Characterization of the Ets-Family Transcription Factor Ets98B in *Drosophila*

by

Beth E. W. Parlikar

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for
the Degree of Master of Science in Biology

at the
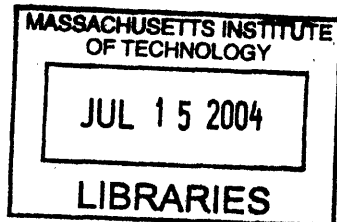
Massachusetts Institute of Technology

June 2004

Signature of Author.. ....

Department of Biology
May 21, 2004

Certified by...

Dr. Ilaria Rebay
Associate Professor of Biology
Thesis Supervisor

Accepted by...

Dr. Alan Grossman
Co-Chair, Committee on Graduate Students
Department of Biology

ABSTRACT

The Ets family of transcription factors is conserved throughout metazoans and plays key roles in normal development and oncogenesis. Prostate-Derived Ets Factor (PDEF), a recently identified family member in humans, is expressed in a subset of normal epithelial tissues in adults. Its expression appears to be altered in many prostate and breast cancers, but its basic biology and normal role in development remain uncharacterized. *Ets98B* appears to be the *Drosophila* homolog of *PDEF*. Ets98B is highly homologous to PDEF in its conserved domains and was found to be expressed in similar tissue types. In order to begin to elucidate the functional characteristics of Ets98B, the phenotypes caused by RNA interference of the *Ets98B* message were analyzed. These phenotypes, as well as expression patterns, point to a role for Ets98B in wing development. Transcription assays in cultured cells suggest that Ets98B can function as a transcriptional activator. In the future it will be interesting to further explore the phenotypes associated with loss and overexpression of *Ets98B* and explore the possibility of connections to signaling pathways. In order to further explore the protein's transcriptional control properties, it may be worthwhile to use chromatin immunoprecipitation to identify target genes, as well as perform structure-function analysis to map the transactivation domain(s).

INTRODUCTION

Ets family transcription factors are found throughout metazoans and play essential roles in cell proliferation, differentiation, survival and migration (Sharrocks 2001). In addition to their diverse roles in development, Ets genes are of great interest because many have been shown to play roles in human oncogenesis. All members of this family contain the ETS DNA binding domain, a highly conserved 85 amino acid structure that recognizes the core consensus sequence 5'-GGAA/T-3' (Karim et al. 1990). Some family members also contain a Pointed/SAM domain (PD), which generally functions in protein-protein interactions (Baker et al. 2001). Many Ets proteins have been shown to be downstream elements of signal transduction cascades and often function in conjunction with members of other transcription factor families. Transcriptional targets of mammalian Ets proteins include genes important for cell cycle, apoptosis, tumorigenesis, signal transduction and differentiation (Sementchenko and Watson 2000).

Drosophila is a powerful model system in which to study the roles and regulation of Ets family members. While eight Ets genes have been identified in the *Drosophila* genome, most have not yet been substantially characterized. Of the Ets genes that have been studied in considerable detail, two have been shown to perform key developmental roles downstream of receptor tyrosine kinases. Yan, a transcriptional repressor functioning downstream of several RTK pathways, blocks premature differentiation of multiple cell types and acts antagonistically in many contexts to the transcriptional activators encoded by *pointed* (*pnt*). Phosphorylation of Yan by MAPK results in its inactivation, export from the nucleus, and presumable degradation (Rebay and Rubin 1995, Tootle et al. 2003). Conversely, the activity of the Pointed P2 isoform is positively regulated by MAPK phosphorylation, while Pointed P1 is a constitutive activator (O'Neill et al. 1994). *yan* and *pnt* thus exemplify many common characteristics of Ets family members: they have key roles in development, function downstream of signaling pathways, and are carefully regulated in their expression and activity.

Ets family proteins have been implicated in many types of cancer (Dittmer and Nordheim 1998). Indeed, the first Ets protein identified was an oncoprotein transduced by the E26 avian retrovirus, consisting of part of the chicken *myb* gene fused to full-length chicken *ets1* (Leprince et al. 1983). Several Ets genes are targets of translocations implicated in leukemias and Ewing tumors. Many of these translocations result in the formation of chimeric proteins in which an oncoprotein is fused to a portion of the Ets protein including the ETS domain (Sorensen et al.

1994). In other instances the Pointed domain of TEL, the human ortholog of Yan, is fused to a kinase involved in signal transduction such as Abl or PDGF-Rβ. Homodimerization via the TEL PD is thought to lead to activation of the associated kinase domains in these cases (Golub et al. 1996). In many other instances Ets proteins are implicated in cancer but are not involved in translocations. Instead, the expression of these proteins is up- or down-regulated in tumors and often seems to correlate with invasive potential; for example, several Ets proteins are overexpressed in breast cancers (Chang et al. 1997, Galang et al. 2004). Furthermore, some Ets proteins have been demonstrated to regulate the expression of genes involved in invasion and angiogenesis, such as urokinase-type plasminogen activator (uPA) and collagenase I (Schneikert et al. 1996). Finally, several Ets proteins are known to be phosphorylated by MAPK; it has been suggested that activation or repression of Ets proteins by activated Ras may contribute to oncogenesis (Dittmer and Nordheim 1998).

A novel Ets family member termed PDEF was recently identified as an EST from a subtracted human prostate hyperplasia cDNA library and as a cDNA from a prostate carcinoma cell line (Oettgen et al. 2000, Nozawa et al. 2000). Its expression in normal tissues seems to be limited to epithelial cells of the prostate, breast, colon and ovary (Oettgen et al. 2000). Curiously, while *PDEF* mRNA was shown by several techniques to be overexpressed in prostate cancer samples and cell lines (Mitas et al. 2002), the PDEF protein is expressed in normal samples yet absent from cancerous samples and transformed cell lines (Nozawa et al. 2000, Tsujimoto et al. 2002). Subsequent studies have found similar results with respect to breast cancer (Mitas et al. 2002, Ghadersohi and Sood 2001, Feldman et al. 2003, Galang et al. 2004). Thus far there is no proposed explanation for these observations.

Recent experiments in human breast cancer cell lines are consistent with a role for PDEF in suppressing tumorigenesis and metastasis. Expression of PDEF leads to decreases in proliferation and invasiveness, activates transcription from the promoter of the tumor suppressor *maspin*, and downregulates *uPA* expression (Feldman et al. 2003). On the other hand, Oettgen et al. (2000) showed that PDEF binds to sites in the prostate-specific antigen (PSA) promoter and can drive its expression alone or synergistically in combination with the androgen receptor. As increased PSA expression is associated with cancer progression, this suggests that PDEF might promote oncogenesis rather than inhibit it. Furthermore, the tumor suppressor NKX-3.1 antagonizes PDEF's ability to drive PSA expression in transcription assays, supporting the

notion that PDEF may function as an oncogene (Chen et al. 2002). However, it is quite possible that PDEF's regulation of PSA expression has no causal role in cancer progression, in which case the weight of the evidence favors the interpretation that PDEF is a potential tumor suppressor.

The *Drosophila* ortholog of *PDEF* is *Ets98B* (Figure 1) (Hsu and Schulz 2000). As with many of the Ets genes, very little has been known about *Ets98B* apart from its embryonic expression pattern. In situ hybridization studies indicated that the mRNA is expressed broadly in embryos and enriched in the pole cells, or germ cell precursors (Chen et al. 1992). The function of *Ets98B* has been investigated only in embryos, where knockdown of *Ets98B* expression via transgenic RNA interference shows it to be required for migration of the pole cells to the developing gonad (Hsouna et al. 2003). In light of its potential relevance to human *PDEF*, experiments were undertaken to study *Ets98B*'s expression, mutant phenotypes, and transcriptional properties. Immunofluorescent staining indicates that, similar to PDEF, Ets98B is found in the nuclei of embryonic epithelial cells, in addition to pole cell nuclei. In larvae the protein seems to be expressed in midgut epithelial cells and perhaps in the brain and eye discs, while the mRNA is strongly expressed in parts of the gut and wing imaginal discs, with lower levels of staining in other discs. Patterning defects were observed in the wing when *Ets98B* expression was knocked down using transgenic RNAi, suggesting that it plays an important role in the development of this organ. Finally, transcription assays showed that like PDEF, Ets98B can activate transcription of reporter genes, but gave mixed results on the potential for regulation by Ras/MAPK signaling. The results of these experiments will hopefully inform future studies of both *Ets98B* and *PDEF*.

RESULTS AND DISCUSSION

Determination of *Ets98B* expression pattern

Ets98B mRNA has previously been shown to be expressed throughout the embryo during germband extension, with enrichment in the pole cells (Chen et al. 1992). However, there was no published report of the subcellular localization or embryonic expression pattern of Ets98B protein. The expression pattern in larvae was also unknown, although Northern blotting indicated that the transcript was present in third-instar larvae (Chen et al. 1992). In order to investigate the expression of Ets98B protein, an antibody raised against the protein was obtained from collaborators and used for immunofluorescent staining of both embryos and larvae. Despite experimenting with a number of different conditions, these experiments did not produce very clear results, probably due to the poor quality of the antibody. In embryonic stages 6 through 9 the antibody detected protein in the nuclei of epithelial cells, and staining was evident in the pole cell nuclei at stages 8 and 9 (Figure 2). In larvae, staining was seen in the midgut epithelial cells, and possibly also in the brain and eye discs (data not shown). No staining above background levels was detected in wing discs.

In order to further investigate the expression pattern of *Ets98B*, in situ hybridization was performed on dissected larvae (Figure 3). Strong and specific staining was found in the wing pouch of the wing imaginal disc, which gives rise to the adult wing blade. In general, staining appeared to be restricted to the ventral half of the pouch. A small number of wing discs exhibited staining in other regions, for example in the presumptive notum or hinge. Variable staining was found in eye imaginal discs; some discs showed staining only in the morphogenetic furrow, while others also had strong staining in various regions of the disc. In contrast, staining in the midgut was always strong, consistent with the detection of Ets98B protein in this tissue by immunofluorescence.

Phenotypes associated with knockdown of *Ets98B* expression

In order to study the biological roles of *Ets98B* in fly development, experiments were performed to look for phenotypes associated with loss of its expression. At this time no mutant alleles or deficiencies were available for *Ets98B*, and thus the technique of transgenic RNA interference was used to "knock down" the gene, i.e. to decrease the abundance of its mRNA transcript (Figure 4A) (reviewed in Hannon 2002). This technique utilizes a transgene which encodes an inverted repeat of a portion of the target coding region. The RNA transcript of this

inverted repeat is predicted to form a hairpin structure. The RNA interference machinery recognizes this structure and cuts it into short double-stranded segments called short interfering RNAs, or siRNAs. These siRNAs mediate the destruction of the homologous endogenous mRNA. By putting the transgene under the control of GAL4 UAS elements, the timing and location of expression of the transgene can be controlled using the binary GAL4/UAS system (Brand and Perrimon 1993).

Based upon the reliable detection of strong expression in the wing it was hypothesized that *Ets98B* might be important in the development of this organ. In looking for phenotypes associated with loss of *Ets98B* function, a variety of GAL4 drivers which drive expression in the wing were therefore used to knock down *Ets98B* expression (Table 1). In order to look for defects in other structures and processes, *ubiquitin-GAL4* was used to drive expression throughout the animal, *nanos-GAL4* in the pole cells, and *GMR-GAL4* and *eyeless-GAL4* in the developing eye. Flies carrying these GAL4 drivers were crossed to flies carrying insertions of the *UAS-Ets98B^{RNAi}* transgene on both the second and third chromosomes (Figure 4B). The progeny of crosses which utilized flies carrying single transgenes on either the second or third chromosome did not exhibit any mutant phenotypes. Because mutant phenotypes were observed when flies with two insertions were used, the gene dosage may be an important factor affecting the potency of the RNA interference. The progeny of these crosses were raised at 25°C and 29°C. The former temperature is generally the ideal for quick growth and health of fly cultures, while GAL4 activity is strongest at 29°C and viability is minimally affected (Duffy 2002). Growth at 29°C thus allows one to detect subtle phenotypes that might not be penetrant at a lower temperature.

As shown in Table 1 and Figure 5, two or possibly three types of defects were seen in wings of progeny grown at 29°C. The most common defect observed was a subtle disruption of patterning in the proximal posterior wing margin (Figure 5C). This was seen in progeny from crosses to *ubiquitin-GAL4*, *67B-GAL4*, and *c768-GAL4*, and was never observed in progeny from control crosses. *67B-GAL4* is expressed uniformly in imaginal discs, while *c768-GAL4* is expressed in the ovary, embryonic central nervous system, larval brain, and in the wing and haltere discs, with especially strong expression in the wing pouch (http://flystocks.bio.indiana.edu/gal4.htm).

The remaining defects were associated only with progeny from the cross to *67B-GAL4*. As shown in Figure 5C, some of these flies lacked most of the L2 longitudinal wing vein, retaining only a small portion of vein tissue near the distal end. Flies with the L2 defect also had some degree of disruption of the distal margin, ranging from a mild nick to large notches. It was not determined whether the margin nicking phenotype was due to the presence of the *TM3 Ser actin-GFP* balancer chromosome, in which case the fly is carrying only the second-chromosome *UAS-Ets98B^{RNAi}* insertion, or whether this is a true phenotype arising from RNA interference of *Ets98B*. It seems unlikely that this phenotype is due to the presence of the *Ser* balancer chromosome because the L2 vein phenotype was not observed when *UAS-Ets98B^{RNAi}/CyO* was crossed to the *67B-GAL4* driver. However, it cannot be ruled out that the combination of the *TM3 Ser actin-GFP* balancer with the *67B-GAL4* driver somehow results in the loss of the L2 vein. If the experiment were repeated one could examine the adult flies for the expression of GFP in order to detect the presence of the balancer chromosome.

The results of these experiments reinforce the notion that *Ets98B* has some role in wing development, possibly with more specific involvement in patterning the wing margin and vein(s). The experiments provide no obvious evidence to support the idea that *Ets98B* might influence cell growth or proliferation. On the other hand, these experiments do not preclude the possibility that *Ets98B* may have other important roles in the wing or in other parts of the animal. Because transgenic RNA interference is a relatively new technique, it is unclear how efficient it is in general, as well as with respect to any given gene. It is preferable to back up results obtained with this technique with the use of genetic mutations in the gene. Mutations in *Ets98B* have recently become available; it will be interesting to see whether these flies show similar defects in wing patterning, as well as other phenotypes in the wing or elsewhere (Figure 6). Ectopic overexpression of *Ets98B* using the GAL4/UAS system may also provide insight into the biological roles of this gene in development.

The signaling events that pattern the wing veins and margin are relatively well-understood and involve most of the major signaling pathways. In order to investigate whether *Ets98B* has a role in the signaling events that pattern the wing veins or margin, one could look for genetic interactions between *Ets98B* and known components of these processes. For example, the dorsal-ventral (D/V) boundary, which gives rise to the wing margin, is defined by Notch signaling (reviewed in Blair 2003). Notch activity induces expression of the morphogen

Wingless, which in turn controls growth and patterning of the cells near this boundary. Ets98B could interact with some component(s) of the Notch and/or Wingless pathways in contributing to patterning of the wing margin. Similarly, patterning of the longitudinal wing veins is controlled by a number of signaling events (reviewed in de Celis 2003). Hedgehog (Hh) signals from the posterior compartment, turning on Decapentaplegic (Dpp) in a stripe along the anterior-posterior (A/P) boundary. Dpp in turn activates expression of the *Spalt* complex genes. The L2 wing provein is induced in a region defined by a certain low level of Spalt activity, which induces the expression of the transcription factor Knirps. Finally, EGFR, Notch and Dpp signaling are all involved in refining the wing vein pattern during pupal development. Given the apparently specific loss of L2 due to knockdown of *Ets98B*, it is possible that Ets98B may interact with components of these signaling pathways as well.

Transcriptional properties of Ets98B

Recent studies have suggested that human PDEF can activate transcription of a small number of target genes (Feldman 2003, Oettgen 2000). Transcription assays were performed in order to ask whether Ets98B might also activate transcription, or might instead act as a transcriptional repressor. The assays were performed in Schneider 2 (S2) *Drosophila* cultured cells using transfected reporter and expression constructs (Figure 7A). Fusions of portions of Ets98B to the Gal4 DNA binding domain (DBD) were used together with a *UAS-luciferase* reporter and *actin-lacZ* to control for transfection efficiency. The fusion proteins were designed to allow some degree of structure-function analysis, such as mapping which region(s) of Ets98B might be important for transcriptional activation or repression (Figure 7B).

The results of these assays clearly showed that full-length Ets98B fused to the Gal4DBD can activate transcription of the *UAS-luciferase* reporter (Figure 8A). This result was consistent in five independent experiments. In contrast, none of the other five Ets98B fusions activated or repressed transcription relative to the activity of the Gal4DBD alone. From these results it appeared that either Ets98B functions properly only when intact, or that there might be a defect in the localization or expression of some of the fusion proteins.

The sequence of Ets98B contains two consensus MAPK phosphorylation sites, defined as P-X-S/T-P (Clark-Lewis et al. 1991). Both sites are located in the amino-terminal portion of the protein, and human PDEF also contains one site in its N-terminus. Many Ets proteins, including Yan and PntP2, are regulated by MAPK phosphorylation in response to activation of the Ras

pathway. In order to ask whether the transcriptional activation potential of Ets98B might be regulated by Ras/MAPK signaling, activated Ras (Ras^{V12}) was added to the transfections (Figure 8B). The results of these experiments were inconsistent. In three experiments, Gal4DBD-Ets98B full-length was activated three-fold, two-fold, or not at all by the addition of activated Ras. The other fusion proteins were relatively unaffected by the presence of Ras^{V12}. It is thus unclear whether or not Ets98B's activity might be regulated by Ras/MAPK signaling. Testing the transcriptional properties of a protein in which the putative phosphoacceptor residues have been mutated to alanine might help to resolve this question.

In order to test whether a failure in protein expression or localization to the nucleus might be responsible for the apparent inactivity or some or all of the Ets98B fusion proteins, transfected cells were stained with an antibody to the Gal4DBD. This revealed that there was indeed a defect in protein localization. While the full-length and PD+ETS fusion proteins were mainly nuclear, as indicated by colocalization with DAPI-stained DNA, the remaining proteins were largely or entirely excluded from the nucleus. This is curious because the Gal4DBD naturally localizes to the nucleus, and moreover an exogenous SV40 nuclear localization signal was also included in the fusion proteins (Figure 11).

Since the full-length and PD+ETS constructs are the only two which localize to the nucleus, and are also the only two which contain the ETS domain, it thus appears that the presence of this domain is important to localize the fusion protein to the nucleus. It is not surprising that the DNA binding domain of the native protein should provide nuclear localization competency. However, these results imply that the C-terminally truncated protein is actively excluded from the nucleus, and that this effect is strong enough to override the other nuclear localization signals in the fusion protein. Examination of the protein sequence does not reveal the presence of any canonical nuclear export signals.

The other implication of these results is that the transcriptional activation domain appears to map to the amino-terminal half of the protein. The PD+ETS fusion protein localizes properly yet has no transcriptional activation potential, while the full-length protein has robust and reproducible activation ability. This logically points to the amino terminus as the location of the transactivation domain. There are several stretches of six to nine amino acid residues in the N-terminus of Ets98B that are conserved in its human and mouse homologs; one or more of these could have a conserved transcriptional activation potential. It may be fruitful to attempt to map

the activation domain by making small deletions within the Ets98B amino terminal region and asking whether any of these proteins lose the ability to activate transcription.

The GAL4/UAS-luciferase assay system was used because it afforded the possibility of also being used in two-hybrid assays for protein-protein interactions. Many Ets proteins are known to homo- or heterodimerize with other Ets proteins, cofactors, or members of other transcription factor families, and it would have been interesting to look for such interactions involving Ets98B. Indeed, fusions of the six portions of Ets98B to the Gal4 activation domain (AD) were constructed, as well as Gal4DBD and Gal4AD fusions to portions of several other Ets family members (Figure 11). Unfortunately, preliminary two-hybrid experiments were inconclusive, probably largely due to problems with fusion protein localization.

In an effort to test whether Ets98B can activate transcription through binding DNA via its own DNA binding domain, transcription assays were performed using FLAG-tagged Ets98B proteins and luciferase reporters under the control of several different binding sites. The assay system is essentially the same as that described above, simply using a different binding site and fusion proteins (Figure 9). The tagged proteins included full-length, PD+ETS, and ΔETS versions, where the ΔETS serves as a negative control because it is not expected to bind DNA. The proteins carry an exogenous SV40 nuclear localization signal (NLS) at the amino terminus in addition to the FLAG epitope. Reporters with three different binding sites were tested. The first is a multimer of consensus Ets binding sites (EBS) containing a core 5'-GGAA-3' sequence which is bound by Yan and Pointed and has been used previously in transcription assays (O'Neill et al. 1994, Tootle et al. 2003). The remaining two sites are fragments of the *mae* locus, each of which contain several potential Ets binding sites and are known to be bound by Yan and Pointed (Vivekanand et al. 2004).

Expression of full-length NLS-FLAG-Ets98B was associated with slight activation of transcription from *EBS-luciferase* (Figure 10A). While of small magnitude, the level of activation was significantly higher than that of the NLS-FLAG-ΔETS or NLS-FLAG-PD+ETS constructs, and the result was similar in two independent experiments. This is consistent with the findings with Gal4DBD-Ets98B full-length but not PD+ETS activates transcription from *UAS-luciferase*, and provides more evidence that the amino terminal half of Ets98B is necessary for transcriptional activation. However, the addition of activated Ras did not seem to have any effect

11

on the ability of FLAG-Ets98B full-length to activate transcription. It therefore remains unclear whether Ets98B's transcriptional activity might be regulated by MAPK phosphorylation. Furthermore, NLS-FLAG-Ets98B had no effect upon transcription from either the *maeB1-luciferase* (Figure 10B) or *maeB2-luciferase* (Figure 10C) reporter relative to the negative control.

In order to ascertain that the FLAG-tagged Ets98B proteins were expressed and properly localized, transfected cells were stained with αFLAG antibody and DAPI. The full-length and PD+ETS proteins were clearly expressed and localized to the nucleus, while the ΔETS protein was both nuclear and cytoplasmic. This localization pattern for the ΔETS protein is not surprising in light of the findings with the Gal4DBD fusion proteins, as the ΔETS construct lacks a DNA binding domain. It is notable that the SV40 NLS was not sufficient to localize the protein to the nucleus. However, there was no evidence that the ΔETS protein was actively excluded from the nucleus, in contrast to the Gal4DBD-Ets98B fusions lacking the ETS domain. This suggests that there may be something unique about the Gal4DBD fusions that leads to their nuclear exclusion; on the other hand, NLS-FLAG-ΔETS may simply be small enough to diffuse into the nucleus while the Gal4DBD fusion proteins are too large to do so.

One concern about the results of these experiments is that the FLAG tag may interfere with the protein's function. The tag is located at the N-termini of the fusion proteins, and is quite close to a consensus MAPK phosphorylation site in the full-length and ΔETS proteins. The negative charge of the FLAG epitope, which has the sequence DYKDDDDK, could potentially mimic the addition of a phosphate at the MAPK site. The charge or conformation of the epitope could also alter the protein's function in other unforeseeable ways. Therefore it would be advisable to repeat these experiments with the HA-tagged and/or untagged versions of Ets98B.

In the future it may be of interest to attempt to identify the DNA sequence(s) bound by Ets98B. This would allow the design of a more effective reporter for transcription assays and could lead to the identification of genes whose transcription is controlled by Ets98B. Chromatin immunoprecipitation (ChIP) could be used to identify DNA fragments that are bound by Ets98B in vivo, thus facilitating both the discovery of target genes as well as a consensus binding site. PDEF has been shown to prefer to bind to sites with the core sequence 5'-GGAT-3' rather than the more common Ets binding site core sequence 5'-GGAA-3' (Oettgen et al. 2000). It was suggested that the altered specificity may be due to several unique amino acid substitutions in the

predicted helix 3 of PDEF's ETS domain, which structural studies have identified as the main recognition helix in Ets proteins (Werner et al. 1997). Notably, the predicted helix 3 of Ets98B is identical to that of PDEF. It will therefore be interesting to see whether Ets98B shares the preference for a 5′-GGAT-3′ core binding sequence with its homolog.

A potential explanation for the observation that PDEF mRNA levels increase while the protein expression decreases in cancers is that PDEF negatively regulates its own expression. In this scenario, the loss of PDEF protein by some means would result in derepression of *PDEF* transcription and a consequent rise in mRNA levels. One interesting supplementary approach to identifying binding sites for Ets98B may thus be to examine the *Ets98B* locus for potential Ets binding sites. One could then use any appropriate fragments in transcription assays to see whether Ets98B can regulate its own expression.

MATERIALS AND METHODS

Drosophila stocks and genetic crosses

Flies were maintained on standard fly medium and raised at 25°C unless otherwise specified. w^{1118} flies were used as wildtype for embryo and larval collections. Flies carrying single RNAi transgenes were *yw; UAS-Ets98B^{RNAi} T1 / SM6a* and *yw; UAS-Ets98B^{RNAi} T1 / TM3 Sb Ser* and were obtained from Dr. Tien Hsu. These flies were double-balanced, then crossed to make a line carrying both transgene inserts: *UAS-Ets98B^{RNAi} / CyO actin-GFP ; UAS-Ets98B^{RNAi} / TM3 Ser actin-GFP*. This double transgene line was crossed to a number of GAL4 drivers obtained from Bloomington Stock Center and to w^{1118} as a negative control (Table 1). w^{1118} was also crossed to each GAL4 line. Crosses were started at 25°C and either maintained at that temperature or shifted to 29°C after two days. Wings were mounted in Aqua Poly/Mount (Polysciences, Inc.).

Immunofluorescence

Embryos were collected and stained according to normal protocols. Rabbit anti–Ets98B 2372 was obtained from Dr. Tien Hsu. The antibody serum was either used directly or pre-adsorbed on embryos, and was tested undiluted and at concentrations of 1:100, 1:500, and 1:2000. The 1:500 concentration gave the best results, and pre-adsorption did not seem to have a noticeable effect. Cy3 goat anti-rabbit (Jackson ImmunoResearch) was used at 1:2000 and DAPI was added to a wash step after the secondary antibody incubation.

Third-instar larvae were dissected and stained according to the standard protocol for larval discs. Rabbit anti–Ets98B 2372 was used at concentrations of 1:50 and 1:250 with Cy3 goat anti-rabbit at 1:2000. The 1:250 dilution gave the better results.

In situ hybridization

Probe template for *Ets98B* was prepared by PCR of *Ets98B* cDNA clone RE41742 using primers Ets98B598S (5-CCTCACAGTCCGCAGCTGAAGCC-3) and Ets98B1333T7A (5-GAA TTAATACGACTCACTATAGGGAGAGTTTGGCATGCTCTAGGGC-3). Template for *Ets96B* was prepared by PCR of cDNA clone SD17695 with primers Ets96B360XS (5-TAGTCT AGACTCGGTGGTTTCCTGTGTCA-3) and Ets96BX1156A (5-TTTTCTAGAGTTGGTGGG CGTAGTGCGAGGGC-3). Template for *Ets97D* was prepared by PCR of clone RE06142 with primers Ets97D100XS (5-TTTTCTAGAGGCAGTTATTTGCGATTTTAAGCC-3) and Ets97D PD SA (5-GCACGAGCTCACGGACACGAAATTACATTCT-3).

Digoxigenin-labeled DNA probes were prepared from the templates. Probe was diluted 1:10 for hybridization and incubated with the dissected larvae at 48°C overnight, then washed and developed as usual.

Plasmid construction

GAL4DBD and AD constructs: Gene fragments were amplified by PCR using Pfu Turbo (Stratagene) and subcloned into the KpnI and SacI sites of pMT-SV40NLS-GAL4AD and pMT-SV40NLS-GAL4DBD or pMT-GAL4DBD (Figure 11A). The template for Ets98B was cDNA clone RE41742; for Ets21C, LP04687; for Ets97D, RE06142; for Mae, pMT-Myc-Mae (Tootle et al. 2003); for PntP2, pMT-PntP2; and for Yan, pMT-Yan. The following fragments were amplified using the indicated primer pair.

Ets98B full-length:

Ets98B598KS 5-CGGGGTACCCCTCACAGTCCGCAGCTGAAG-3

Ets98B2106HSA 5-CTGAGCTCAAGCTTCTGGCTGTAGGGATGGCAGA-3

Ets98B ΔETS:

Ets98B598KS 5-CGGGGTACCCCTCACAGTCCGCAGCTGAAG-3

Ets98B1851HSA 5-CTGAGCTCAAGCTTCCACAGGTGGATGTGGGAGC-3

Ets98B Nterm:

Ets98B598KS 5-CGGGGTACCCCTCACAGTCCGCAGCTGAAG-3

Ets98B 1326HSA 5-CTGAGCTCAAGCTTATGCTCTAGGGCCAGTGAAA-3

Ets98B PD+ETS:

Ets98B PD KS 5-CGGGGTACCGAGCATGCCAAACGAGAGGCG-3

Ets98B2106HSA 5-CTGAGCTCAAGCTTCTGGCTGTAGGGATGGCAGA-3

Ets98B PD+bit:

Ets98B PD KS 5-CGGGGTACCGAGCATGCCAAACGAGAGGCG-3

Ets98B1851HSA 5-CTGAGCTCAAGCTTCCACAGGTGGATGTGGGAGC-3

Ets98B PD:

Ets98B PD KS 5-CGGGGTACCGAGCATGCCAAACGAGAGGCG-3

Ets98B PD SA 5-GCACGAGCTCTGGTCCGCGTAGGCCATCTTC-3

Ets21C PD:

Ets21C PD KS 5-CGGGGTACCCGAAGGAACTCCAGCGACTCC-3

Ets21C PD SA 5-GCACGAGCTCCCGGTGGCGTGGTATAAGCTG-3

Ets97D PD:

Ets97D PD KS 5-CGGGGTACCTGGGTCCTGGACAGCAAGTTT-3

Ets97D PD SA 5-GCACGAGCTCACGGACACGAAATTACATTCT-3

Mae PD:

Mae PD KS 5-CGGGGTACCGATGATGGCACCACCAGTCTG-3

Mae PD SA 5-GCACGAGCTCAGGGCCATTGCTCGGGCGAGT-3

Mae full-length:

Mae1KpnS 5-CGGGGTACCATGCAAGTGGAATCGAGCTAT-3

Mae PD SA 5-GCACGAGCTCAGGGCCATTGCTCGGGCGAGT-3

PntP2 PD:

PntP2 PD KS 5-CGGGGTACCAAGGCATCCTTTGCCTCCTGG-3

PntP2 PD SA 5-GCACGAGCTCGGTTTCTCGCAATCTTTTTGC-3

PntP2 N+PD:

PntP21KpnS 5-CGGGGTACCATGGAATTGGCGATTTGTAAA-3

PntP2 PD SA 5-GCACGAGCTCGGTTTCTCGCAATCTTTTTGC-3

PntP2 1-350:

PntP21KpnS 5-CGGGGTACCATGGAATTGGCGATTTGTAAA-3

PntP21050SA 5-GCACGAGCTCGACAGTCGGCTGCTGCGATTG-3

Yan PD:

Yan PD KS 5-CGGGGTACCTGTCCCCCAGCGCCGTCCAGC-3

Yan PD SA 5-GCACGAGCTCATCATGTGGGACTCTATGATC-3

Yan N+PD:

Yan1KpnS 5-CGGGGTACCATGTCCAAAATGAAAATGCTC-3

Yan PD SA 5-GCACGAGCTCATCATGTGGGACTCTATGATC-3

Yan 1-390:

Yan1KpnS 5-CGGGGTACCATGTCCAAAATGAAAATGCTC-3

Yan1170SacIA 5-GCACGAGCTCCTCAGAATTCTCGGGGAAGAA-3

Tagged Ets98B constructs: Gene fragments were amplified by PCR from cDNA clone RE41742 using Pfu Turbo (Stratagene) and subcloned into the KpnI and SacI sites of pMT-SV40NLS-FLAG and pMT-SV40NLS-HA (Figure 11B). The following fragments were amplified using the indicated primer pair.

Ets98B full-length:

Ets98B598KS 5-CGGGGTACCCCTCACAGTCCGCAGCTGAAG-3

Ets98B2106HSA 5-CTGAGCTCAAGCTTCTGGCTGTAGGGATGGCAGA-3

Ets98B ΔETS:

Ets98B598KS 5-CGGGGTACCCCTCACAGTCCGCAGCTGAAG-3

Ets98B1851HSA 5-CTGAGCTCAAGCTTCCACAGGTGGATGTGGGAGC-3

Ets98B PD+ETS:

Ets98B PD KS 5-CGGGGTACCGAGCATGCCAAACGAGAGGCG-3

Ets98B2106HSA 5-CTGAGCTCAAGCTTCTGGCTGTAGGGATGGCAGA-3

GST fusion protein constructs: Gene fragments were amplified by PCR from cDNA clone RE41742 using Pfu Turbo (Stratagene) and subcloned into the SmaI and HindIII sites of pGEX "Bubba" #3 (Figure 11C). The following fragments were amplified using the indicated primer pair.

Ets98B full-length:

Ets98B598S 5-CCTCACAGTCCGCAGCTGAAGCC-3

Ets98B2106HSA 5-CTGAGCTCAAGCTTCTGGCTGTAGGGATGGCAGA-3

Ets98B ΔETS:

Ets98B598S 5-CCTCACAGTCCGCAGCTGAAGCC-3

Ets98B1851HSA 5-CTGAGCTCAAGCTTCCACAGGTGGATGTGGGAGC-3

Ets98B internal:

Ets98B988S 5-AAGCAGGAGCTGCACATCCTGC-3

Ets98B1753HA 5-CACACAAGCTTAAGTGGTACTGCCGTTCAGGG-3

Ets98B Nterm:

Ets98B598S 5-CCTCACAGTCCGCAGCTGAAGCC-3

Ets98B1326HSA 5-CTGAGCTCAAGCTTATGCTCTAGGGCCAGTGAAA-3

Transcription assays

S2 cells were grown in Schneider's medium (Gibco) supplemented with 12.5% fetal bovine serum (HyClone). Transfections and transcription assays were performed as described (Tootle et al. 2003). Constructs used in the assays are described above and in Figure 11.

Cell stainings

Cell stainings were performed according to standard protocols. Mouse anti-Gal4DBD RK5C1 (Santa Cruz Biotechnology) was used at 1:200, mouse anti-FLAG M2 (Sigma) at 1:7000, and Cy3 goat anti-mouse (Jackson ImmunoResearch) was used at 1:4000. DAPI was added to the last five minutes of the secondary antibody incubation step.

Microscopy and Image Processing

All microscopy was performed using a Zeiss Axioplan 2 and images were processed with Adobe Photoshop.

ACKNOWLEDGEMENTS

APPENDIX

Determination of larval expression patterns for Ets96B and Ets97D

In situ hybridization was used to determine the expression patterns of *Ets96B* and *Ets97D* in larval tissues. *Ets96B* was found to be strongly expressed in all examined imaginal structures, larval tissues which give rise to adult organs (Figure A1). This may point to a general role for *Ets96B* in the development or survival of adult tissues. In contrast, *Ets97D* seems to be expressed at significant levels only in eye discs and anterior midgut (Figure A2). In the eye, one experiment found staining in the morphogenetic furrow and in a regular pattern posterior to the furrow suggestive of expression in a subset of cells in the developing ommatidia. However, this pattern was not evident when the experiment was repeated twice subsequently.

REFERENCES

Baker, D. A., Mille-Baker, B., Wainwright, S. M., Ish-Horowicz, D., and Dibb, N. J. (2001). Mae mediates MAP kinase phosphorylation of Ets transcription factors in Drosophila. Nature *411*, 330-334.

Blair, S. S. (2003). Lineage compartments in *Drosophila*. Current Biology *13*, R548-R551.

Brand, A., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Chang, C.-H., Scott, G. K., Kuo, W.-L., Xiong, X., Suzdaltseva, Y., Park, J. W., Sayre, P., Erny, K., Collins, C., Gray, J. W., and Benz, C. C. (1997). ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. Oncogene *14*, 1617-1622.

Chen, H., Nandi, A. K., Li, X., and Bieberich, C. J. (2002). NKX-3.1 Interacts with Prostate-derived Ets Factor and Regulates the Activity of the PSA Promoter. Cancer Res *62*, 338-340.

Chen, T., Bunting, M., Karim, F. D., and Thummel, C. S. (1992). Isolation and Characterization of Five *Drosophila* Genes That Encode an *ets*-Related DNA Binding Domain. Developmental Biology *151*, 176-191.

Clark-Lewis, I., Sanghera, J., and Pelech, S. (1991). Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. J Biol Chem *266*, 15180-15184.

de Celis, J. F. (2003). Pattern formation in the *Drosophila* wing: the development of the veins. BioEssays *25*, 443-451.

Dittmer, J., and Nordheim, A. U.-h. w. s. c. s. a. B.-T.-f. (1998). Ets transcription factors and human disease. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer *1377*, F1-F11.

Duffy, J. B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis *34*, 1-15.

Feldman, R. J., Sementchenko, V. I., Gayed, M., Fraig, M. M., and Watson, D. K. (2003). Pdef Expression in Human Breast Cancer Is Correlated with Invasive Potential and Altered Gene Expression. Cancer Res *63*, 4626-4631.

Galang, C. K., Muller, W. J., Foos, G., Oshima, R. G., and Hauser, C. A. (2004). Changes in the Expression of Many Ets Family Transcription Factors and of Potential Target Genes in Normal Mammary Tissue and Tumors. J Biol Chem *279*, 11281-11292.

Ghadersohi, A., and Sood, A. K. (2001). Prostate Epithelium-derived Ets Transcription Factor mRNA Is Overexpressed in Human Breast Tumors and Is A Candidate Breast Tumor Marker and A Breast Tumor Antigen. Clin Cancer Res *7*, 2731-2738.

Golub, T., Goga, A., Barker, G., Afar, D., McLaughlin, J., Bohlander, S., Rowley, J., Witte, O., and Gilliland, D. (1996). Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. Mol Cell Biol *16*, 4107-4116.

Hannon, G. J. (2002). RNA interference. Nature *418*, 244-251.

Hsouna, A., Ivey, J., and Hsu, T. (2003). The role of D-Ets-4 in germ cell migration during *Drosophila* embryonic development. Paper presented at: 44th Annual Drosophila Research Conference (Chicago, Illinois).

Hsu, T., and Schulz, R. A. (2000). Sequence and functional properties of *Ets* genes in the model organism *Drosophila*. Oncogene *19*, 6409-6416.

Karim, F. D., Urness, L. D., Thummel, C. S., Klemsz, M. J., McKercher, S. R., Celada, A., Van Beveren, C., Maki, R. A., Gunther, C. V., Nye, J. A., and Graves, B. J. (1990). The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. Genes Dev *4*, 1451-1453.

Leprince, D., Gegonne, A., Coll, J., de Taisne, C., Schneeberger, A., Lagrou, C., and Stehelin, D. (1983). A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. Nature *306*, 395-397.

Mitas, M., Mikhitarian, K., Hoover, L., Lockett, M. A., Kelley, L., Hill, A., Gillanders, W. E., and Cole, D. J. (2002). Prostate-Specific Ets (PSE) factor: a novel marker for detection of metastatic breast cancer in axillary lymph nodes. British Journal of Cancer *86*, 899-904.

Nozawa, M., Yomogida, K., Kanno, N., Nonomura, N., Miki, T., Okuyama, A., Nishimune, Y., and Nozaki, M. (2000). Prostate-specific Transcription Factor hPSE Is Translated Only in Normal Prostate Epithelial Cells. Cancer Res *60*, 1348-1352.

O'Neill, E. M., Rebay, I., Tjian, R., and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell *78*, 137-147.

Oettgen, P., Finger, E., Sun, Z., Akbarali, Y., Thamrongsak, U., Boltax, J., Grall, F., Dube, A., Weiss, A., Brown, L., *et al.* (2000). PDEF, a Novel Prostate Epithelium-specific Ets Transcription Factor, Interacts with the Androgen Receptor and Activates Prostate-specific Antigen Gene Expression. J Biol Chem *275*, 1216-1225.

Rebay, I., and Rubin, G. M. (1995). Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell *81*, 857-866.

Schneikert, J., Peterziel, H., Defossez, P.-A., Klocker, H., Launoit, Y. d., and Cato, A. C. B. (1996). Androgen Receptor-Ets Protein Interaction Is a Novel Mechanism for Steroid Hormone-mediated Down-modulation of Matrix Metalloproteinase Expression. J Biol Chem *271*, 23907-23913.

Sementchenko, V. I., and Watson, D. K. (2000). Ets target genes: past, present and future. Oncogene *19*, 6533-6548.

Sharrocks, A. D. (2001). The ETS-Domain Transcription Factor Family. Nature Reviews Molecular Cell Biology *2*, 827-837.

Sorensen, P. H., Lessnick, S. L., Lopez-Terrada, D., Liu, X. F., Triche, T. J., and Denny, C. T. (1994). A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. Nature Genetics *6*, 146-151.

Tootle, T. L., Lee, P. S., and Rebay, I. (2003). CRM1-mediated nuclear export and regulated activity of the Receptor Tyrosine Kinase antagonist YAN require specific interactions with MAE. Development *130*, 845-857.

Tsujimoto, Y., Nonomura, N., Takayama, H., Yomogida, K., Nozawa, M., Nishimura, K., Okuyama, A., Nozaki, M., and Aozasa, K. (2002). Utility of immunohistochemical detection of prostate-specific Ets for the diagnosis of benign and malignant prostatic epithelial lesions. Int J Urology *9*, 167-172.

Vivekanand, P., Tootle, T. L., and Rebay, I. (2004). MAE, a dual regulator of the EGFR signaling pathway, is a target of the Ets transcription factors PNT and YAN. submitted.

Werner, M. H., Clore, G. M., Fisher, C. L., Fisher, R. J., Trinh, L., Shiloach, J., and Gronenborn, A. M. (1997). Correction of the NMR structure of the ETS1/DNA complex. J Biomol NMR *10*, 317-328.
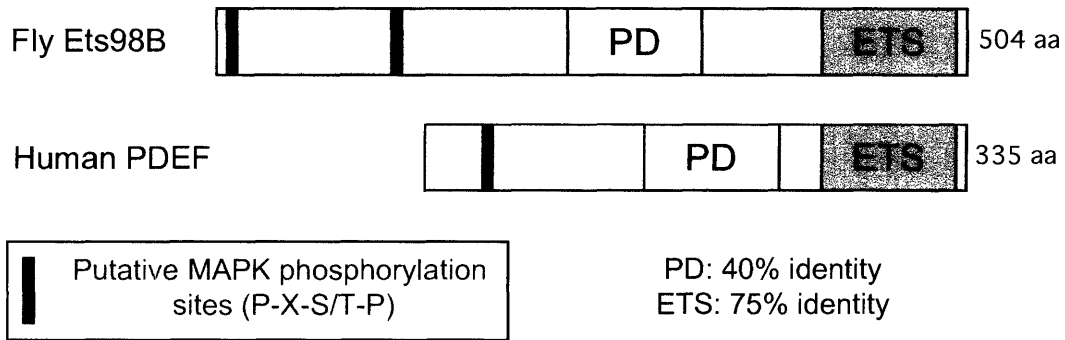
Figure 1. Schematic depiction of the domain structures of Ets98B and its human homolog, PDEF. Both proteins feature a centrally-located Pointed Domain and a C-terminal ETS domain. Ets98B contains two putative MAPK phosphorylation sites near its N-terminus, while PDEF has one. The amino acid sequence identity between the homologs is highest in the PD and ETS domain.
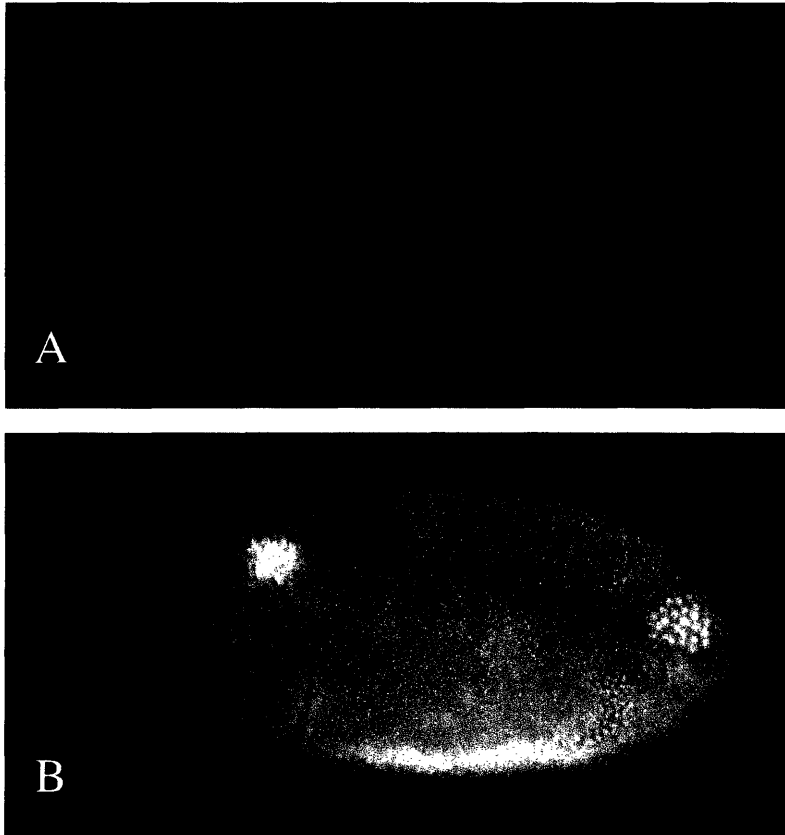
Figure 2. Immunofluorescent staining of Ets98B shows that it is found in the nuclei of epithelial and pole cells in a gastrulation-stage embryo. (A) αETS98B. (B) DAPI staining to reveal the location of nuclei.
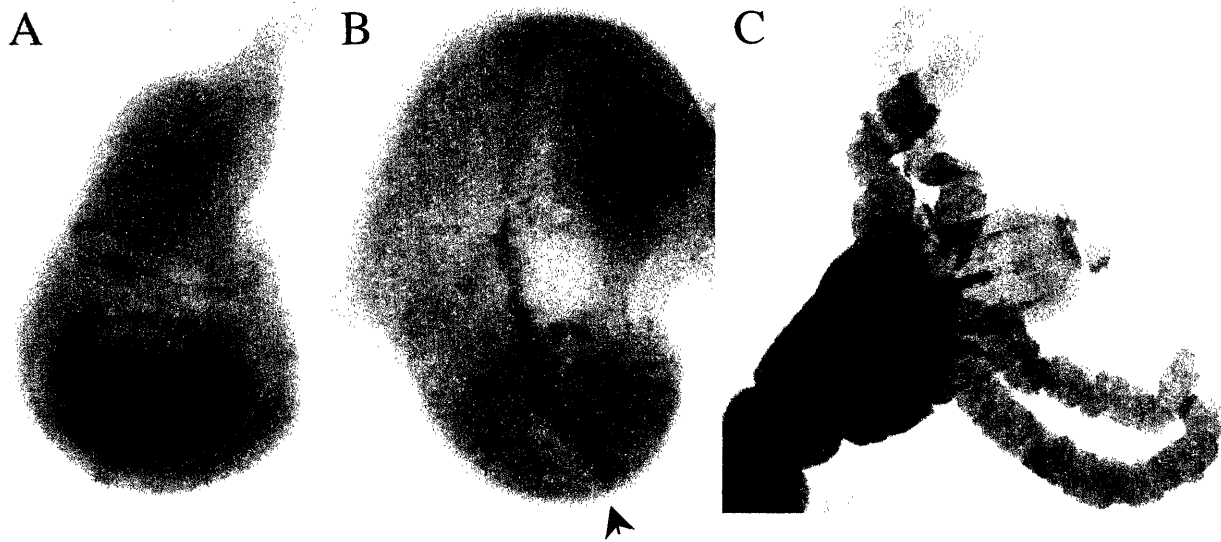
Figure 3. Localization of *Ets98B* mRNA in larval tissues. (A) In wing disc, *Ets98B* message is particularly abundant in the ventral portion of the wing pouch, which gives rise to the adult wing blade. (B) The expression in eye discs was less consistent, but generally appeared stronger in the morphogenetic furrow (arrowhead). (C) Expression was also strong in the larval midgut. A 736-basepair DNA probe to the 5' end of the coding region was used. Posterior is to the left in all photos.

A   GAL4 driver X •••━━━■◄

➡ siRNA
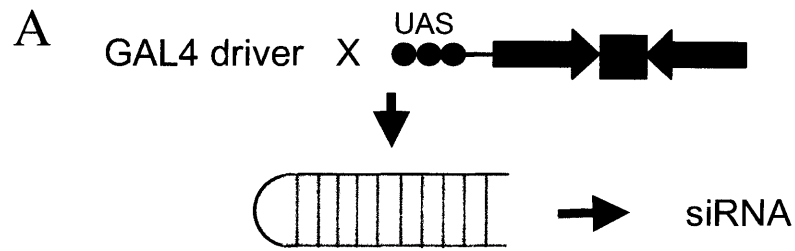
B 1. $\dfrac{UAS\text{-}Ets98B^{RNAi}}{CyO\ actin\text{-}GFP}$; $\dfrac{UAS\text{-}Ets98B^{RNAi}}{TM3\ Ser\ actin\text{-}GFP}$ X *GAL4* driver

2. w^{1118} X *GAL4* driver

Figure 4. Transgenic RNA interference of *Ets98B* has no effect in most parts of the animal, but displays two potentially interesting phenotypes in the adult wing. (A) The bipartite GAL4/UAS system is used to drive overexpression of an engineered hairpin RNA corresponding to the *Ets98B* coding region. This results in knockdown of *Ets98B* mRNA in tissues in which Gal4 is expressed. (B) The experimental (1) and control (2) crosses that were carried out for each GAL4 driver.

Table 1. The phenotypes observed when *UAS-Ets98B^RNAi^/ CyO act-GFP; UAS-Ets98B^RNAi^/ TM3 Ser act-GFP* flies were crossed to the indicated GAL4 drivers. Crosses were incubated at the indicated temperature after eggs were laid at 25°C. Each driver was also crossed to *w^1118^* as a control. Only phenotypes observed in the experimental cross and not the control are indicated.

	GAL4 Driver	At 25°C	At 29°C
1	*ubi-GAL4*	No phenotype	2/17 flies with slightly disrupted proximal posterior wing margin
2	*nos-GAL4*	No phenotype	No phenotype
3	*GMR-GAL4/CyO*	No phenotype	No phenotype
4	*ey-2-1-GAL4/CyO*	No phenotype	No phenotype
5	*vg-GAL4 181/TM6B*	No phenotype	No phenotype
6	*vg-GAL4 111/TM6 Hum*	No phenotype	No phenotype
7	*67B-GAL4*	No phenotype	3 flies missing most of L2 wing vein (most of these also show nicking at distal margin); 1 also displaying slightly disrupted proximal posterior wing margin
8	*69B-GAL4*	No phenotype	No phenotype
9	*c768-GAL4/TM3 Ser*	No phenotype	1/10 flies with slightly disrupted proximal posterior wing margin
10	*459.2-GAL4*	No phenotype	No phenotype
11	*ptc-GAL4*	No phenotype	No phenotype
12	*bs-GAL4*	No phenotype	No phenotype
13	*w^1118^* (negative control)	No phenotype	No phenotype

$67B\text{-}Gal4$
_____
+

$UAS\text{-}Ets98B^{RNAi}$; $UAS\text{-}Ets98B^{RNAi}$
_____   _____
+   +

$UAS\text{-}Ets98B^{RNAi}$; $UAS\text{-}Ets98B^{RNAi}$ or $TM3$ Ser
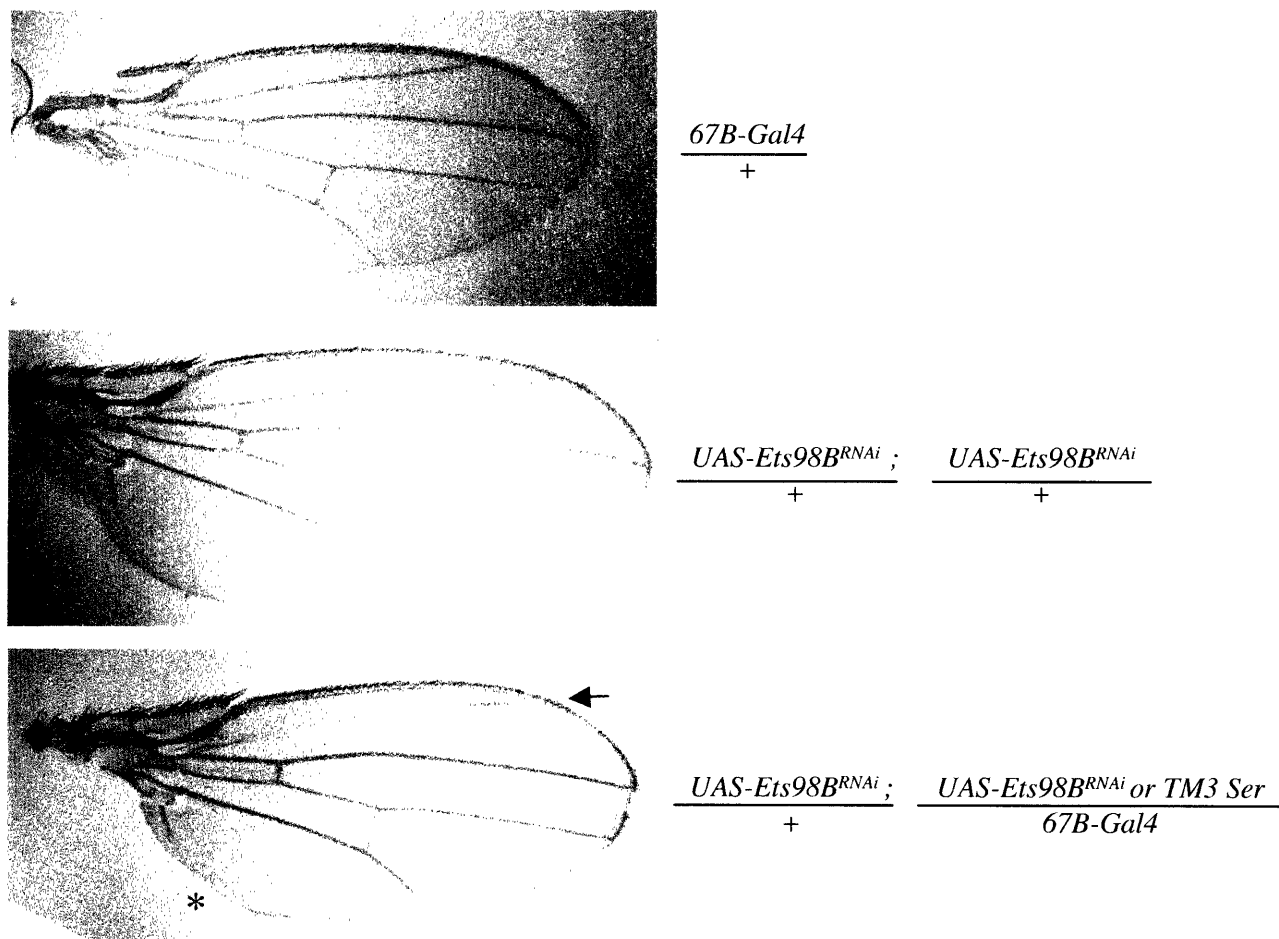_____   _____
+ $67B\text{-}Gal4$

Figure 5. Wing phenotypes associated with knockdown of Ets98B expression. (A) and (B) Wings from flies carrying either the Gal4 driver or UAS-Ets98B^{RNAi} appear wild-type. (C) Wings from flies carrying both *67B-Gal4* and *UAS-Ets98B^{RNAi}* show a loss of most of the L2 wing vein (arrow) and a slight disruption of the proximal posterior margin (asterisk). It is unclear whether the notching of the distal margin is also an associated phenotype or is due to the possible presence of the *TM3 Ser* balancer chromosome. Flies were raised at 29°C.
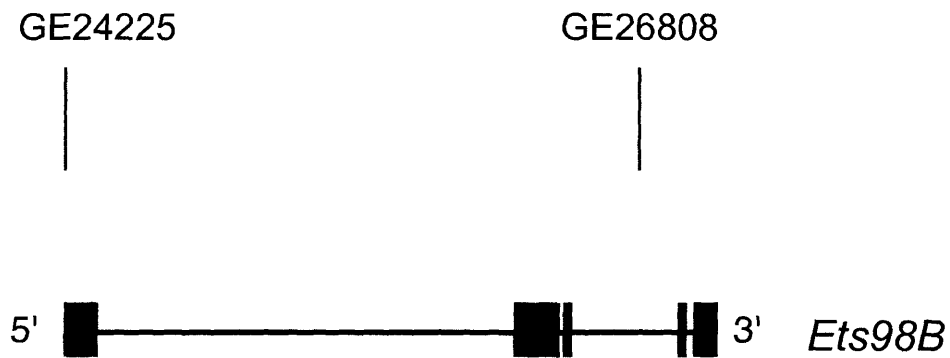
Figure 6. Three P-element insertions in Ets98B have recently become available. GE24225 and GE26808 come from the Genexel GeniSys collection. Their insertion sites are indicated above: GE24225 is just 5' to the first exon of the *Ets98B* locus, while GE26808 is located in the third intron. A third insertion was isolated by Exelixis, Inc. Its exact insertion site is unclear.
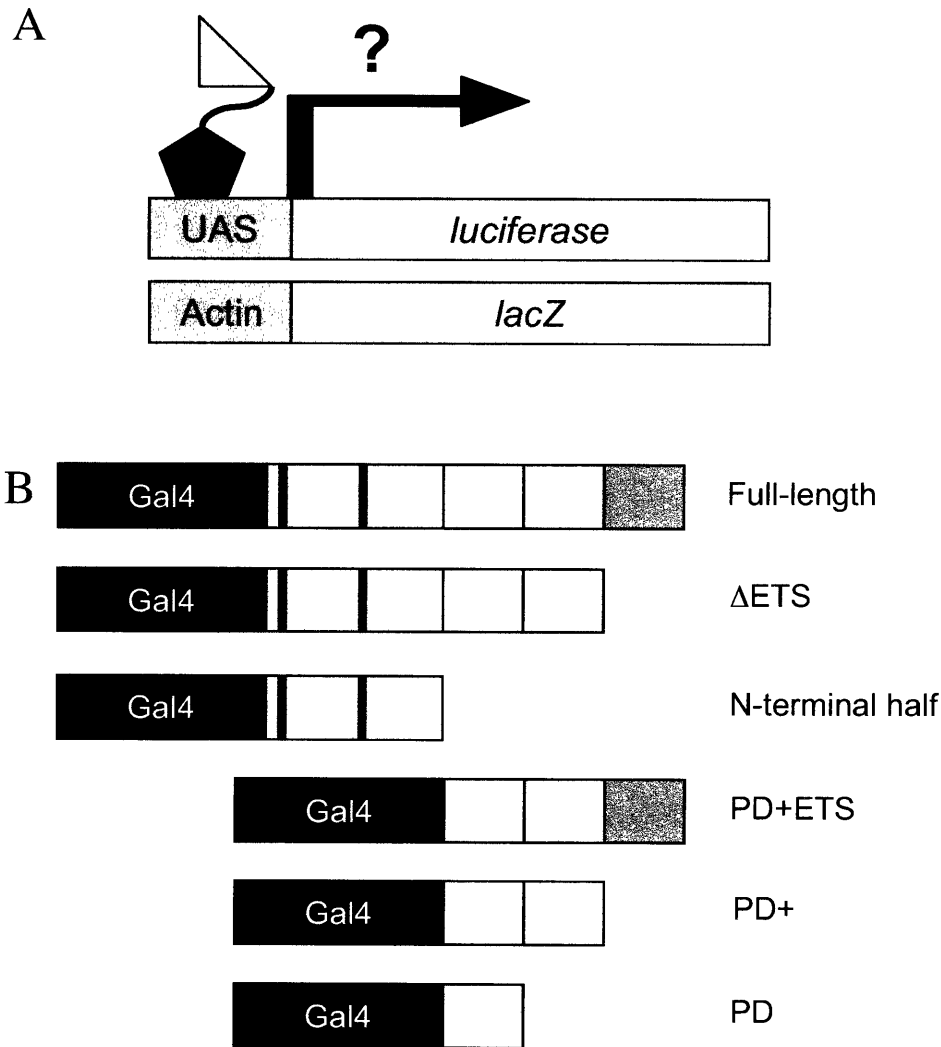
A

B

Figure 7. Schematic depictions of transcription assays and fusion proteins. (A) The transcription assay uses a *luciferase* reporter under control of GAL4 UAS elements, which are bound by the Gal4 DNA-binding domain (DBD). Fusion of a protein with transcriptional activation potential to the Gal4 DBD leads to expression of *luciferase*. *lacZ* expressed under the constitutive *actin* promoter serves as a control for transfection efficiency. (B) A series of proteins fusing portions of Ets98B to the Gal4 DBD and SV40 NLS were constructed for use in this assay.
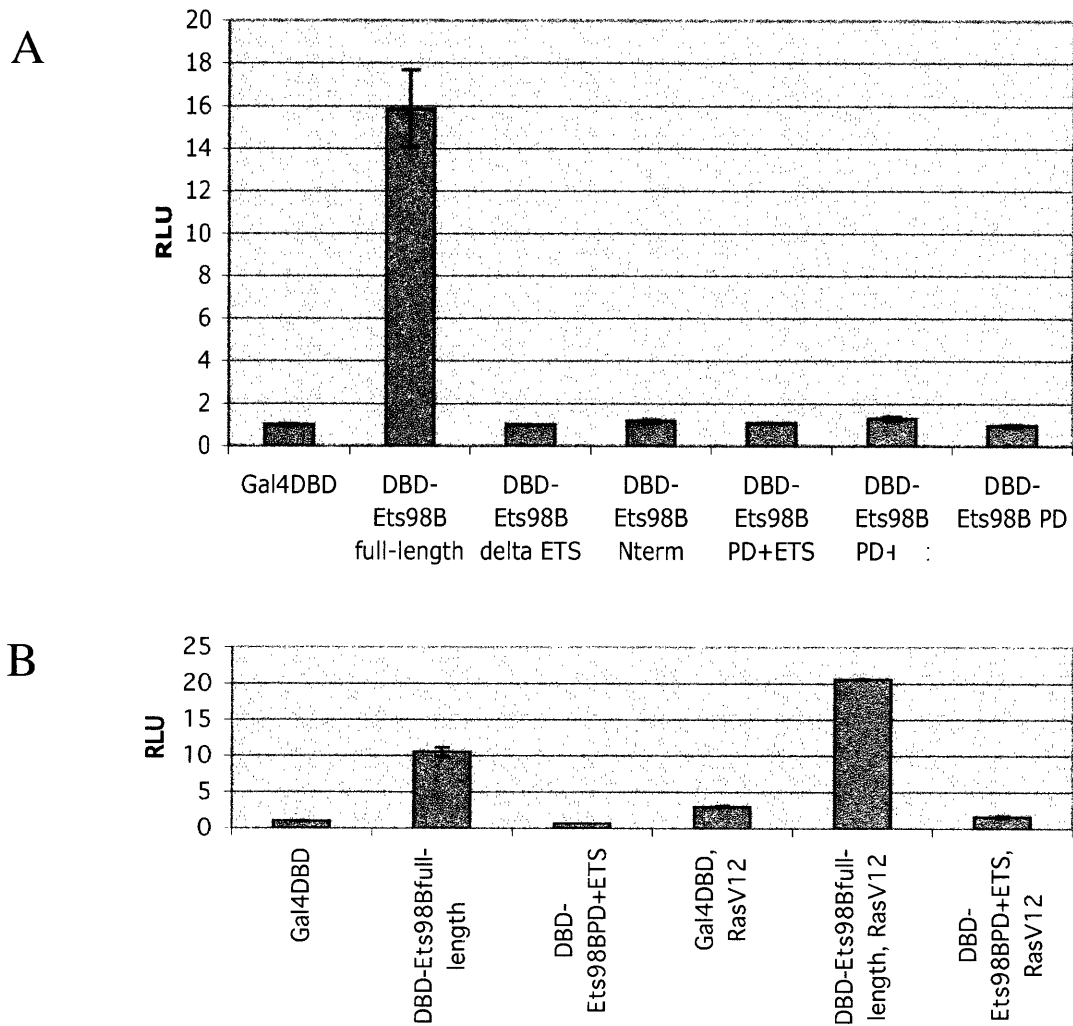
Figure 8. Transcriptional activation properties of Ets98B. (A) Full-length Ets98B fused to the Gal4DBD activates transcription from a *UAS-luciferase* reporter. The remaining Gal4DBD-Ets98B constructs fail to affect transcription, probably largely due to a failure to localize to the nucleus. This result is representative of five independent experiments. RLU refers to the ratio of luciferase to β-galactosidase activity, where this ratio is set to one for the Gal4DBD construct. (B) The addition of activated Ras (Ras^{V12}) had variable results on the activity of Gal4DBD-Ets98B full-length, ranging from no effect to a three-fold increase in activity. Here there is a two-fold increase in activity.
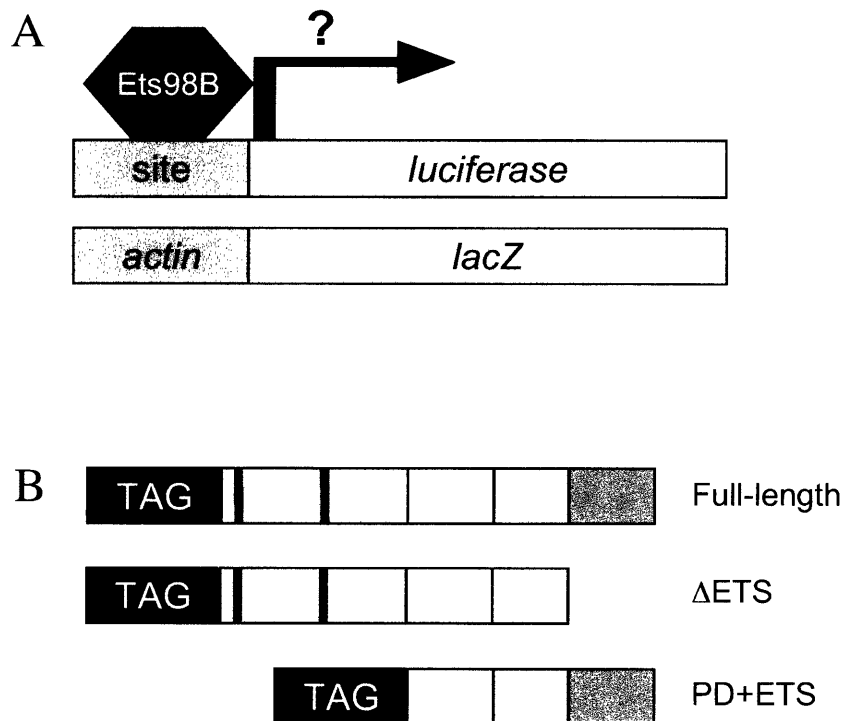
Figure 9. Transcription assay system used to test activity of Ets98B on a variety of binding sites. (A) Tagged forms of Ets98B are tested for their ability to activate transcription of a *luciferase* reporter under the control of a binding site. Transcription of *lacZ* under control of the constitutive *actin* promoter serves as a control for transfection efficiency. (B) Tagged full-length, ΔETS, and PD+ETS constructs were made in two versions, one tagged with FLAG and the other with HA. All proteins also carry the SV40 NLS immediately preceding the tag. The ΔETS construct is not expected to bind DNA and therefore serves as a negative control.
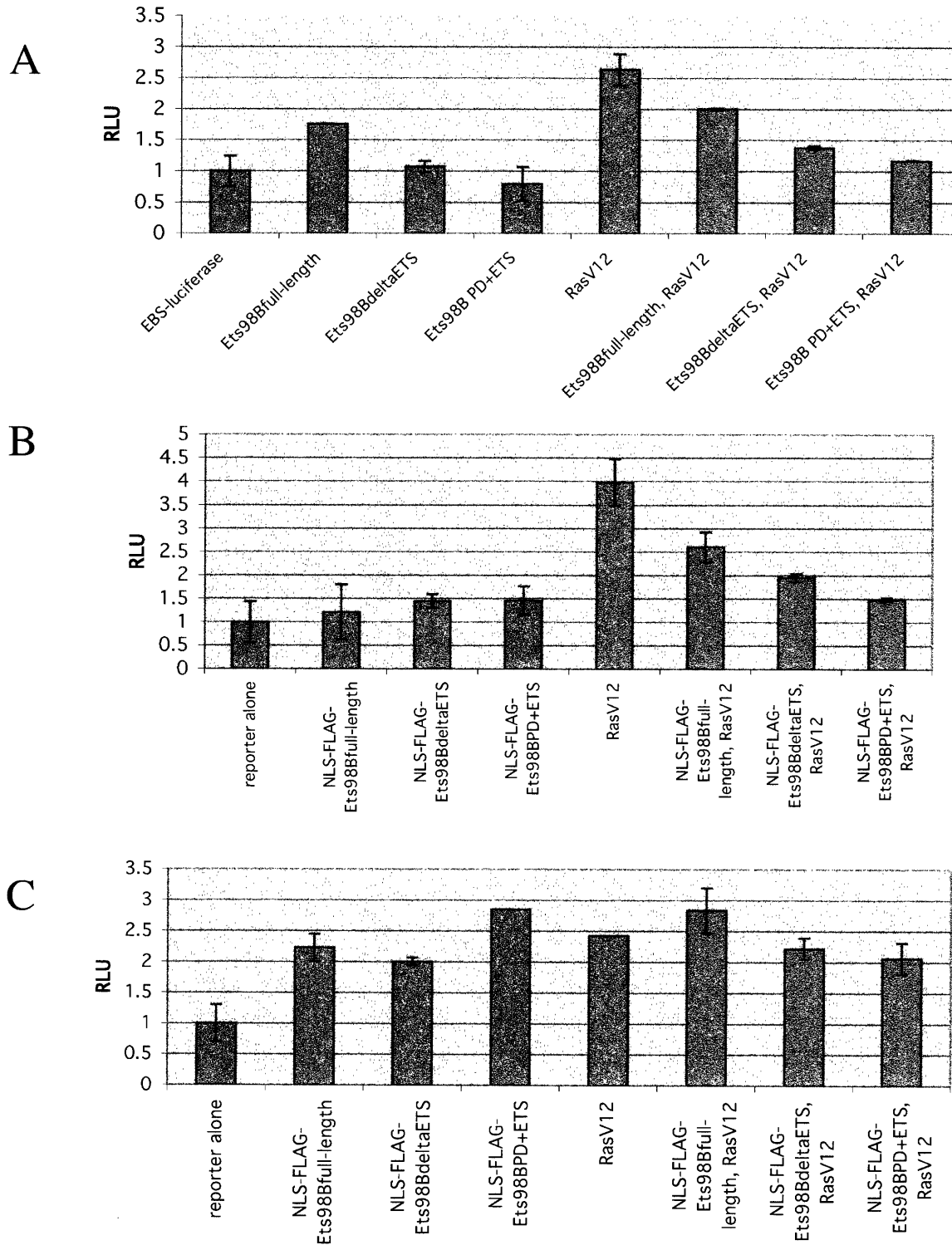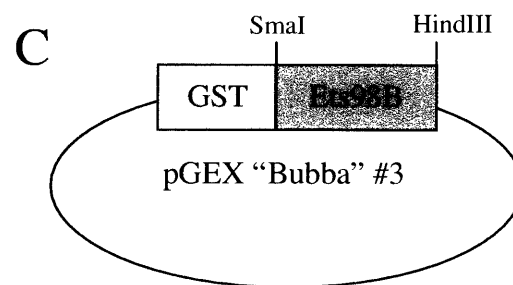
Figure 10. Transcription assay results using FLAG-tagged Ets98B constructs. (A) *EBS-luciferase*, containing a multimerized Ets binding site, is slightly activated by full-length FLAG-NLS-Ets98B. (B) and (C) Two fragments of the *mae* locus, each containing multiple potential Ets binding sites, did not appear to be influenced by Ets98B.

A

KpnI   SacI

nls | GAL4 | Ets

pMT-SV40NLS-GAL4
DBD/AD
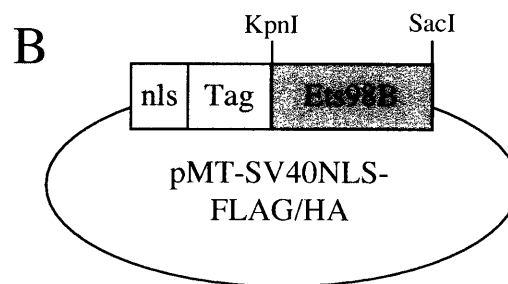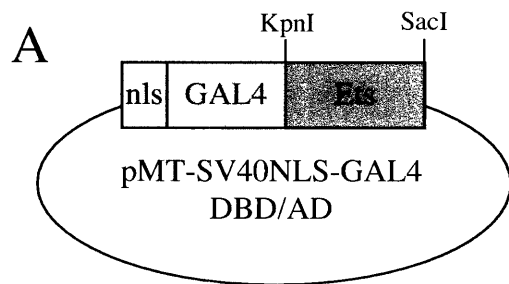
Construct	Amino acid range
Ets98B full-length	2-504
Ets98B ΔETS	2-419
Ets98B Nterm	2-244
Ets98B PD+ETS	243-504
Ets98B PD+bit	243-419
Ets98B PD	243-333
Ets21C PD*	131-221
Ets97D PD*	183-273
Mae PD*	86-176
Mae full-length*	1-176
PntP2 PD*	163-253
PntP2 N+PD*	1-253
PntP2 1-350*	1-350
Yan PD*	28-120
Yan N+PD*	1-120
Yan 1-390*	1-390

B

KpnI   SacI

nls | Tag | Ets98B

pMT-SV40NLS-
FLAG/HA

Construct	Amino acid range
Ets98B full-length	2-504
Ets98B ΔETS	2-419
Ets98B PD+ETS	243-504

C

SmaI   HindIII

GST | Ets98B

pGEX "Bubba" #3

Construct	Amino acid range
Ets98B full-length	2-504
Ets98B ΔETS	2-419
Ets98B internal*	132-386
Ets98B Nterm	2-244

Figure 11. Fusion protein expression vectors. (A) The indicated Ets gene fragments were amplified by PCR and subcloned into both pMT-SV40NLS-GAL4DBD and pMT-SV40NLS-GAL4AD using KpnI and SacI sites. Constructs marked with an asterisk were cloned into pMT-GAL4DBD rather than pMT-SV40NLS-GAL4DBD. (B) The indicated *Ets98B* fragments were amplified by PCR and subcloned into both pMT-SV40NLS-FLAG and pMT-SV40NLS-HA using the KpnI and SacI sites. (C) The indicated *Ets98B* fragments were amplified by PCR and subcloned into pGEX "Bubba" #3 using SmaI and HindIII. The starred construct was PCR amplified but not yet subcloned.
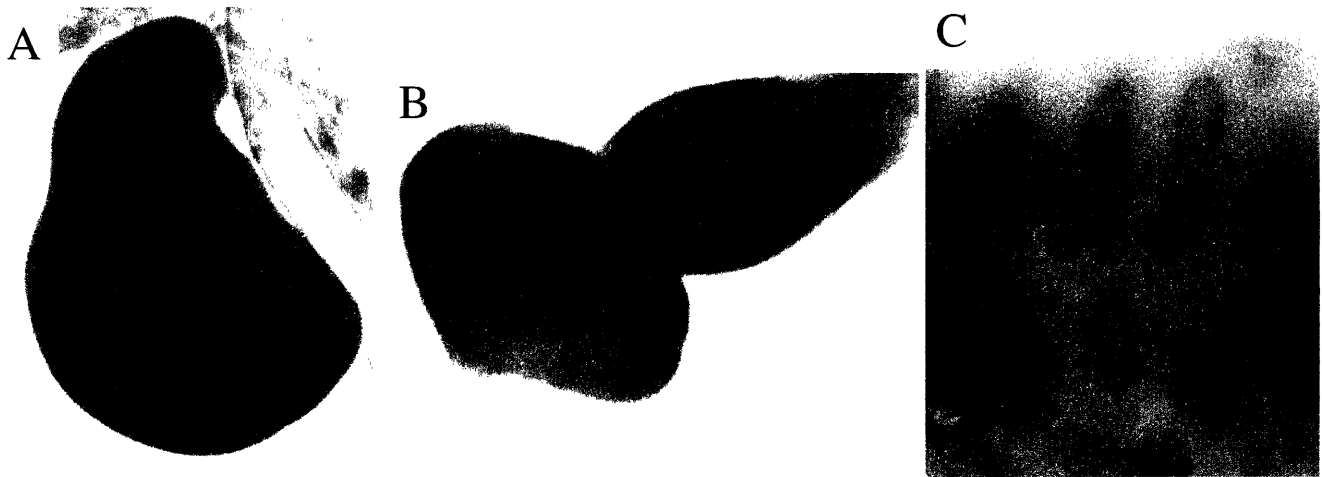
33

Figure A1. *Ets96B* mRNA is abundant in most of the imaginal structures in larvae. (A) *Ets96B* is strongly expressed throughout the wing disc. (B) Expression is also strong throughout the eye and antennal imaginal discs. (C) *Ets96B* is expressed in the midgut histoblasts (imaginal cells found throughout the larval midgut). A 970-basepair DNA probe to the middle of the coding region was used. Posterior is to the left in all photos.
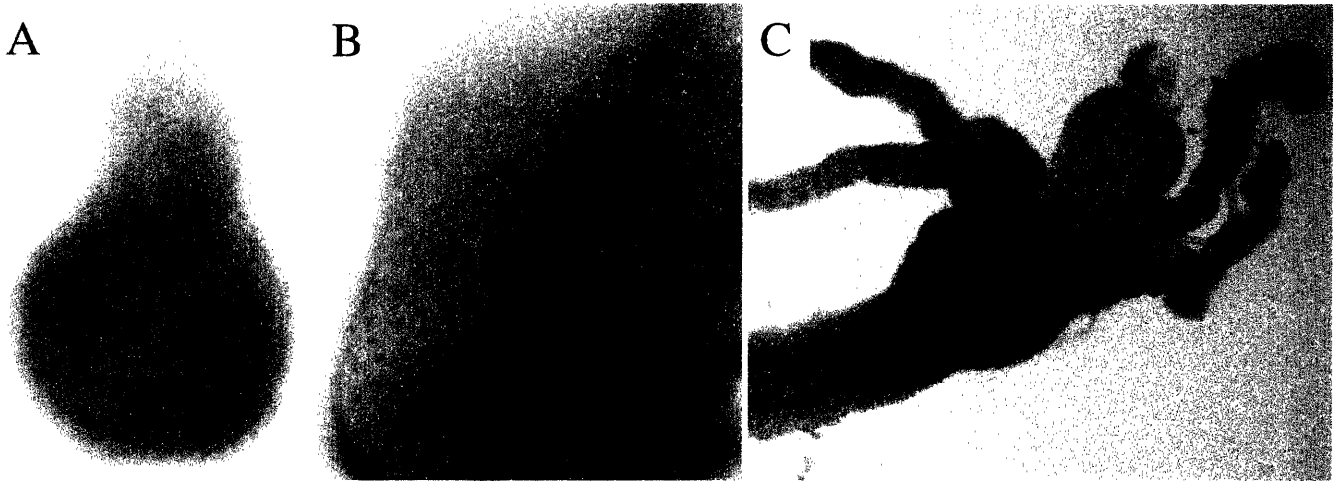
Figure A2. Localization of *Ets97D* mRNA in larval tissues. (A) *Ets97D* expression is undetectable in wing discs. (B) The expression pattern in eye discs was inconsistent, but generally appeared stronger in the morphogenetic furrow (arrowhead) and sometimes in a regular array of spots posterior to the furrow, as shown here. (C) Expression is strong in the anterior end of the larval midgut. An 845-basepair DNA probe to the 5' end of the coding region was used. Posterior is to the left in all photos.