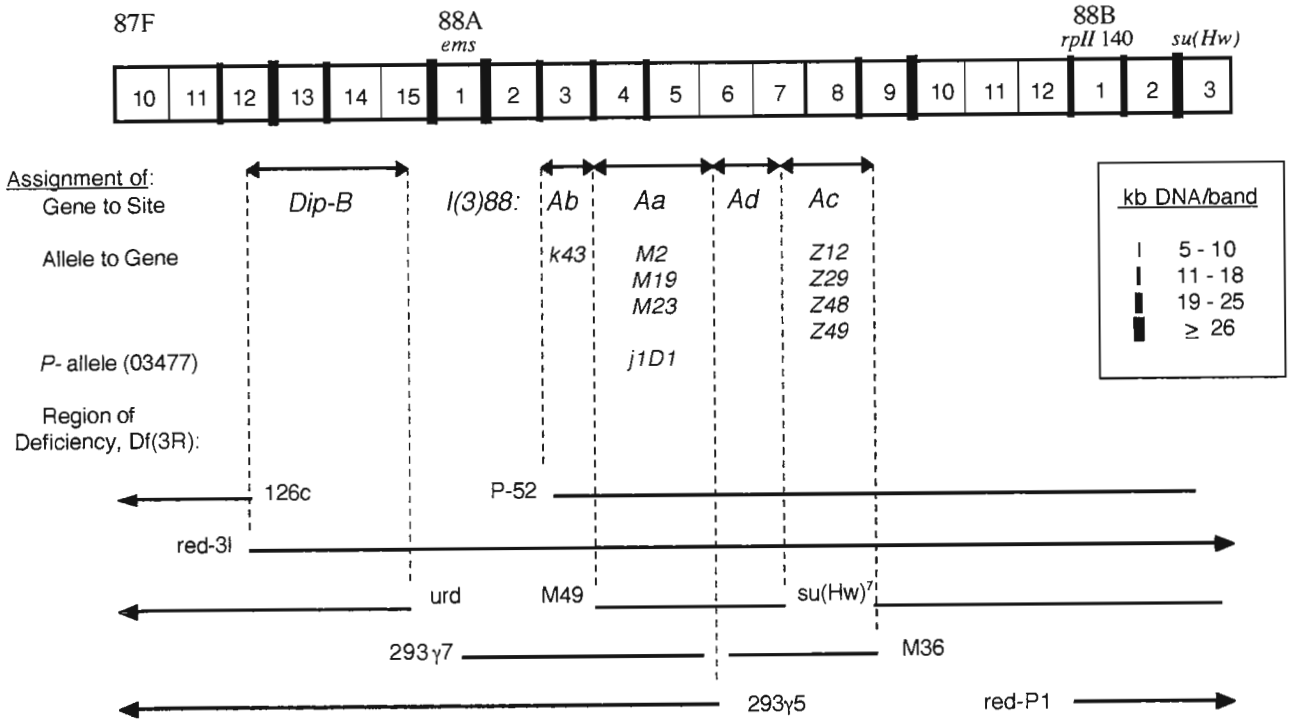

Figure 1. Sites of *Dipeptidase*-B and *l(3)88Aa-Ad* in the region 87F12-88B. The widths of the vertical demarcations of polytene bands represent the constituent amounts of DNA (Heino *et al.*, 1994), as indicated. The genes *ems, rpII140* and *su(Hw)*, assigned to single polytene bands (Flybase, 1997) and used in establishing the positions of deficiency breakpoints are also indicated. Heavy lines indicate the regions of deficiency (see Table 1 and text). Arrows indicate breakpoints in adjoining regions. The M and Z alleles and the *P* - allele *j1D1* of *l(3)03477*, located at 88A4-5 by *in situ* hybridisation (Spradling *et al.*,1995), were assigned by deficiency complementation analysis to the 'recessive lethal' genes *l(3)88Aa-Ac*. In addition, failure of the deficiencies M49 and M36 to complement indicates at least one further 'recessive lethal' gene site, designated l(3)88*Ad*. The vertical lines are guides to the boundaries of the regions of each gene as defined by deficiency breakpoints.

Contribution of the Dipetidases to the Life History of Drosophila, pp.30-41, D.Phil. thesis, University of Sussex, U.K.; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster, p. 876, Academic Press, New York; Mortin, M.A., R. Zuerner, S. Berger, and B.J. Hamilton 1992, Genetics 131:895-903; Onishi, S., and R.A. Voelker 1981, Biochem. Gen. 19:75-85; Parkhurst, S.M., D.A. Harrison, M.P. Remington, C. Spana, R.L. Kelley, R.S. Coyne, and V.G. Corces 1988, Genes and Dev. 2:1205-1215; Spradling, A.C., D.M.Stern, I. Kiss, J.Roote, T. Laverty, and G.M. Rubin 1995, Proc. Nat. Acad. Sci. 92:10824-10830.

Crowley, Thomas E. Department of Biological Sciences, Columbia University, code 2407, 1212 Amsterdam Ave., New York, NY. email: tc45@columbia.edu; phone, 212-854-4835; fax, 212-865-8246. Mutations near the *Trf* cluster cause a premeiotic defect in the *Drosophila* male germ line.

Abstract:

In situ hybridization, P transposase-mediated mutagenesis, and stage-specific markers are used to examine the regulation of expression and function of the *Trf* cluster during spermatogenesis in *Drosophila melanogaster*. The temporal regulation of the presence of the three mRNAs during sperm development is determined, and the effect of various mutations at the *Trf* site on the premeiotic stages is described. The molecular nature of each mutation is then established, and possible functions of *Trf* cluster gene products are discussed, in particular a potential role in cell cycle regulation is suggested.

Introduction:

TBP-Related Factor, or TRF, is a sequence-specific DNA-binding protein in *Drosophila* which resembles the TATA box Binding Protein (TBP) in structure and DNA sequence-specificity (Crowley *et al.,* 1993). TBP binds to the promoter of Pol II transcribed genes in eukaryotic cells in one of the first steps of transcription initiation. In addition TBP plays a critical role in Pol I and Pol III transcription making it a universal general transcription factor (Baumann *et al.,* 1995; Nikolov and Burley, 1994; Burley, 1996). The observed similarity in structure and DNA-binding specificity of TRF and TBP suggested that TRF might be a transcription factor, and in fact the recent findings of Hansen and coworkers (1997) reveal that TRF can substitute for TBP to provide basal level Pol II transcription from an adenovirus or *Drosophila Adh* promoter in an *in vitro* assay which includes the other general transcription factors. They also demonstrate that in the *Drosophila* embryo, TRF is associated with several TRF-Associated Factors (nTAFs) which are distinct from the TBP-Associated Factors and the TRF/nTAF complex can also substitute for TBP to provide basal level transcription in an *in vitro* system. In addition to the *in vitro* experiments, Hansen and coworkers expressed TRF in cultured *Drosophila* cells and observed TRF-mediated activation of a reporter gene by the neurogenic transcription factor, NTF-1.

The fly gene coding for this protein, *Trf*, was discovered in an enhancer-trap screen and cloned by plasmid-rescue via the transposon, P-lacW, which had inserted nearby. Screens of embryo and testis cDNA libraries with genomic DNA probes including 5 kb of sequence on either side of the insertion site revealed that *Trf* is flanked on either side by a gene of unrelated sequence. All three genes are on one side of the insertion and there appear to be no transcripts produced in the embryo or testis from within 5 kb on the opposite side (Figure 1). The transcription reporter in the transposon indicates that this genomic site is transcribed in embryonic neuroblasts which give rise to the CNS, and primary spermatocytes in the adult testis. The transposon insertion at *Trf* causes two phenotypes when homozygous: male-sterility due to a lack of motile sperm and leg-shaking in ether-anesthetized adults (Crowley *et al.,* 1993). This allele is designated P[lacW]Trf for convenience since *Trf* is the best characterized of the three genes, although it is not yet known which gene's expression is actually affected by the insertion. The transcription of the *Trf* cluster in spermatocytes and the male-sterile phenotype of P[lacW]Trf suggest that at least one and possibly all three of the proteins coded at this site play a critical role in spermatogenesis.

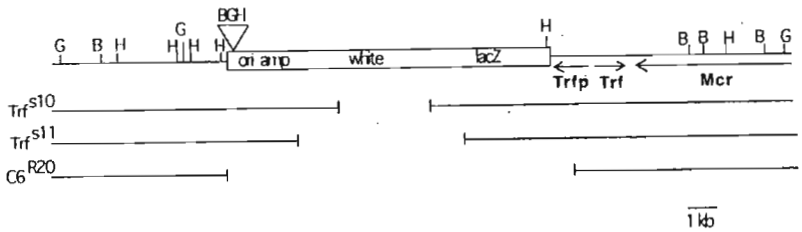

Figure 1. Molecular map of P[lacW]*Trf*. The three transcribed segments are shown and the transposon insertion is depicted as an open rectangle on the left side of the gene cluster. The component genes of P-lacW are indicated within the rectangle. The transcription initiation site for *Mcr* is off the right side of the map, and the direction of transcription for *Trfp* is based on the presence of a 252 codon CDS in a right to left reading of the transcribed segment which is significantly longer than any CDS found in a reading in the opposite direction. The portion of P-lacW removed by Trf^{s10}, Trf^{s11} and $C6^{R20}$ is shown below the map. B = *BamH*I, G = *Bgl*II, H = *Hind*III.

Male germ cell development in *Drosophila* is an excellent system for examining the properties of stem cells, regulation of mitotic proliferation, cell growth, meiosis, alterations in chromatin components, nuclear and cytoplasmic elongation, and the formation of a motile flagellum (Lindsley and Tokuyasu, 1980; Lifschytz, 1987; Fuller, 1993). The study of mutations which cause male-sterility due to inhibition of motile sperm production may shed some light on the mechanisms regulating these processes. To establish the importance of *Trf* cluster expression in spermatogenesis, *in situ* hybridization is used to assay transcription regulation in the male germ cells, P transposase-mediated mutagenesis is used to create new mutations near the *Trf* cluster, and male germ cell-specific markers are used to assay the abundance of the early stages of sperm development in mutant testes. Finally, the molecular nature of each mutation is determined and mechanisms are suggested which may explain how these mutations affect expression of the *Trf* cluster.

Materials and Methods:

Detection of RNA via in situ *hybridization:*

Testes were dissected from *Oregon R* wild type adults, less than 24 h post-eclosion, then fixed in 5% formaldehyde, 0.1 M $NaPO_4$, pH 7.2 for 30 min. Tissue was then washed in 10 mM $NaPO_4$, 140 mM NaCl, pH 7.2 (PBS) four times for 5 min each. The testes were then dehydrated in 1:1 ethanol:PBS for 10 min, followed by four 5 min washes in pure ethanol, and stored at -20°C until hybridization was performed. Proteinase treatment of the tissue, hybridization of the cDNA probe and detection of the signal were carried out as described by Tautz and Pfeifle (1989). The probe was created by labeling a cDNA with digoxigenin-uridine following the supplier's protocol (Boehringer Mannheim Genius Kit).

P element deletion: The source of transposase was the P[ry$^+$ delta 2-3](99B) insertion on the 3rd chromosome described by Robertson *et al.* (1988). This transgene was introduced into the P[lacW]Trf genome by using a *Sp*/CyO; *Sb delta 2-3*/TM6 stock, then removed by standard genetic crosses.

Spermatocyte Labeling with EXU/GFP: An X chromosome carrying a P-element construct which includes the *exu/Gfp* gene fusion was introduced into the *Trf* mutant stock via standard genetic crosses. The expression of the encoded fusion protein, its properties and the method for visualizing it in *Drosophila* oogenesis are described in Wang and Hazelrigg (1994). For images of whole testes, tissue was removed from adults, placed on a slide in a drop of DB, and covered with a coverslip. Images of the fluorescence in the live cells were obtained with a Biorad Confocal Imaging system by performing a z-series, then creating a projection of this series. The z-series consisted of 8 images at 3 μm intervals taken through the 10X objective of the microscope, employing the BHS/fluorescein filter. For individual cysts of primary spermatocytes, the testis epithelium was opened before applying the coverslip in order to allow the premeiotic cysts to flow out. Images were obtained with a conventional fluorescence-detecting microscope using a filter providing 550 nm excitation and 615 nm emission.

Staining for Enhancer-trap Reporter Expression: Testes were dissected from adults in a physiological saline, placed in PBT for 5 min, fixed in 1% glutaraldehyde, 50 mM cacodylate pH 7.3 for 15 min, washed in PBT 3x5 min then stained in X-gal as described in Hiromi *et al.* (1985).

Characterization of Transposase-induced Deletions: Genomic DNA was purified from adult flies, digested with restriction enzymes, and Southern blots performed with standard techniques. The genotypes examined were:
y w; P[lacW]*Trf*
y w; *Trf*$^{\delta 10}$
y w; *Trf*$^{\delta 11}$
and probes consisted of genomic subclones from the *Trf* region or P-lacW plus the *Trf* region which includes the three transcribed segments.

Results and Discussion:

Detailed cytological description of *Drosophila* spermatogenesis can be found in Lindsley and Tokuyasu (1980), Lifschytz (1987), and Fuller (1993), so only a brief summary will be given here. Each testis is a long narrow tube, closed at the end where the germ line stem cells are found (apical), and open at the basal end where mature elongated spermatids pass into the seminal vesicle. As their name indicates, the stem cells divide asymmetrically, regenerating themselves and producing a spermatogonium which then undergoes 4 rounds of mitosis, resulting in a cyst of 16 primary spermatocytes which then enter a 90 h growth period in the absence of cell division. The enlarged primary spermatocytes, each 25 μm in diameter, are now prepared to enter meiosis, which will give rise to clusters of 64 haploid spermatids. The early round spermatids now begin the process of elongation, by which the streamlined mature sperm will be formed, with each batch of 64 sister spermatids held together in a bundle by the two somatic cyst cells which have enclosed the developing cluster of germ cells since just after stem cell division. As development of the sperm proceeds, the cells move through the testis tube from apical to basal end, so that the mitotic spermatogonia are clustered near the apical end while the enlarged primary spermatocytes fill the region 2-27% of the testis length measured from the apical end, and the elongated spermatid bundles fill the remaining volume down to the basal end.

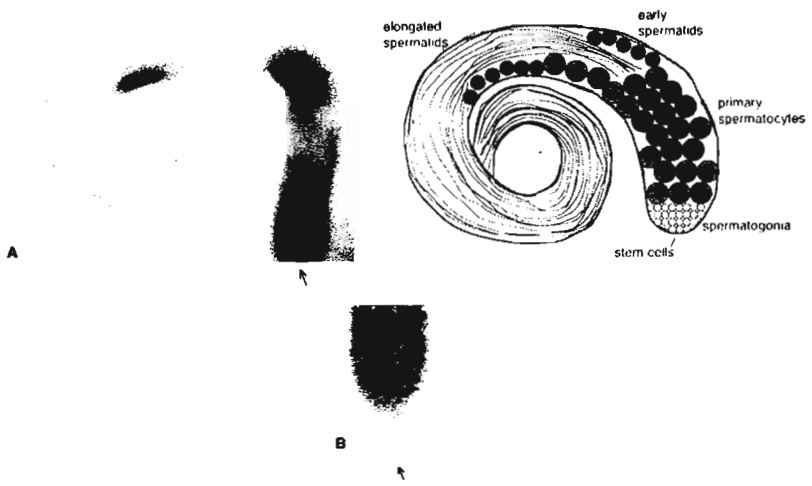

Figure 2. **A,B** Distribution of the *Trf* mRNA in developing germ cells of the adult testis. **A,** Whole testis, in same orientation as the drawing to the right. The apical end is left out of frame in order to include all of the remainder of the tissue. **B,** Higher magnification photo of the apical end of the testis shown in panel **A**.

To determine the temporal and spatial regulation of expression of *Trf* cluster genes during spermatogenesis, a cDNA derived from the *Trf* message was labeled with digoxigenin and hybridized to a whole mount preparation of a wild type testis. As shown in Figure 2, the probe hybridized to RNA in the cytoplasm of the enlarged primary spermatocytes, and this hybridization signal was much stronger than any produced by control probes made of non-*Drosophila* sequence (data not shown). The apical tip of the testis tube, which is the location of the germline stem cells and

the spermatogonial cells going through mitotic divisions, is not stained and there is clearly no signal in the elongated spermatids at the basal end of the testis. Similar results were obtained when cDNAs derived from the mRNAs encoded by the other two genes of the *Trf* cluster were used as probes (data not shown).

Since transcription of the *Trf* cluster in the wild type is restricted to the stage which precedes meiosis, one would expect that the male-sterile effect of the P[lacW]*Trf* transposon insertion would be caused by a defect in the entry into meiosis, meiosis itself, elongation of the haploid spermatids, individualization of the spermatids from the cyst or lack of motility of the mature sperm. Examination of the reproductive tracts of adult males homozygous for P[lacW]*Trf* reveals that no sperm are present in the seminal vesicles. The preelongation spermatogenic stages, such as the early round spermatid, the metaphase I spermatocyte with condensed chromosomes preparing to undergo meiosis, and the enlarged primary spermatocyte all appear unaffected by P[lacW]*Trf* (data not shown).

Since no defects in sperm maturation are visible until long after the time of transcription of the *Trf* cluster, it seems likely that P[lacW]*Trf* is a hypomorph and that stronger *Trf* alleles might show premeiotic defects. The method to test this hypothesis involves inducing deletion of the P-lacW transposon at its insertion site at *Trf* via the introduction of the gene coding for the transposase enzyme (Johnson-Schlitz and Engels, 1993). Flies carrying a copy of the 2nd chromosome which have had a deletion of the transposon are identified by scoring for loss of eye pigment produced by the *white* minigene within P-lacW, and stocks are then established with each mutant chromosome over a balancer. These alleles are designated [w^{-}]*Trf*, although as explained above this is only for convenience and does not imply that associated phenotypes are necessarily due to alterations in expression of *Trf* rather than the other two genes of the cluster. The reproductive tracts of males homozygous for these [w^{-}]*Trf* chromosomes are then examined for defects in spermatogenesis which cause male-sterility. Any new mutant which is male-sterile is then tested for lack of complementation of P[lacW]*Trf* to ensure that the mutation causing sterility is in fact at *Trf* and not somewhere else on the 2nd chromosome. A new lethal is tested for lack of complementation of the $C6^{R20}$ lethal deletion described in Crowley *et al.* (1993), and shown in Figure 1. Mutations which fail to complement the previously characterized *Trf* alleles are designated Trf^{s} for a male sterile or Trf^{l} for a lethal.

Sixty-nine [w^{-}]*Trf* alleles have been generated; one of these is homozygous lethal, 16 cause male-sterility when homozygous and the remainder have no known phenotype. Some of the male-sterile alleles do in fact show more severe spermatogenic defects than P[lacW]*Trf*, in particular Trf^{s10} and Trf^{s11} show an alteration in the distribution of the stages of spermatogenesis. The number, distribution and structure of the premeiotic enlarged primary spermatocytes in testes of Trf^{s11} and control flies was studied by introducing a gene fusion coding for the EXU/GFP fusion protein which provides a fluorescent marker in the cytoplasm of these cells. The *exu* gene, originally identified and studied due to its importance in oogenesis, also has a critical function in the male germ line. Eight of the nine *exu* alleles which have been tested cause sterility in males when homozygous, and immunohistochemistry experiments have shown EXU to be first expressed in the enlarged primary spermatocytes of the germ line and not in the somatic cells of the testis. The *exu/Gfp* fusion contains all the *cis*-regulatory sequence required for proper transcription regulation so that the EXU/GFP fusion protein is expressed in the same pattern as endogenous EXU in the testis, being localized to the cytoplasm of the spermatocytes, and in fact can carry out the function of the endogenous protein as well (Wang and Hazelrigg, 1994).

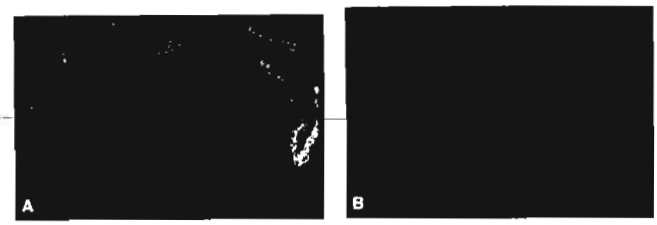

Figure 3. **A,B** Reduction in number of enlarged primary spermatocytes in the adult testis shown by fluorescence of the EXU/GFP marker. **A,** *w exu/Gfp*; **B,** *w exu/Gfp*; Trf^{s11}. The primary spermatocytes are 25 μm diameter spherical cells with a 12 μm diameter nucleus; only the cytoplasm is labeled with EXU/GFP resulting in a donut-like appearance in these photos. The apical end of the testis is in the lower right in each panel, and the variation in shape of the testes is due to the pliability of the tissue and not part of a mutant phenotype.

Confocal imaging of the fluorescently labeled live cells in whole testes reveals that Trf^{s11} reduces the number of enlarged primary spermatocytes to approximately 10% the wild type level (Figure 3), resulting in a cluster of these cells close to the apical end of the testis, but not extending nearly as far towards the basal end as in wild type. The size and shape of these spermatocytes, and the expression of one marker gene, *i.e. exu*, have not been disrupted by this mutation. The testes of the Trf^{s10} allele show a similar phenotype, although not quite as extreme as Trf^{s11} (data not shown).

The reduced number of enlarged primary spermatocytes in [w^{-}]*Trf* testes could be the result of a reduced number of mitotic divisions in the spermatogonial cells which arise from stem cell division, failure of the gonial cells to enlarge after the last (*i.e.,* fourth) mitosis, death of some spermatocytes early in this 90 h stage, or an early entry into meiosis of all the enlarged primary spermatocytes. To address the first possible explanation, a chromosome carrying a reporter construct

known to label the mitotic male germ cells was introduced into the $Trf^{\delta 11}$ genome, and testes from these flies stained to detect reporter expression. As shown in Figure 4, equal numbers of gonial cells are found at the apical end of control and $Trf^{\delta 11}$ testes, indicating that this mutation is not inhibiting mitosis during spermatogenesis.

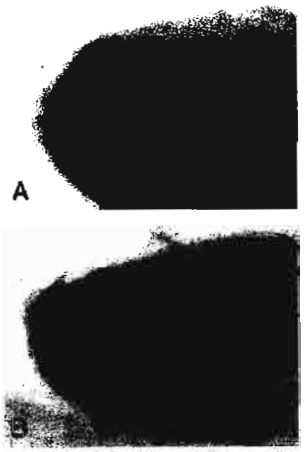

Figure 4. **A,B** Staining of mitotic cells in $Trf^{\delta 11}$ testis. **A,** Apical end of testis from a $S_3$46 adult, fixed and stained for reporter expression as described in Materials and Methods. **B,** Apical end of testis from a $S_3$46; $Trf^{\delta 11}$ adult. $S_3$46 is an X chromosome with an enhancer-trap P element insertion known to give expression in the spermatogonial cells (Gonczy *et al.,* 1992). The reporter gene codes for a fusion protein containing a portion of the P element transposase linked to beta-galactosidase, which results in the enzyme activity being localized to the nucleus of cells in which the reporter is transcribed (Bier *et al.,* 1989).

If the observation made with the enhancer-trap marker of the spermatogonial cells, that $Trf^{\delta 11}$ does not reduce the number of mitotic divisions during sperm development, is correct, then it is expected that each cyst of primary spermatocytes should contain 16 cells which have resulted from the four mitoses after stem cell division. To address this issue, individual cysts of enlarged primary spermatocytes in mutant and control testes were examined under higher magnification than was used previously for examining the entire testis. Again, the cytoplasm of these cells is labeled with the fluorescent EXU/GFP fusion protein; the results are shown in Figure 5. The fluorescence throughout the cytoplasm in these cells provides a clear outline of the nucleus, and by counting nuclei in the images, it is clear that the cysts in the mutant testis contain 16 cells just like the control. This observation indicates that $Trf^{\delta 11}$ does not inhibit the mitotic divisions, consistent with the conclusion drawn from the staining of mitotic cells. These mutant spermatocytes do appear to go through meiosis since orcein-staining of condensed chromosomes in metaphase I nuclei shows the typical set of bivalents, and the spermatids eventually elongate in bundles as in a wild type testis (data not shown).

The loss of eye pigment in the [w^r]*Trf* mutants indicates that at least a portion of the *white* gene has been eliminated from the P-lacW transposon, but provides no further information regarding the nature of these mutations. To establish exactly how much of the transposon and flanking genomic DNA are missing in each [w^r]*Trf* stock, genomic Southern blots were performed as described in Materials and Methods, the results of which are summarized in Figure 1. The original allele P[lacW]*Trf*, has an insertion which is close to the transcribed regions of three genes, but does not interupt any of the transcribed sequence. $Trf^{\delta 10}$ removes 3 kb from the center of P-lacW, while $Trf^{\delta 11}$ eliminates the same 3 kb missing in $Trf^{\delta 10}$ plus some adjacent sequence to give a 4 kb deletion overall. Neither deletion extends beyond the ends of P-lacW, so none of the flanking genomic DNA has been lost. The P[lacW]*Trf* male-sterile phenotype is probably due to alteration in *cis*-acting transcription regulatory sequence at the *Trf* site, and this effect becomes more severe when a portion of the transposon is removed.

The importance of the *Trf* cluster in *Drosophila* sperm development first became clear with the observation that males homozygous for a transposon insertion at this site are unable to produce functional sperm, *i.e.,* no motile sperm are present in the seminal vesicles of these animals. More detailed cytological studies and the generation and examination of more *Trf* mutant alleles traces the function of this gene cluster back to the premeiotic stages of spermatogenesis. As is often the case, a defect in production of a particular type of differentiated cell is due to events which have occured in precursor cells, and it is in these precursor cells where many important developmental decisions are made. In the *Drosophila* male germ line, regulated steps include: stem cell division which determines the rate at which cells enter the sperm development pathway, spermatogonial mitosis which affects the number of primary spermatocytes generated subsequent to each stem cell division, the halt of the cell cycle for 90 h in the primary spermatocyte to allow for the dramatic increase in cell volume, the entry into meiosis, the two meiotic divisions and the beginning of the elongation process in the postmeiotic early round spermatid. The first *Trf* allele, P[lacW]*Trf*, appears to prevent elongated spermatids from entering the seminal vesicles and acquiring motility without affecting regulation of the progression through the developmental stages, while the transposon-deletion alleles whose generation is described in this work are clearly affecting a regulatory decision before meiosis.

The observation that a *Trf* mutation reduces the total number of enlarged primary spermatocytes per testis without reducing the number of spermatogonial cells or primary spermatocytes per cyst, or affecting the production of elongated spermatid bundles, suggests that in this mutant the amount of time the male germ cells spend in the enlarged primary spermatocyte stage has been reduced. Apparently these mutant spermatocytes are entering meiosis before the typical 90 h timepoint after the last mitotic division.

How might alterations in the expression of the *Trf* cluster genes affect the regulation of the primary spermatocyte stage? To address this question the arrangement of the genes of the *Trf* Cluster and the structure and implied function of the encoded proteins must be considered. The insertion in P[lacW]*Trf* lies on one side of the *Trf* cluster, and the cluster is arranged with *Trf* in the middle, being transcribed away from the insertion site, while the most distal gene which produces an 8 kb mRNA, is transcribed towards the insertion. The sequence of the distal gene contains a coding sequence (CDS) whose hypothetical translation shows similarity to several proteins of the mammalian immune system: alpha$_2$Macroglobulin, a serum proteinase-inhibitor, and the complement proteins. The *Drosophila* protein is named Macroglobulin Complement-Related (MCR) and the gene *Mcr*. The transposon-insertion lies 2.8 kb downstream of the 3' end of *Mcr*, so the size of the mRNA transcribed from this gene assures that the transcription initiation site and promoter lie at least 10.8 kb away from the site of mutation in the various *Trf* alleles. This means that effects on *Mcr* expression are the least likely explanation for the observed phenotypes in the *Trf* mutants described in this work, which points to altered regulation of *Trf* and/or the gene most proximal to the insertion site, *Trf-proximal* (*Trfp*), as the probable explanation for the defective male germ cells. Since the TRF protein is a transcription factor, altered expression of *Trf* would probably result in some sort of effect on transcription in spermatocytes, while *Trfp* harbors a CDS for a protein of 252 residues which does not resemble any yet characterized, so it is impossible to predict the consequences of changes in its expression.

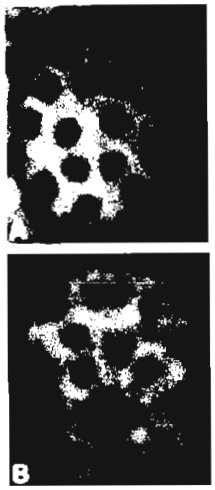

Figure 5. **A,B** Cysts of enlarged primary spermatocytes visualized with the EXU/GFP fluorescent fusion protein. **A,** A cyst from a *w exu/Gfp* testis. **B,** A cyst from a *w exu/Gfp* ; *Trf*^{s11} testis.

The phenotypes created by the *Trf* mutations discussed here are most likely due to a *cis*-regulatory transcription mechanism which is somehow different in these mutants than in wild type. There are several possible explanations for how the P-lacW insertion at the *Trf* site might alter transcription control of the three genes coded there, including: destruction of an endogenous enhancer or repressor by the insertion, displacement of a regulatory sequence (by 10.5 kb) to a point where it can no longer function properly, or fortuitous regulatory action of a sequence within the transposon. Some of the sequence within P-lacW is *Drosophila* sequence, but none of it is normally found at the *Trf* locus. This last possible mechanism for the *Trf* mutation effects is particularly relevant due to the observation that deletions internal to the transposon, *i.e.*, the [w^{r}]*Trf* alleles, result in appearance of a much more dramatic phenotype in the male germ line than was present in P[lacW]*Trf*. The finding that deletions of less than 50% of the transposon sequence generate a new phenotype, hints at the possibility that a sequence within the P-element construct is counteracting the effect of a second sequence within the element (not deleted in the [w^{r}]*Trf* alleles), or the disruption or displacement of an endogenous *cis*-acting sequence described above. Perhaps two effects on transcription of the *Trf* cluster, antagonistic to one another, occur in P[lacW]*Trf*, and when the sequence for one of these effects is removed by deletion as in [w^{r}]*Trf*, only one of the altered regulatory mechanisms remains and its effect is now much more pronounced.

Quantitative Northern blots of testis RNA with cDNA probes for each of the three genes will eventually show if in fact these mutations do affect transcription at this site; however, signals have not been obtained for any of the three genes in preparations from 50 pairs of testes although strong signals for the *exuperantia* transcript (Crowley and Hazelrigg, 1995) were detected on these filters. Apparently the *Trf* cluster RNAs are expressed at a very low level in this tissue and higher specific activity probes or more tissue will be needed to produce detectable signals.

Acknowledgments: I would like to thank Sheng Wang and Tulle Hazelrigg for providing the chromosome carrying *exu/Gfp*, and Stephen Dinardo for the S$_3$46 chromosome carrying the spermatogonial marker. Also, thanks to Bethany Slater for introducing the S$_3$46 chromosome into the *Trf*^{s11} stock.

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Struc. Bio. 1:621-637; Robertson, H.M., C.R. Preston, R.W. Phillis, D.M. Johnson-Schlitz, W.K. Benz, and W.R Engels 1988, Genetics 118:461-470; Tautz, D., and C. Pfeifle 1989, Chromosoma 98:81-85; Wang, S., and T. Hazelrigg 1994, Nature 369:400-403.

Breitmeyer, C., and G. Hocutt. Arizona State University, Tempe, AZ 85287. Alternative feeding sites in desert *Drosophila*: fly-ant interactions.

Host plant specificity has long been the cornerstone of our understanding of the basic ecology of the cactophilic *Drosophila* of the Sonoran Desert (Heed, 1978). Sonoran Desert *Drosophila* use specific host cacti necroses as sites for feeding and breeding. Oviposition and larval development take place within the necrotic tissue while the adults feed on the surface. In the case of one of these species, *D. pachea* (Patterson and Wheeler, 1942), a unique sterol found in the decaying tissue of its host *Lophocereus schottii* (senita), is actually required for successful larval development (Heed and Kircher, 1965).

One assumption made by many investigators is that the necrotic cactus tissue and its microbial flora are the sole nutritional source for the adult flies. However, this necrotic tissue contains many physiologically taxing secondary compounds and, therefore, may not provide an optimum source of nutrition. A source of free sugars, such as those produced by extrafloral nectaries would provide a much richer energy source as well as free amino acids at a lower metabolic cost (Baker *et al.,* 1978). Data on spatial resource availability indicates that rot distribution for these cactophilic species is patchy and for some species suitable substrates are rare (Breitmeyer and Markow, 1998). Sonoran Desert *Drosophila* have the ability to disperse over 2 km in a 24 hour period (Johnston and Heed, 1976; Breitmeyer, unpub.). Flights of this distance would require a significant expenditure of energy. Ganter, Starmer, Lachance and Phaff (1986) hypothesized that non-cactus food sources may be used by dispersing flies. Until now there have been no previous reports of cactophilic *Drosophila* utilizing alternative feeding sites. Here we present observational data indicating that alternative food sources are available to, and are utilized by, at least some of these cactophilic *Drosophila* species.

We observed flies of two species, *D. pachea* and *D. arizonae* (Ruiz, Heed and Wasserman, 1990), feeding at the extra floral nectaries of senita cacti during two separate periods in October 1995 and May 1996 near San Carlos, Sonora, Mexico. Observations were made at each cactus for approximately 10 minutes. Flies were concentrated on the terminal end of a single cactus arm in groups of 5-10 individuals, and these clusters were always associated with a necrosis found on another arm of the same cactus. The observations of *D. arizonae* occurred where its preferred host, *Stenocereus alamosensis*, is absent from the local area. *Drosophila arizonae* is the sister species of the Sonoran desert endemic *D. mojavensis*, both of which are considered generalists. Observed along with the feeding *Drosophila* were large ants of the Genus *Pseudomyrex*. Ants in this genus have been reported to have an association with both barrel and prickly pear cactus nectaries (Pickett and Clark, 1979; Ruffner and Clark, 1986). These ants associated with the extrafloral nectaries of senita were observed to prey upon the *Drosophila* and appeared to be tending the extra-floral nectaries, perhaps feeding on them or promoting nectar flow to bait in more *Drosophila* prey. This behavior is an ant actively chasing a fly found near the nectaries.

With the exception of one cactus, *D. arizonae* were more numerous than *D. pachea* (Table 1). While these numbers are not large they are comparable to the numbers of *D. pachea* aspirated from cactus rots during the same time period. Population sizes of *D. pachea* have been estimated by mark-recapture to be as low as 15-20 individuals (Breitmeyer and Markow, 1998). The individuals observed feeding at the nectaries may account for 5 –20 percent of the *D. pachea* population at a given plant. Additional *Drosophila* have been observed feeding at the nectaries since the initial discovery, but their numbers have not been quantified. When flies were observed at nectaries they were never more than 30 cm away, unless being pursued by an ant. The *Drosophila* did not resort to aerial escape immediately and pursuits lasted 15-30 seconds.

Ant behavior was distributed evenly over the three categories (Table 2). Table 2 does not reflect the amount of time spent in each activity. Although no direct measurements of time spent in each behavior were made, approximately 90 percent of ant behavior was split between tending and guarding with short bouts of pursuit. The prescence of a fly near the nectary did not always illicit a pursuit response. A few *Drosophila* were able to feed successfully and escape unmolested. Two ants were observed with flies in their mouthparts and were scored as pursuing.

Discussion: Because of its ability to detoxify Senita sterols and its unique dependence on one of them for development, *D. pachea* is considered the extreme specialist of the four Sonoran desert endemic *Drosophila*. These data represent the first confirmed use of an alternative feeding site for *D. pachea*, demonstrating that at least one species of cactophilic *Drosophila* is not strictly dependent on the host plant for all its nutritional requirements. The absence of the preferred host of *D. arizonae* from the local area of our observations indicates that this species may use extrafloral nectaries during dispersal. It is reasonable to expect that *D. mojavensis*, the sister species, has these same resources