DOI: 10.1534/genetics.106.061556

Molecular Characterization of *teflon*, a Gene Required for Meiotic Autosome Segregation in Male *Drosophila melanogaster*

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Manuscript received June 1, 2006
Accepted for publication June 27, 2006

ABSTRACT

Drosophila melanogaster males lack recombination and have evolved a mechanism of meiotic chromosome segregation that is independent of both the chiasmatic and achiasmatic segregation systems of females. The *teflon* (*tef*) gene is specifically required in males for proper segregation of autosomes and provides a genetic tool for understanding recombination-independent mechanisms of pairing and segregation as well as differences in sex chromosome *vs.* autosome segregation. Here we report on the cloning of the *tef* gene and the molecular characterization of *tef* mutations. Rescue experiments using a *GAL4*-driven *pUAS* transgene demonstrate that *tef* corresponds to predicted Berkeley Drosophila Genome Project (BDGP) gene *CG8961* and that *tef* expression is required in the male germ line prior to spermatocyte stage S4. Consistent with this early prophase requirement, expression of *tef* was found to be independent of regulators of meiotic M phase initiation or progression. The predicted Tef protein contains three C2H2 zinc-finger motifs, one at the amino terminus and two in tandem at the carboxyl terminus. In addition to the zinc-finger motifs, a 44- to 45-bp repeat is conserved in three related Drosophila species. On the basis of these findings, we propose a role for Tef as a bridging molecule that holds autosome bivalents together via heterochromatic connections.

THE pairing and subsequent segregation of homologous chromosomes to opposite poles at meiosis I are key events required to ensure the equal distribution of chromosomes to gametes. In most organisms, these events are intimately associated with meiotic recombination. During prophase, a recombination-associated proteinaceous structure called the synaptonemal complex (SC) assembles along the chromosome arms of paired chromosomes, establishing a physical connection between homologs. This structure disassembles later in prophase, after which homolog connections are relegated to sites of reciprocal exchange, or chiasmata, that serve to maintain homolog adhesion until their resolution at anaphase I. Chiasmata are stabilized by sister chromatid cohesion proteins (cohesins), and connections made by cohesins between sisters distal to crossover events act to prevent resolution of chiasmata. The regulated destruction of cohesins along chromosome arms initiates anaphase I by allowing resolution of chiasmata and the resulting coordinated separation of homologs (Buonomo *et al.* 2000). In addition to physically uniting paired homologs, chiasmata also balance opposing poleward forces across the bivalent, and the resulting tension is an important component of a cell cycle checkpoint that monitors proper homolog orientation and alignment prior to anaphase I (Nicklas 1997).

Not all organisms, however, depend on recombination for meiotic chromosome segregation. In the female fruit fly *Drosophila melanogaster*, alternative genetic pathways exist to segregate chromosomes that have not undergone reciprocal exchange. A distributive, or achiasmate, pathway depends on heterochromatic homologies to segregate nonexchange homologs, while a second achiasmate pathway exists for segregating nonhomologous chromosomes (Hawley *et al.* 1992). Male Drosophila completely lack meiotic recombination, yet do not appear to use the same achiasmate systems as do females, as mutations in components of the female systems do not affect chromosome segregation in the male. Thus, males appear to have evolved a separate system for pairing and/or homolog adhesion.

A comparison between the female achiasmate and male segregational systems reveals both similarities and differences. Heterochromatic homologies play an

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY840216–AY840221

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important role in both sexes. In females, genetic studies of chromosome rearrangements (Karpen *et al.* 1996) and cytological studies involving *in situ* hybridization to heterochromatin sequences (Dernburg *et al.* 1996) indicate a role of heterochromatin in partner choice and adhesion. Male sex chromosome pairing also relies on heterochromatic homology; the XY pairing sites lie within the rDNA repeats (McKee *et al.* 1992), which are located in the heterochromatin of both the X and Y chromosomes. Observations of meiosis in males using the LacO/LacI-GFP system to visualize specific loci suggest that heterochromatic pairing may also be critical for maintaining autosomal adhesion. In G_2, paired homologs move into discrete nuclear compartments and euchromatic associations between both homologs and sister chromatids are dissolved or relaxed. Associations between homologous centromeres are also lost in G_2, and as chromosomes condense and move toward the metaphase plate during prometaphase, neither homologous centromeres nor euchromatic loci appear tightly associated. It is unclear how the bivalents are held together at this point, but it has been suggested that adhesion is maintained either through heterochromatic associations or via topoisomerase-resolvable entanglements (Vazquez *et al.* 2002). Whatever these associations are, they must be sufficient to balance poleward forces in lieu of chiasmata. In the absence of chiasmata, the role of cohesins in regulating homolog adhesion/separation in males is also unclear, but if they are involved in regulating anaphase onset it seems that their activity may be limited to heterochromatic regions.

Recently, a number of male-specific meiotic mutants have been described that identify genes involved in regulating homolog pairing and adhesion (Hirai *et al.* 2004; Wakimoto *et al.* 2004). Two of these, *mod(mdg4)in meiosis* (MNM) and *stromalin in meiosis* (SNM), have been characterized at the molecular and cytological level (Thomas *et al.* 2005). These genes are required for maintaining adhesion between paired homologs at late prophase. MNM and SNM proteins have been colocalized to the sex chromosome pairing sites at this stage, suggesting that they act in an adhesion complex to maintain associations between paired homologs. MNM has also been localized to a small number of foci on each autosomal bivalent, and this latter localization is dependent on an additional gene, *teflon* (*tef*) (Thomas *et al.* 2005). Mutations in *tef* result in precocious separation of autosomal bivalents during late prophase and early prometaphase of meiosis I, whereas the sex bivalent is unaffected by *tef* (Tomkiel *et al.* 2001). Together, these genetic and cytological observations suggest that there are separate protein complexes that mediate homolog adhesion in males: an autosomal complex that is *tef*-dependent, and a sex chromosome-specific complex that is not. Toward a further understanding of the nature of the differences between autosome and sex chromosome pairing and adhesion, we have cloned and characterized the *tef* gene. On the basis of our results, we suggest a direct physical role of the Tef protein in maintaining adhesion between paired autosomes.

MATERIALS AND METHODS

Stocks: Drosophila stocks were grown at 25° on standard media consisting of cornmeal, molasses yeast, and agar. All *D. melanogaster* mutations and chromosomes are described in Lindsley and Zimm (1992) or in FlyBase (http://flybase.bio.indiana.edu). The wild-type *D. pseudoobscura* strain MV2-25 was obtained from the Tucson stock center.

Assay for nondisjunction: Tests for sex and fourth chromosome nondisjunction were performed as previously described (Tomkiel *et al.* 2001). Males homozygous for the recessive fourth chromosome mutation *sparkling-poliert* (*spa^{pol}*) and bearing a *yellow* (*y*) X chromosome and *In(1)YsX.YL*, *y*$^+$ were mated to tester *y w sn^3; C(4) ci ey* females. Errors in reductional segregation of sex chromosomes were detected as y w sn sons (resulting from *nullo-XY* sperm) or y$^+$ daughters (resulting from *diplo-XY* sperm). Reductional or equational fourth chromosome nondisjunction was detected as spa progeny (from *diplo-4* sperm) or ci ey progeny (from *nullo-4* sperm).

Genomic PCR: Genomic DNA was isolated from 40 adult flies for each genotype, as described (http://www.fruitfly.org/about/methods/index.html). PCR amplification was performed using the following primer pairs corresponding to *D. melanogaster* predicted gene *CG8961*: 5′-CCTCCGCAGTAATGGTAA-3′ and 5′-CTTCTGAGCCTTCGATGG-3′, 5′-GGTGACTATAGTTCACTG-3′ and 5′-GGTACACAGTTCAGTACG-3′, 5′-CATACCGAGCTCATCTC-3′ and 5′-CAAGTGCTTACTCCAACCGC-3′, 5′-ACAGCACAAAGATCCGCA-3′ and 5′-GTAAGCAAAACTAACAGG-3′, and 5′-CTGGCGAACAGCGATATA-3′ and 5′-ATCCAGAGCAGATGGTGA-3′.

Amplification of genomic DNA corresponding to the homologous region from *D. pseudoobscura* strain MV2-25 was performed using the following primer pairs: 5′-CACATATGTCCTCGTTTCTTGAT-3′ and 5′-TTTCAAGCGATCGCATGATACGCT-3′, 5′-AGCGTATCATGCGATCGCTTGAAA-3′ and 5′-GATTGTATGGTACGTATG-3′, and 5′-CATACGTACCATACAATC-3′ and 5′-TAGCGGCCGCTTGGCACGTTATGGCTTA-3′.

Inverse PCR to analyze transgene insertions was performed as described (http://www.fruitfly.org/about/methods/index.html).

RT–PCR: Total RNA was isolated from tissues or whole organisms using the RNeasy mini kit (QIAGEN, Palo Alto, CA). Reverse transcription (RT) was performed using Superscript II (Invitrogen, Carlsbad, CA) using 1 μg total RNA and 10 pmol of a gene-specific primer at 42° as per manufacturer's instructions. Full-length *teflon* cDNA was recovered by RT of total RNA isolated from 1- to 3-day-old adult male testis. For *D. melanogaster tef*, RT was performed using 5′-TTGCGGCCGCAGAATGAGCGTTTCGCAA-3′. The cDNA was amplified by PCR using the same reverse primer in combination with the 5′-TAGGATCCATATGTCTAAGTTTCTGGA-3′ forward primer. The resulting product was subcloned into pT7-7 (Tabor and Richardson 1985) at the *Nde*I and *Not*I sites and verified by DNA sequencing.

For *D. pseudoobscura tef* cDNA, RT was performed as above using a mixture of the three reverse primers above, and the resulting cDNA was amplified by PCR using the following primer pairs: 5′-ATGTCCTCGTTTCTTGATATTC-3′ and 5′-GAGATGACCGTTAGCGAAGA-3′, 5′-GCCACCCATCAATATAAAAC-3′ and 5′-CCTGTTTACCTCCTCGGTGA-3′, and 5′-GCGGCCAAGTTTAGCAAAAT-3′ and 5′-TGGCACGTTATGGCTTAAGA-3′. PCR cycling conditions were 40 cycles of 30 sec at

94°, 1 min at 51°, and 1 min at 72°. The resulting three overlapping cDNA fragments, each spanning at least one intron, were assembled into a contig.

For *tef* expression analysis, RT–PCR was performed using primers 5′-GCCCAATGAAACCTGAAC-3′ and 5′-GGTACAC AGTTCAGTAGC-3′. These primers flanked an intron in the *tef* gene, allowing differentiation between PCR products resulting from amplification of cDNA *vs.* possible contaminant genomic DNA. For *fat-spondin* expression analysis, 5′-CGGCAAGACGT ATAATTTACT-3′ and 5′-CAGGCGCAGCATTCCTT-3′ primers were used.

Creation of transgenic flies: Full-length *teflon* cDNA was PCR amplified using 5′-TAGGATCCATATGTCTAAGTTTCTG GA-3′ forward and 5′-TTGCGGCCGCAGAATGAGCGTTTCG CAA-3′ reverse primers. A *Bam*HI–*Not*I fragment of the resulting PCR product was cloned into the *Bgl*II- and *Not*I-digested pUAST vector (van Roessel and Brand 2000). The gene encoding enhanced green fluorescent protein (EGFP, Clontech, Palo Alto, CA) was amplified using 5′-TTGCGGCCGCTCT GGTGAGCAAGGGCGA-3′ and 5′-CCTCTAGATTACTTGTAC AGCTCG-3′ forward and reverse primers. PCR cycling conditions for both reactions were as follows: 30 cycles of 94° for 30 sec, 46° for 30 sec, 72° for 30 sec. The amplified *EGFP* gene was cloned into *Bgl*II- and *Not*I-digested pUAST-*tef* vector. The resulting pUAST-*tef::*GFP construct produces a fusion protein containing full-length Teflon protein with EGFP fused to its carboxyl terminus. The start methionine codon is absent from the GFP gene, ensuring GFP production only as part of a Tef fusion protein. The plasmid was purified using a DNA purification kit (QIAGEN) and verified by DNA sequencing. All sequencing was performed by MWG (High Point, NC).

Pooled 0- to 1-hr embryos were dechorionated in 50% bleach, rinsed in water, then aligned on double-stick tape. Embryos were dehydrated in chambers containing dri-rite for 5–10 min, then covered with halocarbon oil prior to injection. pUAST-*tef::*GFP along with the transposase source P{SB2.1} (DeCicco and Spradling 1984) were injected through Femtotip II glass needles (Eppendorf, Hamburg, Germany) at a concentration of 500 μg/μl each into 1956 embryos. From 183 surviving larvae, 20 adults (11 males and 9 females) were obtained. From these, 16 were fertile (9 males and 7 females). Twenty-four transgenic individuals were obtained from a single G_0 adult.

Genomic DNA was extracted from adult flies using the Quick Fly Genomic DNA Prep (http://www.fruitfly.org/about/methods/inverse.pcr.html). The *tef::GFP* transgene was amplified by PCR as above using the same *EGFP* primers as above and the following *teflon* primer pairs: 5′-TAGGATCCATATGT CTAAGTTTCTGGAC-3′ and 5′-CGGCAATCCAACCGCTT-3′, and 5′-GCCCAATGAAACCTGAAC-3′ and 5′-CACTGAAACC GAACTTGG-3′. These *tef*-specific primers were designed to bridge the two introns in the teflon gene such that only the transgene cDNA was amplified. PCR fragments were gel purified using the QIAquick Spin columns (QIAGEN) and were sequenced to verify the integrity of the transgene.

Confocal microscopy: Testes from 0- to 3-day-old adults were dissected in Schneider's Drosophila tissue culture media (Sigma, St. Louis), transferred to 95% ethanol for 1 min, then stained in 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml for 1 min. Tissues were either splayed open or mounted whole in 50% glycerol and examined at ×40–60 using an Olympus Fluoview FV500 confocal laser scanning microscope.

RESULTS

Molecular mapping of *tef*: We determined the location of the *tef* gene using a combination of deletion mapping, complementation testing, and DNA sequencing. The *tef* gene had previously been mapped to salivary chromosome bands 53F–54A (Tomkiel *et al.* 2001). We used *P* element-mediated male recombination (Preston *et al.* 1996) to generate deletions in this vicinity, starting with a *P* element inserted at 53E, *P{w[+mC]=lacW}GstS1[k08805]*. From 42 male recombination events, a single deletion, *P803Δ15*, was recovered that extended proximally from the *P*-insertion site. The presence of the *w[+mC]* marker gene indicated that the deletion chromosome retained the original *P* insertion. Flanking sequences were recovered by inverse PCR and DNA sequencing revealed that 64,420 base pairs had been deleted from the proximal side of the *P* element. This deletion was used in a complementation test with five EMS-induced *tef* alleles that had been isolated from the Zuker collection (Koundakjian *et al.* 2004) by screening for mutations that elevated the frequency of fourth chromosome loss (Wakimoto *et al.* 2004). Four of these were previously described (Tomkiel *et al.* 2001). The fifth allele, tef^{Z3455}, is described here. Assays of meiotic nondisjunction in males heterozygous for *P803Δ15* and each of these *tef* alleles revealed that this deletion fails to complement the *tef* male meiotic defect. The frequency of nondisjunction in tef^{Z5684}/*P803Δ15* males does not significantly differ from that previously reported for tef^{Z5684} homozygotes (Table 1) (Tomkiel *et al.* 2001), indicating that tef^{Z5684} is a null allele. We previously found that all heteroallelic combinations of the EMS *tef* alleles behave similarly (Tomkiel *et al.* 2001). Together, these observations suggest that all five EMS-induced *tef* alleles behave as null alleles with respect to meiotic chromosome transmission.

Complementation tests were performed using the tef^{Z5864} allele and a *P* element, *P{w[+mC]=lacW}l(2)k15914[k15914]*, that maps within the sequences deleted

TABLE 1

Sex and fourth chromosome disjunctional data from crosses of *y/y⁺Y; tef; spa^pol* males to *y w sn; C(4)EN ci ey/0* females

	Paternal genotype		
Recovered male gametes	tef^{z5864}/ *Df803Δ15*	$tef^{l(2)k15914}$/ $tef^{l(2)k15914}$	$tef^{l(2)k15914}$/ tef^{z5864}
Y;4	160	688	684
X;4	204	860	484
0;4	0	0	0
X/Y;4	0	0	0
Y;0	57	0	9
X;0	65	0	12
Y;4/4	48	1	16
X;4/4	70	0	16
0;0	0	0	0
0;4/4	0	0	0
X/Y;0	0	0	0
X/Y;4/4	0	0	0
Fourth nondisjunction (%)	39.7	0.1	4.3

by *P803Δ15*. This insertion failed to complement *tef*, but *trans*-heterozygous males exhibited only a low frequency of fourth chromosome nondisjunction (~4%, Table 1), rather than the near-random segregation observed in males homozygous for tef^{Z5864}. This suggests that this *P* insertion results in a hypomorphic *tef* allele, and will henceforth be referred to as $tef^{l(2)k15914}$. Although this element was originally characterized as a lethal insertion, we were able to establish a viable line of flies homozygous for this insertion. Males homozygous for $tef^{l(2)k15914}$ are wild type with respect to meiotic chromosome transmission (Table 1), consistent with the interpretation that the insertion results in a nearly wild-type, hypomorphic *tef* mutation.

The *P* insertion of $tef^{l(2)k15914}$ is located in the first exon of predicted gene *CG8961*, which is entirely contained with the first intron of a second predicted gene, *CG6953*, a Drosophila *fat-spondin* homolog (http://flybase.bio.indiana.edu/). To determine which of these putative genes might be affected in *tef* mutants, we sequenced PCR-amplified genomic DNA from flies homozygous for each of the five EMS-induced *tef* alleles. Genomic sequences were compared to that of the original isogenic chromosome on which these mutations were induced. Each mutant allele was found to contain a single base pair substitution in the predicted open reading frame of *CG8961*. Four of these are nonsense mutations, predicted to cause truncations in the resulting protein, and the fifth was a missense mutation (Figure 1). *CG8961* is predicted to encode a 649-amino acid protein that contains three predicted classical C2H2 zinc fingers—one at the amino terminus and two in tandem at the carboxyl terminus. C2H2 zinc fingers are frequently involved in DNA binding (Wolfe *et al.* 1999), although they may also act as RNA- or protein-binding domains (Shastry 1992; Mackay and Crossley 1998). The tef^{z5864} allele is a missense mutation predicted to disrupt the amino-terminal zinc finger, changing the first conserved cysteine residue to a tyrosine [C(38)Y] (Figure 1). Sequencing of RT–PCR-recovered *CG8961* cDNA from homozygous $tef^{l(2)k15914}$ males indicated that, despite *P* insertion within the coding region of the gene, the processed message is wild type. This suggests that the hypomorphic nature of P-insertion alleles may reflect reduced transcription or inefficient splicing to remove P sequences from the *CG8961* message.

Transgene rescue of *tef*: We used RT–PCR to isolate wild-type *CG8961* cDNA from Oregon-R wild-type flies and sequenced it to confirm the predicted exon/intron structure (Figure 1). To establish that *CG8961* indeed corresponded to *tef*, we created transgenic flies that expressed this cDNA to test for rescue of the *tef* meiotic defect. We used the *GAL4-UAS* conditional expression system (Brand and Perrimon 1993) to express full-length *CG8961* cDNA as a fusion protein with a carboxy-terminal EGFP. In addition to testing for rescue, this system allowed us to query temporal and spatial requirements for *tef* expression by utilizing a collection of driver lines that express *GAL4* in various male reproductive tissues and at various times with respect to spermatogenesis (Hrdlicka *et al.* 2002). The GFP fusion also potentially allows visualization of the expressed protein, although efforts to do so have been unsuccessful.

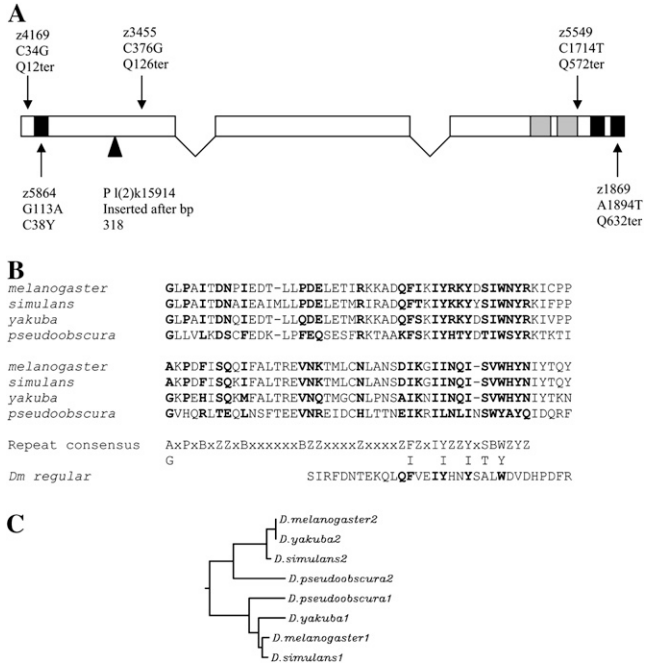

Figure 1.—(A) Diagram of *D. melanogaster tef* transcript and mutations, with solid boxes indicating the positions of consensus sequences for C2H2 zinc-finger motifs and shaded boxes indicating a conserved repeat motif. For each mutation, the allele designation, alteration in DNA sequence, and predicted alteration in amino acid sequence are indicated. The insertion site of the *P* allele is indicated by a solid triangle. (B) Sequence alignment of a conserved tandem repeat, which resides in amino acid residues 478–573 in *D. melanogaster* (Z, hydrophilic; B, hydrophobic). The homologous repeat from the *D. melanogaster regular* gene is indicated below the consensus sequence. (C) ClustalW-generated dendrogram showing the degree of similarity between the two repeat domains identified in the carboxyl half of the predicted Tef protein. Branch lengths are proportional to degree of differences.

Detailed expression patterns for these GAL4 drivers with respect to spermatocyte development and meiotic progression were not previously reported. We were unable to detect the Tef::GFP fusion protein and therefore could not directly define the spatial and temporal expression of Tef from these drivers. As an alternative, we characterized the ability of each of the driver lines to express an easily detected *pUAS CD8::GFP* reporter gene. The CD8 protein is localized to the cell membrane. This same reporter system was used originally to define the gross expression of these driver lines in living testis (Hrdlicka *et al.* 2002). To more precisely define expression patterns here, we examined fixed testis that had been stained with the DNA-specific dye DAPI. This allowed use of nuclear morphology to accurately stage

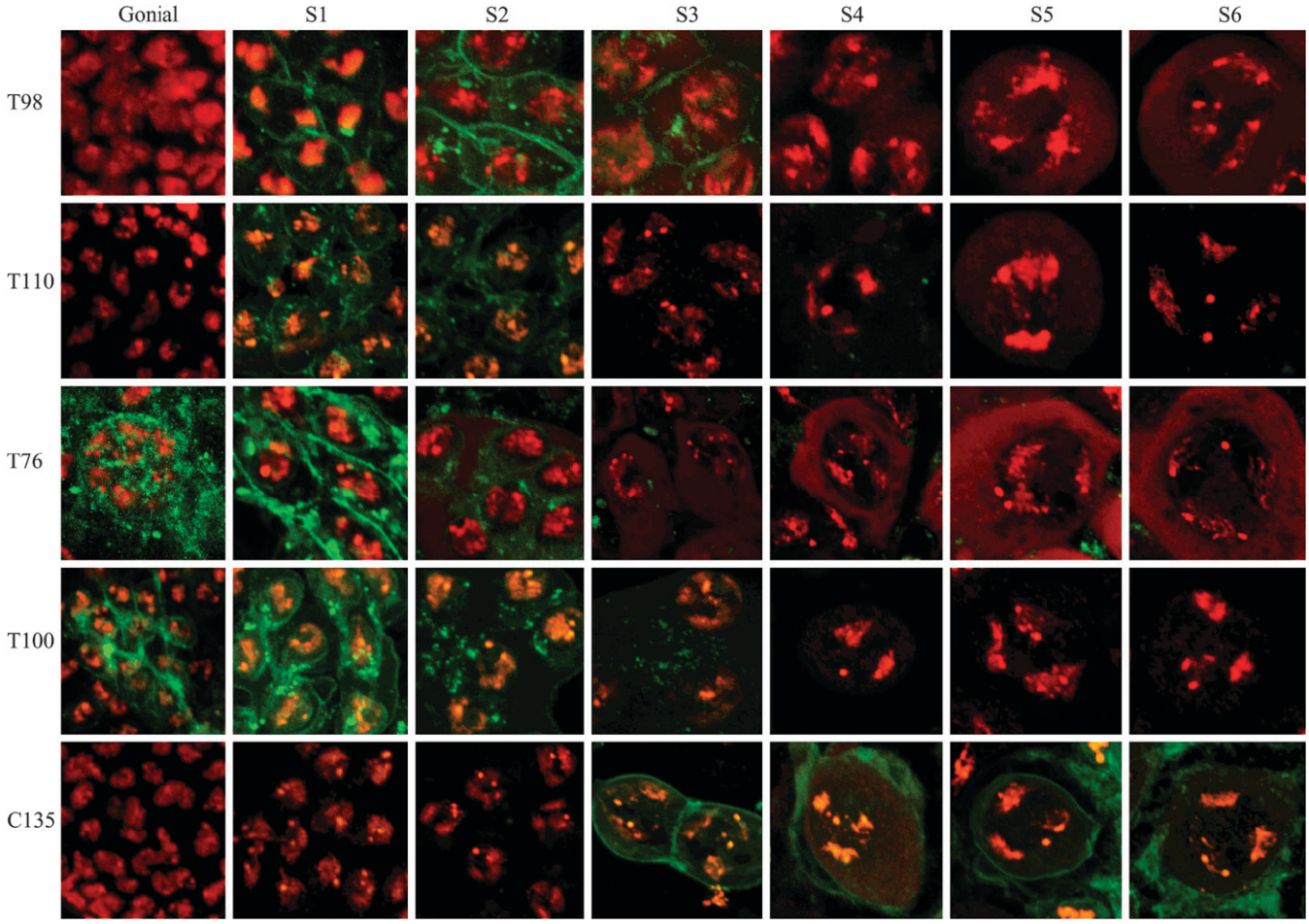

FIGURE 2.—Testis expression patterns of GFP from a *pUAST CD8::GFP* reporter transgene under the control of the indicated *GAL4* drivers. DAPI-stained DNA (red) and GFP images (green) of testis from males bearing the indicated GAL4 driver in combination with *pUAST CD8::GFP*.

expressing cells. Two Gal4 drivers, T155 and C729, caused expression of the GFP reporter exclusively in somatic cells. Among the Gal4 drivers that caused germline reporter gene expression, *T76*, *T98*, *T100*, and *T110* all drove expression at or prior to the earliest stages of primary spermatocytes (S1–S3) (CENCI *et al.* 1994). In contrast, *C135* showed detectable expression only during later (S4–6) stages of primary spermatocyte development (Figure 2 and Table 2).

Male flies homozygous for *tef*^{z5864}, heterozygous or homozygous for a chromosome 2 insertion of the *pUAST CG8961::GFP* transgene, and heterozygous for

TABLE 2

Summary of expression patterns

	Cell type assayed for GFP expression			Rescue of *tef*-induced nondisjunction
GAL4 driver	Cyst cells	Spermatogonia	Spermatocytes	
T155	+	–	–	–
C729	+	–	–	–
C135	+	–	+ (S4–6)	–
T76	+/–	+	+ (S1–2)	+
T100	–	–	+ (S1–3)	+
T98	–	–	+ (S1–3)	+
T110	–	+	+ (S1–2)	+

Expression patterns include cyst cell expression patterns reported by HRDLICKA *et al.* (2002), which were confirmed here (data not shown).

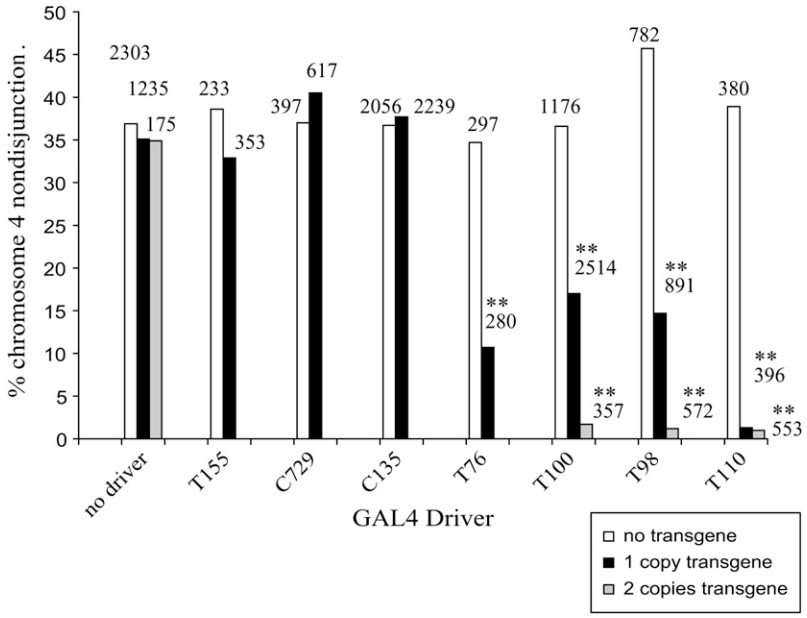

FIGURE 3.—Complementation of the *tef* meiotic defect by a *pUAS CG8961::GFP* transgene under the transcriptional regulation of various *GAL4* drivers. Fourth chromosome nondisjunction frequencies among progeny of *tef* males bearing the indicated *GAL4* driver and transgene combinations are shown. Numbers above bars indicate progeny scored. **Significantly different, $P < 0.01$.

one of the *GAL4* driver lines were assayed for fourth chromosome nondisjunction. We monitored segregation of the fourth chromosome because it appears to be more sensitive than the major autosomes to changes in *tef* gene product, as males bearing the hypomorphic *P* allele show low frequencies of fourth chromosome nondisjunction, whereas the major autosomes segregate normally (data not shown). These crosses also allowed us to directly calculate nondisjunction frequencies, as viable progeny that are euploid or aneuploid for the fourth chromosome are both recovered from these crosses. Results are shown in Figure 3. The *CG8961* transgene alone (in absence of a *GAL4* driver) and each *GAL4* driver alone (in the absence of the *CG8961* transgene) had no effect on fourth chromosome meiotic segregation. Similarly, no rescue was observed in flies with both the *CG8961* transgene and either *GAL4* driver line *T155* or *C729*. Neither of these lines expresses *GAL4* in germ-line cells. In contrast, significant decreases in *tef*-induced nondisjunction were observed in flies bearing the *CG8961* transgene and one of the following *GAL4* drivers: *T76*, *T98*, *T100*, or *T110*. Each of these lines is reported to drive expression in spermatocytes. Nearly complete rescue was seen in flies bearing two copies of the *CG8961* transgene in combination with *T98*, *T100*, and *T110*.

We also verified that the transgene reduced nondisjunction of the major autosomes both genetically and cytologically. Males homozygous for the tef^{z5684} and the *tef* transgene and heterozygous for the *T98*, *T100*, or *T110* GAL4 driver were mated to *C(2)EN* females and the number of progeny produced were counted. The only progeny that survive from such a mating are those that receive either two or zero second chromosomes from their father, and therefore progeny numbers are a direct reflection of the frequencies of paternal nondisjunction. From matings of 50 homozygous tef^{z5684} males lacking the transgene, 145 viable F_1 were produced. In contrast, in each case <5 viable progeny were produced from equal numbers of matings of *GAL4/+ ; pUAST CG8961::GFP* males bearing the *T98*, *T100*, or *T110* drivers.

The *tef* cytological defect observed in late spermatocytes (S6), a separation of autosomal homologs, was also ameliorated by transgene expression in early stage spermatocytes. Autosomal pairing appeared normal in *T98*, *T100*, and *T110* males bearing two copies of the transgene (Figure 4).

We conclude that predicted gene *CG8961* corresponds to *tef* and that *tef* expression is required in the germ line to effect proper meiotic chromosome transmission. These observations further suggest that expression of *tef* is required prior to the S4 stage of primary spermatocyte development to influence chromosome behavior.

Identification of *tef* homologs in related species: BLAST homology searches (http://www.ncbi.nlm.nih.gov/) using the full-length Tef primary amino acid sequence failed to identify obvious homologs in nondrosophilids. Significant regions of homology to proteins in other species were limited to the zinc-finger domains. Searches performed using only the zinc-finger domains revealed that the best non-Drosophila matches were to two putative genes from *Anopheles gambiae* (XM319648 and XM317117), a tumor suppressor gene in *Homo sapiens* (AF294278), and the transcriptional repressor *deltaEF1* in *Gallus gallus* (D76434). This suggests that Tef, as proposed for MNM and SNM (THOMAS *et al.* 2005), may have evolved to perform a function unique to Drosophila.

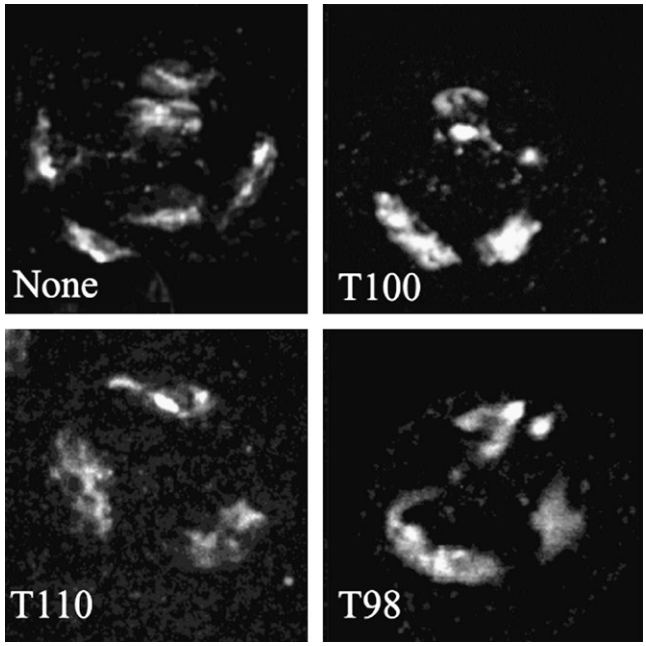

FIGURE 4.—Cytological evidence of rescue of the autosomal pairing defect by expression of the *UAS tef::GFP* transgene. A representative late prophase (S6) spermatocyte from a male bearing two copies of the *UAS tef::GFP* transgene and the indicated GAL4 driver are shown. Each cell is oriented with the sex bivalent uppermost. Note the unpaired major autosomes in the cell lacking a Gal4 driver.

Homologs of *tef* in other Drosophila were identified through BLAST searches of the *D. pseudoobscura* (http://www.hgsc.bcm.tmc.edu/projects/drosophila/), *D. simulans*, and *D. yakuba* databases (http://www.genome.wustl.edu/projects/simulans, ftp://genome.wustl.edu/pub/seqmgr/yakuba). Searches of *simulans* and *yakuba* databases prior to their publication were kindly performed by J. C. Yasuhara and B. T. Wakimoto. In all species, the nested arrangement of *tef* within *fat-spondin* was found to be conserved. The *tef* intron/exon structures and predicted Tef proteins in *D. simulans* and *D. yakuba* are extremely conserved and are nearly identical to that of *D. melanogaster*, whereas the *D. pseudoobscura* gene contains additional introns (see accession AY840221). A ClustalW (http://clustalw.genome.jp/) alignment of all four Tef proteins revealed that they share a similar organization. All contain a single amino terminal zinc-finger motif. *D. melanogaster*, *D. simulans*, and *D. pseudoobscura* also each have two zinc-finger motifs in tandem at the carboxyl end, while *D. yakuba* lacks the last conserved histidine residue in the second carboxy-terminal zinc-finger motif. In addition to the zinc-finger motifs, this alignment also revealed a conserved 44- to 45-bp motif repeated in tandem adjacent to the carboxy-terminal zinc fingers, ([FI]-X-X-I-[IY]-X(0,1)-[ST]-X-[WY]-X-Y, Figure 1B). Greater similarity was found between the first repeat from all four species than between the first and second repeat within any of the species (Figure 1C). This suggests that the duplication of this motif in Tef occurred in an ancestral species. The conservation between the tandem copies also suggests that this motif may be functionally significant.

Within *D. melanogaster*, the protein most similar to Tef was found to be Regular (Rgr), a putative zinc-finger-containing transcription factor. The *rgr* message shows oscillation in abundance in concert with circadian rhythms (CLARIDGE-CHANG *et al.* 2001). Rgr and Tef have a similar amino and carboxyl location of C2H2 zinc fingers. Each protein has a single zinc-finger motif at the amino terminus, but Rgr has seven rather than two at the carboxyl terminus. In addition, Rgr contains homology to a single copy of the Tef tandem repeat, and this homology resides in a similar relative position adjacent to the carboxy-terminal zinc fingers. The overall identity of Rgr to Tef is 22.4% and the similarity is 54.3%. These sequence and structural similarities, considered together with the location of *tef* within an intron of another gene, suggest that *tef* may have evolved from an ancient duplication of *rgr*. Consistent with a more ancient function for Rgr, the Rgr homolog is more highly conserved between *D. melanogaster* and *D. pseudoobscura* (72% identical and 77% similarity) than is Tef (35% identical and 43% similarity).

Pattern of *tef* expression: We examined the expression pattern of the *tef* gene in *D. melanogaster* by *in situ* hybridization and RT–PCR. We could not detect *tef* expression in adult testis by *in situ* hybridization but could detect its presence by RT–PCR. Using this latter method, we assayed for expression in testis of wild-type and *tef* males (Figure 5A). We also examined expression in testis of males homozygous for mutations that affect entry into meiotic M phase, including the *boule* (EBERHART *et al.* 1996), *always early*, the transcriptional activating factors *cannonball* (*can*), *spermatocyte arrest* (*sa*), and *meiosis I arrest* (*mia*) (HILLER *et al.* 2004), and the cell cycle regulators *twine* (*twe*) (ALPHEY *et al.* 1992) and *pelota* (*pelo*) (EBERHART and WASSERMAN 1995). We detected *tef* message in males of each genotype, indicating that each of the *tef* EMS alleles produces transcript and *tef* expression is not dependent upon initiation of meiotic M (Figure 5B).

By *in situ* hybridization in embryos, we observed a uniform distribution of *tef* message prior to cycle 13, but were unable to detect a signal after cellularization (data not shown). This confirms the embryonic expression pattern for *CG8961* previously reported (http://www.fruitfly.org/cgi-bin/ex/insitu.pl). Expression of *tef* in embryos prior to activation of zygotic transcription suggests a possible role for *tef* in early development; however, there is no detectable developmental or chromosomal phenotype in embryos from *tef* null mothers. Furthermore, the severity of the meiotic defect in *tef* hypomorphic males is not influenced by maternal *tef* levels (data not shown).

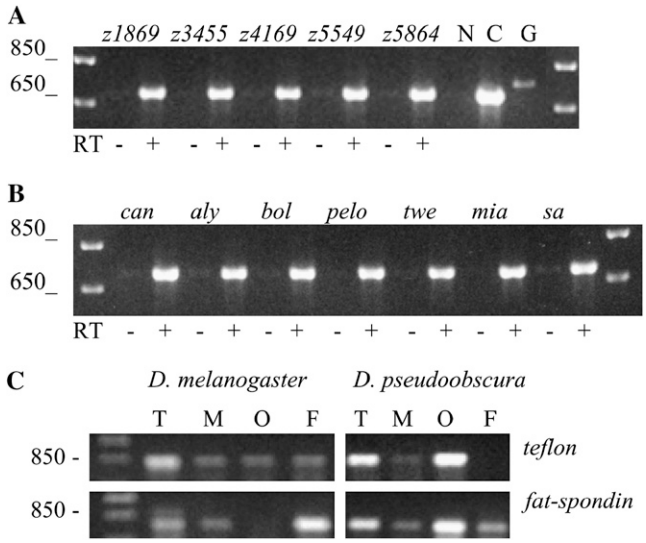

FIGURE 5.—RT–PCR of *tef* mRNA agarose gel electrophoresis analysis of RT–PCR products. (A) RT of testis RNA isolated from males homozygous for the indicated *tef* alleles, amplified with *tef*-specific primers that flank the first intron. N, no template; C, cDNA template; G, genomic DNA template. (B) RT of testis RNA isolated from males homozygous for mutations that affect entry into or progression through meiosis, amplified using the same primers as in A. (C) RT of total RNA isolated from *D. melanogaster* or *D. pseudoobscura* testis (T), male soma minus testis (M), ovaries (O), and female soma minus ovaries (F). Amplifications were done on the same reverse-transcribed RNA samples using primers specific either for *tef* (top) or for the Drosophila *fat-spondin* homolog (bottom).

The *tef* message could also be detected by RT–PCR in 3- to 24-hr embryos, larvae, pupae, and both adult males and females, confirming prior microarray expression analysis (http://genome.med.yale.edu/Lifecycle/).

RT–PCR of the same *D. melanogaster* RNA samples using primers specific to *fat-spondin* revealed that its expression was neither reciprocal nor identical to *tef*. A similar result was obtained in *D. pseudoobscura* (Figure 5C). This suggests that *tef* and *fat-spondin* expression are not coregulated as a consequence of their nested arrangement, although it must be cautioned that this analysis represents an average expression at the tissue level and may not reveal coregulation at the cellular level.

DISCUSSION

Our molecular characterization of *tef* mutations in combination with transgene rescue experiments reveals that *tef* corresponds to BDGP predicted gene *CG8961*. The *tef* gene, as well as its location within the first intron of a *fat-spondin* homolog, is conserved in at least three additional Drosophila species. This suggests that *tef* may have arisen from an ancient duplication and transposition event. The similarity of *tef* to the *rgr* gene in *D. melanogaster* suggests that *rgr* may be the original ancestral gene. It is interesting to note that *rgr* was identified as a transcript that is expressed in correlation with circadian rhythms. The *Caenorhabditis elegans TIM-1* gene, a paralog of the Drosophila clock gene *timeless*, provides precedence for a link between circadian rhythm and meiotic chromosome segregation. TIM-1 associates with the cohesin complex and is required for the assembly of cohesin subunits onto meiotic chromosomes (CHAN *et al.* 2003). The homology between *tef* and *rgr* may be an indication of an additional relationship between genes involved in circadian rhythm and chromosome segregation.

No obvious *tef* homologs could be identified by amino acid sequence homology in organisms other than Drosophila. This may reflect that *tef* evolved specifically in response to a need for meiotic chromosome segregation in the absence of recombination. Conservation of *tef* in related Drosophila species may reflect a conserved role in segregating noncrossover chromosomes in male meiosis. Like *D. melanogaster*, *D. simulans* has been reported to have extremely low rates of male meiotic recombination (WOODRUFF and BORTOLOZZI 1976). Male meiotic recombination frequencies have not been reported for *D. yakuba* or *D. pseudoobscura*.

Alternatively, proteins analogous to *tef* may exist but simply not share sufficient primary sequence homology to permit detection. A conserved, repeated motif in Tef is found in a variety of proteins from other species (data not shown), but there is no apparent functional similarity shared by these proteins, and thus the role of this conserved region is enigmatic.

RT–PCR analysis indicated that *tef* is expressed in a variety of tissues in addition to the male germ line and at various stages of development. The functional significance of this expression is unclear, as there are no detectable somatic phenotypes at any stages of development in *tef* mutants and no effect on chromosome segregation in females (TOMKIEL *et al.* 2001). The Tef protein may have an additional, redundant function outside of male meiosis, or this expression pattern may be an indirect consequence of *fat-spondin* regulation, in which the *tef* gene is nested. That is, establishment of an open chromatin conformation to facilitate *fat-spondin* expression may also result in *tef* expression. Such coregulation of nested genes has been observed in mice for the transcription factor *Rbpsuhl* and the extracellular matrix protein *Matn4*. Similar to the arrangement of *tef* within *fat-spondin*, *Rbpsuhl* resides within an intron of *Matn4* and is coded on the antiparallel strand (WAGENER *et al.* 2001).

The expression of *tef* in the male germ line is independent of transcriptional regulators of meiosis and is also independent of genes that control meiotic cell cycle progression. This suggests that *tef* may be expressed early in meiosis. Results of transgene rescue experiments are consistent with this interpretation, as amelioration of the *tef* segregational defect required transgene

expression prior to the S4 stage of spermatocyte development. This requirement suggests Tef is present in wild-type males during the early spermatocyte stages when homologs are paired at both euchromatic and centromeric regions. This is prior to the phenocritical period observed in *tef* mutants, which occurs at stages S5–6. In wild-type S5–6 spermatocytes, euchromatic and close centromeric associations between homologs are no longer observed, and each bivalent has been sequestered into a unique nuclear domain. It is during these later stages that it has been proposed that the homologs remain associated through heterochromatic connections (Vazquez *et al.* 2002). In *tef* mutants, homolog associations are disrupted at S5 and S6 and homologs prematurely separate. Our observations support a model in which Tef functions during pairing stages to ensure bivalent adhesion after euchromatic associations are disrupted. Tef might be loaded onto the bivalent, perhaps at heterochromatin, prior to the dissolution of euchromatic pairing.

This model is well accommodated by the domain organization of the predicted Tef protein. We suggest that the antithetically located zinc fingers in the Tef protein may act as part of a bridging structure, analogous to a synaptonemal complex that physically connects homologs. Tef's terminal zinc fingers may recognize specific DNA sequences or chromatin-associated proteins at one end and mediate self-interaction at the other end, either directly or through an adhesion complex. In this manner, Tef's function might be analogous to the yeast SC protein Zip1 (Sym *et al.* 1993; Sym and Roeder 1995; Dong and Roeder 2000). This model is supported by our molecular analysis of *tef* mutants, which revealed that the zinc-finger domains at either end of the protein are required for its function. Either truncation of the carboxy-terminal zinc finger or alteration of a conserved cysteine residue in the amino-terminal finger results in a null mutation. A variation of this model is that, rather than acting as the bridge itself, chromatin-associated Tef protein may play an essential role in tethering an MNM-containing adhesion complex, as suggested by Thomas *et al.* (2005). This might be considered analogous to the role proposed for the zinc-finger-containing yeast lateral element SC component Hop1 in tethering other SC components (Hollingsworth and Ponte 1997; de los Santos and Hollingsworth 1999).

This model makes the testable prediction that Tef will reside between paired homologous autosomes at late prophase metaphase of meiosis I. It further suggests that Tef may be a useful tool for identifying the *cis*-acting sequences that mediate autosomal pairing and/or adhesion.

We thank Margaret Fuller and Steve Wasserman for providing fly stocks, Dennis Lajeunesse for use of injection facilities, and Jiro Yasuhara and Barbara Wakimoto for assistance with BLAST searches of *D. yakuba* and *D. simulans* libraries. This work was funded by National Science Foundation grant MCB-0350707. Confocal microscopy was performed on instrumentation funded by National Science Foundation grant DBI-0319021, the North Carolina Biotechnology grant 2003-IDG-1011, and the University of North Carolina at Greensboro Department of Biology.

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Communicating editor: A. Villeneuve