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Genetics

Ets oncogene-related gene *Elg* functions in *Drosophila* oogenesis

(female sterile mutation/complementation rescue/follicle cells/chorion/chromosome decondensation)

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ABSTRACT **Members of the *ets* gene family encode transcription factors that regulate the expression of a variety of cellular and viral genes including several protooncogenes. We have utilized *Drosophila* to elucidate the *in vivo* function of one family member. We show by complementation rescue and sequence analysis that the female sterile mutant tiny eggs (*tne*) is an allele of the *Drosophila Ets*-related gene *Elg* (also called *D-elg*). The mutation of a highly conserved tyrosine residue in the ETS DNA-binding domain of the *Elg* gene product demonstrates that normal gene function is required for proper follicle cell migration, chorion formation, and nurse cell–chromosome decondensation during *Drosophila* oogenesis.**

The c-*ets* gene family is a group of cellular genes related to the viral v-*ets* oncogene (1, 2). v-*ets* is present, along with the v-*myb* oncogene, in the avian leukemia virus E26 (3). Cellular genes homologous to v-*ets* have been characterized in a variety of organisms ranging from *Drosophila* (*Ets*) to humans (*ETS*) (1). The major conserved sequence found in members of this family encodes the ETS domain, an ≈85 amino acid region that functions as a sequence-specific DNA-binding domain (4). The ETS protein binding site is a purine-rich sequence, containing a G-G-A core (4, 5), that has been identified in the transcriptional control elements of a broad spectrum of cellular and viral genes, including several protooncogenes (1, 2). Numerous studies have shown that members of the *ets* gene family encode proteins that function as transcriptional activators (1, 2). Thus, ETS proteins are an emerging group of regulators that have been implicated in the control of cell growth and differentiation.

Despite the considerable knowledge of the DNA-binding properties of ETS proteins, relatively little is known of the functions of c-*ets*-related genes in normal cellular processes. To the best of our knowledge, the functions of the murine *Ets* genes have yet to be elucidated using the gene knock-out approach (6) that would create, by homologous recombination, *Ets* gene-null mouse lines. Clearly, *Drosophila* is an amenable system for the study of gene function because of the wealth of genetic approaches available. So far, mutants have been obtained in two members of the *Drosophila Ets* gene family. Analyses of the mutant phenotypes have demonstrated that the *yan* gene is involved in photoreceptor development in the compound eye (7), while the pointed (*pnt*) gene functions in the development of the midline glial cells during embryonic nervous system formation (8).

As an additional approach toward elucidating the functions of c-*ets* genes in normal cellular processes, we have initiated the characterization of the *Drosophila Elg* gene (also called *D-elg*) (9, 10). *Elg* is one of seven *Ets*-related genes present in the *Drosophila* genome, and it produces a 2.0-kilobase (kb) transcript that is detected in all stages of fly development (9, 11). During early development, the *Elg* transcript is maternally contributed to the egg and maintains a uniform distribution pattern throughout embryogenesis. The gene maps within the genetically well-characterized 97D region of *Drosophila* chromosome 3 (9, 11). Other genes located in this interval include Toll (*Tl*), a maternal-effect gene required for the establishment of dorsal-ventral polarity in the embryo (12), the histone variant *His2AvD* (13), and rough (*ro*), a homeobox gene required for inductive interactions in the eye (14). In addition, the female sterile mutant tiny eggs (*tne*) has been mapped to the distal half of the 97D region (15).

Based on *Drosophila Elg*'s location in 97D and its expression during early development, we considered the possibility that *tne* represented a mutation in the *Elg* gene. In this report, we correlate the two by rescuing the *tne* phenotype with *Elg* sequences and determining the molecular mutation present in the *Elg* mutant allele. Our analysis of the female sterile phenotype of *tne* demonstrates that normal *Elg* function is required for several important processes of *Drosophila* oogenesis. Overall, these results give us a valuable genetic entrance into the detailed study of an ETS protein that may function as a transcriptional regulator in egg chamber development and cellular differentiation.

MATERIALS AND METHODS

***Drosophila* Strains.** The 97D-deficiency stocks *Df(3R)Tl*^{9QRX} [Df(3R)97B;97D1,2] (12), *Df(3R)ro*^{XB3} [Df(3R)-97D1-2;97D9] (14, 15), and *Df(3R)ro*^{80b} [Df(3R)97D1;97D15] (15) and the female sterile stock tiny$^{4.14}$ were obtained from Kathryn Anderson (University of California, Berkeley). Note that "tiny" was the original name for this female sterile mutation (16) but has since been renamed "tiny eggs" (*tne*) (15). The *y w*^{67c23} strain was utilized as our "wild-type" stock for the molecular and phenotypic analyses described in this report.

Southern Analysis. Genomic DNA was isolated from adult flies by using a standard protocol described by Ashburner (17), and Southern analysis was by published procedures (18). The *Elg* probe was generated by using the elg^{M} fragment (9) of the pcD-elg cDNA (10). A 1.2-kb *Hin*dIII fragment from the 5′ flanking region of the *mex1* gene (19) was used as a quantitative reference probe for the *Elg* copy number determination. Membranes were exposed to XAR-5 film (Kodak) for autoradiography or analyzed with a Molecular Dynamics PhosphorImager system to quantitate hybridizing DNA bands.

***In Situ* Hybridization to Whole-Mount Ovaries.** *In situ* hybridization of the digoxigenin-labeled elg^{M} DNA probe to ovaries isolated from wild-type adults was as described by Tautz and Pfeifle (20) with minor modifications. For tissue fixation, ovaries were fixed in freshly prepared 4% paraformaldehyde without heptane. Ovaries were fixed in this manner to optimize staining in both the germ-line nurse cells and somatic follicle cells. For random prime probe synthesis, *Elg* template DNA was incubated with a 10-fold greater concentration of the random hexamer primers to reduce probe size.

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The *Drosophila* bicoid gene was used in parallel in the *in situ* experiment as a control probe; bicoid transcripts are detected in a spatially restricted manner during oogenesis (21). After staining, the ovaries were dehydrated in xylene, mounted in Permount (Fisher), and photographed with a Nikon Diaphot microscope equipped for differential interference contrast microscopy.

Germ-Line Transformation and Complementation Rescue. A 5.4-kb *Hin*dIII–*Bam*HI *Drosophila Elg* genomic fragment (10) was cloned into the CaSpeR transformation vector (22) and stably introduced into the *Drosophila* genome by *P* element-mediated germ-line transformation (23). The $y\ w^{67c23}$ strain was used for embryo injections and *Elg*-CaSpeR-transformed flies were identified by w^+ selection. Multiple transformant lines were obtained, and strains with the *Elg* sequence integrated on chromosome 2 were identified by chromosome segregation away from the *CyO* balancer. Males from two different transformant lines homozygous for the *Elg* transposon on chromosome 2 were mated to *tne, ru st e ca/TM3, Ser* females. All progeny from this cross should contain a single copy of the *Elg* transposon, with about half of the progeny heterozygous for the mutant allele. *tne* heterozygous males were scored as non-*Ser* and were crossed to $Df(3R)ro^{80b}$, *st e/TM3, Sb* females. About half of the progeny from this cross should possess a $tne/Df(3R)ro^{80b}$ genotype, while a quarter of the progeny should carry the chromosome 2-linked *Elg* sequence in the hemizygous mutant background. Hemizygous females were selected as *st e*, non-*Sb* flies and were tested for fertility by mating to $y\ w^{67c23}$ males.

PCR Amplification and Sequence Analysis. The *Drosophila Elg* gene was cloned from genomic DNA obtained from $y\ w^{67c23}$ or $tne/Df(3R)ro^{80b}$ adults in three overlapping fragments using PCR amplification. Three separate PCR reactions were carried out with the following primer pairs: fragment A, primers 31 (GCCCTGTACCAACGCC) and 26 (GTGCATGTGTCCATTCG); fragment B, primers 11 (GCCGCTCAGCATGCTCA) and 25 (GCACTACGGACACGAAA); and fragment C, primers 14 (CTGCCCCGTGATCCAGG) and 22 (GAGCTTCAGCTCGCCAT). Reactions contained 10 ng of genomic DNA, 300 ng of each primer, and components of the GeneAmp PCR reagent kit (Perkin–Elmer/Cetus) as specified by the kit instructions. Forty amplification cycles [94°C (97°C for the first four cycles), 1 min; 60°C, 0.5 min; 72°C, 2 min] were performed, and the resulting products were cloned into the PCRII vector (Invitrogen). The subcloned amplified DNA fragments were sequenced by using the Sequenase kit (United States Biochemical) and synthetic *Elg* primers (Genosys, The Woodlands, TX). To confirm the point mutation observed in the *tne* allele, a second amplification, subcloning, and sequencing of *Elg* fragment C was accomplished.

Mutant Phenotype Analyses. For chorion analysis, eggs were collected from $y\ w^{67c23}$ or $tne/Df(3R)ro^{80b}$ mothers on yeasted grape juice–agar plates, washed with PBT (phosphate-buffered saline with 0.1% Tween-20), mounted directly in Hoyer's medium (17), and photographed with a Nikon Optiphot microscope equipped with dark-field optics. For egg chamber analysis, ovaries were dissected from wild-type or mutant mothers in *Drosophila* Ringer's solution (17) and fixed in 4% paraformaldehyde in PBT. After rinsing with PBT, the tissues were stained with 0.1 μg of fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma) per ml for 20 min. The ovaries were rinsed with PBT, stained with 1 μg of Hoechst 33258 (Sigma) per ml for 5 min, rinsed again with PBT, and examined with a Zeiss 18 microscope equipped with filter sets 10 and 02 for FITC and Hoechst 33258 detection, respectively.

RESULTS AND DISCUSSION

Cytological Mapping of the *Drosophila Elg* Gene. Both the *Elg* gene and the *tne* mutant have been mapped to the 97D region of chromosome 3 (9, 11, 15). *tne* is located in the 97D9-15 interval, as it is uncovered by $Df(3R)ro^{80b}$, which deletes sequences from 97D1 to 97D15, but not by $Df(3R)ro^{XB3}$, which deletes sequences from 97D1-2 to 97D9 (Fig. 1*C*). To more precisely map the location of the *Elg* gene, we initiated a Southern analysis of genomic DNAs isolated from *Drosophila* stocks that carried deletions in the 97D region. DNAs obtained from $Df(3R)T1^{9QRX}$, $Df(3R)ro^{XB3}$, and $Df(3R)ro^{80b}$ flies were analyzed for the number of copies of the *Elg* gene present within each strain. Fig. 1*A* shows that *Elg* was present in two nondistinct copies in $Df(3R)T1^{9QRX}$ (lane 1), two heterozygous copies in $Df(3R)ro^{XB3}$ (lane 2; see also Fig. 1*B*, lane 2), and one copy in $Df(3R)ro^{80b}$ (lane 3).

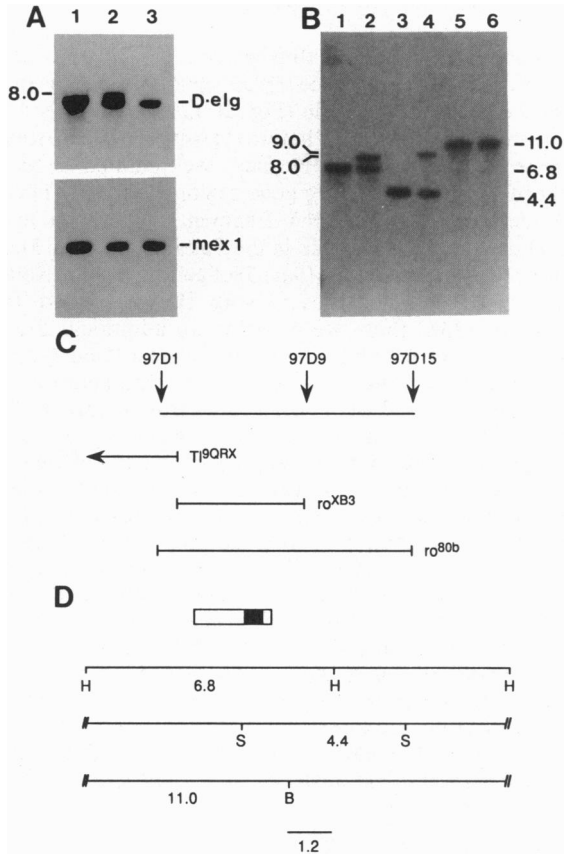

FIG. 1. Southern analysis of the *Drosophila Elg* gene region. (*A*) Determination of the *Elg* copy number in genomic DNAs from the 97D-deficiency strains. $Df(3R)T1^{9QRX}$ (lane 1), $Df(3R)ro^{XB3}$ (lane 2), and $Df(3R)ro^{80b}$ (lane 3) DNAs were digested with *Hin*dIII and hybridized with *Elg* or *mex1* probes. "D·elg" indicates the expected 6.8-kb *Drosophila Elg* DNA, 8.0 indicates the size of the polymorphic *Elg* fragment in the $Df(3R)ro^{XB3}$ DNA, and "mex1" indicates the 1.2-kb *mex1* control DNA band. (*B*) Chromosomal alteration in the *Drosophila Elg* gene region. Genomic DNA from control flies was digested with *Hin*dIII, *Sac* I, or *Bam*HI (lanes 1, 3, and 5) and from $Df(3R)ro^{XB3}$ flies was digested with *Hin*dIII, *Sac* I, or *Bam*HI (lanes 2, 4, and 6) and hybridized with an *Elg* probe. Sizes of the wild-type *Elg* DNAs are given on the right; polymorphic DNAs are indicated on the left. (*C*) Schematic of the 97D-deficiency chromosomes. Deleted sequences are indicated by the horizontal bars; corresponding chromosomal breakpoints are indicated by arrows on the top line. (*D*) Map of the *Drosophila Elg* region. The *Elg* gene is indicated with the elg^M probe shown in black. The sizes of hybridizing *Elg* fragments from digests of control DNA are indicated. H, *Hin*dIII; S, *Sac* I; B, *Bam*HI. The 1.2-kb region marks the site of the DNA alteration in the $Df(3R)ro^{XB3}$ chromosome.

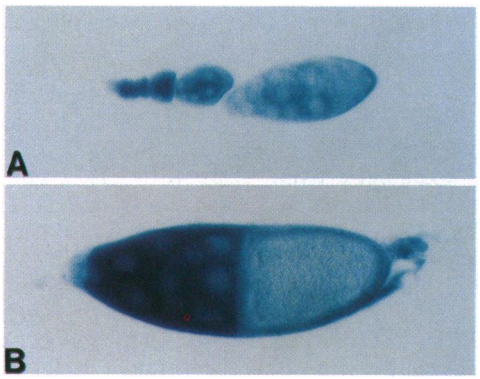

FIG. 2. *Drosophila Elg* expression during oogenesis. *Drosophila Elg* mRNA was localized in wild-type ovaries by *in situ* hybridization with a digoxigenin-labeled *Elg* cDNA probe during early oogenesis (*A*) and in a stage 10B egg chamber (*B*).

This quantitative analysis thus placed *Elg* along with *tne* within the distal part of the *Df(3R)ro*^{80b} deficiency in the 97D9-15 chromosome region (Fig. 1*C*).

To further investigate the heterozygosity of the *Drosophila elg* gene in the *Df(3R)ro*^{XB3} flies, we initiated a second Southern analysis of the *Elg* gene region (Fig. 1*B*). The *Elg* probe detected unique DNA fragments of 6.8 kb in the *Hin*dIII digest (lane 1), 4.4 kb in the *Sac* I digest (lane 3), and 11.0 kb in the *Bam*HI digest (lane 5) of genomic DNA isolated from *+/TM3* control flies. Using DNA isolated from *Df(3R)ro*^{XB3}*/TM3* flies, we detected an additional *Elg* hybridizing band of 8.0 kb in the *Hin*dIII digest (lane 2) and of 9.0 kb in the *Sac* I digest (lane 4). The same heterozygous pattern was observed when using DNA from *Df(3R)ro*^{XB3}*/+* animals, obtained from an outcross of the deficiency stock to wild-type flies (data not shown). The presence of two *Elg*-hybridizing bands in the *Hin*dIII and *Sac* I digests, coupled with a normal *Bam*HI pattern (lane 6), identified a genomic DNA alteration in the *Df(3R)ro*^{XB3} chromosome that mapped between the *Bam*HI and *Hin*dIII sites 3′ of the *Elg* gene (Fig. 1*D*). It is possible that this alteration corresponds to the distal breakpoint of the *Df(3R)ro*^{XB3} deletion, thus allowing us to tentatively map the *Elg* gene to the 97D9 region.

***Elg* Expression During Oogenesis.** Based on the female sterile phenotype of *tne* mutant mothers, we examined the pattern of *Elg* gene expression during oogenesis. Elg transcripts accumulated early in oogenesis with initial RNA staining observed in the posterior part of the germarium and in very young egg chambers (Fig. 2). Thereafter, *Elg* RNA was detected in all stages of egg chamber development in germ-line nurse cells and somatic follicle cells. Previous studies have shown this RNA was maternally contributed to the egg with the RNA globally distributed in the early embryo (9). Thus, *Elg* expression during oogenesis and early embryogenesis was consistent with a potential function of the gene in female fertility.

Table 1. *Drosophila Elg* DNA can rescue the *tne* female sterile phenotype

Rescue construct	Fertility*	Molecular confirmation†
None	0/25 (0%)	NA
5.4-kb *Elg* DNA		
Construct 1	23/36 (64%)	10/10 (100%)
Construct 2	21/32 (66%)	10/10 (100%)

*Fertility test of *tne/Df(3R)ro*^{80b} females in the absence or presence of the introduced 5.4-kb *Elg* DNA integrated at two different sites (Construct 1 or 2) on chromosome 2.
†Southern analysis of DNA obtained from adult progeny of fertile *tne/Df(3R)ro*^{80b} females to confirm the presence of the *Elg* rescue sequence. NA indicates that progeny were not available.

***Elg* Rescue of the *tne* Female Sterile Phenotype.** To directly test the hypothesis that *tne* was a mutant allele of the *Elg* gene, we attempted a complementation rescue of the *tne* female sterile phenotype with a transposed *Elg* genomic sequence. This 5.4-kb DNA included the complete *Elg* transcription unit along with 3.0 kb of *Elg* 5′ flanking sequence. The *Elg* sequence integrated on chromosome 2 was crossed into a *tne/Df(3R)ro*^{80b} genetic background. Complementation was assessed by mating *tne* females with wild-type males. Based on the genetic crosses, about 50% of these females would carry a single copy of the *Elg* sequence in the hemizygous mutant background, allowing a possible rescue to fertility, and about 50% would fail to carry a copy of the *Elg* sequence, precluding any rescue possibility.

The *tne* mutation resulted in a fully penetrant female sterile phenotype as 100% of the hemizygous females tested failed to generate progeny (Table 1). Most females did lay tiny eggs that failed to initiate embryogenesis. The introduction of the 5.4-kb *Elg* DNA into the *tne* mutant background resulted in fertility in >60% of the animals in both rescue experiments (Table 1). Adult progeny from the fertile females were then analyzed for the presence of the introduced *Elg* sequence by Southern analysis. All 10 pools of progeny from both rescue experiments yielded DNA fragments corresponding to the

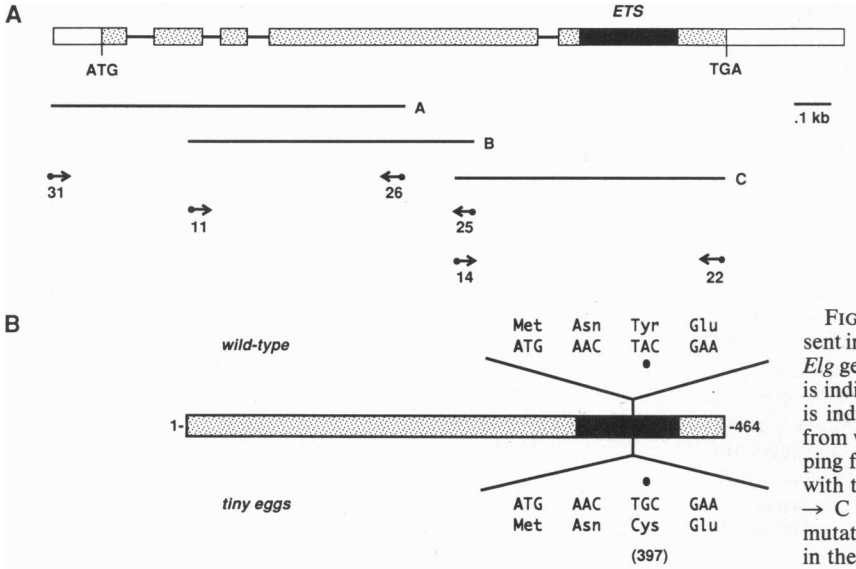

FIG. 3. Determination of the molecular mutation present in the *tne* allele of *Drosophila Elg*. (*A*) The *Drosophila Elg* gene is schematized at the top; the open reading frame is indicated by the stippled region, while the ETS domain is indicated by the black box. The *Elg* gene was cloned from wild-type and *tne/Df(3R)ro*^{80b} flies in three overlapping fragments (lanes A, B, and C) by PCR amplification with the primer pairs indicated. (*B*) Identification of an A → C base change in codon 397 of the *Elg* gene. This mutation changes Tyr-397 in the wild-type gene to cysteine in the *tiny eggs* allele.

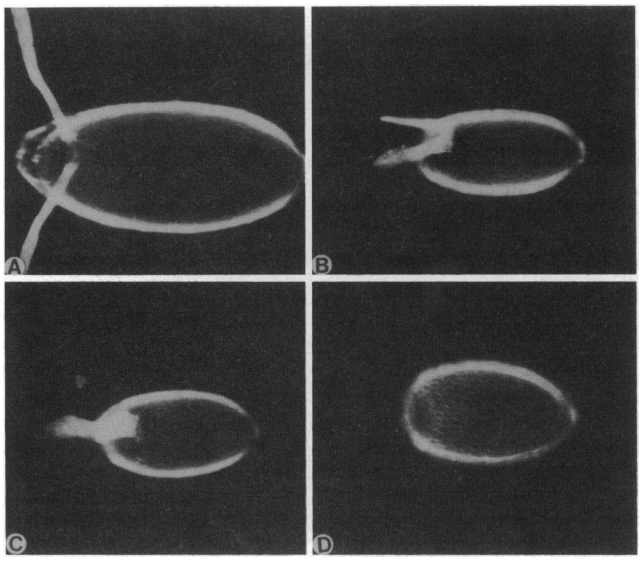

FIG. 4. Comparison of chorions obtained from eggs laid by wild-type and *tne* mutant mothers. (*A–D*) Dark-field micrographs of chorions from wild-type (*A*) and *tne* (*B–D*) eggs.

endogenous and introduced *Elg* genes. Thus, 100% of the rescued hemizygous mothers assayed transmitted the *Elg* transposon (Table 1).

Determination of the *tne* Molecular Mutation. These results showed that the 5.4-kb *Elg* DNA could rescue the *tne* female sterile phenotype. To prove that *tne* was a mutant allele of *Elg* and to determine the molecular mutation present in this allele, we cloned *Elg* genes from wild-type and hemizygous mutant flies and compared their protein coding sequences (Fig. 3). In the *tne* allele, an A → G base change was observed in codon 397, generating a cysteine residue instead of the tyrosine residue found in the wild-type gene. This specific mutation was the only change found in the 464-amino acid open reading frame of the *Elg* gene. Tyr-397 resides in the ETS DNA-binding domain and a survey of ETS proteins revealed that this amino acid was completely conserved, being present in all *Drosophila* and mammalian ETS proteins characterized to date (1, 11). The possibility of this invariant tyrosine residue being a phosphorylation target in ETS proteins remains to be investigated.

Abnormal Oogenesis in *tne* Mutant Females. We next initiated an analysis of the *tne* female sterile phenotype. Females of the hemizygous genotype laid eggs that were about half the size of eggs obtained from wild-type mothers, as was evident from the comparisons of chorions prepared from eggs laid by the different mothers (Fig. 4). Additionally, most eggs obtained from *tne* mothers showed a defect in chorion formation. This variable chorion phenotype ranged from eggs with reduced (Fig. 4*B*) or fused (Fig. 4*C*) dorsal appendages to eggs that had completely open-ended chorions lacking the dorsal appendages (Fig. 4*D*). A group of mutations that cause similar variable abnormalities in dorsal appendage formation have been identified in a screen for female sterile loci mapping on the second chromosome (24). It has been suggested that such defects are a result of mutations that interfere with the normal patterns of follicle cell migration in egg chamber development.

Based on this observation, we analyzed the pattern of follicle cell migration in egg chambers obtained from the ovaries of wild-type and *tne* flies by staining for filamentous actin and DNA. In the early stages of normal oogenesis, the follicle cells surround the nurse cell–oocyte complex in a uniform monolayer (25). During stages 8 and 9, about 95% of the follicle cells take on a columnar morphology and migrate posteriorly to cover the oocyte. About 5% of the cells remain over the nurse cell cluster and assume a flattened morphology. By stage 10B, a clear spatial arrangement of the two sets of cells is seen as the boundary of the anterior and posterior follicle cells aligns precisely with the boundary of the posterior nurse cells and the oocyte (Fig. 5 *A*, *C*, and *E*). Thereafter, centripetal follicle cells will migrate inward from the posterior group to cover the anterior end of the oocyte; some of these cells will be involved in forming the dorsal appendages.

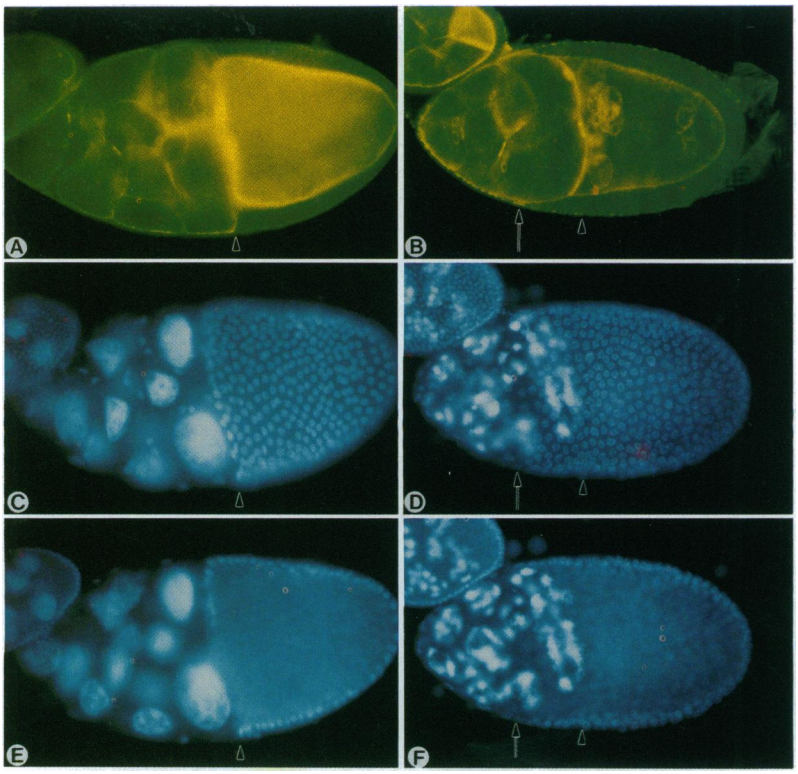

FIG. 5. Staining patterns of filamentous actin and DNA in wild-type and *tne* mutant egg chambers. (*A*) Phalloidin staining of a wild-type stage 10B egg chamber showing the alignment of the anterior-most columnar follicle cells along the nurse cell–oocyte boundary (arrowhead). (*B*) Phalloidin staining of a mutant stage 10B egg chamber showing the abnormal positioning of the columnar follicle cells over the nurse cells (arrow) relative to the nurse cell–oocyte boundary (arrowhead). (*C–F*) Hoechst 33258 DNA staining patterns of the wild-type (*C* and *E*) and mutant (*D* and *F*) egg chambers as viewed from surface and sagittal focal planes.

By staining egg chambers from *tne* mothers, it was clear that the *Elg* mutation caused a defect in follicle cell migration (Fig. 5 *B*, *D*, and *F*). While the exact position of the anterior–posterior follicle cell boundary can vary from one stage 10 egg chamber to the next, we often observed egg chambers with columnar follicle cells surrounding the most posterior nurse cells. In the example shown, about eight rows of follicle cells have failed to complete their migration to a position over the oocyte, and these cells have established an abnormal positional boundary. The location of this boundary coupled with an apparent failure of the centripetal follicle cells to migrate between the nurse cells and oocyte could account for the generation of an open-ended egg shell (24).

These studies also revealed an abnormal nurse cell chromosome morphology within the *tne* egg chambers. During normal oogenesis, the nurse cell chromosomes undergo several rounds of endomitotic doublings, eventually reaching a haploid DNA value of 1024C (25). Through egg chamber stages 3–5, the polyploid chromosomes show somatic pairing with distinct chromosome arms visible within the nucleus. By stage 6, the homologous chromosomes asynapse and continue replicating, with nurse cell nuclei of this and later stages showing a dense, amorphous DNA staining (Fig. 5*E*). In the mutant egg chambers, it appeared that the nurse cell chromosomes failed to decondense, exhibiting a bulbous DNA staining pattern as observed in Fig. 5*F*. An analysis of nurse cell nuclei of younger egg chambers clearly revealed the staining of five large masses and one small DNA mass, presumably corresponding to individual *Drosophila* chromosomes and chromosome arms (data not shown).

A final, less understood mutant phenotype was also observed in these studies. The staining of *tne* egg chambers for filamentous actin showed the proper formation of the ring canals in the nurse cell–oocyte complex (Fig. 5 *A* and *B*). However, this analysis revealed the staining of "spherical vesicles" of an unknown origin in the oocytes of the mutant egg chambers. This finding may be related to the decreased staining of the actin-rich layer around the oocyte periphery and the nurse cell junctions. Alternatively, this unusual staining may be a consequence of the breakdown of nurse cell nuclei in the mutant egg chambers. Further ultrastructural studies are required to better assess this phenotype.

Concluding Remarks. We have demonstrated that *tne* is a mutant allele of the *Drosophila Elg* gene. Normal *Elg* function is required for proper follicle cell migration, chorion formation, and nurse cell chromosome decondensation. The Tyr-397 → Cys mutation in the conserved DNA-binding domain suggests that the *Elg* gene product is functioning as a transcription factor in *Drosophila* development.

Our experimental findings on *Drosophila Elg* and the results of others on the *Ets*-related genes *yan* (7) and *pnt* (8) demonstrate the utility of the *Drosophila* system in elucidating the functions of c-*ets* genes in normal cellular processes. Studies on these genes could add significantly to our knowledge of ETS proteins as transcriptional regulators in cellular differentiation. Specifically, one could take advantage of the available genetics to position each gene within genetic regulatory hierarchies that control either egg chamber, photoreceptor, or midline glial cell development; such studies may provide important insights relevant to the elucidation of the functions of ETS proteins in mammalian systems.

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