Requirement for two nearly identical TGIF-related homeobox genes in 
Drosophila spermatogenesis

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SUMMARY
The genetic analysis of spermatogenesis in Drosophila melanogaster has led to the identification of several genes that control the onset of meiosis, spermatid differentiation, or both. We described two tightly linked and nearly identical homeobox genes of the TGIF (TG-interacting factor) subclass called vismay and achintya that are essential for spermatogenesis in Drosophila. In flies deficient for both genes, spermatogenesis is blocked prior to any spermatid differentiation and before the first meiotic division. This suggests that vismay and achintya function at the same step as two previously characterized meiotic arrest genes, always early and cookie monster. Consistent with this idea, both always early and cookie monster are still expressed in flies deficient in vismay and achintya. Conversely, Vismay and Achintya proteins are present in always early mutant testes. Co-immunoprecipitation experiments further suggest that Vismay and Achintya proteins exist in a complex with Always early and Cookie monster proteins. Because Vismay and Achintya are likely to be sequence-specific DNA binding factors, these results suggest that they help to specify the spermatogenesis program by recruiting or stabilizing Always early and Cookie monster to specific target genes that need to be transcriptionally regulated during testes development.

Key words: Spermatogenesis, Meiosis, Homeobox genes, TGIF, TALE genes, Drosophila melanogaster

INTRODUCTION
The homeodomain is a DNA-binding motif present in a large number of eukaryotic transcription factors. To date, over 1,000 homeodomain proteins from over 100 different organisms have been described, making this family of DNA binding proteins one of the largest in biology (Banerjee-Basu et al., 1999; Banerjee-Basu et al., 2000; Gehring et al., 1994). Many homeodomain-containing factors, such as members of the Hox family, play critical roles in animal development (Cavodeassi et al., 2001; Hobert and Westphal, 2000; Lawrence and Morata, 1994; McGinnis and Krumlauf, 1992; Panganiban and Rubenstein, 2002; Trainor and Krumlauf, 2001). Perhaps in part because of their central role in animal development, many human diseases and genetic disorders are linked to mutations in homeodomain proteins (Abate-Shen, 2002; Buske and Humphries, 2000; Goodman and Scambler, 2001; Wallis and Muenke, 2000). Moreover, changes in the expression patterns, target genes and functions of homeodomain proteins are also thought to be driving force in animal evolution (Mann and Carroll, 2002; Vervoort, 2002).

Homeodomain proteins are classified into different groups depending upon the sequence of the homeodomain and immediately flanking amino acids, and the presence of other protein domains elsewhere in the protein (Gehring et al., 1994). One group of homeodomain proteins, called the TALE (for three amino acid loop extension) group, has an additional three amino acids separating the first and second alpha helices, resulting in a 63, instead of the more typical 60, amino acid homeodomain (Burglin, 1997). The TALE group is noteworthy for several reasons. Two TALE subgroups, MEIS and PBC, function as cofactors for the Hox homeodomain proteins (Mann and Affolter, 1998). At least for the regulation of some Hox target genes, a Hox/PBC/MEIS trimer is critical (Ferretti et al., 2000; Jacobs et al., 1999; Ryoo et al., 1999). In the PBC subgroup, the TALE-specific amino acids are Leu Ser Asn, which form part of a hydrophobic pocket that is directly contacted by the peptide Tyr-Pro-Trp-Met present in many Hox proteins (Passner et al., 1999; Piper et al., 1999). In addition to their role as Hox cofactors, the MEIS and PBC genes homothorax (hth) and extradenticle (exd) carry out several additional functions during Drosophila development. These genes are important for eye and antennal development and help to form the proximodistal axis in the thoracic appendages (Abu-Shaar and Mann, 1998; Bessa et al., 2002; Casares and Mann, 1998; Gonzalez-Crespo and Morata, 1996; Pai et al., 1998; Wu and Cohen, 1999). The Iroquois genes, which constitute a third TALE subgroup, are also important for patterning the appendages, as well as in neural and organ development (Cavodeassi et al., 2001). The TG interacting factors (TGIF) make up another interesting TALE subgroup. The founding member of this subgroup, TGIF, can be recruited to DNA directly, by binding to its target site, or indirectly, by virtue of its interaction with...
Smad proteins, which are key transcription factors that are activated by TGFβ signaling (Bertolino et al., 1995; Sharma and Sun, 2001; Wotton et al., 2001; Wotton et al., 1999a; Wotton et al., 1999b; Wotton and Massague, 2001). In humans, mutations in TGFβ cause holoprosencephaly (HPE), a severe genetic disorder affecting craniofacial development (Gripp et al., 2000; Wallis and Muenke, 2000). HPE is also caused by mutations in the Nodal pathway, which is related to TGFβ, thus linking TGFβ with Nodal signaling in vivo (Gripp et al., 2000; Wallis and Muenke, 2000). TGFβ appears to be a transcriptional repressor protein with at least two repressor domains that recruit either histone deacetylases (HDACs) or C-terminal binding protein (CTBP), a general corepressor (Sharma and Sun, 2001; Wotton et al., 2001; Wotton et al., 1999a; Wotton et al., 1999b; Wotton and Massague, 2001). The genome sequence of the fruit fly, Drosophila melanogaster, predicts the existence of eight TALE genes: exd (a PBC gene), hth (a MEIS gene), three linked Iroquois genes (mirror, araucana and caupolican), two tightly linked TGFβ-like genes, vismay and achintya (achi), and one predicted gene, CG11617 (Adams et al., 2000; Misra et al., 2002). Of these, only CG11617 and the two TGFβ-like genes have not yet been characterized. To analyze a potential role for the TGFβ-like genes in Drosophila development, we generated a deficiency that uncovers both vis and achi. Surprisingly, flies homozygous for this deletion are viable, suggesting that these genes play no essential role in embryonic or larval development. However, homozygous males are sterile. Further analysis shows that in the absence of vis and achi, spermatogenesis is blocked prior to the meiotic divisions with no signs of differentiation, placing these genes in the category known as male meiotic arrest genes. Genomic rescue and epistasis experiments suggest that Vis and Achi are redundant transcription factors that function at the same step in spermatogenesis as two other meiotic arrest genes, always early (aly) and cookie monster (comr) (Dwivedi and White-Cooper, 2003; White-Cooper et al., 2000). Moreover, Aly and Comr co-immunoprecipitate with Vis and/or Achi, suggesting that these proteins may function together as a complex to control normal male meiosis and spermatid differentiation.

MATERIALS AND METHODS

Fly stocks
A stock carrying EP(2)2107, a P element inserted close to the 5’ end of achintya (Fig. 1C), was obtained from Exelexis/BDGP. Stocks carrying aly1, sde1 or CycB2 were ordered from Bloomington stock center. aly2 and.cam12 mutant stocks were kindly provided by Helen White-Cooper. All stocks were raised at 25°C, at which temperature these alleles behave as null mutations (Jiang and White-Cooper, 2003; White-Cooper et al., 2000; White-Cooper et al., 1998).

Generation and identification of a deficiency that removes vis and achi
Although the EP(2)2107 insertion is homozygous viable, some sterility is observed in this stock. Among six males tested, two did not give any progeny after being crossed to uly females for 10 days. The four fertile males yielded an average of 56 progeny.

To generate a deletion of vis and achi, we employed a P-element-mediated male recombination strategy (Preston et al., 1996). We screened for recombination between a proximal marker (hook or aristaleless) and EP(2)2107, which is marked by the mini white gene. Out of six recombinant lines, one deleted vis and achi. Because of its resulting male sterile phenotype, we named this deficiency Df(2R)pingpong (Fig. 1C), and refer to it here simply as pingpong. The other recombinants did not disrupt vis or achi as determined by PCR mapping, and they are homozygous viable and fertile. Other potential lethals on the Df(2R)pingpong chromosome were removed by meiotic recombination.

In the pingpong deficiency the original EP insertion is still present. We therefore mapped the deletion molecularly by P-element-mediated plasmid rescue. The sequence at the deletion junction is as follows: TGAATGTAGTGTGTGGAAGCCCTGATTCTCATGAAA-TAACATAAGGTGGTCCCCCT (genomic sequences are in normal type and P-element sequences are in bold type).

Approximately 40 kb was deleted (Fig. 1C). The absence of vis, achi, CG8824, CG30044 and CG12370 was verified by Southern analysis and PCR mapping (data not shown).

Rescue constructs
Genomic DNA fragments containing either vis or achi were generated by PCR using a BAC clone (24H9). Research Genetices/BDGP as the template. The primers used to generate the genomic rescue fragments of vis or achi are:

\[\text{5'-vis ccccgctcatcagataccgg}\]
\[\text{3'-vis tcactgctgaagctctgc}\]
\[\text{5'-achi gcagcctagctggc}\]
\[\text{3'-achi gttgagctgctgctc}\]

P[vis] contains a 4.7 kb fragment extending from 362 bp upstream of the first exon to 1563 bp downstream of the last exon of vis. P[achi] contains a 4.8 kb fragment ranging from 900 bp upstream of the first exon to 700 bp downstream of the last exon of achi. These PCR products were cloned into a shuttle vector and sequenced for verification. Finally, vis or achi rescue fragments were cloned into the pP(W8) vector (FlyBase) and transgenic flies were generated by standard methods. Although P[achi] and, to some extent, P[vis] rescue the infertility of Df(2R)pingpong males, Df(2R)pingpong; P[achi] and Df(2R)pingpong; P[vis] flies are not as healthy as wild type.

tub-AchiS2 and tub-AchiL were constructed by cloning the EcoRI/Xbal fragments of LD25085 and LP02076 (from BDGP EST collection), respectively, into an α-tubulin (tub; 6αTub484 – FlyBase) cassette described previously (Chen and Struhl, 1996; Zecca et al., 1995).

Antibodies and immunofluorescence
A polyclonal antibody was raised against 6His-AchiS (full length) in guinea pigs (Cocalico Biologicals, Inc., Reamstown, PA). No staining above background was observed in Df(2R)pingpong embryos, imaginal discs or germline tissues. In contrast, testes from either Df(2R)pingpong; P[vis] or Df(2R)pingpong; P[achi] males showed a normal expression pattern, demonstrating that this antibody recognizes both Vis and Achi proteins (data not shown). We therefore refer to the immunoreactivity as Vis/Achi when both genes are present. Rabbit anti-Aly and rabbit anti-Comr antibodies were generously provided by Helen White-Cooper. Rabbit antibodies against Cyclin A or Cyclin B were gifts from Christian Lehner and David Glover. Mouse monoclonal antibody against Polo was a gift from Claudio Sunkel. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Embryos and ovaries were fixed and immunostained according to published protocols (Patel, 1994; Verheyen and Cooley, 1994). Third instar larval discs were dissected in PBS and fixed in 4% formaldehyde. Adult testes were dissected in PBS, frozen in liquid nitrogen before being fixed by either the methanol/acetone method or in 4% paraformaldehyde (Bonaccorsi et al., 2000). Using the paraformaldehyde fixation we observed Vis/Achi throughout the testes, including in mitotic cells at the apical tip (shown in Fig. 3); the methanol/acetone fixation revealed a more limited Vis/Achi pattern in
which only the primary spermatocytes were labeled. Anti-Achi antibody was used at 1:1000, Aly antibody at 1:2000, Comr antibody at 1:1000, Cyclin A antibody at 1:600, Cyclin B antibody at 1:2000 and Polo antibody at 1:40 dilutions. All secondary antibodies were diluted to 1:400. To stain DNA with propidium iodide (Molecular Probes), 100 µg/ml of RNase A was included in the secondary antibody incubation.

In situ hybridization
We carried out RNA in situ hybridization according to the protocol described by White-Cooper et al. with the following modifications (White-Cooper et al., 1998). Prehybridization, hybridization and washes were all done at 60°C, except for twist in situ, which were at 55°C. Hybridized Dig-RNA probes were detected by AP-conjugated anti-Dig antibody at a 1:2500 dilution. To ensure equivalent conditions for both wild-type and pingpong mutant testes (easily distinguished by their morphology), they were kept in the same incubation well in all the in situ experiments. cDNA clones of boule and mst87F were kindly provided by Helen White-Cooper. Digoxigenin-labeled RNA probes were synthesized by in vitro transcription according to manufacturer’s instruction (Roche).

BrdU labeling
Testes were dissected in Ringer’s solution and bathed in 100 mg/ml BrdU (Sigma) in Ringer’s. After labeling for 60 minutes, testes were washed 3 times in PBT (PBS + 0.1% Triton X-100) and subsequently fixed in 4% formaldehyde/PBT for 30 minutes. To make labeled DNA more accessible to immunodetection, fixed testes were treated with 50 U/ml DNAseI (Roche) for 60 minutes at 25°C (Gonczy and DiNardo, 1996). BrdU incorporation was detected by 1:100 mouse anti-BrdU (Becton Dickinson) and followed by FITC-conjugated secondary antibody.

Western analysis
Whole-testes lysates were prepared by putting dissected testes directly into 2× SDS sample buffer then vortexing vigorously. Adult carcasses were saved and ground in 2× SDS sample buffer. Primary antibodies were used at 1:7,500-10,000 for AchiS, at 1:10,000 for Aly, and at 1:5,000 for Comr. Signals were detected by a secondary antibody conjugated to peroxidase followed by the ECL reaction (Amersham). Secondary antibody was used at 1:7,500-10,000 for AchiS, at 1:10,000 for Aly, and at 1:5,000 for Comr. To stain DNA with propidium iodide (Molecular Probes), 100 µg/ml of RNase A was included in the secondary antibody incubation.

Co-immunoprecipitation
20-30 testes of each genotype were ground up in 100 µl RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 20 mM MgCl2, 0.5% NP40, and 1 mM PMSF) containing a protease inhibitor cocktail (Roche) and 2.5 mM Na3VO4 (Sigma). Testes extracts and α-Achi antibody at 1:500 dilution were mixed and incubated at 4°C overnight. 10 µl of proteinA/G-agarose beads (Santa Cruz Biotechnology) was added and incubated for an additional 3 hours. Samples were then washed three times and all bound proteins were eluted with 2× SDS sample buffer.

RESULTS
vis and achi encode members of the TGIF subgroup of TALE homeodomain proteins
vis (CG8821) and achi (CG8819) are both predicted to encode TALE homeodomain proteins (Fig. 1A). Analysis of genomic and cDNA sequences suggests that both genes have the potential to encode two protein products, which we refer to as long (L) and short (S) isoforms (Fig. 1A,B). The long and short forms of both Vis and Achi, which have been directly observed by immunoblot analysis (see below), differ in length because of the presence or absence of an alternatively spliced exon (Fig. 1B). Blast and ClustalW analyses indicate that Vis and Achi are almost identical. For example, AchiS and VisS are predicted to be identical in 406 of 424 residues (96% identity). In addition, the alternatively spliced exons in Vis and Achi are 129 residues in length and are predicted to be 100% identical to each other. The high degree of identity between these proteins is unusual for homeobox genes.

Blast and ClustalW analyses also reveal that the mammalian proteins most similar to Vis and Achi are human TGIF and TGIF2 (Fig. 1A). The similarity to TGIF and TGIF2 is most obvious in the homeodomain (TGIF is ~78% identical to Achi), but the identity extends to residues C-terminal to the homeodomain. A protein predicted from the genome sequence of the mosquito, Anopheles gambiae, shows several additional regions with similarity to Vis and Achi. Other members of the TGIF subgroup have homology to Vis and Achi primarily within the homeodomain (Fig. 1A).

vis and achi are required for male fertility
vis and achi are located on the right arm of chromosome 2 in the Drosophila genome, in cytological position 49A. Starting with a P element (EP(2)2107) inserted close to the 5’ end of achi, we used P element-mediated male recombination to generate a ∼40 kilobase deletion that removes both vis and achi (Fig. 1C). Because this deficiency deletes two nearly identical genes and results in male sterility, we named it Df(2R)pingpong and refer to it here simply as pingpong. In addition to deleting vis and achi, pingpong also removes four additional genes (Fig. 1C). However, homozygous flies carrying this deficiency are viable, suggesting that none of these genes are required for embryonic or larval development. Moreover, pingpong homozygous females are fertile and can give rise to viable pingpong progeny, eliminating the possibility that any of these genes have an essential maternal function. The only highly penetrant phenotype observed in pingpong homozygous flies is male sterility which can be rescued by transgenes carrying genomic regions for either vis (P{vis}) or achi (P{achi}) (Fig. 1C and see below). Thus, we conclude that vis and achi encode redundant functions required for male fertility.

We quantitated the role of vis and achi in male fertility by comparing the fertility of pingpong males with pingpong; P{vis} and pingpong; P{achi} males. We crossed individual males to wild-type females and counted the number of progeny after 15 days. In this assay wild-type (yw) males yielded an average of 132 progeny/male (n=2). pingpong males were completely infertile (0 progeny; n=26). In contrast, pingpong; P{achi} males were nearly as fertile as wild type (100.5 progeny/male; n=10) and pingpong; P{vis} males were partially rescued (27.8 progeny/male; n=16). Although both P{vis} and P{achi} appear to generate a wild-type Vis/Achi expression pattern in the male germline (see below and data not shown) these results suggest that achi is better able to rescue the mutant phenotype than vis.

Vis and Achi are widely expressed during development
To analyze the vis and achi expression patterns we generated
Fig. 1. *vis* and *achi* encode two highly related TGIF-like proteins. (A) Sequence alignment of the predicted VisS and AchiL proteins with other members of the TGIF family. Residues conserved in all family members are highlighted in pink; residues conserved in all but one sequence are highlighted in yellow; residues conserved in a smaller subset of sequences are highlighted in blue; and similarities to Vis/Achi are shown in purple. The alternatively spliced exon in AchiL is indicated by the green arrowheads (also in B). * marks the predicted end of each protein.

hTGIF, human TGIF; hTGIF2, human TGIF2; hTGIF2LX, human TGIF2-like on X; mTex1, mouse Tex1; fTGIF, Fugu TGIF; AgTGIF, Anopheles gambiae TGIF. The extent of the homeodomain and three alpha helices (1, 2, 3) is indicated above the sequence by the black and grey bars; the red box marks the three TALE residues in between helices 1 and 2. The residues 'AYP', also in the loop between helices 1 and 2, are one of the hallmarks of the TGIF family. (B) Genomic organization of the *vis/achi* locus. cDNAs predicted to encode VisS (SD01238), AchiS2 (LD25085) and AchiL (LP02076) have been sequenced; transcripts for VisL and AchiS1 are predicted from the analysis of the genome sequence. The alternatively spliced exons are indicated with an apostrophe. Lighter shading indicates non-coding sequences, and the black boxes indicate the homeoboxes in *vis* and *achi*. (C) *Df(2R)pingpong* is a deletion (indicated by the dashed line) of *vis* and four neighboring genes. The extent of the two rescue P elements for *vis* (P(*vis*)) and *achi* (P(*achi*)) are indicated.
a polyclonal antibody against AchiS. Using this antibody, we detected nuclear expression in nearly all cells and stages of development, including cells in the testes (Fig. 2). The specificity of this antibody was confirmed by observing only background staining in the *pingpong* deficiency (Fig. 2B and data not shown). Moreover, a wild-type testes staining pattern is generated by either the P{vis} or P{achi} transgenes (data not shown and see below) demonstrating that this antibody recognizes both Vis and Achi proteins.

Because of the male sterile phenotype of homozygous *pingpong* flies we examined the Vis/Achi expression pattern in more detail in wild-type testes and compared its pattern to that of Aly, a chromatin-associated protein that is required for male meiosis in *Drosophila* (White-Cooper et al., 2000). At the most apical tip of the testes are somatic ‘hub’ cells, germline stem cells, and mitotically dividing spermatogonia (Gonczy et al., 1992). Further from the tip is a zone of larger primary spermatocytes organized into 16-cell cysts. These cells, which grow in volume ~25-fold over a period of ~3.5 days, remain in meiotic prophase, a specialized stage of the meiotic cell cycle that precedes the meiotic divisions. Like Aly (White-Cooper et al., 2000), Vis/Achi is most strongly observed in the nuclei of primary spermatocytes (Fig. 3A-C). In addition, weaker nuclear Vis/Achi staining is also observed in the mitotically dividing cells at the apical tip of the testes (Fig. 3B). This is not a background staining because it is not observed in the *pingpong* deficiency, which still have these cells (data not shown and Fig. 4B). In addition, we also observed weak Aly staining at this stage (Fig. 3B).

The various stages of meiosis can be identified by the state of the chromosomes, as revealed by a fluorescent DNA dye such as propidium iodide (Fig. 3). At the large primary spermatocyte stage, when both Aly and Vis/Achi are nuclear, the three major chromosome pairs can be seen as three separate but diffuse signals, indicating that the chromosomes are partially condensed (Fig. 3C). At metaphase of meiosis I the chromosomes are fully condensed and appear as nuclear dots (Fig. 3D). At this stage, Aly appears to associate with the spindle and Vis/Achi is predominantly in the cytoplasm. A similar pattern is seen for both proteins at metaphase of meiosis II (Fig. 3E). Following the second meiotic division spermatid differentiation begins with a stage known as the onion stage, in which each cell has a characteristically round and specialized mitochondrion adjacent to its haploid nucleus. At this stage, the anti-Aly antibody labels a single dot per nucleus that co-localizes with the DNA (Fig. 3F). In contrast, Vis/Achi is observed throughout these cells except that it is excluded from the large and specialized mitochondrion next to each nucleus (Fig. 3F). Finally, at an even later stage of spermatid differentiation, Aly is barely detected above background and Vis/Achi partially co-localizes with the DNA (Fig. 3G). Vis/Achi is not detected in the somatic cyst cells that surround the primary spermatocytes (data not shown).

**Spermatogenesis in Df(2R)*pingpong* males arrests before the first meiotic division**

The male sterile phenotype, together with its strong nuclear expression in primary spermatocytes, suggested that *vis* or *achi* may be required for meiosis. We therefore examined *pingpong* and *pingpong*; P{achi} testes by phase contrast microscopy and by staining the chromosomes with propidium iodide. In contrast to wild type, *pingpong* mutant testes are much smaller and are blocked before the first meiotic division (Fig. 4). Specifically, no evidence of any elongated or onion stage spermatids are observed in the *pingpong* mutant (Fig. 4A-D). Instead, the testes are filled with cells that appear to remain at the primary spermatocyte stage. 16-cell cysts are still present, suggesting that the four mitotic divisions proceed normally in this mutant. However, the cells are smaller and not as round as in wild type (Fig. 4C,D). Far from the apical tip of the testes cells appear to degenerate. In addition, some of the chromosomes fail to fully condense in the absence of Vis and Achi (Fig. 4F,H). Typically, we observe three spots of DNA per cell, but the appearance of these spots ranges from diffuse (partially decondensed) to fully condensed (Fig. 4H). This phenotype indicates that the normally synchronized events leading to chromosome condensation fail in the *pingpong* mutant. Furthermore, these results suggest that although the *pingpong* mutant initiates meiosis in the male, meiosis is blocked before the first meiotic division, probably prior to the G2 to M transition. In addition to being blocked in meiosis, *pingpong* testes do not show any signs of spermatid differentiation, such as the distinctive onion stage cysts or spermatid elongation.

We also used bromodeoxyuridine (BrdU) labeling to determine if the 16-cell cysts in *pingpong* testes undergo DNA
synthesis before arresting. As seen (Fig. 4I,J), BrdU-labeled 16-cell cysts were observed in both wild-type and pingpong testes. Thus, the block in meiosis occurs after DNA synthesis but before the first meiotic division.

vis and achi act in parallel with aly and comr

The genetic analysis of spermatogenesis in Drosophila has identified several genes that are required for the normal progression through meiosis in males (Fuller, 1998). Two of these genes, aly and cookie monster (comr) can be distinguished from the others because they are required for the expression of twine, which encodes a cdc25-like phosphatase, and boule, an ortholog of the human gene Deleted in Azoospermia (Alphay et al., 1992; Eberhart et al., 1996; Jiang and White-Cooper, 2003; White-Cooper et al., 2000; White-Cooper et al., 1998). In contrast, the gene cannonball (can) is also required for meiosis in males, but is not required for the expression of twine or boule mRNAs (Hiller et al., 2001; White-Cooper et al., 1998). To gain additional insight into the pingpong mutant phenotype we stained mutant testes for several markers known to be expressed in testes, including twine and boule. Like aly and comr, but unlike can mutant testes, the pingpong mutant does not express twine or boule mRNAs (Fig. 5A-D). Also like aly and comr mutants, pingpong testes do not express mst87F, a gene that is required for spermatid differentiation (Kuhn et al., 1988) (Fig. 5E,F).
These results suggest that vis and achi function at a similar step as aly and comr.

To determine if vis and achi act upstream of or in parallel with aly and comr we stained pingpong mutant testes with antibodies for the aly and comr gene products, which are also highly expressed in primary spermatocytes (Jiang and White-Cooper, 2003; White-Cooper et al., 2000). Strikingly, both Aly and Comr were observed in the nuclei of most cells in pingpong mutant testes (Fig. 4H and Fig. 5G-J). Thus, vis and achi are not required for the expression or nuclear localization of these genes. Conversely, Vis/Achi proteins are still observed in aly mutant testes (as is Comr) (Fig. 5K, L). Consistent with these results, Vis and Achi are also expressed and nuclear in testes mutant for spermatocytes arrest (sa) and can, genes that appear to act downstream of aly and comr (Fig. 5M-P). Thus, neither sa, can, or aly are required for the expression or nuclear localization of Vis or Achi. Taken together, these data suggest that Vis and Achi function in spermatogenesis in parallel with Aly and Comr.

The expression of cell cycle regulators in pingpong mutant

The lack of twine expression in pingpong mutant testes is consistent with it causing a meiotic arrest phenotype (Courtot et al., 1992). In addition to twine, meiosis is also controlled by the availability of Cyclins A and B, which are required to activate Cyclin dependent kinase 1 (Cdk1) (Knoblich and Lehner, 1993; Lehner and O’Farrell, 1990; Sigrist et al., 1995). Both Cyclins A and B are normally expressed during male spermatogenesis, at high levels in the mitotically dividing cells at the apical tip of the testes, at lower levels during the primary spermatocyte stage, and at higher levels in 16-cell cysts just
prior to the G2 to M transition (Fig. 6A,C). The cyclins are rapidly degraded at the end of metaphase (Lin et al., 1996; White-Cooper et al., 1998). We therefore examined cyclin levels in pingpong mutant testes (Fig. 6A-D). Although there is some variation between individual testes, in general we observed intermediate levels of both Cyclin A and Cyclin B throughout pingpong testes (Fig. 6A-D). Cyclin A and B levels persisted, although at lower levels, up to the point when the cells appear to degenerate. In addition, although there is a transient nuclear localization of both Cyclins prior to cell division in the wild type, both Cyclins were predominantly observed in the cytoplasm in the pingpong mutant. Therefore, unlike in the wild type, Cyclin levels are not modulated in the pingpong mutant, consistent with a block prior to the G2/M transition. In addition, the levels of Polo, a protein kinase required for cytokinesis during meiosis (Herrmann et al., 1998), indicate that in pingpong testes the block occurs prior to the first meiotic division (Fig. 6E,F).

Either AchiL or AchiS is sufficient to partially rescue Df(2R)pingpong

The characterization of vis and achi cDNAs suggested the existence of two isoforms that differ because of the presence or absence of an alternatively spliced exon (Fig. 1A,B). To analyze the protein products derived from vis and achi we used the anti-Achi antibody in immunoblot experiments. Two bands with apparent molecular masses of ~60 kDa and ~80 kDa were detected in whole lysates of wild-type testes (Fig. 7A). In contrast, only a ~60 kDa band was detected in wild-type ovaries. Neither band was detected in the testes or ovaries from the pingpong mutant, suggesting that both are derived from vis and achi (Fig. 7A). Furthermore, based on the sizes of proteins observed in pingpong; P[vis] or pingpong; P[achi] flies we deduce that VisL and AchiL both migrate at ~80 kDa and both VisS and AchiS migrate at ~60 kDa (Fig. 7B). This assignment was confirmed by expressing the AchiL or AchiS cDNAs under the control of the α1-tubulin promoter in Df(2R)pingpong flies.
fully rescue (data not shown). Furthermore, in wild-type flies the slower migrating forms (AchiL and VisL) are testes specific because only the ~60 kDa species was observed in male or female somatic tissues (Fig. 7A). Although additional bands were observed in wild-type extracts, they were still present in extracts derived from Df(2R)pingpong flies, suggesting that they are not encoded by vis or achi (data not shown).

We also tested if either AchiL or AchiS2 is sufficient to rescue the pingpong male infertility phenotype by ubiquitously expressing these isoforms under the control of the tub promoter. Flies containing either tub-AchiL or tub-AchiS2 in an otherwise wild-type background appear normal. In addition, although the long isoforms are testes specific, both Df(2R)pingpong; tub-AchiL and Df(2R)pingpong; tub-AchiS2 males had normal appearing testes, as demonstrated by both phase contrast microscopy and immunostaining with several markers (Fig. 7C,D and data not shown). However, in both cases these males were only weakly fertile (Df(2R)pingpong; tub-AchiL males gave rise to an average of 10 progeny/male; n=24). One explanation for this result is that the tub promoter fails to provide accurate levels or timing of Achi expression to fully rescue pingpong sterility. However, males in which both long and short forms of Achi are co-expressed exhibit better fertility (Df(2R)pingpong; tub-AchiL; tub-AchiS2), suggesting that expression of both isoforms is required for complete rescue (data not shown).

Vis/Achi, Aly and Comr exist in a complex in wild-type testes

Many of the phenotypes we observe in the pingpong deficiency are also observed in aly and comr mutants (Jiang and White-Cooper, 2003; White-Cooper et al., 2000; White-Cooper et al., 1998). In addition, immunolocalization studies suggest that Vis, Achi, Aly and Comr proteins are co-expressed in the nuclei of primary spermatocytes. These observations prompted us to test if these proteins may be present as a complex in wild-type testes. We tested this by carrying out immunoprecipitation (IP) experiments with the anti-Achi antibody and determining if either Aly or Comr is co-immunoprecipitated. Interestingly, both Aly and Comr can be co-immunoprecipitated with Vis/Achi from wild type, but not from pingpong testes (Fig. 8). These results suggest that Vis and Achi proteins are present in a complex with Aly and Comr during wild-type testes development.

DISCUSSION

These results suggest that the vis and achi genes encode at least four highly related and partially redundant TGIF-like homeodomain proteins that are required for spermatogenesis in Drosophila. The block in spermatogenesis occurs during the primary spermatocyte stage, which is a growth phase prior to either meiotic division. No evidence of spermatid differentiation is observed in a deficiency that removes both genes, suggesting that the block in development affects both meiosis and differentiation. Below, we discuss three main points relevant to these results, specifically, potential mechanisms of Vis/Achi action, the similarities and differences between Vis, Achi and mammalian TGIFs, and a potential role for TGIF-like proteins in mammalian spermatogenesis.

vis and achi encode homeodomain proteins required for male meiosis in Drosophila

Previous work has identified a handful of meiotic arrest genes that are required for the completion of meiosis in males (Fuller, 1998; Hiller et al., 2001; Jiang and White-Cooper, 2003; Lin et al., 1996; White-Cooper et al., 2000; White-Cooper et al., 1998). Some of these genes, including aly and comr, have been proposed to work at an early step in
spermatogenesis because they are required for both meiosis and spermatid differentiation. In contrast, other meiotic arrest genes, such as boule, twine and polo, block only some aspects of the spermatogenesis program (Eberhart et al., 1996; Fuller, 1998; Herrmann et al., 1998; Hiller et al., 2001; Lin et al., 1996). Our analysis of the Df(2R)pingpong mutant phenotype suggests that vis and achi act at the same or earlier step as aly and comr. Most significantly, we found that, like aly and comr, the expression of boule, twine and mst87f mRNAs is absent in the pingpong mutant. In addition, the mutant phenotype is more consistent with an early block in spermatocyte development because no sign of spermatid differentiation can be observed in the Df(2R)pingpong mutant. Also like aly and comr, the Df(2R)pingpong mutant arrests prior to the G2 to M transition of meiosis I. Moreover, based on our ability to label 16-cell cysts in the Df(2R)pingpong mutant with BrdU, the block in meiosis apparently occurs after DNA synthesis.

Despite the many similarities to aly and comr, our results also suggest that vis and achi play a role in spermatogenesis that is different from these genes. First, unlike Aly or Comr, the Vis and Achi proteins are homeodomain-containing proteins of the TGIF subclass, making it likely that they have the ability to bind DNA in a sequence-specific manner. In contrast, aly encodes a chromatin-associated protein that is related to the C. elegans lin-9 gene and comr encodes a novel nuclear protein (Jiang and White-Cooper, 2003; White-Cooper et al., 2000). Thus, of the known meiotic arrest genes, vis and achi are the best candidates for encoding sequence-specific transcription factors necessary for the regulation of specific genes during Drosophila spermatogenesis.

Our results further suggest, however, that there are additional transcription factors required for male meiosis in Drosophila that have yet to be identified. First, we found that vis and achi are not required for the expression of aly or comr, indicating that there are other factors that activate the expression of these genes in the testes. Second, vis and achi are widely expressed during development. Thus, the expression of these genes cannot be sufficient to trigger the male meiosis program. Instead, these results suggest that Vis and Achi must work together with other factors during testes development. Two of these factors are likely to be Aly and Comr, an idea that is supported by our ability to co-immunoprecipitate Vis/Achi, Aly, and Comr with an anti-Achi antibody. However, it is probable that there are additional, currently unknown
factors that activate aly and comr expression and that work with Vis/Achi in primary spermatocytes.

**Similarities and differences to mammalian TGFIs**

Mammalian TGF is known to interact with three co-repressors, CtBP, Sin3 and HDAC1, and TGF2 interacts with HDAC1 (Melhuish et al., 2001; Wotton et al., 2001; Wotton et al., 1999a; Wotton et al., 1999b; Wotton and Massague, 2001). These interactions, together with the ability of TGF to antagonize TGFβ-mediated gene activation, strongly suggest that both TGF and TGF2 are transcriptional repressors. However, the sequences in TGF and TGF2 required for the interactions with these co-repressors map to sequences that are poorly, if at all, conserved in Vis or Achi. Thus, it is unclear at present if Vis/Achi recruit these or other co-repressors. However, we have shown that Vis/Achi proteins interact with Aly in vivo. Interestingly, an aly homolog in C. elegans, lin-9, has been genetically linked to components of the NURD complex, which is a chromatin remodeling complex with both ATPase and HDAC activities (Solarli and Ahrringer, 2000; Unhavaithaya et al., 2002; von Zelewsky et al., 2000; Xue et al., 1998). Moreover, the NURD complex has been implicated in gene repression. Thus, for some targets Vis/Achi may repress transcription indirectly, by helping to recruit Aly or stabilize its association with chromatin. Aly, in turn, may be able to recruit or activate a NURD-like complex, resulting in repression.

We observe a loss of expression of specific target genes in the Df(2R)pignong mutant, suggesting that Vis/Achi directly or indirectly activate these genes. However, as yet, none of the TGF family members have been shown to activate transcription. Thus, it remains an open question whether Vis/Achi play a direct role in the activation of genes such as boule and twine or, alternatively, if it acts indirectly by repressing the expression of a repressor of these genes.

Another well characterized feature of the mammalian TGF protein is its ability to directly interact with Smad2, a transcription factor that is activated by TGFβ signaling (Wotton et al., 1999a). The interaction between TGF and Smad2 is thought to modulate the response to TGFβ from gene activation to repression. Interestingly, Drosophila spermatogenesis requires TGFβ signaling (Matunis et al., 1997). However, this pathway, which utilizes the receptor encoded by punt and the transcription factor encoded by schnurri, is active in the somatic cyst cells that surround the germline spermatocytes. Once this pathway is activated, the somatic cyst cells are thought to release a second, unknown signal that limits the proliferation of the underlying germ cells (Matunis et al., 1997). At present, there is no known TGFβ-like pathway activated in primary spermatocytes, where Vis/Achi are maximally expressed. However, the available data do not rule out that a TGFβ-like pathway, which functions independently of punt and schnurri, may be active in these cells.

There are also connections between TGF factors and epidermal growth factor (EGF) signaling. Specifically, TGF and TGF2 have consensual MAPK phosphorylation sites that are phosphorylated in response to EGF signaling, and phosphorylation appears to stabilize these proteins (Lo et al., 2001; Melhuish et al., 2001). Although the same phosphorylation sites do not appear to be conserved in Vis/Achi, it will nevertheless be interesting to determine if the activity and/or stability of Vis/Achi are modulated by a Ras/MAPK pathway that may be activated in primary spermatocytes.

**Potential role of mammalian TGFIs in spermatogenesis**

In summary, our results demonstrate that vis and achi play a critical role in spermatogenesis in Drosophila, but that they are dispensable for other aspects of fly development. The highly restricted role for these homeobox genes contrasts with the widespread roles for the TALE group homeobox genes in the fly, including exd, hth and the Iro-C genes. It also contrasts with the apparently important role that the TGF genes play in TGFβ and Nodal signaling in vertebrates. It is possible that the original function of this gene family was in male meiosis but that they duplicated in vertebrates, allowing some family members to evolve into modulators of TGFβ signaling. Consistent with this idea, there are two TGF family members, Tex-1 and TGIFlx (TGF-like on X) that have recently been found to be specifically expressed in mouse or human testes (Blanco-Arias et al., 2002; Lai et al., 2002). Given the many other similarities between fly and mammalian spermatogenesis (Fuller, 1998; Zhao and Garbers, 2002), it is plausible that these genes play an analogous role in mammalian spermatogenesis as the vis and achi genes play in Drosophila.

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