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Drosophila TGIF is essential for developmentally regulated transcription in spermatogenesis

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SUMMARY

We have investigated the role of TGIF, a TALE-class homeodomain transcription factor, in *Drosophila* development. In vertebrates, TGIF has been implicated, by in vitro analysis, in several pathways, most notably as a repressor modulating the response to TGFß signalling. Human TGIF has been associated with the developmental disorder holoprosencephaly. *Drosophila* TGIF is represented by the products of two tandemly repeated highly similar genes, *achintya* and *vismay*. We have generated mutations that delete both genes. Homozygous mutant flies are viable and appear morphologically normal, but the males are completely sterile. The defect lies at the primary spermatocyte stage and differentiation is blocked prior to the onset of the meiotic divisions. We show that mutants lacking TGIF function fail to activate transcription of many genes required for sperm manufacture and of some genes required for entry into the meiotic divisions. This groups TGIF together with two other genes producing similar phenotypes, *always early* and *cookie monster*, as components of the machinery required for the activation of the spermatogenic programme of transcription. TGIF is the first sequence-specific transcription factor identified in this pathway. By immunolabelling in mouse testes we show that TGIF is expressed in the early stages of spermatogenesis consistent with a conserved role in the activation of the spermatogenesis transcription programme.

Key words: *Drosophila* development, Spermatogenesis, Homeodomain, Spermatocyte

INTRODUCTION

TALE-superclass homeodomains are characterised by the presence of an additional three amino acids (Three Amino-acid Loop Extension) between helices 1 and 2 (Burglin, 1997). They are an ancient family represented from yeast to humans and they act as transcription factors, often in collaboration with other homeodomain proteins. Members of the PBC and Meis classes of TALE proteins function as cofactors of Hox homeodomain proteins (reviewed by Mann and Affolter, 1998). TGIF (TG-Interacting Factor) is a transcription factor of the TALE homeodomain class that has been implicated in a number of distinct pathways. TGIF was first identified as a competitor of the retinoic acid receptor for binding to retinoic acid response elements (Bertolino et al., 1995). Subsequently TGIF was demonstrated to interact with Smads and a role has been proposed for it as a negative regulator of TGFβ signalling based on in vitro and cell culture experiments (Wotton et al., 1999a). The findings that TGIF binds transcriptional repression proteins such as HDAC, mSin3A and CtBP and that TGIF can displace the CBP/p300 co-activator from Smad complexes suggest that it acts to build a repression complex on Smad target gene promoters (Wotton et al., 1999b; Wotton et al., 2001; Wotton and Massague, 2001). TGIF acting as a Smad co-repressor has been proposed to impose a response ceiling on transcription from TGFß response genes. TGIF has also been suggested to act as a competitive inhibitor of the TALE-class homeodomain protein Meis2 in neuronal cell lines (Yang et al., 2000).

Consistent with an in vivo role in TGFβ/BMP signalling, TGIF has been identified as one of a small group of genes implicated in the human developmental disorder holoprosencephaly (HPE) (Gripp et al., 2000). This failure of forebrain formation is a relatively common developmental disorder affecting 1 in 250 conceptuses and 1 in 16000 live-born infants (Muenke and Beachy, 2000). Loss-of-function mutations in TGFβ family members in the mouse and zebrafish exhibit holoprosencephaly phenotypes (Conlon et al., 1994; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). Four regions in the human genome (HPE 1-4) have been correlated with HPE and HPE 4 has been mapped to a 6 Mb region on chromosome 18 at p11.3, which includes the TGIF locus. Collections of HPE families have revealed TGIF alleles with mutations that affect protein function, which provide a plausible case for the relevance of TGIF to HPE but surprisingly these mutations do not appear to be more prevalent in the HPE group (Nanni et al., 2000). Another potentially relevant gene, *twisted gastrulation* has also been recently found to be located at 18p11.3 (Graf et al., 2001).

TGIF shows strong evolutionary conservation and similar

sequences are present in the genomes of chicken, *Drosophila*, man and mouse. However, there has been no investigation of the in vivo developmental consequences of deleting TGIF from the genome. Here we present an analysis of the effects of deleting TGIF gene function in *Drosophila*. We show that the major developmental defect arising from loss of TGIF is a failure in spermatogenesis. TGIF mutants are male sterile with an *aly*-class meiotic arrest phenotype. We show that *Drosophila* TGIF is required for transcription of many spermatogenic target genes, although not for the expression or normal localisation the other *aly*-class meiotic arrest proteins Always early (Aly) and Cookie monster (Comr). TGIF represents the first sequence-specific transcription factor to be shown to be required for this spermatogenesis transcription programme.

MATERIALS AND METHODS

Drosophila culture and stocks

Drosophila were maintained on standard cornmeal/agar/sucrose medium at 25°C. Wild-type flies were Oregon R for molecular biology and *y w* or *red e* for immunolabelling. Markers are described in FlyBase (FlyBase, 1999). Stocks used were *aly*^{5} *red e/TM6C, mia*^{1} *st/TM3, cn achi*^{Z3922} *vis*^{Z3922} *bw/CyO, cn comr*^{z1340} *bw/CyO*. The deletions *Df(2R)vg-C* and *Df(2R)BSC3* were obtained from the Bloomington stock centre.

Mutagenesis

The fly strain EP(2)2107 obtained from BDGP was identified as an insertion in the 5′-UTR of *achi* and used to generate the *achi*^{1} deletion by standard P-element excision. P-element induced male recombination (Preston et al., 1996) against the *dp b cn bw* chromosome was used to generate the *achi*^{2}, *achi*^{3} and *achi*^{4} alleles. The extents of the deletions were confirmed by PCR or inverse-PCR sequencing. The *achi*^{Z3922} *vis*^{Z3922} chromosome was isolated in a large scale EMS mutagenesis for new viable mutants conducted by Charles Zuker and colleagues (E. Koundakjian, R. Hardy, D. Cowen and C. Zuker, personal communication). All the lines were tested for male sterility by Barbara Wakimoto and Dan Lindsley and these were then re-screened for a meiotic-arrest phenotype in the laboratory of Margaret Fuller. *achi*^{Z3922} *vis*^{Z3922} has been previously referred to as *zaa*.

Sequence analysis

Similarity searches were performed using the tBLASTn, BLASTn and BLASTp algorithms (Altschul and Lipman, 1990; Altschul et al., 1997) via www.ncbi.nlm.nih.gov/BLAST/. Protein sequence alignments were performed using ClustalW (Thompson et al., 1994) at www.ebi.ac.uk/clustalw. Genomic and EST sequences were obtained from GenBank. The ESTs LD25085, GM01582, LP02076 and SD01238 were sequenced completely and used to make intron-exon assignations; RT-PCR was used to confirm this. Genomic Southern blotting was used to confirm the gene duplication (data not shown).

To identify the defect in *achi*^{Z3922} *vis*^{Z3922}, PCR primers were designed to amplify all of the predicted ORFs from candidate genes, as sets of overlapping products. The amplified fragments were sequenced from both ends using BigDye terminator cycle sequencing reagent (ABI), reactions were run on an ABI 377 automated DNA sequencing system. Sequence alignments were carried out using Sequencher 3.1 (GeneCodes Corp).

Expression analysis

For *achi/vis* developmental time course analysis RNA was extracted from various tissues using the TRIzol reagent (Life Technologies). 1 μg of this RNA was used for reverse transcription using appropriate primers and SuperScript^{TM} II RNAseH Reverse Transcriptase (Life Technologies). PCR amplification (30 cycles) was performed using Taq DNA polymerase (Roche) on a Perkin Elmer Gene Amp PCR system 9700. For analysis of expression in the testis compared with that in the carcass, total RNA was prepared, using TRIzol reagent, from testes of wild-type and mutant male flies, the whole bodies of wild-type females and male carcasses (testes were removed by dissection). Total RNA was treated with DNAse I to remove possible contamination of genomic DNA. RT-PCR was carried out using Superscript Preamplification System (Invitrogen) in which a pair of oligo primers (5′-ATGATCTCGCCGGAACAAGAGGA and 5′-GTCTCCCATGTAAACGAAATCG) was used to amplify the whole open reading frame of *vis* and *achi* on a Biometra personal thermal cycler.

For in situ hybridisation, single-stranded RNA probes were generated by in vitro transcription (Roche) and were used to detect *achi/vis* transcripts as described in White-Cooper et al. (White-Cooper et al., 1998).

For the analysis of expression in the wild-type and the mutant, equal amounts of RNA (3 μg) from dissected testes was used for reverse transcription with oligo(dT) primers. PCR amplification with appropriate primers was for 28 cycles. Primers used were: TGIF.f1 CCAGGACATGATGCACGAG, TGIF.r3 TCGCTCGGATAGGCGTTATAG, TGIF.f3 AAGTCCTGCTTCCGAAGTGG, TGIF.r2 ACTTGTGCCCTGCGACATAG, aly.1 ATTTTCGGCCGCCTTCATC, aly.2 TACTCGACCAGGTAGTCG, can.1 GGCTTGTGAAGAACTTCCC, can.2 CCGCAAAAACCGAATCCTC, twe.1 CGCCAAGGATTTGGCAATC, twe.2 CTGGGATACATGCTTAGGC, bol.1 AAACGCATCGTATCTGGG, bol.2 TGAAGGTGGGTAGATGGC, cycB.1 AGCGTCTGCCTATCTTCG, cycB.2, GAACTGCAGGTGGACTTC, cycA.1 AGAGCATAATCGGACTCC, cycA.2 TAAGCAGTCGGTGTGCAC, fzo.1 TGGAGGCCATTGCAAAGG, fzo.2 AAACGCACCGACCTACTC, janB.1 CTTTGCAACTGCTCGCAC, janB.2 AGTGGTCCACGCTTGAAG, dj.1 AGGAAGCCGATGACCTTC, dj.2 TAAAGCCGCGTTGAACAG, gdl.1 GGGCAGCCAAACTGATTG, gdl.2 AATGTGGCGCAACTCCTC, esc.1 AGTCGCGGCCTAATTTGG, esc.2 ACAATGCGATCTCCACGC, hay.1 TCGAGAAAGGATCGCAGC, hay.2 TGCTGTGACACCCACTAG, taf24.1 ACAATGGCTTCCGATGGC, taf24.2 ACGAAGTACTGCGGCTTG.

Microscopy and immunolabelling

Testis squashes were performed as described previously (Cenci et al., 1994). Immunolabelling was as described by Jiang and White-Cooper (Jiang and White-Cooper, 2003). Primary antibodies used were anti-histone (1:1000 dilution, Chemicon), anti-Esc mAb E53.1 used at 1:5 dilution (Gutjahr et al., 1995), anti-Aly at 1:1000 dilution (White-Cooper et al., 2000) and anti-Comr at 1:1000 dilution (Jiang and White-Cooper, 2003). Secondary antibodies used were Alexa488-conjugated anti-mouse antibody (Molecular Probes) or FITC-conjugated anti-rabbit Ig (Jackson). Testes were counterstained with DAPI (1 μg/ml; Molecular probes) or propidium iodide (1 μg/ml; Sigma) and mounted in CitiFluor (Agar Scientific). Samples were analysed on a Leica TCS confocal microscope or a Bio-Rad Radiance Plus confocal microscope. Paraffin wax sections of mouse testes were labelled using anti-TGIF (I:10 dilution; Santa Cruz Biotech).

RESULTS

TGIF in *Drosophila* is represented by two tandemly repeated genes, *achi* and *vis*

To identify *Drosophila* TALE class homeodomain genes we performed BLAST searches of *Drosophila* genomic sequence

databases. This analysis revealed an uncharacterised TALE sequence, STS Dm2587, which showed strong sequence similarity to the TGIF family. ESTs homologous to this sequence were also found in cDNA libraries from several tissues and developmental stages. The complete *Drosophila* genome sequence revealed that these EST sequences are in fact the products of two closely juxtaposed tandemly-repeated transcription units. We have named these genes *achintya* (*achi*) and *vismay* (*vis*); 'achintya' is a Sanskrit word meaning 'that which is beyond thought and contemplation' relating to initial difficulties in interpreting the mutant analysis and 'vismay' is a Hindi word meaning 'surprise' when the genome sequence revealed the tandem duplication (Ayyar and White, 2001). The two genes are highly similar (93% at nucleotide level and 97% at protein level).

A comparison of the protein encoded by this locus with vertebrate TGIF sequences reveals that the sequence similarity exists both within the homeodomain (including RYN as the TALE amino acids characteristic of the TGIF family) and for about 30 amino acids on the carboxy-terminal side of the homeodomain (Fig. 1). Such a C-terminal domain is also found in the yeast TALE protein MATalpha2 and members of the PBC family, where these residues fold into additional helices required for the proper functioning of the homeodomain (Phillips et al., 1994; Sprules et al., 2000). The complete genome sequence of the mosquito *Anopheles gambiae* has recently been published and also reveals the presence of a TGIF homologue (AgCG54405). This gene encodes a protein (AgCP3385) with 63% similarity to Achi/Vis (Fig. 1). In addition to the sequence conservation in the homeodomain and C domain there are additional blocks of homology between these invertebrate sequences.

Sequencing ESTs made possible the generation of intron-exon maps for *achi*/*vis* and revealed alternative spliced products from the two transcription units (Fig. 2A). The major difference between splicing products is the presence or absence of exon6, which is 387 bp in length and encodes an additional 129 aa. We then investigated the expression profiles of these transcripts by RT-PCR. The *achi*/*vis* transcripts are present from embryogenesis through to adulthood (Fig. 2B). Interestingly, adult males predominantly expressed the larger (class 2) splice variant and females predominantly the smaller (class 1) (Fig. 2C). Using RNA derived from gonadectomised males compared to RNA derived from testes we determined that the larger splice isoform was testis specific, whereas the shorter was present in both the carcass and the testis (Fig. 2D). Sequencing of subclones of this testis-specific large isoform confirmed that both *achi* and *vis* class 2 transcripts are expressed in the testis.

Deletion of *Drosophila* TGIF results in male sterility

To examine the in vivo role of *Drosophila* TGIF we generated deletions by imprecise excision of a P-element that mapped within the 5′-UTR of the *achi* transcription unit (Fig. 3). Several small deletions were obtained together with a larger deletion, *Df(2R)achi*^{1}, that removes the whole of the *achi* transcription unit and part of the *vis* unit. In addition, using P-element induced male recombination, we produced several large deletions that removed *achi* and *vis* together with some neighbouring genes. The largest of these deletions *Df(2R)achi*^{4} is homozygous lethal. In contrast the smaller deletions *Df(2R)achi*^{2} and *Df(2R)achi*^{3} have the same phenotype as *Df(2R)achi*^{1}: they are homozygous viable, the males are completely sterile and females crossed to wild-type males exhibit a delay in egg laying. All three deletions failed to complement *Df(2R)BSC3* with respect to the male-sterile phenotype.

Examination of the testes from homozygous mutants revealed complete absence of mature sperm. Developmental stages up to late primary spermatocyte were present but no meiotic stages could be seen (compare Fig. 4B,C). A mutant with the same phenotype, Z3922 was identified in a screen of EMS mutants generated in the laboratory of C. Zuker (Fig. 4D). We mapped the mutation in Z3922 by meiotic recombination to 63.3±1.8 mu. The Z3922 chromosome failed to complement *Df(2R)BSC3*, placing the mutation in the 48F-49A chromosomal region. Fine scale deficiency and recombination mapping relative to P-element insertions in this region located the Z3922 locus to within 7 genes immediately distal to the P-element insertion *P{w*$^{+}$*}l(2)k17040*. PCR sequencing of these candidate genes from Z3922 revealed mutations in both *achi* and *vis*; there is a premature stop in exon 5 of *achi* together with a 56 bp deletion just 5′ of the *vis* homeodomain. The Z3922 chromosome (*achi*^{Z3922} *vis*^{Z3922}) fails to complement the *Df(2R)achi*^{1} allele.

Timing of the defect

We then examined in more detail the effects of the *achi*/*vis* mutations on spermatogenesis. In wild type, the stem cells located at the tip of the testes divide asymmetrically to give a stem cell daughter and a primary spermatogonial cell (Fig. 4A) (Fuller, 1993; Fuller, 1998). The primary spermatogonial cells divide four times mitotically to give a cluster of 16 spermatogonia encased by two somatic cyst cells. After the fourth mitotic division, the cells undergo DNA replication and, now called primary spermatocytes, they enter a long (approximately 3.5 days) G_2 phase which is a period of extensive transcription preceding the first meiotic metaphase. During this period the cells enlarge 25-fold. Upon entry into meiosis, transcription is shut down (Olivieri and Olivieri, 1965) The spermiogenesis genes, required to build functional sperm, tend to be transcribed during the long G_2 phase and then held under translational control for later protein production after meiosis (Schäfer et al., 1995).

Labelling the DNA with DAPI provides an easy way to visualise the stages of spermatogenesis. In wild-type whole mounts, cells at the apical tip of the testis label strongly, but during the primary spermatocyte stage the intensity decreases correlating with chromosomal reorganisation and increasing cell size (Fig. 5A). An expanded zone of high DAPI labelling was observed in *Df(2R)achi*^{1} mutant testes (Fig.5B). This experiment was repeated using confocal microscopy with anti-histone labelling to study the chromatin morphology (Fig. 5C,D). The expanded zone of cells with higher DAPI labelling correspond to an expanded population of small primary spermatocytes with diffuse chromatin surrounding a prominent nucleolus (Fig. 5D).

Squashed preparations of cells from the testes were used to study the defects in greater detail using phase contrast microscopy and DAPI labelling (Fig. 6A-D). Spermatocytes from stages S1 to S6 (Cenci et al., 1994) were identified in *Df(2R)achi*^{1} mutant testes. However, a marked defect was observed in the chromatin of the mature primary spermatocytes

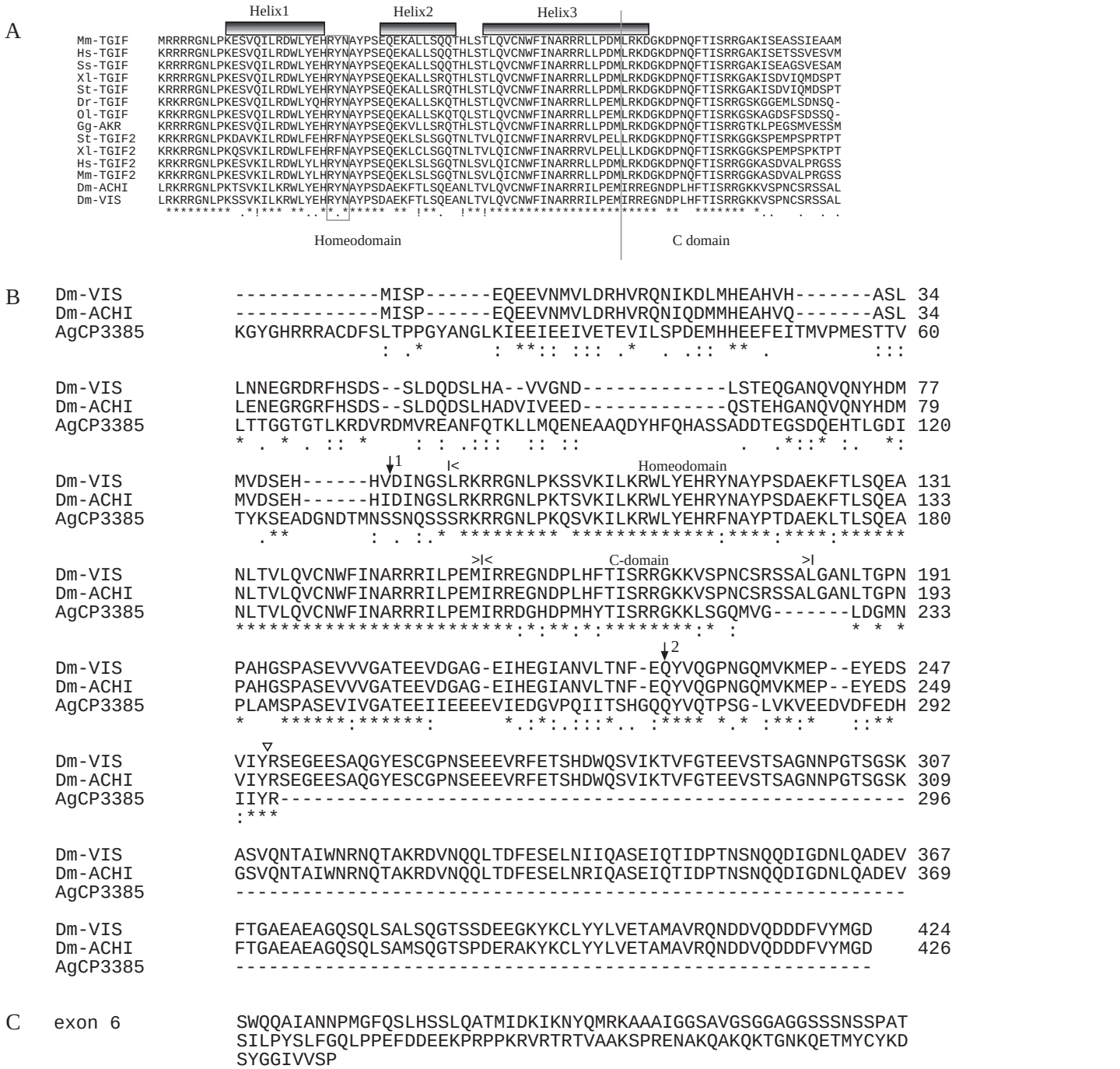

Fig. 1. TGIF is represented in *Drosophila* by Achi and Vis. (A) Sequence comparison shows the high TGIF similarity within the homeodomain and extending into the C-domain. The TALE amino acids, RYN, characteristic of the TGIF family of TALE homeodomains, are boxed. The extended helix 3 is shown as predicted by PSIpred. The vertical line indicates the COOH-terminal limit of the homeodomain; * indicates identical residues or highly conserved substitutions in all sequences; . indicates less conserved sequences and ! indicates conservation only between Achi/Vis and TGIF2. (B) Achi and Vis are highly similar and comparison with the *Anopheles* TGIF homologue (AgCP3385) indicates conserved regions in addition to the homeodomain and C domain. * indicates identity; : indicates highly conserved and . indicates conserved residues. The extents of the homeodomain and C domain are indicated above the sequence. The position of the insertion of exon 6 sequence is indicated by arrowhead. Arrows: number 1 indicates start of deletion in vis^{Z3922} and number 2 indicates site of stop codon in $achi^{Z3922}$. (C) Exon 6 sequence from class 2 transcripts. Exon 6 sequence is identical in *achi* and *vis*.

(stages S5 and S6). While these cells were large and had prominent nucleoli characteristic of this stage, the chromatin was condensed into tight central blobs normally observed only in later meiotic cells (Fig. 6D). Hence, in these mutants there appears to be an uncoupling between the nuclear/nucleolar differentiation and the chromatin condensation events of meiosis. Similar results were observed comparing wild-type and mutant histone-labelled preparations (Fig. 6E,F).

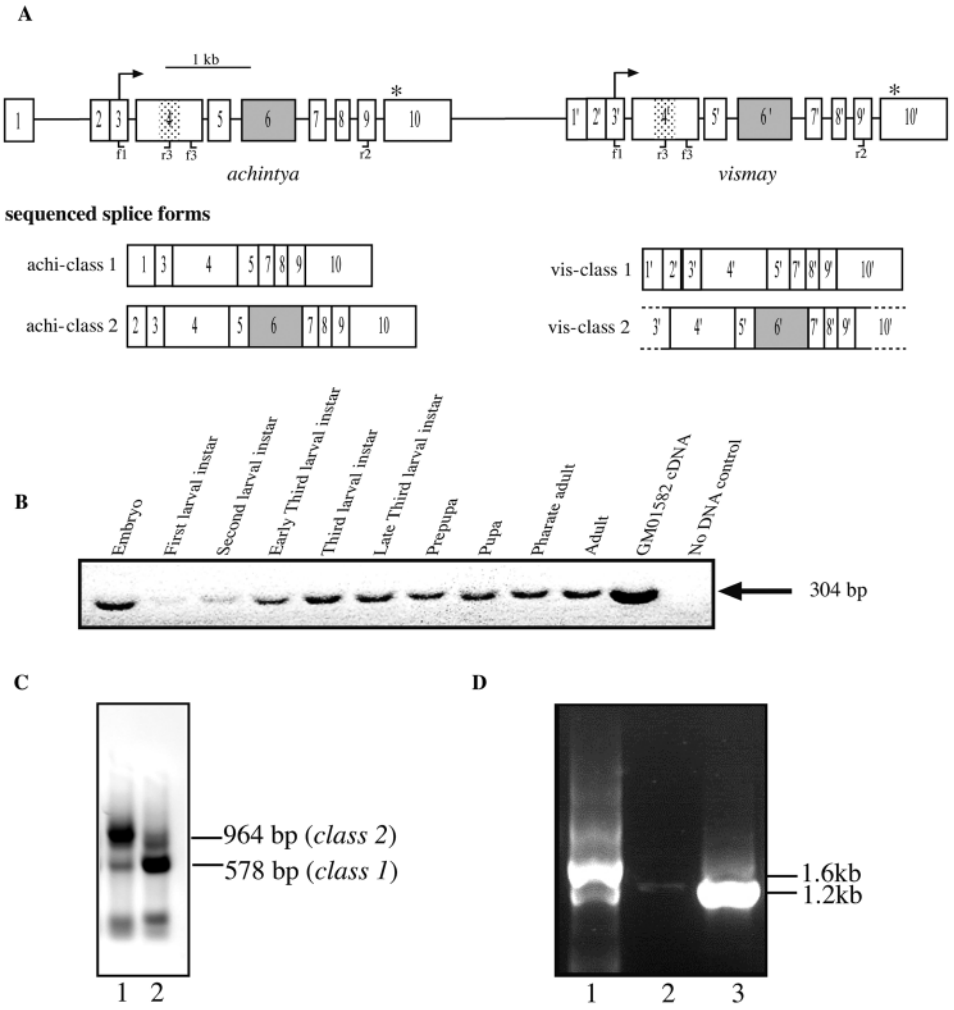

Fig. 2. Gene structure and expression. (A) *achi* and *vis* are tandem duplications. The intron/exon structure is shown together with the alternative splice products determined from completely sequencing the following cDNAs: achi-class 1 LD25085,GM01582; achi-class 2 LP02076; vis-class 1 SD08875, SD01238. vis-class 2 intron/exon structure was determined by sequencing a subclone of the ORF RT-PCR shown in D. Translation is initiated in exon3 (arrow) and terminates in exon10 (asterisk). (B) RT-PCR analysis with primers (f1 and r3) common to both *achi* and *vis* indicates expression throughout development. A similar result was obtained with primers specific for *achi*. The RP49 control gave approximately equal levels with RNAs from all the different developmental time points. (C) Sex-specific splice differences of *achi*/*vis*. Class 2 products predominate in testes (lane 1) whilst class 1 products (lane 2) predominate in ovaries (primers: f3 and r2). Similar results are seen comparing male and female whole fly RNA (not shown). (D) Class 2 transcripts are testes specific. Lane 1: testes; lane 2: male gonadectomised carcass and lane 3: adult females (*achi*/*vis* ORF primers).

In keeping with the meiotic arrest phenotype, RNA in situ analysis of *achi*/*vis* expression showed strong expression in the primary spermatocyte stage, which decayed as the cells progressed through meiosis (Fig. 4E). There was no detectable expression in the tip of the testis where the stem cells and spermatogonial cells reside.

Achi/*vis* are members of the *aly*-class of meiotic arrest genes

The control pathway underlying spermatogenesis is, as yet, poorly defined but a few 'meiotic-arrest' mutants have been identified. All the meiotic arrest mutants have a similar phenotype; mature primary spermatocytes arrest development, and fail to enter either the meiotic divisions or spermatid differentiation. The currently identified meiotic arrest genes have been subdivided into two classes. The *aly*-class genes (*aly* and *comr*) appear to be higher in the control hierarchy and regulate

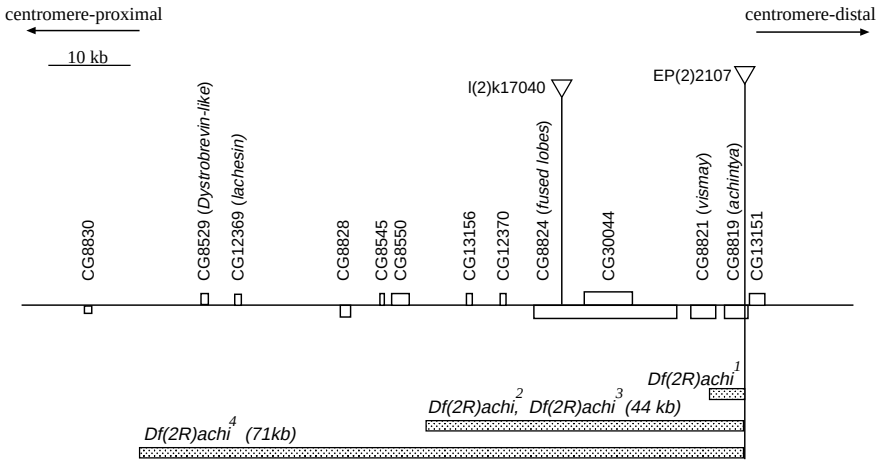

Fig. 3. Deletion mutants. Position of the starting transposon insertion (EP(2)2107) is indicated. EP(2)2107 homozygotes are viable and fertile although expression of *achi* class 1 transcripts is reduced. The *Df(2R)achi*^{1} deletion extends at least to the 3′ end of *vis* exon 4. Prediction of genes in the region is based on release 3 of the genome annotation. Genes transcribed left to right in the figure are above the line, those in the right to left orientation are below the line.

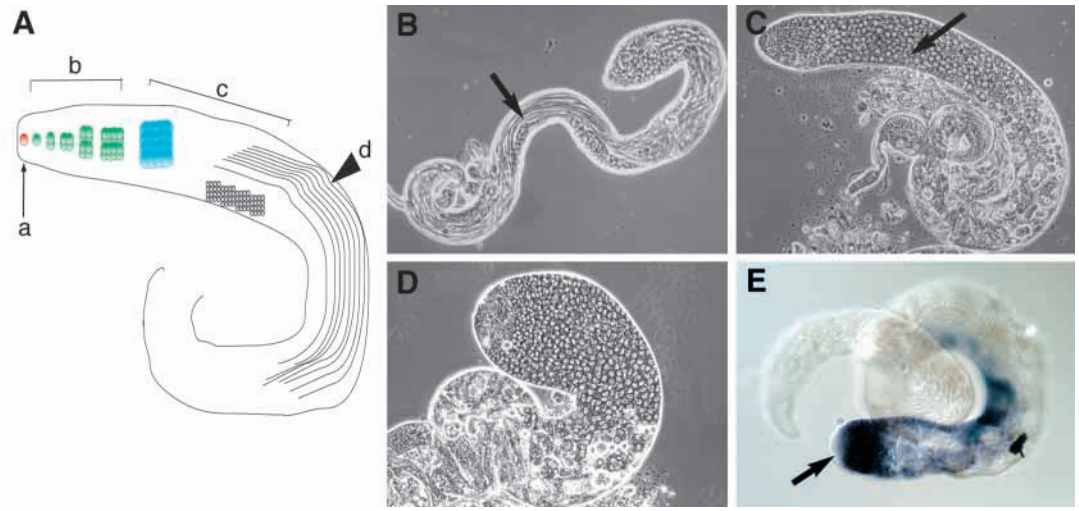

Fig. 4. Loss of *achi/vis* function results in male infertility. (A) Schematic of spermatogenesis; (a) stem cell at apex of testis, (b) zone of mitotic expansion of spermatogonia, (c) primary spermatocytes and meiotic divisions, (d) differentiated sperm. (B-D) Phase contrast image of (B) wild type (arrow indicates bundles of differentiated sperm); (C) *Df(2R)achi*^{1} homozygous testis (note absence of sperm and abundance of undifferentiated primary spermatocytes; arrow); (D) *achi*^{Z3922} *vis*^{Z3922}; same phenotype as *Df(2R)achi*^{1}; (E) In situ hybridisation with probe recognising the products of both *achi* and *vis*. Expression is low/undetectable at the apex (arrow), high in the primary spermatocyte stage and persists into the meiotic divisions.

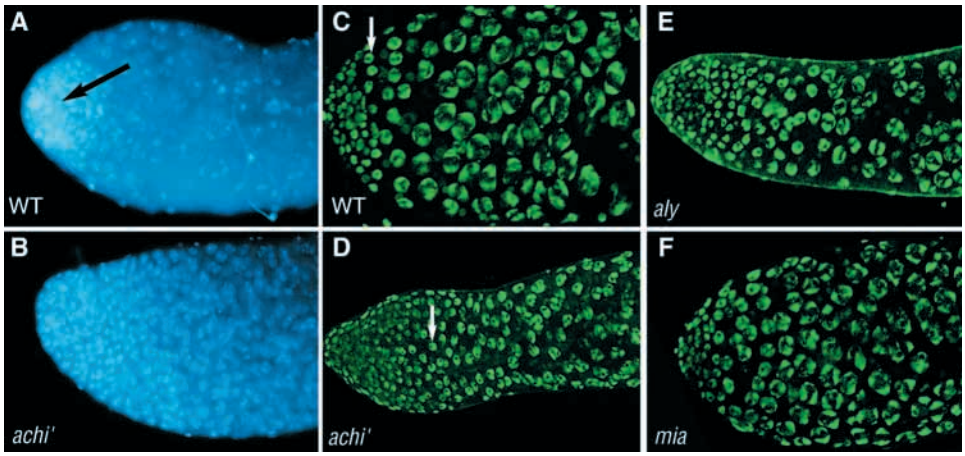

Fig. 5. The meiotic arrest phenotype. A. DAPI labelling is strong in the cells at the apex (arrow) and in *Df(2R)achi*^{1} homozygotes this zone is expanded (B). (C-F) Anti-histone labelling. In the wild type (C) and in *mia* homozygotes (F) there is a small population of early primary spermatocytes (arrow in C) and this population is markedly expanded in *Df(2R)achi*^{1} (D; arrow) and *aly* homozygotes (E).

transcription of some genes involved in entry into meiosis (*boule*, *twine*, *Cyclin B*) and also of many spermiogenesis genes (e.g. *fuzzy onions*, *janus B*, *don juan*, *gonadal*) required for the differentiation of functional sperm. In contrast, *can*-class meiotic arrest genes (including *cannonball*, *meiosis 1 arrest (mia)* and *spermatocyte arrest*) do not affect transcription of the meiosis cell-cycle genes but are required for spermiogenesis gene transcriptional activation (White-Cooper et al., 1998; Jiang and White-Cooper, 2003).

To place *achi*/*vis* within this scheme we examined the expression of a set of meiosis-related genes and a selected set of spermiogenesis genes in *Df(2R)achi*^{1} homozygous mutant testes by RT-PCR analysis, and in homozygous mutant males by in situ hybridisation (Fig. 7 and data not shown). Both the set of spermiogenesis genes tested (*fuzzy onions*, *janus B*, *don juan*, *gonadal*) and the meiosis-related cell-cycle genes (*boule*, *twine*, *Cyclin B*) showed strongly reduced expression in the mutant, placing *achi*/*vis* in the *aly* class of meiotic arrest genes. Transcription of other genes (*RP49*, *polo* and *Cyclin A*) was not affected in the mutants. To determine whether *Drosophila* TGIF is required upstream in the pathway for transcription of other meiotic arrest genes, we examined the expression of *aly* and *comr* in *achi/vis* mutant testes. In situ hybridisation on *achi*^{Z3922} *vis*^{Z3922} mutant testes revealed *aly* and *comr* transcripts at similar levels to wild type, and RT-PCR analysis on *Df(2R)achi*^{1} demonstrated robust expression of *aly* and *can* transcripts. In the RT-PCR analysis the levels of *aly* and *can* actually appeared somewhat higher than wild type. We do not, however, interpret this as indicative of a regulatory interaction but rather as a reflection of the altered cellular composition of the mutant testes. Similarly, *aly* and *comr* are not required for the expression of *achi/vis* as normal levels of *achi/vis* transcripts were found, by RT-PCR, in *aly* and *comr* homozygous mutant testes (data not shown).

Neither of the two previously described *aly*-class meiotic arrest genes contain a predicted DNA binding domain, yet they are both chromatin associated, and are clearly required for transcriptional activation. A simple model would be that the gene products, Aly, Comr and Achi/Vis all act together as components of a single mechanism required for gene activation in spermatogenesis. If this were true we would

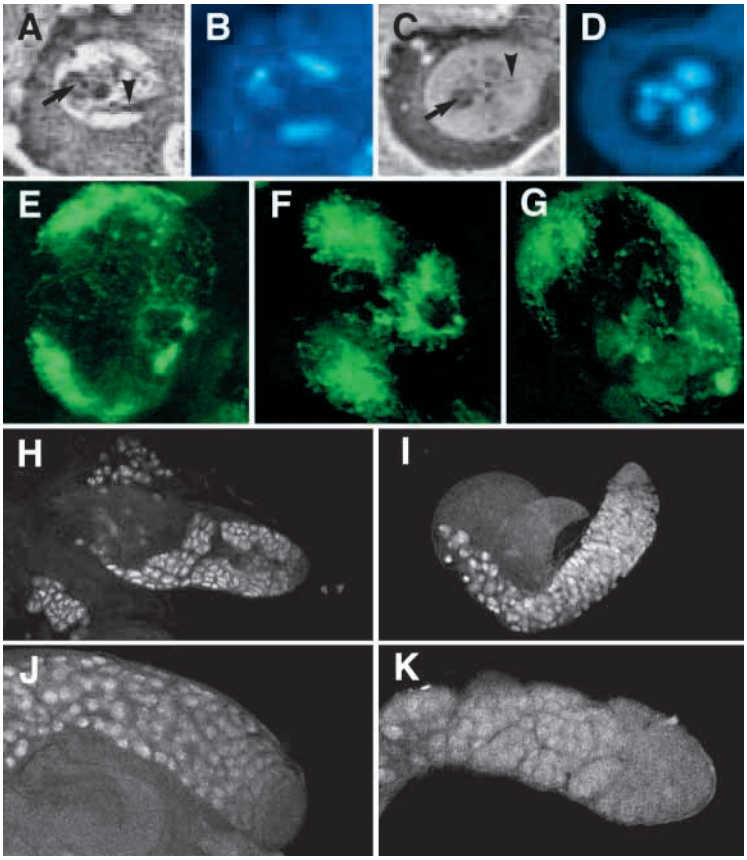

Fig. 6. *achi*/*vis* phenotype. (A-D) The late stage primary spermatocyte chromatin phenotype. (A) Phase contrast image of wild-type stage 5 primary spermatocyte. Arrow indicates nucleolus, arrowhead indicates Y-chromosome loops. (B) DAPI labelling reveals the chromatin peripherally located against the nuclear membrane. (C) Primary spermatocyte from *Df(2R)achi*^{1} homozygote testis demonstrating stage 5 characteristics: nucleolus (arrow), Y-loops (arrowhead) and intact nuclear membrane. (D) DAPI labelling of cell in C reveals chromatin clumps reminiscent of meiotic division stages (Cenci et al., 1994). (E-G) Anti-histone labelling reveals the chromatin configuration in stage 5 primary spermatocytes in wild type (E), *Df(2R)achi*^{1} (F) and *aly* (G). The chromatin morphologies in F and G are clearly distinct; the *aly* configuration resembles that of wild type, but is more diffuse. (H-K) Expression and localisation of Aly and Comr in *achi*^{Z3922} *vis*^{Z3922} homozygote testes. (H) Wild type, anti-Aly; (I) *achi*^{Z3922} *vis*^{Z3922}, anti-Aly, (J) wild type, anti-Comr, (K) *achi*^{Z3922} *vis*^{Z3922}, anti-Comr. Aly and Comr are present in the nucleus in *achi*^{Z3922} *vis*^{Z3922} testes but the localisation appears more diffuse than in the wild type.

predict that the phenotype of *aly* and *comr* mutations might be indistinguishable from the *achi/vis* loss-of-function phenotype. To test this we examined the phenotypes in detail. As noted above the *Df(2R)achi*^{1} phenotype includes an expansion of early primary spermatocytes, indicative of an early role for *achi*/*vis* in the primary spermatocyte stage. A similar cellular defect has not previously been described for *aly* but, as shown in Fig. 5E, *aly* mutants also display expansion of the early primary spermatocyte population presumably due to a defect in progression through the primary spermatocyte differentiation programme. This phenotype is not common to all meiotic arrest mutants and progression through the primary spermatocyte stages in *mia* mutants appears similar to wild type (Fig. 5F). In both *aly* and *achi*/*vis* mutants the primary spermatocytes do exhibit some spermatocyte differentiation; they increase in size and chromosomal reorganisation occurs, giving clear chromatin clumps, as visualised by either DAPI or anti-histone labelling. However, as described previously, the chromatin fails to organise as tightly in the *aly* mutant as in the wild type and the cells arrest with peripheral chromatin clumps with a fuzzy appearance (Lin et al., 1996) (Fig. 6G). The chromatin morphology of *comr* was identical to that of *aly*. In *achi*/*vis* mutant testes (*Df(2R)achi*^{1} or *achi*^{Z3922} *vis*^{Z3922}) the chromatin appears to follow a wild-type programme up to the generation of mature primary spermatocytes with peripheral chromatin clumps, however, the cells arrest with rounded chromatin clumps that are not apposed to the nuclear

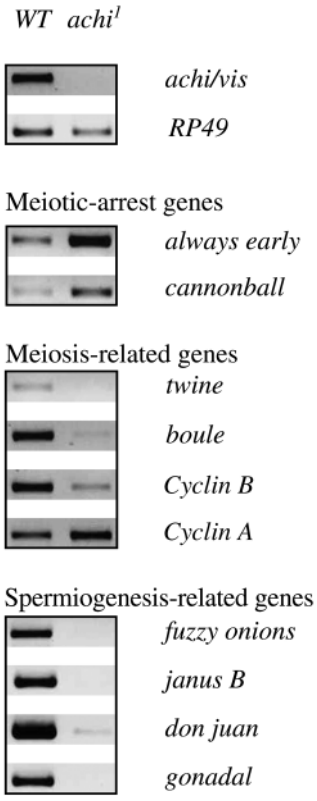

Fig. 7. Gene expression profile in *Df(2R)achi*^{1} mutants. RT-PCR analysis on RNA samples from wild-type and *Df(2R)achi*^{1} mutant testes.

periphery and that resemble the chromatin configuration in meiotic stages (Cenci et al., 1994). We conclude that the *achi*/*vis* phenotype is similar but not identical to that of *aly* and *comr*.

Drosophila TGIF is not required for the normal subcellular localisation of Aly or Comr

The normal chromatin association of Aly and Comr proteins is essential for their function, and the localisation of these two proteins is mutually dependent, i.e. in an *aly* mutant Comr protein remains cytoplasmic, and vice versa. In contrast, both Comr and Aly proteins localise to chromatin in testes mutant for the downstream, *can*-class, genes. To determine whether TGIF plays a role in the production or localisation of the other *aly*-class proteins, we examined the levels and localisation of Aly and Comr proteins in $achi^{Z3922}$ vis^{Z3922} mutant testes. Both Aly and Comr proteins were detected by western blotting in $achi^{Z3922}$ vis^{Z3922} mutant testes (data not shown). Immunofluorescent staining revealed that Aly and Comr proteins were nuclear in $achi^{Z3922}$ vis^{Z3922} testes (Fig. 6H-L). This places TGIF downstream of, or parallel to, *comr* and *aly*.

Drosophila TGIF: evidence for repressor function?

TGIF has been extensively characterised as a transcriptional repressor in vertebrates, yet in *Drosophila* the predominant effect we see in *achi*/*vis* mutants is failure to activate a testis-specific developmentally regulated transcriptional programme. In *Drosophila*, TGIF must be acting either directly as a transcriptional activator, or indirectly, as a repressor of a repressor. To investigate the transcriptional effects of lack of *achi*/*vis* in more detail we undertook RNA expression profiling looking particularly for genes whose expression was increased in the mutants. This system is well suited to expression analysis as a viable infertile phenotype allows the easy generation of mutant tissue for comparison with wild type. Our preliminary microarray analysis, comparing RNA from $Df(2R)achi^{1}$ mutant testes to wild-type, demonstrated, as expected, a large number of transcripts (including the spermiogenesis genes) showing decreased expression relative to wild-type. However, we also observed many transcripts with increased levels in the mutant. Comprehensive analysis will be presented elsewhere, however we were particularly intrigued by the strongly increased transcripts of *extra sex combs (esc)*, in the $Df(2R)achi^{1}$ mutant testis and we further investigated *esc* as a possible candidate for repression by TGIF. The microarray result was supported by RT-PCR comparison of *esc* transcript in wild type and $Df(2R)achi^{1}$ (Fig. 8A). Esc is a component of the transcription silencing machinery (Struhl, 1981; Gutjahr et al., 1995; Jones et al., 1998; Tie et al., 2001) and hence provided a possible link between *Drosophila* TGIF and gene repression. If TGIF normally represses *esc* transcription in primary spermatocytes this might allow the activation of the spermatogenesis transcription programme. According to this model TGIF mutants would overexpress *esc* and this would prevent the programme activation.

The interpretation of altered levels of transcripts in the mutant RNA population is complicated by the gross disruption of the RNA and cellular content of the mutant testes due to the failure of spermatogenesis at the primary spermatocyte stage. We therefore further investigated the expression of *esc* in the testes at a cellular level using an antibody against Esc protein. This analysis did not support the model proposing repression of *esc* by TGIF. In wild-type testis, Esc was expressed in the early mitotic cells and was also robustly expressed in early primary spermatocytes; clearly *esc* expression is not normally switched off in primary spermatocytes by *achi*/*vis* (Fig. 8B). Esc immunolocalisation also revealed the unexpected observation that as the primary spermatocytes mature, the Esc labelling, at first rather evenly distributed in the nucleus (but excluded from the nucleolus) progressively accumulates in nuclear spots. These nuclear spots are highly reminiscent of the labelling of a variety of functional silencing complexes in various cell types (Messmer et al., 1992; Alkema et al., 1997; Satijn et al., 1997; Saurin et al., 1998). Levels of Esc protein diminished as wild-type primary spermatocytes matured.

In the *achi*/*vis* mutant testes the overall expression levels of Esc protein were similar to wild type (Fig. 8C). How then do we explain the increase in *esc* transcript level? Esc protein level appears highest in early primary spermatocytes and this cell

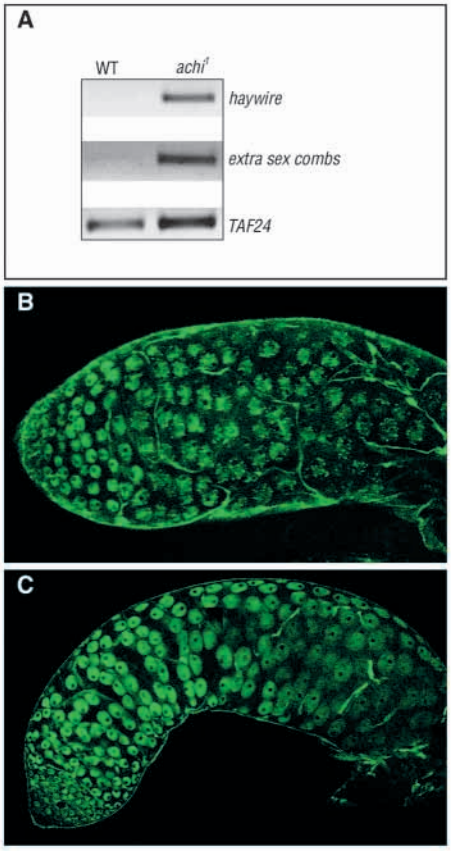

Fig. 8. Expression of *esc* in $Df(2R)achi^{1}$ mutants. (A) RT-PCR analysis on RNA samples from wild-type and $Df(2R)achi^{1}$ mutant testes reveal that *esc* is amongst the genes showing enhanced transcript abundance in $Df(2R)achi^{1}$ mutants, other enhanced transcripts include *haywire* and *Taf24*. (B) Anti-Esc immunolabelling on wild-type testes. Esc accumulates in nuclear speckles during primary spermatocyte differentiation. (C) Anti-Esc immunolabelling on $Df(2R)achi^{1}$ testis. Esc is clearly expressed and demonstrates the expansion of the early primary spermatocyte population. Nuclear speckles do not form.

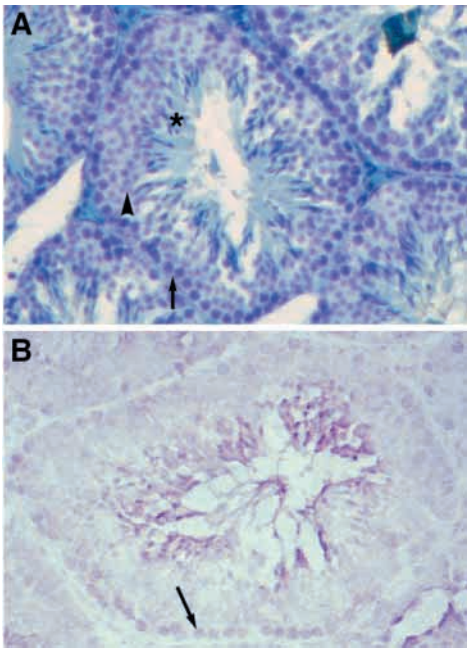

Fig. 9. Expression of TGIF in mouse testes. (A) Toluidine Blue stained cross section of adult mouse seminiferous tubules. Arrow, spermatogonia; arrowhead, dividing primary spermatocytes; asterisk, elongating spermatids. (B) Immunolabelling with anti-TGIF. Arrow indicates nuclear labelling in peripheral cells (spermatogonia).

population is markedly expanded in *achi*/*vis* mutants. Our interpretation is that expansion of cells with the highest level of *esc* is the likely cause of the observed increase in *esc* transcript abundance.

Despite no change in the apparent level of Esc protein in mutant cells, the Esc localization was strikingly altered in *Df(2R)achi*^{1} mutant testes (Fig. 8C). Although in *achi*/*vis* mutants the primary spermatocytes apparently differentiate to the final primary spermatocyte stage, the concomitant accumulation of Esc in nuclear spots failed to occur. It appears *achi*/*vis* function is required for assembly of the Esc complexes.

TGIF in mouse spermatogenesis

As *achi*/*vis* mutants in *Drosophila* primarily affect spermatogenesis we were interested in examining the potential for a similar role of vertebrate TGIF. The expression profile of TGIF in the mouse indicates widespread expression including strong expression in the testes (Bertolino et al., 1996). We used an antibody raised against human TGIF to examine the protein localisation of TGIF in the mouse testis (Fig. 9). In the mouse seminiferous tubules the spermatogonial stem cells and mitotic spermatogonial cells are found at the periphery of the tubules. Older cells are displaced towards the centre of the tubule so that a transect across the tubule gives a time-line of development with more mature meiotic stages towards the centre. TGIF was most prominently expressed in cells in more peripheral regions of the tubules, including spermatogonia and primary spermatocytes, and the labelling was restricted to cell nuclei. Therefore TGIF is available in the mouse in appropriate cells for the activation of the spermatogenesis transcriptional programme, as in the fly.

DISCUSSION

Drosophila TGIF is required for a specific transcriptional programme in *Drosophila* spermatogenesis

The extended G_2 phase that primary spermatoctyes undergo prior to entry into meiotic division is an important period in spermatogenesis (reviewed in Fuller, 1998). It lasts for about 3.5 days and during this time the cells increase in volume 25-fold, execute the transcription programme required to produce all the transcripts necessary for sperm differentiation and undergo a striking sequence of chromatin reorganisation. The switch in transcriptional activity that occurs in primary spermatocytes is one of the most dramatic of any differentiation pathway, with a great many genes being expressed exclusively in this cell type. At the end of this stage virtually all transcription is switched off; many of the transcripts produced during this period are held under translational inhibition to be released after meiosis in a co-ordinated programme of protein production that mediates sperm differentiation. Most male sterile mutations affect these later stages of sperm manufacture and typically result in a block in spermiogenesis at the very late stage of sperm individualisation (Fuller, 1993). A relatively small set of mutations have been characterised that block spermiogenesis during the extended primary spermatocyte G_2 phase, fail to enter meiotic division and fail to initiate spermatid differentiation (Lin et al., 1996). These meiotic arrest genes are required to initiate the primary spermatocyte-specific transcriptional activation programme. Previous genetic and biochemical analyses have suggested that the *aly*-class genes act before the *can*-class genes, at the top of a regulatory hierarchy (White-Cooper et al., 1998). Therefore the *aly*-class meiotic arrest genes provide an entry point into the mechanisms that initiate and orchestrate the transcriptional programme of spermiogenesis, that control spermatocyte differentiation and that regulate the entry into the meiotic divisions.

The relatively small number of these meiotic arrest genes currently identified presents the beguiling prospect that the transcriptional programme of spermiogenesis may be controlled by an ancient simple mechanism. Interestingly, two of the *can*-class meiotic arrest genes (*cannonball* and *no hitter*) encode testis-specific components of the general transcription factor TFIID suggesting that the genes activated during the primary spermatocyte stage may share a distinct core promoter type (Aoyagi and Wassarman, 2000; Aoyagi and Wassarman, 2001; Hiller et al., 2001). The homology of *aly* to a *C. elegans* gene implicated in a pathway leading to chromatin remodelling factors suggests that *aly* may have a role in chromatin reorganisation to allow access for specific transcription factors and the testis specific TFIID to target promoters (Beitel et al., 2000; White-Cooper et al., 2000). Our characterisation of *Drosophila* TGIF is the first description of a sequence-specific transcription factor implicated in this pathway. We show here that *achi* and *vis* are tandemly duplicated genes with close sequence similarity to the vertebrate TALE-class homeodomain transcription factor TGIF. Combined mutations in *achi* and *vis* or deletion of both genes lead to a recessive male sterile meiotic arrest phenotype. We found markedly decreased expression of both the spermiogenesis genes and

also of *CycB* and *twine*, required for entry into meiosis, placing *achi/vis* into the *aly*-class of meiotic arrest mutants. *Drosophila* TGIF does not appear to be required for the transcriptional activation of other meiotic arrest genes as *aly*, *comr* and *cannonball* are all expressed in *achi*/*vis* mutants. Similarly function of other meiotic arrest genes is not required for transcription of *achi/vis*.

Although the gross transcriptional consequences of loss of either *achi*/*vis* or *aly* appear similar, the mutant phenotypes are distinct. Both show effects on early primary spermatocytes with an expansion of this cell type presumably due to a slowing of the progress of differentiation through this stage. All the *aly*-class mutants, *aly*, *comr* and *achi*/*vis* exhibit defects in chromatin organisation but whereas the *aly* and *comr* mutant spermatocytes arrest with 'fuzzy' chromatin condensation (Lin et al., 1996), in *achi*/*vis* mutants the chromatin rounds up in condensed 'blobs' which are reminiscent of meiotic pro-metaphase. This difference in the phenotype is consistent with our finding that *Drosophila* TGIF is not required for the normal localisation of Aly and Comr proteins, and suggests that TGIF is also not required for the chromatin remodelling mediated by Aly and Comr. This raises the question of whether the *aly*-class genes all act together as components of a simple transcription activation switch or whether they may be a somewhat heterogeneous collection with more diverse roles within the spermatogenesis transcriptional programme.

Is *Drosophila* TGIF a transcriptional repressor or activator?

Although we have shown that *achi*/*vis* are required for the activation of both the spermiogenesis and meiosis genes in *Drosophila*, vertebrate TGIFs have been extensively characterised in vitro as repressors of transcription (Wotton et al., 1999a; Wotton et al., 1999b; Wotton and Massague, 2001). Human TGIF has been shown to bind 5′-TGTCA-3′ and repress activation of retinoic acid responsive genes by competing for this binding site with the retinoic acid receptor (Bertolino et al., 1995). TGIF and TGIF2 are both able to bind to the TGFβ-responsive Smad transcription factors, and act in competition with co-activators such as CBP/p300. TGIF recruits the co-repressor mSin3, and TGIF, but not TGIF2, interacts with the co-repressor CtBP. Additionally both TGIF and TGIF2 have been shown to bind histone deacetylase1 and thereby repress transcription by directing histone deacetylase activity to particular chromosomal regions. Apart from the CtBP interaction site (Melhuish and Wotton, 2000), the co-repressor interaction sites have not been precisely defined but they have been mapped outside the region that is clearly conserved between *Drosophila* and vertebrates. The comparison between *Drosophila* and mosquito sequences revealed conserved regions outside the homeodomain and C-terminal extension but these were not obviously related to the vertebrate sequences. We do not know whether Achi/Vis acts directly as a transcriptional activator, or as a repressor of a repressor, to regulate transcriptional activity in primary spermatocytes. Clearly our data are more simply interpreted if *Drosophila* TGIF acts as a transcriptional activator, and we have, as yet, no evidence to support a model of TGIF as a transcriptional repressor in *Drosophila*.

Our investigation of TGIF function led us to examine Esc expression in spermatogenesis and revealed a potential link between *achi/vis* and gene repression. Esc has been shown to play a key role in the establishment of silencing complexes in the embryo as a component of a repressor complex with histone H3 methyl transferase activity that marks chromosomal sites for silencing (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002). In wild type, Esc protein underwent a profound change in sub-cellular localisation during the primary spermatocyte stage. Initially Esc was rather evenly distributed throughout the nucleus but absent from the nucleolus, however, as the primary spermatocytes differentiated, Esc progressively accumulated in nuclear spots. Similar nuclear spots have been reported in a variety of cell types for components of the gene silencing machinery (Messmer et al., 1992; Alkema et al., 1997; Satijn et al., 1997; Saurin et al., 1998). Interestingly, in *achi*/*vis* mutants the accumulation of Esc in these complexes failed to occur. This does not appear to be simply because the mutant primary spermatocytes arrest prior to the appearance of the Esc nuclear spots; these complexes are visible in the wild type at about stage 3 of primary spermatocyte differentiation whereas in *achi*/*vis* mutants the primary spermatocytes attain the size and several characteristics of the final stage 6 primary spermatocyte. The activation of TGIF expression precedes the relocalisation of Esc, consistent with a link between *achi*/*vis* function and the formation of Esc silencing complexes. This may be a very indirect link but it is interesting that vertebrate TGIF has been proposed to provide the specific DNA binding protein to enable the co-repressors CtBP and HDAC to initiate silencing complexes at specific sites (Melhuish and Wotton, 2000). Clearly much work remains to be done to investigate this link and it will be interesting to see whether mutants in other meiotic arrest genes also show the same effect on Esc localisation. Whilst Esc relocalisation may be an important downstream event it is not an essential intermediary in *achi/vis* function as the activation of several *achi/vis* target genes (e.g. *Cyclin B*) precedes the formation of Esc complexes.

TGIF in spermatogenesis

Achi/Vis are the only TGIF homologues in *Drosophila* and thus deletions of *achi*/*vis* completely remove TGIF-homologous function. *achi*/*vis* deletion primarily affects spermatogenesis and this may have implications for the role of TGIF in vertebrates and the conservation of the mechanism of regulation of spermatogenesis. The picture is complicated by the presence of several TGIF-like genes in vertebrates. The expression of the original TGIF gene has been studied in adult mouse and it was found to be highly expressed in liver, kidney and testis (Bertolino et al., 1996). The distribution of human TGIF2, which shares 77% identity with TGIF in the homeodomain but only 49% similarity elsewhere, has been examined and it is expressed at highest levels in the heart, kidney and testis (Imoto et al., 2000). Recently the testis-specific expression of two TGIF-like genes has been reported in humans (Blanco-Arias et al., 2002) and a more distant relative in the mouse, *Tex1*, has been described, which shares 50% identity in the homeodomain with mouse TGIF and which is exclusively expressed in testis germline cells (Lai et al., 2002). We have observed immunolabelling of spermatogonia in the mouse testis using an antibody raised against a human TGIF peptide. Altogether the expression of TGIF and its family members points to a strong connection between vertebrate TGIF and spermatogenesis. Neither human nor mouse

infertility has yet been associated with TGIF family genes. However meiosis1 maturation arrest, the most common cause of human idiopathic male infertility, shows striking similarities to the *Drosophila* meiotic arrest phenotype (Meyer et al., 1992; Lin et al., 1996). Vertebrate and *Drosophila* spermatogenesis follow a similar pathway of cellular differentiation and it seems likely that many aspects of underlying regulatory mechanisms are conserved. Indeed, molecular conservation has been found for *boule*/DAZL, a regulator of *twine*/cdc25 translation required for meiotic entry (Eberhart et al., 1996; Ruggiu et al., 1997; Maines and Wasserman, 1999). We anticipate that the TGIF family will play a role in the activation of the vertebrate spermatogenesis transcription programme.

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