not impaired at any of the five loci examined by loss of DRH1 zygotic expression.

Mutants that fail to perform IES excision typically fail to carry out chromosome breakage, a second DNA rearrangement that couples chromosome fragmentation at specific sites with de novo telomere addition. To investigate whether this process was impaired by loss of DRH1 expression, we examined breakage at the Tt819 locus in single ΔDRH1mic exconjugants. We used nested primer pairs flanking Tt819 cbs, which can amplify the unbroken chromosome, together with a telomere-specific primer that amplified the telomerized ends after fragmentation. More than 90% of the exconjugants tested failed to fragment their chromosomes at this telomerized ends after fragmentation. More than 90% of the exconjugants tested failed to fragment their chromosomes at this locus, results comparable to exconjugants from ΔDCL1 or ΔPDD1 crosses (Fig. 5A; Table 2). In other control tests of wild-type exconjugants, the unrearranged locus was only detected in 4 of 25 exconjugants (Table 2), which likely resulted from amplification of the micronuclear copies.

To determine whether other sites of chromosome breakage were affected, we used Southern blot analysis of total DNA isolated from wild type and ΔDRH1mic populations post-conjugation to examine fragmentation downstream of the LIA1 locus (Fig. 5B). In wild-type cells, the appearance of a ~2.3 kbp terminal fragment (generated by cleavage but with minimally elongated telomeres) and under-representation of the 10.5 kbp unbroken germline locus is indicative of de novo chromosome breakage. By contrast in the ΔDRH1 cell lines, we detected a larger ~2.6 kbp terminal fragment with fully elongated telomeres (2.6 kbp=2.2 kbp plus 0.4 kbp of telomere sequence) derived from parental macronuclei of the unmated cells remaining in the population and any parental macronuclei not degraded within the mating pairs. Because of the high copy number of macronuclear chromosomes, even a relatively small fraction of unmated cells can result in a significant representation of the parental chromosomes in the DNA analyzed. Additionally, we observed an increased abundance of the 10.5 kbp band corresponding to the unrearranged locus. Thus chromosome breakage was blocked upon loss of zygotic DRH1 expression at both loci tested. This represents the first identification of a protein that appears to be required for chromosome breakage but does not affect the efficiency of IES excision. This observation further links Drh1p as an essential protein critical for ensuring development of proper chromosome structure.

DISCUSSION

Our studies of DRH1 and LIA2 indicate that they are paralogs related to the p68-family of DExD/H box helicases and have diversified in both expression and function during the Tetrahymena evolution. They are best hits in reciprocal blast searches of the Tetrahymena genome and the top hits when searching the genome for putative p68 homologs. This is consistent with these both being orthologous to the p68 helicases. DRH1 expression is essential, but LIA2 expression is not; thus, like paralogs p68 and p72 in mammalian cells, the proteins encoded by these Tetrahymena genes have distinct roles. Drh1p has essential functions during both growth and development and localizes to multiple cellular compartments: near basal bodies during growth, in micronuclei in pre-zygotic development and in differentiating macronuclei during post-zygotic development, all features that are consistent with the multi-functional role of the p68 family of DEAD box proteins (Fuller-Pace, 2006; Linder, 2006; Cordin et al., 2006).

When we tried to delete DRH1 from the macronuclear genome, we were unable to obtain cells lacking all copies of the gene from this polygenic somatic nucleus. This observation indicates that Drh1p is essential for growth. We were able to eliminate all copies of the endogenous DRH1 gene by introducing and expressing an inducible GFP-DRH1 fusion allele. The fusion protein localized to the p68-family of DExD/H box helicase and have diversified in both expression and function during the Tetrahymena evolution.

The temporal and mechanistic relationships between internal DNA elimination and chromosome fragmentation remain to be elucidated. Cells with deletions of genes involved in the IES excision pathway, including DCL1 and PDD1, exhibit failure of chromosome breakage, but otherwise, these two processes are not mechanistically linked. The strains lacking genes critical for IES...
excision arrest in development near the time period when chromosome breakage occurs, so it is plausible that the arrest associated with failed IES excision stops chromosome breakage from occurring. ΔDRH1mic cells that lack DRH1 expression from developing macronuclei also arrested late in development (Fig. 3B), but IESs were excised normally and only chromosome breakage was blocked (Figs 4 and 5). Clearly, these two chromosomal rearrangement events have separate requirements, and Drh1p is specifically required for chromosome breakage.

We have yet to determine whether Drh1p acts directly at sites of chromosome breakage or is more indirectly involved by facilitating the assembly of RNA-protein complexes that are responsible for fragmentation. The protein’s localization within meiotic micronuclei suggests a direct role in interacting with chromatin. In prophase, Drh1p localized to the regions of the elongated nucleus where centromeres and telomeres are positioned (Loidl and Scherthan, 2004; Cervantes et al., 2006). Later in pro-zygotic development, Drh1p localized in foci and fibers, possibly with the spindle apparatus. The mouse p68 helicase binds to satellite DNA and also localizes to fibers and in puncta in the nucleus (Gavrilo et al., 2009). These similarities lead us to suggest that the interaction of p68 proteins with chromosomes has been conserved through these proteins’ evolution and indicate that they possess a fundamental role in chromosome structure and/or maintenance.

Because DRH1 was essential for growth, and the inducible GFP-DRH1 we introduced was too leaky to abrogate expression, even without induction, we could not determine how absence of Drh1p affected meiotic chromosome behavior. It remains to be determined whether the mechanism by which Drh1p interacts with meiotic chromosomes is similar to its role in chromosome fragmentation. At this point, we can only speculate on the possible roles of this RNA helicase in fragmentation. In Drosophila melanogaster, the P68 RNA helicase promotes release of RNA transcripts from chromatin (Buszczak and Spradling, 2006). There is no direct evidence to date that RNAs release of RNA transcripts from chromatin (Buszczak and Spradling, 2006). There is no direct evidence to date that RNAs release of RNA transcripts from chromatin (Buszczak and Spradling, 2006). There is no direct evidence to date that RNAs release of RNA transcripts from chromatin (Buszczak and Spradling, 2006). 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recombined into the pBS_MTTGFPGTW vector with LR Clonase Plus. The vector was linearized with HindIII and biologically introduced into both of the macronuclear knockout strains. Successful transformants were selected for by growth in media containing both paromomycin and cycloheximide. Fertile strains were selected and subsequently subcloned until full macronuclear assortment had occurred. Assortment was monitored by using a three-primer PCR assay. The primers were: p68_140RF (TTTCTTTTTGGATACTTTAGA), and Tel1 (CCCCAACCCCAACCCCAA).

Tt819-2 (TCAAAACTTATCCAGGATTAAAG), Tt819-4 (ATTTTATTA-CACTATC), Tt819-3 (GATCAATTCATTTTAATTAATTTAG), and Tel1

Round one used the following primers: Tt819-1 (GATCAAAAATCA-CTGAGACT--This was performed as a nested PCR reaction on cells 30+ hours post mixing.

Total genomic DNA was isolated 30+ hours post mixing via Southern blot. A small fragment upstream of the CaM gene was used as a

via Southern blot. Nuclear assortment had occurred. Assortment was monitored by using a

gene was analyzed

The germ line limited M element of


References


