Genetic Analysis of the Drosophila 63F Early Puff: Characterization of Mutations in *E63-1* and *maggie*, a Putative Tom22

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ABSTRACT

The 63F early puff in the larval salivary gland polytene chromosomes contains the divergently transcribed *E63-1* and *E63-2* ecdysone-inducible genes. *E63-1* encodes a member of the EF-hand family of Ca^{2+}-binding proteins, while *E63-2* has no apparent open reading frame. To understand the functions of the *E63* genes, we have determined the temporal and spatial patterns of E63-1 protein expression, as well as undertaken a genetic analysis of the 63F puff. We show that E63-1 is expressed in many embryonic and larval tissues, but the third-instar larval salivary gland is the only tissue where increases in protein levels correlate with increases in ecdysone titer. Furthermore, the subcellular distribution of E63-1 protein changes dynamically in the salivary glands at the onset of metamorphosis. *E63-1* and *E63-2* null mutations, however, have no effect on development or fertility. We have characterized 40 kb of the 63F region, defined as the interval between *Ubi-p* and *E63-2*, and have identified three lethal complementation groups that correspond to the *dSc-2, ida*, and *mge* genes. We show that *mge* mutations lead to first-instar larval lethality and that Mge protein is similar to the Tom22 mitochondrial import proteins of fungi, suggesting that it has a role in mitochondrial function.

THE steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) directs the post-embryonic events of molting and metamorphosis in *Drosophila melanogaster* (reviewed in Riddiford 1993). According to the current model, the hormone initiates these events by first binding to an intracellular receptor, and the steroid/receptor complex directly affects the patterns of target gene expression. Since many aspects of steroid/nuclear-hormone signaling are conserved from insects to mammals (reviewed in Mangelsdorf *et al.* 1995; Cherbas and Cherbas 1996), Drosophila, with its powerful molecular and genetic tools, is an important experimental organism for understanding the details of how these hormones affect changes in physiology and development. The salivary glands of third-instar larvae in particular remain a favorable assay system since one can monitor global levels of gene expression in response to hormone by observing the pattern of puffs that form on the large polytene chromosomes of this epithelial tissue (reviewed in Ashburner and Berendes 1978; Richards 1997).

The early puffs form within minutes of ecdysone exposure (Becker 1959; Ashburner 1972), and the genes residing there are direct targets for the ecdysone/receptor complex. Their induction constitutes the primary response. Many of the early puff genes thus far identified encode DNA-binding proteins (Chao and Guild 1986; Burtis *et al.* 1990; Segraves and Hogness 1990; DiBello *et al.* 1991), and they in turn direct the expression of a large number of secondary-response genes manifested as the late puffs (Ashburner *et al.* 1974; Fletcher and Thummel 1995; Urness and Thummel 1995; Crossgrove *et al.* 1996). The interaction of primary- and secondary-response gene products then directs appropriate late larval and prepupal developmental responses, including glue secretion and programmed cell death in the salivary glands (reviewed in Richards 1997; Henrich *et al.* 1999). However, the molecular details of how these changes in gene expression lead to the specific physiological response in the target tissue are not well understood.

Uncharacteristically, the 63F early puff contains two ecdysone-regulated genes designated *E63-1* and *E63-2* that do not encode DNA-binding proteins. Both genes are induced in the salivary gland by the major pulse of ecdysone that triggers puparium formation (the prepupal pulse). They are divergently transcribed and are separated from each other by 1.1 kb of 5′ sequence (Figure 4; Andres and Thummel 1995).

E63-1 gives rise to three RNA species (*E63-1A*, *E63-1B*, *E63-1C*) that use different Poly(A) addition sites. All are induced by ecdysone in the salivary gland, and all encode the same 193-amino-acid high-affinity calcium-binding protein of the EF-hand family. In addition to

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its hormonal regulation in the salivary gland, *E63-1* is constitutively expressed in many larval tissues (Andres and Thummel 1995).

E63-2 appears to be expressed only in the salivary gland, but it does not contain an AUG-initiated open reading frame, and no significant matches to proteins or RNAs in the databases have yet been identified (Andres and Thummel 1995).

The major physiological response of the larval salivary gland to the premetamorphic pulse of ecdysone is to secrete glue proteins (reviewed in Berendes and Ashburner 1978). Glue secretion occurs 3–6 hr after hormone exposure and requires *de novo* protein synthesis (Zhimulev and Kolesnikov 1975; Boyd and Ashburner 1977; Farkas and Sutakova 1998). Thus, it is reasonable to assume that products of the early genes direct this process. Furthermore, since changes in calcium have been correlated with the secretory state of most epithelial cells that undergo a regulated exocytosis (reviewed in Muallem and Lee 1997; Avery and Jahn 1999; Lang 1999), we hypothesized that E63-1 might function as an integral component of glue secretion, perhaps by transducing the ecdysone stimulus to a Ca^{2+}-mediated vesicle migration/fusion event.

Here we show that E63-1 protein is localized to many larval tissues where it does not appear to be regulated by hormone. In addition, E63-1 protein accumulates to high levels in salivary gland cells only after exposure to the premetamorphic pulse of ecdysone. Initially, the protein becomes localized to cell membranes and nuclei. Several hours later, when glue vesicles are fusing with the apical membrane and releasing their contents, E63-1 becomes excluded from the nucleus and is localized primarily in the cytoplasm. This change in the subcellular localization is consistent with a role for E63-1 in the secretion process.

To test this hypothesis, we undertook a comprehensive genetic and molecular analysis of the 63F region. However, small deficiencies that remove *E63-1* and *E63-2* have no measurable effect on development, viability, or glue secretion. In addition, we identified and characterized three genes located between the previously mapped *Polyubiquitin (Ubi-p)* gene (Arribas *et al.* 1986; Lee *et al.* 1988) and *E63-1*. One of these genes shows sequence similarity to the SC-2 mammalian synaptic glycoprotein (Johnston *et al.* 1992) and was therefore renamed *dSc-2*. The other two genes were studied in detail because their null mutant phenotypes are reminiscent of mutations in components of ecdysone signaling (a failure to molt or undergo metamorphosis). *ida* encodes a protein required for imaginal disc development and will be described separately (A. M. Bentley, B. Williams, M. Goldberg and A. J. Andres, unpublished results). *mge* encodes a protein with similarity to mitochondrial protein Tom22, a component of translocase of the outer membrane complex (reviewed in Neupert 1997; Pfanner *et al.* 1997; Ryan *et al.* 2000). We show that mutations in this gene cause early larval lethality and that the protein co-localizes with a mitochondrial marker in cultured cells.

MATERIALS AND METHODS

Antibody production and Western blotting: Recombinant E63-1 protein was generated using the pet5 (GCCGTCGAC CAT ATG AGC CCA ATG AGC GGA C) and pet3ht (GGCGTCGACCTCGAG GAG CAA AAG TCT CGT GAA TTC) primers to amplify the *E63-1* open reading frame (ORF). This product was cloned into the pET-23a expression vector (Novagen, Madison, WI) after being digested with *Nde*I and *Xho*I. E63-1 protein produced from this clone contains a His-tag at its carboxy terminus. Essentially pure protein was prepared from crude bacterial extracts on a His-Bind column (Novagen) and was used for the generation of polyclonal rabbit antibodies (East Acres Biologicals, Southbridge, MA). The antiserum R4694 was tested on Western blots and was shown to be specific for E63-1 because one major band of the appropriate molecular weight (22 kD) was detected in extracts from staged salivary glands. This band is overproduced in heat-treated animals containing a heat-inducible *E63-1* construct, and no E63-1 protein is detected (by Western analysis or immunohistochemistry) in animals homozygous for an *E63-1* deficiency. This antiserum does not cross-react with other His-tagged or Ca^{2+}-binding proteins tested by immunoblotting.

Third-instar larval tissues were dissected from animals staged relative to the premetamorphic pulse of ecdysone using the blue-food method as described previously (Andres and Thummel 1994). In standard practice, four stages have been designated as hours relative to puparium formation (0 hr). They include animals naïve to the premetamorphic ecdysone pulse (−18), those recently exposed to ecdysone (−8), and those exposed to the hormone for several hours (−4). Dissected tissues were collected in PBS (3 mm Na_2PO_4, 7 mm Na_2HPO_4, 130 mm NaCl, pH 7.2) and homogenized in 2× Laemmli buffer (4% SDS, 20% glycerol, 120 mm Tris pH 6.8, 10% β-mercaptoethanol, and 0.002% bromphenol blue). Tissue extracts were boiled, sonicated, and boiled a second time (5 min each) before being stored at −20°. For whole animals, 10 third-instar larvae were homogenized, and 1/10 volume of the extract was loaded per lane. For salivary glands, an extract from 10 pairs was loaded per lane. Protein samples were separated on 12% SDS-PAGE gel according to standard methods (Laemmli 1979) and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Immunodetection was performed using R4694 primary antibody at a dilution of 1:50,000. For loading controls, a mouse anti-tubulin antibody (Amersham, Buckinghamshire, UK) was used at a dilution of 1:4000. Horseradish peroxidase-coupled anti-rabbit and anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at dilutions of 1:7500. Blots were blocked in 5% dry milk in PBT (PBS with 0.05% Tween 20) for 1 hr, incubated for 1 hr with primary antibody diluted in blocking solution, followed by an incubation with secondary antibody in PBT for 30 min. Blots were developed using the ECL immunodetection kit (Amersham) according to manufacturer's instructions.

Immunohistochemistry and microscopy: Whole-mount antibody staining of embryos was performed using a modification of the procedure as described (Mahaffey and Kaufman 1987). Embryos were dechorionated, fixed in a 1:1 mixture of 4% paraformaldehyde-PBS:heptane, and devitellinized with methanol. Embryos were washed in PBS with Triton-X-100 (0.05%), followed by a 1-hr block in 10% NGS (normal goat serum) in PBS with Triton-X-100. Primary antibody incubation

was performed overnight at 4° with R4694 antibody diluted 1:5000 in block solution. Embryos were washed in PBS with Triton-X-100, blocked for 1 hr, and incubated with goat anti-rabbit-FITC secondary antibody (Jackson ImmunoResearch) diluted at 1:200. Samples were mounted in glycerol with 1% *n*-propyl gallate and viewed using a Bio-Rad confocal laser microscope.

Third-instar larval tissues were dissected from animals staged relative to the premetamorphic ecdysone pulse as described above. Tissues were dissected in PBS, fixed in 4% paraformaldehyde/PBS, and incubated in block [10% NGS in PBT (0.3% Triton-X in PBS)] for 1 hr at 22°. R4694 antibody was diluted at 1:10,000 in block solution and incubated with tissues at 4° overnight. Secondary antibodies, dilutions, and mounting conditions were as described above. Dissected central nervous systems were stained simultaneously for E63-1 and EcR proteins using the conditions as described above for R4694. An EcR common region antibody (DDA2.7), a generous gift from Dr. Scot Munroe (described in Talbot *et al.* 1993), was diluted at 1:5 in block solution and detected with goat anti-mouse-Texas Red secondary antibody (Jackson ImmunoResearch) diluted at 1:200.

Drosophila stocks and media: All flies were reared on standard cornmeal molasses medium supplemented with baker's yeast. Stocks containing chromosome III markers, balancers, and a genomic source of transposase (*Δ2-3*) were obtained from the Bloomington Stock Center (Bloomington, IN). Also obtained from Bloomington were the following stocks that carry deficiencies that affect all or part of the 63F region: *Df(3L)GN19*, *Df(3L)GN24*, *Df(3L)GN34*, and *Df(3L)GN50* (see Figure 3C for cytological endpoints). *l(3)05634* is a homozygous-lethal *P[ry$^+$, PZ]* element inserted into 63F, and *disembodied (dib)* is an EMS-induced embryonic lethal mutation that maps to the region (Jürgens *et al.* 1984).

Dr. Charles Zucker generously provided the *P[w$^+$, lacW]y49* stock. It was mapped by *in situ* hybridization to 63F1,2. Several alleles of each of the lethal complementation groups *l(3)63Ea/Awh*, *l(3)63Eb*, *l(3)63Ed*, *l(3)63Fa*, *l(3)63Fb*, *l(3)63Fc*, and *l(3)63Fd* (Harrison *et al.* 1995) were generously provided by Dr. Stephen Harrison. All stocks were balanced in a *w* background using *TM6B*, *Tb*, and in a *y w* background maintained over the *TM3*, *y*$^+$ balancer to simplify complementation and developmental analyses.

Lethal phase studies: Approximately 300 females of the genotype *y w; Df(3L)GN19/TM3, y*$^+$ were crossed to ~100 *y w* males carrying a parental chromosome III (from the *CS* or *w^{1118}* stock) or mutations in the 63F region balanced over *TM3, y*$^+$. Crosses were maintained in population cages and embryos were collected on yeasted hard-agar molasses plates. The number of *y* or *y*$^+$ animals that hatched was scored. If no embryonic lethality occurred, a frequency of 0.25 (yellow larvae/total eggs) was expected and was interpreted as 100% embryonic viability. Animals of the appropriate genotype were also selected as hatching first-instar larvae, transferred at a density of 100 animals/10 ml of standard cornmeal molasses medium and scored for puparium formation and eclosion.

***In situ* hybridization to polytene chromosomes:** Stocks carrying the large deficiencies *Df(3L)GN19*, *Df(3L)GN24*, *Df(3L)GN34*, and *Df(3L)GN50* (balanced over *TM6B*, *Tb*) were outcrossed to wild-type Canton-S animals. *Tb*$^+$ wandering third-instar larvae were selected for analysis. Probes used for hybridization were as indicated in Figure 4 (asterisks). Probe fragments were labeled using digoxygenin from the Genius (Boehringer Mannheim, Indianapolis) kit, and squashes were prepared and hybridized as reported elsewhere (Chen *et al.* 1992).

***P*-element rescue:** Constructs for generating transgenic animals were inserted into the CaSpeR 4 vector (Thummel *et al.* 1988) and injected into dechorionated *w^{1118}* animals containing a genomic source of transposase, *P[ry$^+$, Δ2-3](99B)* (abbreviated *Δ2-3*), for the generation of *w*$^+$ transformants as described previously (Robertson *et al.* 1988; Andres and Cherbas 1994). The four constructs used are described below and are diagrammed in Figure 4. *P[w$^+$, E63-1/K2]* consists of an *E63-1A* cDNA fused to 1.5 kb of 5′ genomic sequence (including part of *E63-2*). *P[w$^+$, E63-2]* consists of a 7.5-kb genomic fragment containing the intergenic region between *E63-1* and *E63-2*, the 1.3 kb of coding information for *E63-2*, and 5 kb of 3′ sequence flanking *E63-2*. *P[w$^+$, 63F/K1]* contains 1 kb of noncoding sequence from exon 7 of *E63-1* and 1.5 kb of genomic DNA containing the *mge* gene without its untranslated 3′ DNA. *P[w$^+$, 63F/dc]* consists of 1 kb of sequence from exon 7 of *E63-1* and an additional 6 kb of sequence downstream from *E63-1*, including information for the *mge* and *ida* genes. At least three independent lines were generated for each transgene.

***P*-element insertion/deletion mutagenesis:** By Southern analysis, we determined that the *P[w$^+$, lacW]y49* element resides ~40 kb from the intergenic region between *E63-1* and *E63-2* (Figure 4). We used this as a starting stock to isolate second-site insertions of the initial element into the *E63* genes, using a modified procedure for generating local transpositions from a starting element (Hamilton *et al.* 1991; Tower *et al.* 1993). Briefly, *w^{1118}; P[w$^+$, lacW]y49* males were crossed to *w^{1118}; Δ2-3, Dr/TM6B, Tb* females. F_1 males that were *w; P[w$^+$, lacW]y49/Δ2-3, Dr* were crossed to the double-balancer stock *w; TM3, Sb/TM6B, Tb*. *Dr*$^+$ male progeny (containing either *TM3, Sb* or *TM6B, Tb*) from the above cross that had wild-type red eyes (presumably due to a second-site insertion of the *P[w$^+$, lacW]* element) were selected. These males were crossed to *w; TM3, Sb/TM6B, Tb* females to establish a temporary line that could be tested for an insertion of the *P* element into *E63* coding information. We isolated and established 910 temporary second-site insertion lines and screened them in batches of 25 by inverse PCR (Yeo *et al.* 1995) for insertions within the *E63-1* neighborhood. Six lines were identified that had an insert within 5 kb of the *E63* genes. However, five of the inserts had transposed to a "hotspot" 5 kb from the 5′ end of *E63-2*. One line, *P[w$^+$, lacW]I056* (Figure 4), was used for further analysis. The sixth line, *P[w$^+$, lacW]J021* (Figure 4), had an insert 5 kb from the 3′ end of *E63-1*. These new insertion lines were subsequently used to generate small deletions [*Df(3L)337*, *Df(3L)449*, and *Df(3L)107*] within the 63F region by excising the *P[w$^+$, lacW]* element(s) and selecting for changes in eye color (Cooley *et al.* 1990). The endpoints of each of these deletions (Figure 4) were determined by Southern analysis.

cDNA library screening and sequence analysis: A directionally cloned cDNA library prepared using RNA from third-instar larval tissues (Hurban and Thummel 1993) was screened for *mge* cDNAs using 1.0 kb of non-*E63-1* sequence from the *P[w$^+$, 63F/K1]* rescue construct (Figure 4). Three cDNA clones were recovered and all had identical 3′ sequence, differing only in the amount of 5′ extensions. The largest clone isolated was 1.1 kb in length, the size expected for a full-length cDNA based on the size of *mge* mRNA determined from Northern blots (Figure 7). This clone was sequenced and compared to genomic sequence obtained from the *pBS63F.15* and *pBS63F.16* subclones generated from a 63F chromosomal walk (Andres and Thummel 1995). Sequence analysis was performed using the automated sequencing facilities at the University of Chicago and at Northwestern University Medical School. Sequence-specific primers were commercially produced (Integrated DNA Technologies, Coralville, IA) and used in each reaction. Both strands were sequenced to ensure accuracy. Database searches were performed using BLAST

2.0.1 (Altschul *et al.* 1997), and sequence alignments were done using CLUSTAL W 1.74 (Thompson *et al.* 1994).

Taq DNA polymerase (GIBCO BRL, Gaithersburg, MD) and primers specific for the 5′ (Fc-FA = CTC CGC TCA AGC TCA ATT) and 3′ (Fc-3A = GTG TGG ATG CGT AAA TGC) ends of the *mge* gene were used to amplify genomic DNA isolated from mge^{b10} and mge^{d13} homozygotes, as well as the *st*-derived parental chromosome (Harrison *et al.* 1995). The 1.5-kb amplified products were subcloned into Bluescript vectors and sequenced from both strands using internally derived gene-specific primers. A comparison among the *st* parental chromosome, the longest *mge* cDNA, and the genomic clones from a cosmid library (kindly provided by Dr. John Tamkun), indicated that no polymorphisms are found in *mge* coding information. Two independent rounds of DNA isolation and PCR were performed for each mutant allele to confirm that sequence differences were due to *bona fide* mutations and not errors in the PCR process.

Northern blot analysis: The developmental blot presented in Figure 7A contains 20 μg of total RNA/lane. Animals were synchronized at three time points: egg deposition for the embryonic blot, hatching for the larval blot, and puparium formation for the prepupal/pupal blot. All animals were grown at 25°, and the time points (in hours) are relative to the synchronization points described above. The blot was prepared for a previous study (Andres and Thummel 1995), and the *rp49* loading control is presented in that work. In this analysis, the blot was hybridized using the *mge* cDNA (described above) that was random prime labeled using the Prime It II (Stratagene, La Jolla, CA) kit.

The developmental blot presented in Figure 7B was prepared from animals staged relative to pupariation (see above) using 30 μg of total RNA/lane. *mge* cDNA and *rp49* genomic probes were labeled using the Prime It II kit, and RNA extractions and blot-hybridization conditions were as described previously (Andres and Cherbas 1992).

Epitope-tagged Maggie and mitochondrial staining: The *mge-myc* construct was generated by inserting annealed oligonucleotides nsp5 (GAA CAA AAG CTT ATT TCT GAA GAA GAC TTG TCCATG) and nsp3 (GA CAA GTC TTC TTC AGA AAT AAG CTT TTG TTC CATG) into the *mge* coding region at a unique *Nsp*I site (Figure 5). The resulting construct encodes a protein with the c-Myc epitope (Wilson *et al.* 1984) plus two additional residues (Ser, Met) between amino acids 16 and 17 of the wild-type Mge protein (Figure 5). The insertion of the epitope in the correct orientation and translational frame was verified by DNA sequencing. The *mge-myc* construct was cloned into the pPAc-PL vector (L. Urness and C. S. Thummel, unpublished results) under the control of the *Actin5C* promoter. The S2 Drosophila cell line (Schneider 1972) was grown at 25° in M3 medium (Sigma, St. Louis) supplemented with 2.5 mg/ml Bacto-Peptone (Difco, Detroit), 1 mg/ml tissue-culture grade Yeastolate (Difco), and 10% heat-inactivated fetal calf serum (GIBCO BRL). Plasmid DNA was prepared using the QIAGEN (Chatsworth, CA) plasmid kit. Transient transfections were performed using 20 μg of plasmid DNA per 10 ml of cells grown to a density of 3–5 × 10^6 cells/ml as described (Fehon *et al.* 1991), except that BSS (140 mm NaCl, 0.75 mm Na_2HPO_4, 25 mm BES, pH 6.95) was used in place of HBS.

MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) was a generous gift from Dr. Phillip Hockberger. Anti-c-Myc hybridoma supernatant was a gift from Dr. Jeffrey Nye. Between each of the following steps, cells were washed three times (15 min each) in PBS. Following transfection, cells were allowed to recover for 15 hr prior to incubation with 0.3 μm MitoTracker Red CMXRos for 1 hr at 22°. Treated cells were fixed in 2% paraformaldehyde in PBS for 15 min at 22°. Cells were incubated overnight at 4° with anti-Myc hybridoma supernatant diluted at 1:20 in PBS with 1% NGS and 0.1% saponin. Treatment with secondary antibody (FITC-conjugated goat anti-mouse; Jackson ImmunoResearch), diluted 1:250 in PBS containing 1% NGS and 0.1% saponin, was performed for 2 hr at 22°. Mounting was done in 80% glycerol in 0.5 m Tris pH 8.8, and samples were viewed on a Zeiss LSM510 confocal microscope. Images were processed using Photoshop 3.05 (Adobe Systems, San Jose, CA).

RESULTS

E63-1 protein displays a dynamic subcellular localization in the salivary glands: We have previously demonstrated that the *E63-1* gene is induced in third-instar larval salivary glands in response to the premetamorphic pulse of ecdysone (Andres and Thummel 1995). In an effort to examine the localization of E63-1 during salivary gland secretion, we generated a polyclonal antibody against the full-length protein and demonstrated its specificity (see materials and methods; Figures 1C and 2E). We used this antiserum on Western blots to monitor the accumulation of E63-1 in developing salivary glands. As expected, based on RNA induction in salivary glands (Andres and Thummel 1995), E63-1 protein levels increase in response to the premetamorphic pulse of ecdysone in the salivary gland (Figure 1A). Also consistent with our previous RNA analysis is a failure to detect any major differences in protein levels when extracts from whole larvae are examined (Figure 1B). These results support our conclusion that the induction of *E63-1* by ecdysone is tissue restricted.

During embryogenesis, E63-1 protein is first detected at stage 15, primarily in the somatic musculature (Figure 1D), the pharynx (Figure 1E, arrowheads), and in the cytoplasm of cells of the anterior part of the midgut including the gastric caeca (Figure 1, E and F, arrows). These patterns of protein expression persist throughout larval development (data not shown). In addition, we detect lower levels of E63-1 localized to the postembryonic central nervous system (CNS). This pattern is easily visualized in the late third instar (Figure 1G). During this time, it is known that ecdysone regulates the transformation of the larval CNS into that of the adult. In addition, specific patterns of EcR (a component of the ecdysone receptor) isoform expression are correlated with larval CNS remodeling and apoptosis (Truman *et al.* 1994). At the onset of metamorphosis, we detect E63-1 protein in a subset of the EcR-expressing, as well as nonexpressing, cells (Figure 1, H and I). However, further analysis is needed to address the significance of this pattern or the fate (remodeling *vs.* apoptosis) of the E63-1-expressing cells.

Since we detected induction of E63-1 in third-instar larval salivary glands by Western analysis (Figure 1A), we examined the pattern of protein localization more carefully in this tissue following ecdysone exposure. As expected, E63-1 is not expressed in gland cells dissected

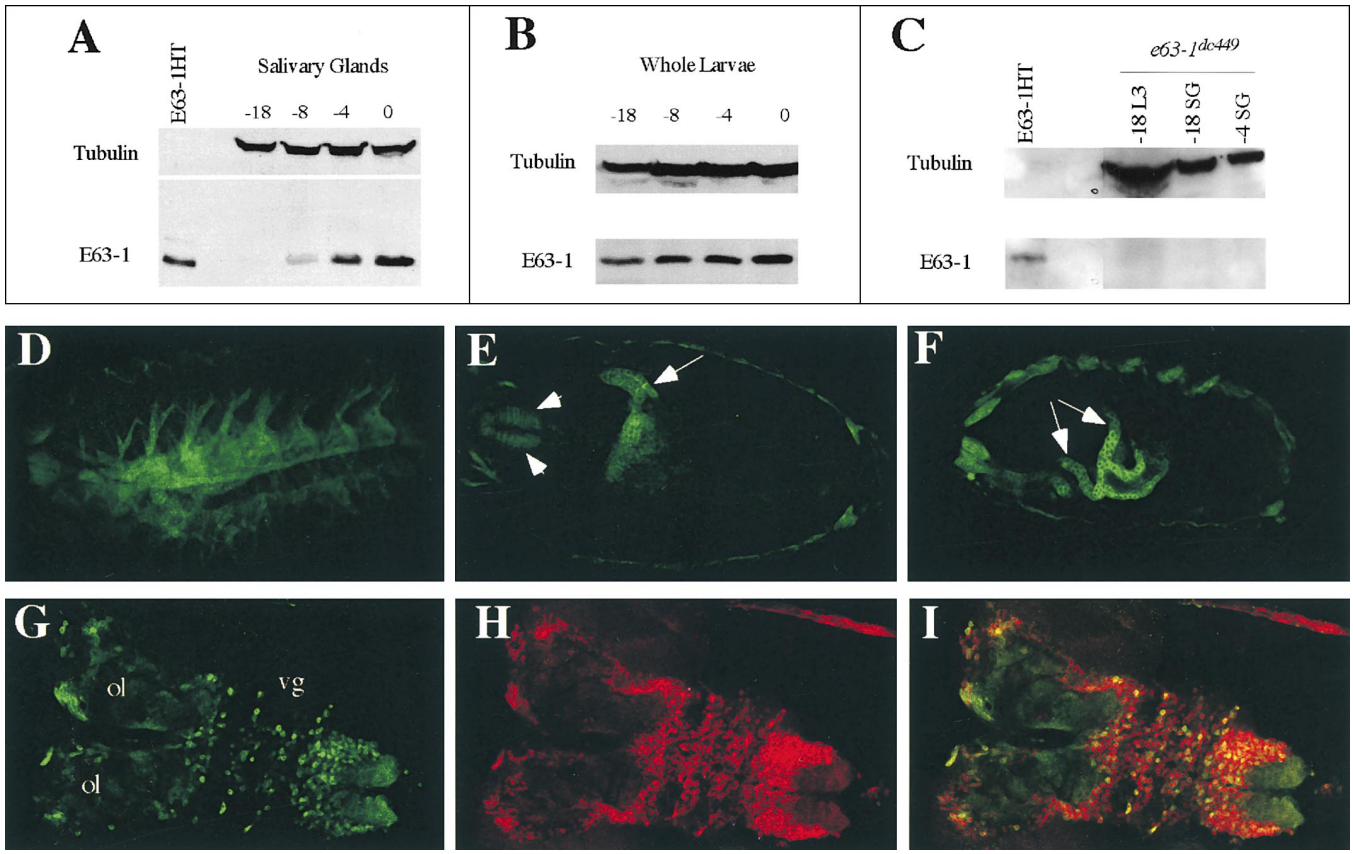


FIGURE 1.—E63-1 protein is widely expressed in embryonic and larval tissues. (A–C) Western blots of staged animals and salivary glands. Animals were staged as mid-third-instar larvae (−18, −8 hr relative to puparium formation), late-third-instar larvae (−4 hr), or newly formed puparia (0 hr; see MATERIALS AND METHODS). (A) E63-1 protein is induced in staged salivary glands in parallel with the premetamorphic pulse of ecdysone, which occurs 6–8 hr prior to pupariation. The top part of the blot was incubated with an anti-tubulin antibody as a loading control. E63-1HT is a purified bacterially expressed E63-1 protein included as a positive control. (B) E63-1 induction is not detected when extracts from whole third-instar larvae are examined. This pattern reflects the high levels of constitutive expression in nonsalivary gland tissues (see text for details). Note that when whole animals are used for extracts, three tubulin bands are usually detected in younger (−18, −8 hr) larvae. Only the top isoform is presented here. (C) E63-1 protein is not detected in *E63-1* mutant animals. Extracts from whole mid-third-instar wandering larvae (−18 L3) and dissected staged salivary glands (−18 SG; −4 SG) are represented from animals completely missing *E63-1* coding information (*e63-1*^{dc449}). (D–F) Optical sections showing E63-1 protein localization in embryonic tissues. E63-1 is detected in the somatic musculature (D), the pharynx (E, arrowheads), and the gastric caeca of the anterior midgut (E, F, arrows). (G–I) Optical sections showing that E63-1 and EcR co-localize in a subset of third-instar larval cells. E63-1 is detected in the CNS of third-instar larvae (G). Note that it is expressed in cells of the ventral ganglia (vg), but it is not detected in cells of the optic lobe (ol). EcR localization to the same CNS (H). Note that more cells are labeled, and only nuclei stain. Merged images of E63-1 and EcR staining (I). Note colocalization of EcR and E63-1 in a subset of cells from the ventral ganglia.

prior to the ecdysone pulse (Figure 2A). The staining detected in the nonsecreting duct cells is nonspecific as it has been observed in all salivary glands examined (>100 wild-type animals from all developmental stages), as well as in mutants ($N > 50$) carrying deletions of the *E63-1* locus (Figure 2E). However, ~1 hr after ecdysone exposure, E63-1 is detected near or on cell membranes of the gland cells proper, in nuclei (Figure 2B, arrow), and in the cytoplasm between secretory granules (Figure 2B, arrowheads). This pattern of localization is always seen in tissues that have not yet secreted glue. After glue is secreted into the lumen (note the dramatic decrease in the number of secretory granules in Figure 2C, arrowheads), the entire gland begins to swell in size, and E63-1 becomes excluded from nuclei (Figure 2C, arrow). E63-1 remains excluded from nuclei and is retained in the cytoplasm even after the glue is expelled from the gland (Figure 2D).

This dynamic pattern of E63-1 localization in the salivary gland during secretion led us to speculate about its function in these cells. One model involves a mechanism whereby E63-1 modulates Ca^{2+} levels by allowing ions to enter the cell through a membrane-localized pore or channel (membrane staining). Alternatively, E63-1 may control the expression of downstream effector molecules, perhaps by regulating transcription factors (nuclear staining), or E63-1 might be involved in transporting or docking secretory granules. To test these

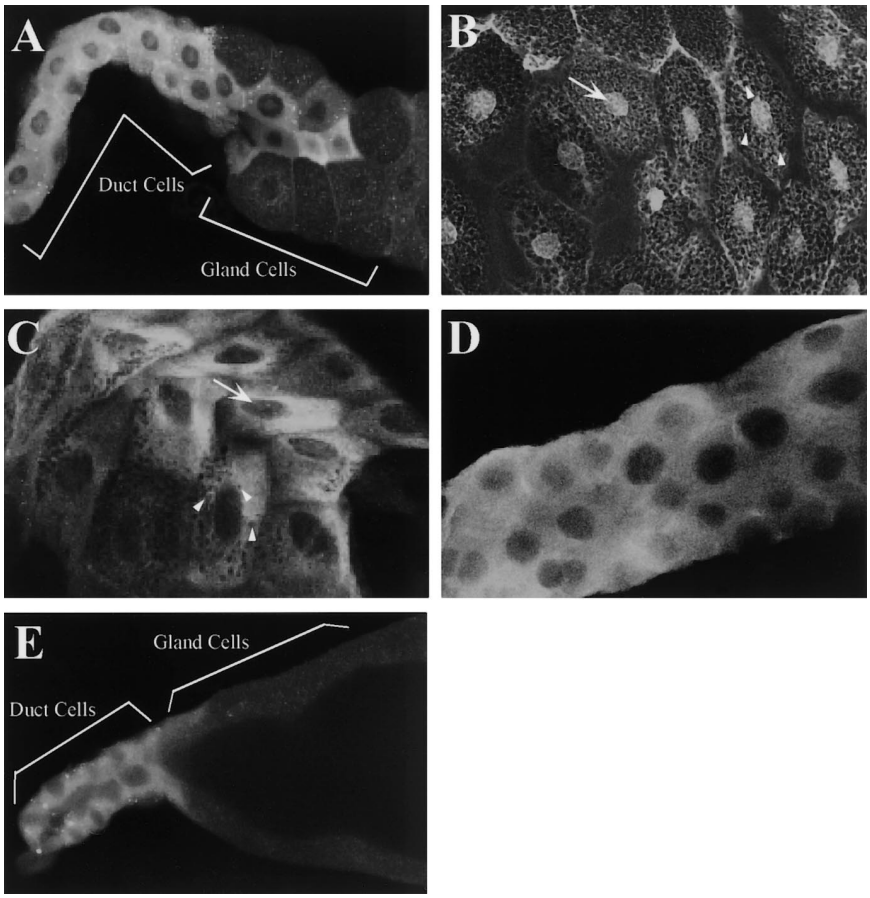


FIGURE 2.—E63-1 undergoes changes in subcellular localization during salivary gland secretion. (A–E) Confocal micrographs showing the localization of E63-1 protein in wild-type and *e63-1*^{dc449} salivary glands staged relative to the premetamorphic pulse of ecdysone. High levels of protein are not detected in the gland cells before the tissue is exposed to ecdysone (A). When E63-1 is first detected in gland cells (B), they are not yet secreting glue, and the protein is localized primarily to nuclei (arrow), near cell membranes, and around secretory granules (arrowheads). Once the salivary gland secretes glue (C), fewer secretory granules are detected in the cytoplasm (arrowheads), and E63-1 is no longer detected in nuclei (arrow); overall levels of E63-1 protein are also higher at this stage, consistent with the data presented in Figure 1A. High levels of cytoplasmic E63-1 protein remain in the salivary glands after the glue has been expelled (D). Note that the staining detected in duct cells (A) is nonspecific. This was verified when glands dissected from the *e63-1*^{dc449} null animals were stained (E). Note the enlarged lumen (filled with glue) of the mutant gland. At this stage, E63-1 is always expressed at high levels in the cytoplasm of gland cells.

hypotheses, we undertook a genetic analysis of the 63F region in an effort to isolate and characterize loss-of-function mutations in *E63-1*.

A genetic analysis of the 63F3-7 region reveals that *E63-1* and *E63-2* are not necessary for viability: The location of the *E63-1* gene relative to the endpoints of four large chromosomal deletions (Figure 3C) was determined. We hybridized probes specific for the 5′ and 3′ ends of the *E63-1* primary transcript to chromosomal preparations from animals carrying one copy of each deficiency. The analyses for *Df(3L)GN19* and *Df(3L)GN24* are the most informative. Figure 3, A and B, shows examples of this analysis using a 3′-specific probe (Figure 4, asterisk). The 3′ probe fails to hybridize to the *Df(3L)GN19*-bearing homolog (Figure 3A, arrow), but hybridizes to the homolog containing the *Df(3L)GN24* deletion (Figure 3B, arrows). The same result is observed with a probe specific to the 5′ end (data not shown). On the basis of these data, we conclude that *E63-1* resides between the distal endpoints of the *Df(3L)GN19* and *Df(3L)GN24* deletions in an area defined as 63F3-7 (Figure 3C).

Eight lethal complementation groups identified in a previous EMS screen were mapped to 63F (Figure 3C; HARRISON *et al.* 1995). Using a complementation analysis with the *Df(3L)GN19* and *Df(3L)GN24* deficiencies described above, we found that only four of these complementation groups [*l(3)63Eb*, *l(3)63Fa*, *l(3)63Fb*, and *l(3)63Fc*] map to the region where *E63-1* is localized. However, a construct containing an *E63-1A* cDNA fused to 1.5 kb of 5′ sequence (*P[w*$^{+}$*, E63-1/K2]*, Figure 4) or a 7.5-kb genomic fragment containing all of the coding information for *E63-2* (*P[w*$^{+}$*, E63-2]*, Figure 4) fails to rescue the lethality associated with these complementation groups. There are several possible explanations for these results. First, the *E63-1* rescue construct may not function as the endogenous gene since potentially important regulatory information contained in introns and 3′ flanking DNA might be missing. Second, the EMS screen might not have identified all the lethal genes within the 63F region. Third, mutations in the *E63* genes might not cause lethality.

To address the above possibilities concerning the *E63* genes, we generated mutations by mobilizing the *P[w*$^{+}$*, lacW]y49* element. This insert maps 38 kb from the intergenic region between *E63-1* and *E63-2* (Figure 4). This strategy allowed us to screen for insertions into the *E63* coding regions without having to rely on the detection of specific phenotypes. After screening nearly 1000 second-site insertions, we were unsuccessful in disrupting the coding information of *E63-1* or *E63-2*, but found *P* elements inserted ~5 kb from the 3′ end of each gene (Figure 4, *P[w*$^{+}$*, lacW]J021* for *E63-1* and *P[w*$^{+}$*, lacW]I056* for *E63-2*). We mobilized these inserts

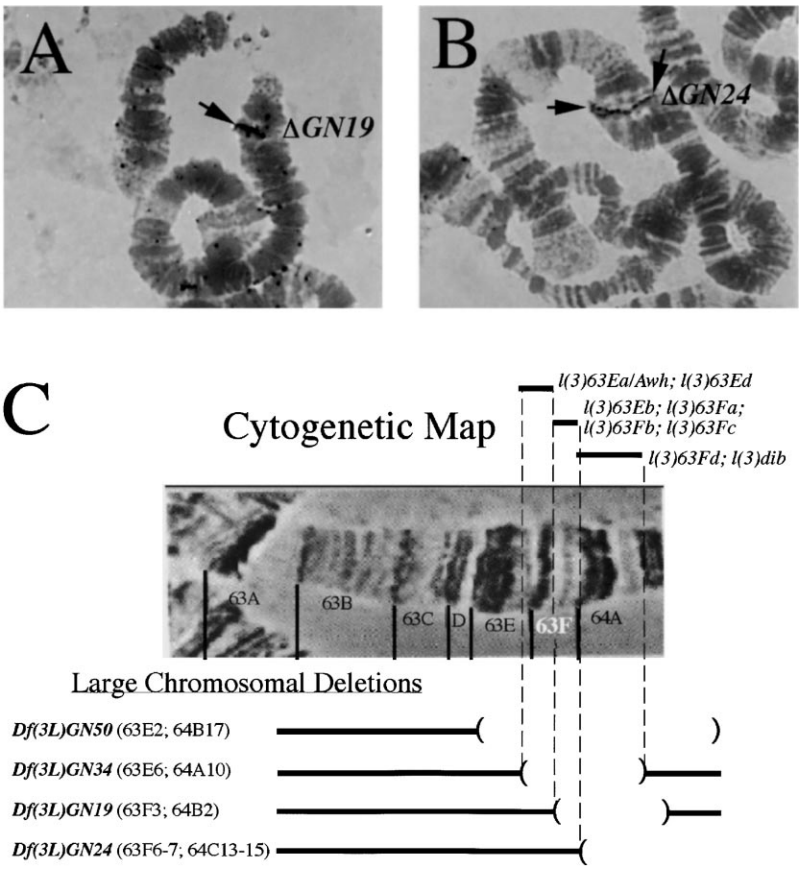


FIGURE 3.—Deletion mapping of the 63F region. (A and B) *In situ* hybridization to polytene chromosomes carrying deficiencies of 63F. Probes were derived from the 5′ and 3′ ends of *E63-1* (Figure 4, asterisks). Hybridization of the 3′ probe (arrow) to a chromosome squash carrying the *Df(3L)GN19* deficiency is shown (A). Note the lack of signal on the deleted homolog (Δ) indicating that *E63-1* maps to the area defined by the deficiency. Hybridization of the same probe (arrow) was performed on a *Df(3L)GN24*-containing squash (B). Note that the signal is detected on both homologs, indicating that *E63-1* does not map to the DNA removed by this deletion. The results of a similar analysis using a 5′ probe indicated that E63-1 maps to the area defined as 63F3-7, determined by the cytological endpoints of the *Df(3L)GN19* and *Df(3L)GN24* deficiencies (C). Lethal complementation groups were assigned to a chromosomal position based on the results of a standard complementation test among all reagents presented in C (see text for details). Note that four lethal genes, *l(3)63Eb*, *l(3)63Fa*, *l(3)63Fb*, and *l(3)63Fc*, mapped to the 63F3-7 region where *E63-1* was localized by *in situ* hybridization. The chromosomal photograph and the endpoints reported for the deficiencies were obtained from Flybase.

in a second screen, selecting for flies that had lost one or both elements along with flanking genomic DNA. The endpoints of two deletions that are homozygous lethal [*Df(3L)449* and *Df(3L)107*] and one that is homozygous viable [*Df(3L)337*] were mapped by Southern analysis and are depicted in Figure 4.

Three important genetic tools were generated in this analysis. The first, *Df(3L)337* (Figure 4), is a 6-kb deficiency that removes all *E63-2* coding information and 4.5 kb of its 3′ flanking sequence. However, the entire *E63-1* gene remains intact and is properly expressed as demonstrated by Northern and Western analyses on homozygous mutant animals (data not shown). Flies homozygous for *Df(3L)337* or flies carrying this deficiency in combination with a larger deletion [*Df(3L)337/Df(3L)GN19*] develop normally and are fertile, demonstrating that the *E63-2* gene is not required for viability (Table 1).

The second reagent, *Df(3L)449*, is a deletion that removes *E63-1* and *E63-2* as well as 5 kb of flanking DNA on each side. Animals missing the *Df(3L)449* region die as first-instar larvae. Two of the four lethal complementation groups that we had previously mapped to 63F3-7 [*l(3)63Fb and l(3)63Fc)*] fail to complement this deletion, indicating that *Df(3L)449* is not specific for *E63-1* and *E63-2*. In addition, both lethal complementation groups can be rescued with constructs containing DNA between the distal end of the deficiency and the 3′ end of *E63-1* (see below).

The third reagent is a stock that carries a genomic segment of DNA spanning the gap from the 3′ end of *E63-1* through the distal end of the *Df(3L)449* deletion (Figure 4, *P[w$^+$, 63F/dc]*). This transgene was recombined onto the *Df(3L)449* chromosome to create *Df(3L)449, P[w$^+$, 63F/dc]* (abbreviated *e63-1^{dc449}*). Animals homozygous for this recombinant chromosome are completely lacking the *E63* genes but are rescued for *l(3)63Fb* and *l(3)63Fc* functions as determined by subsequent complementation analyses. The *e63-1^{dc449}* stock is completely deficient for *E63-1* and *E63-2*; however, these animals display only minor reductions in viability (Table 1). Surviving animals display no major abnormalities in salivary gland glue secretion and are fully fertile (T.-V. Do, A. BIYASHEVA, Y. LU, M. VASKOVA and A. J. ANDRES, unpublished results). We therefore conclude that both *E63-1* and *E63-2* are not essential for viability or fertility.

Disruption of *maggie* results in early larval lethality: In the course of generating the *e63-1^{dc449}* synthetic deficiency and by analyzing the genomic sequence of the region, we determined that the *l(3)63Fc* lethal complementation group (HARRISON *et al.* 1995) is separated from the *E63-1* gene by 395 bp (Figure 5). Two EMS-induced alleles, *l(3)63Fc^{a1}* and *l(3)63Fc^{b10}*, are recessive

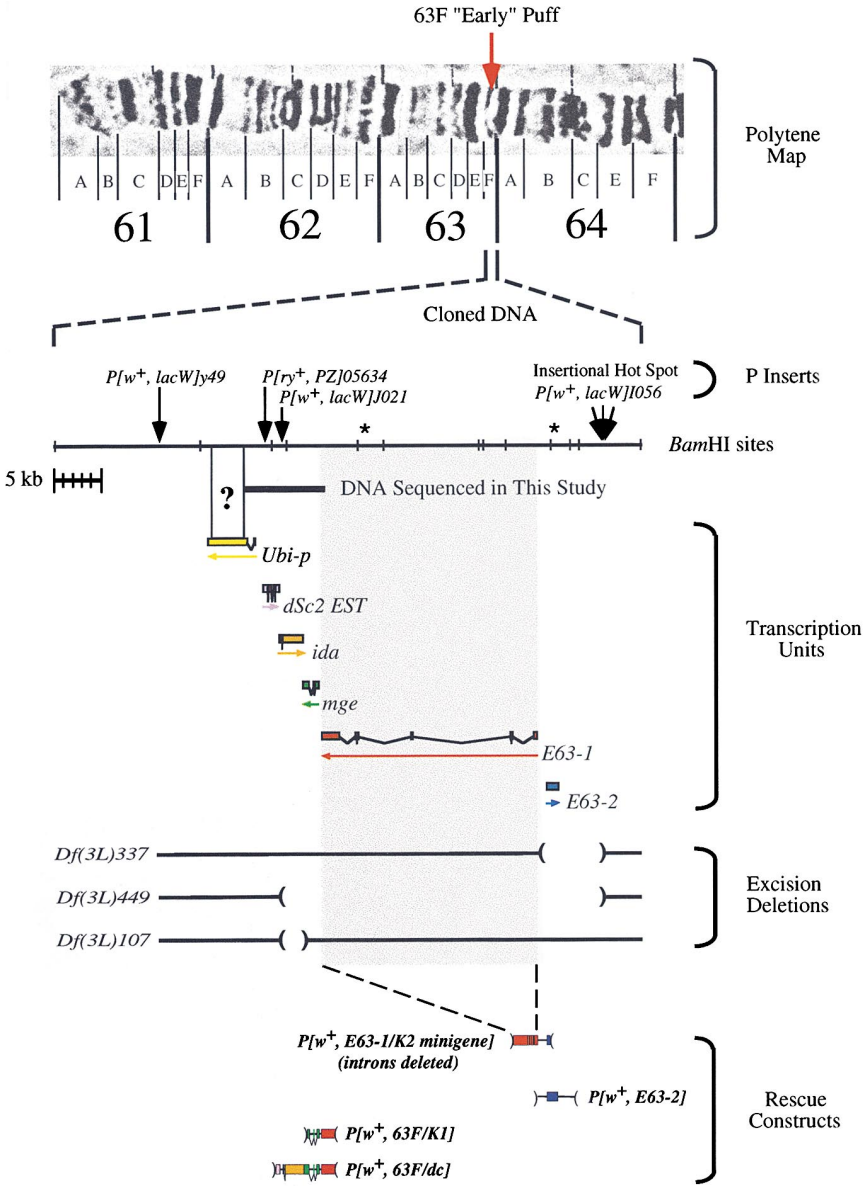


FIGURE 4.—Genetic and molecular map of the 63F region. The position of the 63F early puff is marked (red arrow) relative to a photograph of the 61–64 interval of chromosome III (downloaded from Flybase). Below the photograph is a *Bam*HI restriction map of cloned DNA from the 63F puff. Positions of the $P[w^+]$ and $P[ry^+]$ elements indicated in the text are marked by arrows. Asterisks (*) indicate DNA fragments used as probes for *in situ* hybridization to polytene chromosomes (Figure 3, A and B). A section of the chromosome sequenced before the genome project was completed is represented by the black bar. The box containing the question mark represents 14 ubiquitin repeats that are not present in the current version of the sequenced genome (see text for details). Below the restriction map are the gene structures of *Ubi-p*, *dSc-2*, *ida*, *mge*, *E63-1*, and *E63-2*; the directions of transcription are indicated by arrows. Deletions that were identified in *P*-element excision screens are indicated with boundaries marked by parentheses. At the bottom of the figure are diagrams of transgene constructs that were used in rescue experiments.

lethal null mutations resulting in growth arrest and 100% lethality during the first larval instar. Animals of the genotypes presented in Table 1, as well as *Df(3L)449/Df(3L)449*, $l(3)63Fc^{a1}/Df(3L)449$, $l(3)63Fc^{b10}/Df(3L)449$, $l(3)63Fc^{b10}/l(3)63Fc^{b10}$, and $l(3)63Fc^{a1}/l(3)63Fc^{b10}$ progress normally through embryogenesis and hatch on schedule. However, mutant larvae do not grow but remain as first instars for 5–9 days (the normal first-instar period is 24 hr). No gross morphological defects are observed, but mutant larvae appear to move more slowly than wild type. Homozygous $l(3)63Fc^{a1}/l(3)63Fc^{a1}$ animals are embryonic lethal, presumably due to an unrelated second-site mutation on that chromosome.

Animals hemizygous or homozygous for a hypomorphic allele, $l(3)63Fc^{d13}$, exhibit significantly slower growth/developmental rates, as compared to wild type, and take twice as long to pupariate (8 days), with only 22% of the mutant animals reaching that stage (Table 1).

Since *l(3)63Fc* null mutants appear to arrest in the first-instar stage of development, we have renamed this gene *maggie (mge)* after a cartoon character in "The Simpsons," who is also arrested in early development.

***mge* encodes a putative mitochondrial outer membrane protein:** Since it is known that pulses of ecdysone precede and initiate the molting process (reviewed in RIDDIFORD 1993), one possible explanation for the failure of *mge* null mutants to progress to the second instar is that they are defective in ecdysone synthesis or signaling. To test this possibility, we molecularly characterized the gene responsible for the *mge* mutant phenotype.

Figure 5 summarizes the gene structure of the mge transcription unit as determined from a comparison between cDNA and genomic sequences, as well as a number of expressed sequence tags (ESTs) derived from a variety of RNA sources that are listed with the Berkeley Drosophila Genomic Project (BDGP). As shown in Figure 5, mge contains two introns of 179 bp

TABLE 1

Lethal phases of mutant alleles that map to 63F3-7

			Hatched			Pupariated		
Gene	Allele		No. eggs	No. *y* larvae exp.	No. *y* larvae obs.	% viable	No. *y* larvae hatch	No. *y* larvae pupar.	% viable
		Parental from w^{1118}:	760	190	166	87.4	341	233	68.3
		Parental from *CS*:	609	152	132	86.8	196	157	80.1
l(3)63Eb/dSc-2	*l(3)63Eb*^{A4}		446	112	84	75.0	0	0	0.0
	l(3)63Eb^{E15}		442	110	97	88.2	0	0	0.0
	l(3)05634		410	102	100	98.0	0	0	0.0
l(3)63Fa	*l(3)63Fa*^{D9}		435	109	99	90.8	199	155	77.0
l(3)63Fb/ida	*Df(3L)107*		545	136	123	90.4	206	176	85.4
	l(3)63Fb^{b4}		639	160	154	96.2	200	161	80.5
	l(3)63Fb^{d14}		678	170	147	86.5	200	150	75.0
l(3)63Fc/mge	*Df(3L)449*		416	104	88	84.6	0	0	0.0
	l(3)63Fc^{a1}		410	102	97	95.1	0	0	0.0
	l(3)63Fc^{b10}		349	87	97	111*	0	0	0.0
	l(3)63Fc^{d13}		591	148	107	72.3	202	44	21.8
E63-1	*e63-1*^{dc449}		456	114	78	68.4	195	133	68.2
E63-2	*Df(3L)337*		483	121	95	78.5	199	135	67.8

The viability of animals hemizygous for each mutant allele that mapped to 63F3-7 was assayed at two developmental stages, hatching to the first instar and puparium formation. In all cases, males of the genotype *y w; Test/TM3,* y^+ (where *Test* is the allele represented in the table) were crossed to y *w; Df(3L)GN19/TM3,* y^+ females. The percentage of viable *y* progeny (test allele/deficiency for the 63F3-7 region) is recorded. Note that if no lethality occurs during the embryonic period, the number of *y* larvae should equal one quarter the total number of eggs examined. In one case (marked by an asterisk), the observed number was greater than the expected number, resulting in a percentage viability >100. *CS* (Canton-S) and w^{1118} represent wild-type controls. Although not listed, all animals mutant for *l(3)63Fa* and *ida* failed to eclose; 2% of *mge*^{d13} prepupae eclosed, but were sterile; and 90% of *CS*, w^{1118}, and *e63-1*^{dc449} eclosed and were fertile. Both the original (Harrison *et al.* 1995) and renamed versions of each gene are listed in the left column. When available, multiple alleles were assayed.

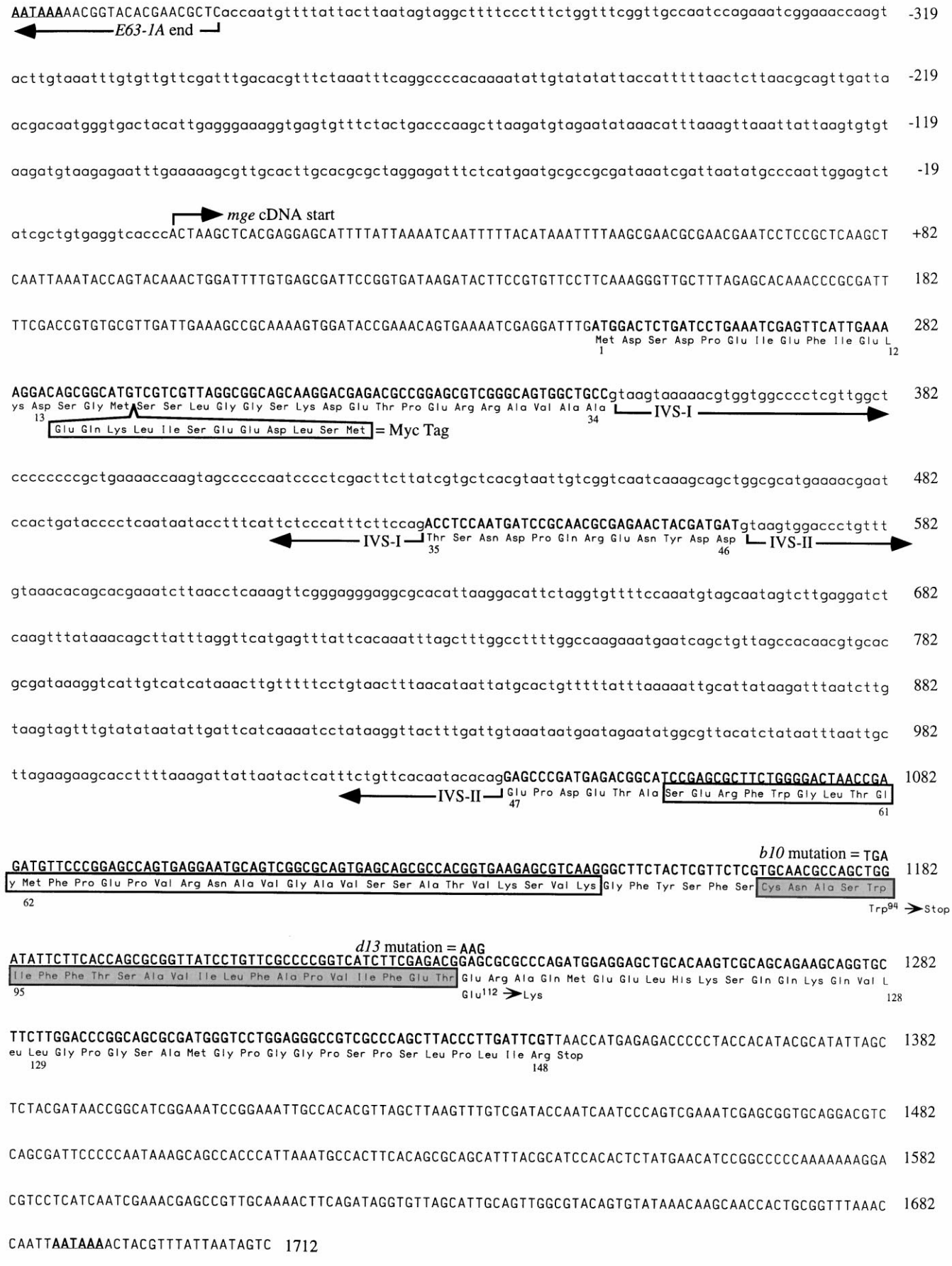
and 473 bp. The cDNA contains a putative ORF of 447 nucleotides, encoding a hypothetical protein of 148 amino acids with a calculated molecular weight of 16.2 kD. The predicted Mge amino acid sequence shows similarity to Tom22 proteins from *Saccharomyces cerevisiae* (20% overall identity; Lithgow *et al.* 1994; Nakai and Endo 1995) and *Neurospora crassa* (25% overall identity; Kiebler *et al.* 1993; Figure 6A).

Tom22 is a component of the preprotein translocase of the mitochondrial outer membrane (TOM complex), and its disruption is lethal in both *S. cerevisiae* and *N. crassa* (Lithgow *et al.* 1994; Nargang *et al.* 1995). In addition, we believe the 20–25% identity between fungal Tom22s and Mge to be significant as the same degree of similarity is observed when the functional homologs of *S. cerevisiae* and *N. crassa* are compared (Lithgow *et al.* 1994; Nakai and Endo 1995).

Like other Tom22 proteins, Mge contains a stretch of 21 uncharged hydrophobic amino acids (residues 90–109), suggesting that it has the same membrane insertion topology as Tom22 (Figure 6, A and B). The N-terminal cytosolic region of Mge also contains two sequence motifs identified from the fungal Tom22s: an acidic region that may provide a binding site for mitochondrial precursor proteins (Lithgow *et al.* 1994) and a positively charged sequence that has been shown to be required for Tom22 import into mitochondria (Rodriguez-Cousino *et al.* 1998).

To confirm that mutations in *mge* are responsible for the *l(3)63Fc* phenotypes, we sequenced two of the EMS-induced alleles. In *mge*^{b10} null mutants, the Trp-94 codon (TGG) is converted to a stop codon (TGA), truncating the Mge protein at the beginning of the transmembrane domain (Figures 5 and 6B). We predict that this truncation affects the ability of the protein to be inserted into the mitochondrial membrane. The hypomorphic *mge*^{d13} allele results in a single amino acid change from the acidic Glu-112 (GAG) to a basic Lys (AAG) residue in the carboxyl-terminal portion of the protein that projects into the intermembrane space (Figures 5 and 6B). Tom22 proteins contain an acidic domain in their carboxyl terminus, and it has been suggested that this domain may bind the targeting sequences of mitochondrial precursors (Moczko *et al.* 1997; Komiya *et al.* 1998).

***maggie* is expressed throughout development and localizes to mitochondria:** To determine the developmental expression profile of *mge*, we performed a Northern analysis using total RNA samples extracted from all stages of development. As shown in Figure 7A, *mge* mRNA is most abundant in embryos, early larvae, and late pupae, with a decrease (but not elimination) of


AATAAAAACGGTACACGAACGCTCaccaatgttttattacttaatagtaggcttttccctttctggtttcggttgccaatccagaaatcggaaaccaagt -319
E63-1A end
acttgtaaatttgtgttgttcgatttgacacgtttctaaatttcaggccccacaaaatattgtatatattaccatttttaactcttaacgcagttgatta -219
acgacaatgggtgactacattgagggaaaggtgagtgtttctactgacccaagcttaagatgtagaatataaacatttaaagttaaattattaagtgtgt -119
aagatgtaagagaatttgaaaaagcgttgcacttgcacgcgctaggagatttctcatgaatgcgccgcgataaatcgattaatatgcccaattggagtct -19
mge cDNA start
atcgctgtgaggtcacccACTAAGCTCACGAGGAGCATTTTATTAAAATCAATTTTTACATAAATTTTAAGCGAACGCGAACGAATCCTCCGCTCAAGCT +82
CAATTAAATACCAGTACAAACTGGATTTTGTGAGCGATTCCGGTGATAAGATACTTCCGTGTTCCTTCAAAGGGTTGCTTTAGAGCACAAACCCGCGATT 182
TTCGACCGTGTGCGTTGATTGAAAGCCGCAAAAGTGGATACCGAAACAGTGAAAATCGAGGATTTGATGGACTCTGATCCTGAAATCGAGTTCATTGAAA 282
Met Asp Ser Asp Pro Glu Ile Glu Phe Ile Glu L
1 12
AGGACAGCGGCATGTCGTCGTTAGGCGGCAGCAAGGACGAGACGCCGGAGCGTCGGGCAGTGGCTGCCgtaagtaaaaacgtggtggcccctcgttggct 382
ys Asp Ser Gly Met Ser Ser Leu Gly Gly Ser Lys Asp Glu Thr Pro Glu Arg Arg Ala Val Ala Ala
13 34
IVS-I
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Ser Met = Myc Tag
ccccccccgctgaaaaccaagtagcccccaatcccctcgacttcttatcgtgctcacgtaattgtcggtcaatcaaagcagctggcgcatgaaaacgaat 482
ccactgataccctcaataataccttcattctcccatttcttccagACCTCCAATGATCCGCAACGCGAGAACTACGATGATgtaagtggaccctgttt 582
IVS-I
Thr Ser Asn Asp Pro Gln Arg Glu Asn Tyr Asp Asp
35 46
IVS-II
gtaaacacagcacgaaatcttaacctcaaagttcgggagggaggcgcacattaaggacattctaggtgttttccaaatgtagcaatagtcttgaggatct 682
caagtttataaacagcttatttaggttcatgagtttattcacaaatttagctttggccttttggccaagaaatgaatcagctgttagccacaacgtgcac 782
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IVS-II
Glu Pro Asp Glu Thr Ala Ser Glu Arg Phe Trp Gly Leu Thr Gl
47 61
b10 mutation = TGA
GATGTTCCCGGAGCCAGTGAGGAATGCAGTCGGCGCAGTGAGCAGCGCCACGGTGAAGAGCGTCAAGGGCTTCTACTCGTTCTCGTGCAACGCCAGCTGG 1182
y Met Phe Pro Glu Pro Val Arg Asn Ala Val Gly Ala Val Ser Ser Ala Thr Val Lys Ser Val Lys Gly Phe Tyr Ser Phe Ser Cys Asn Ala Ser Trp
62 Trp94 Stop
d13 mutation = AAG
ATATTCTTCACCAGCGCGGTTATCCTGTTCGCCCCGGTCATCTTCGAGACGGAGCGCGCCCAGATGGAGGAGCTGCACAAGTCGCAGCAGAAGCAGGTGC 1282
Ile Phe Phe Thr Ser Ala Val Ile Leu Phe Ala Pro Val Ile Phe Glu Thr Glu Arg Ala Gln Met Glu Glu Leu His Lys Ser Gln Gln Lys Gln Val L
95 Glu112 Lys 128
TTCTTGGACCCGGCAGCGCGATGGGTCCTGGAGGGCCGTCGCCCAGCTTACCCTTGATTCGTTAACCATGAGAGACCCCCTACCACATACGCATATTAGC 1382
eu Leu Gly Pro Gly Ser Ala Met Gly Pro Gly Gly Pro Ser Pro Ser Leu Pro Leu Ile Arg Stop
129 148
TCTACGATAACCGGCATCGGAAATCCGGAAATTGCCACACGTTAGCTTAAGTTTGTCGATACCAATCAATCCCAGTCGAAATCGAGCGGTGCAGGACGTC 1482
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CGTCCTCATCAATCGAAACGAGCCGTTGCAAAACTTCAGATAGGTGTTAGCATTGCAGTTGGCGTACAGTGTATAAACAAGCAACCACTGCGGTTTAAAC 1682
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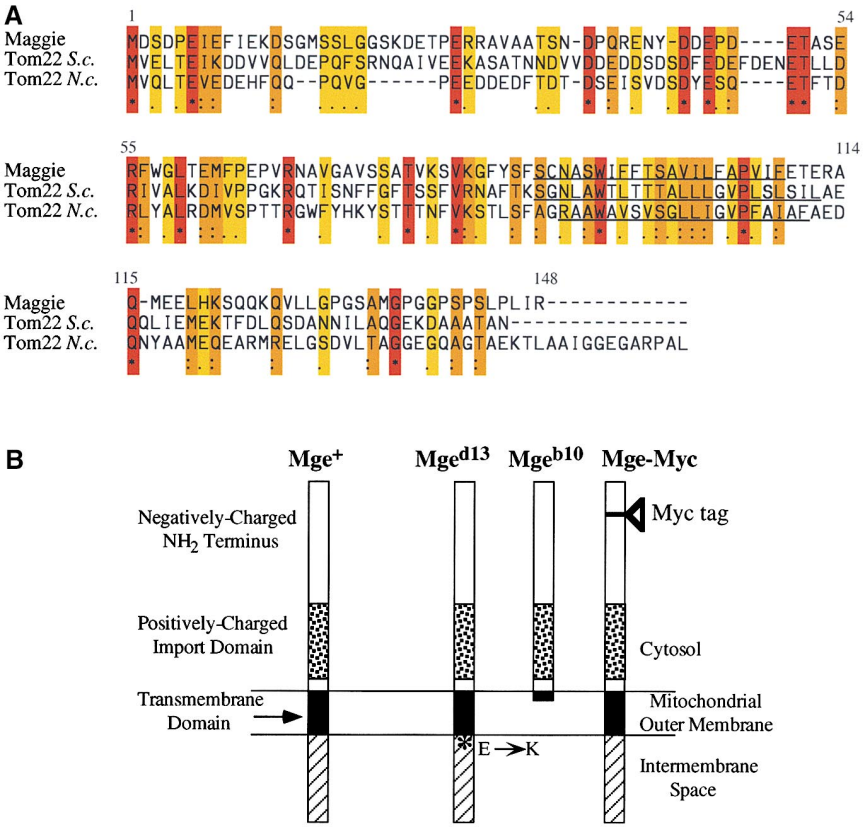

FIGURE 6.—Mge has sequence similarity to Tom22 proteins. (A) An alignment of Mge with full-length sequences of Tom22 from *S. cerevisiae (S.c.)* and *N. crassa (N.c.)* shows that there is similarity along the entire length of the protein. Amino acid numbers are relative to the Mge sequence. Identical residues among all three sequences are shown in red and marked by an asterisk (*), and conservative changes are shown in orange (:) with less conserved changes shown in yellow (·). The putative transmembrane domain is underlined. (B) A diagram of the putative structure of wild-type and mutant Mge proteins is presented. Mge^{b10} behaves as a genetic null and Mge^{d13} as a hypomorph (see text for details).

mRNA during the late-second-instar through early-pupal stages (Figure 7, A and B). This pattern of expression at all developmental stages is not unexpected for an mRNA encoding a mitochondrial import protein, although it is not clear why a drop in mge mRNA occurs during the third instar. One possible explanation might be that it correlates with a period during development when little cell division is occurring relative to the size of the animal (ASHBURNER 1989). At this time, growth is mostly a consequence of enlargement of larval cells that may have acquired an adequate supply of functional mitochondria.

Although the sequence similarity and predicted protein motifs make a compelling argument that Mge could function as a Tom22, Mge localization to mitochondria would strengthen our hypothesis that it is a functional Drosophila homolog. To address this question, we transfected Drosophila S2 cells, which constitutively express endogenous *mge* RNA (data not shown), with a *myc*-tagged *mge* construct under the control of the *Actin5C* promoter (see MATERIALS AND METHODS; Figures 5 and 6B). We observed that Mge-Myc is detected within punctate spots in the cytoplasm of transfected cells (Figure 8A). When cells are simultaneously treated with MitoTracker Red CMXRos (Figure 8B), a compound that specifically labels mitochondria (WHITAKER *et al.* 1991; IYENGAR *et al.* 1999), we detect fluorescent colocalization in the same punctate pattern (Figure 8C). This observation is consistent with the hypothesis that Mge is part of the TOM complex in Drosophila.

The *l(3)63Fb/ida* locus is required for imaginal disc development: As described above, the *l(3)63Fb* complementation group also maps to the region missing in the *Df(3L)449* deletion, since two EMS alleles, $l(3)63Fb^{b4}$ and $l(3)63Fb^{d14}$, fail to complement it. Both alleles behave as genetic nulls since animals hemizygous or homozygous for each exhibit the same lethal mutant phenotype—an arrest in development during the prepupal period (Table 1). This is also the phenotype observed in animals missing DNA from the *Df(3L)107* deletion [*Df(3L)107/*

FIGURE 5.—Sequence analysis of the *mge* gene. Genomic sequence is shown in lowercase letters, cDNA sequence is capitalized, and the coding sequence is in boldface letters. The numbers on the right represent the nucleotide position relative to the *mge* cDNA start. The positions of the two introns (IVS-1 and IVS-II) are marked by arrows below the sequence. A putative poly(A) site is underlined. Numbers below the sequence indicate amino acid positions in the *mge* open reading frame. The open box shows the position of the putative mitochondrial targeting sequence, and the shaded box depicts the position of the putative transmembrane domain. Positions of the mge^{b10} and mge^{d13} mutations, as well as of the Myc insertion, are also shown. The GenBank accession number is AF241222.

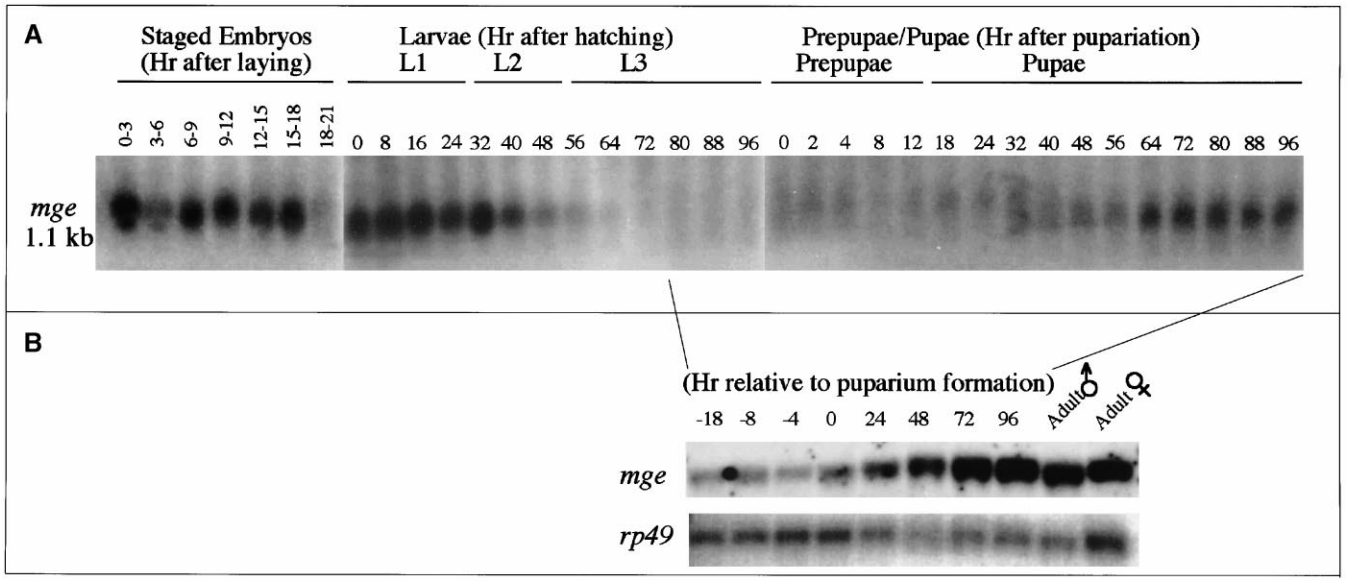

FIGURE 7.—Temporal pattern of *mge* expression throughout development. A comprehensive developmental Northern blot was probed for *mge* mRNA (A). Twenty micrograms of total RNA per lane was loaded and hybridized with a *mge* cDNA insert. Only one transcript was observed. The blot contains equal amounts of RNA (except for an underloaded embryonic lane marked 18–21) as noted by an *rp49* hybridization performed on the same blot in a previous analysis (ANDRES and THUMMEL 1995). A separate late larval/pupal blot (B) containing 30 μg of total RNA/lane was prepared to confirm that *mge* transcript is present during all stages of development.

Df(3L)107 or *Df(3L)107/Df(3L)GN19*]. This deletion was generated by a *P*-element excision that removes DNA encoding *l(3)63Fb* (Figure 4).

Sequence analysis demonstrated that the 3′ untranslated region of *mge* and *l(3)63Fb* gene abut each other with no overlap (Figure 4).

Since larvae require the product of the *l(3)63Fb* locus for metamorphosis, we have studied this gene in more detail and have shown that it is necessary for imaginal disc development (A. M. BENTLEY, B. WILLIAMS, M. GOLDBERG and A. J. ANDRES, unpublished results). We have renamed the *l(3)63Fb* complementation group *ida*, for *i*maginal *d*iscs *a*rrested.

The 63F3-7 region contains at least one additional transcription unit: In the course of studying *E63-1*, *mge*, and *ida*, we have sequenced 10 kb of genomic DNA contained within the *pBS63F.14*, *pBS63F.15*, and *pBS63F.16* subclones from the 63F locus (ANDRES and THUMMEL 1995; Figure 4, black bar). We have identified an additional transcription unit between the previously mapped *Ubi-p* gene and *ida*, which gives rise to a single RNA band on Northern blots (data not shown). Sequence analysis of this genomic DNA and an EST identified by BDGP revealed a single open reading frame. This ORF (translated from four putative exons) contains 42% amino acid identity to a rat synaptic glycoprotein (SC-2; JOHNSTON *et al.* 1992) believed to be involved in axonal guidance (SAKURAI *et al.* 1994). Therefore, we have designated this gene *dSc-2*. A total of 874 nucleotides separate the *Ubi-p* mRNA start site from the 5′ end of the dSc-2 EST, and the two genes are divergently transcribed as illustrated in Figure 4. Furthermore, there are 331 nucleotides that separate the end of the putative *dSc-2* ORF from the longest EST/cDNA clone of *ida* (Figure 4).

In addition to the molecular analysis of *dSc-2*, we have completed a preliminary genetic analysis of this locus. One lethal *P*-element insert, *P[ry*$^{+}$*, PZ]05634* [abbreviated *l(3)05634*], has been shown by the BDGP to map within the *dSc-2* gene. This insert resides 98 nucleotides downstream from the putative *dSc-2* AUG-initiation codon. It should be noted that the *l(3)05634* element has

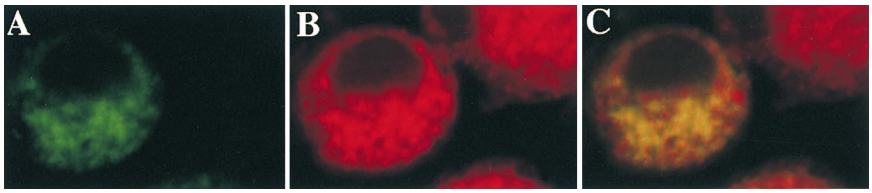

FIGURE 8.—Maggie protein localizes to mitochondria. Drosophila S2 cells were transiently transfected with the *mge-myc* construct and subsequently incubated with MitoTracker Red. Shown in A is anti-Myc staining of cells that express Mge-Myc protein. Only ~10% of the cells stained, reflecting the transfection efficiency. Note the punctate staining in the cytoplasm. Shown in B is the same field stained with MitoTracker to visualize mitochondria. In C, the merged image of A and B is presented. The yellow color indicates co-localization of Mge-Myc with MitoTracker.

been reported to inactivate the *Ubi-p* gene based solely on its proximity to that locus (Spradling *et al.* 1999). However, we believe this conclusion to be in error since the position of this element is 982 nucleotides away from the transcriptional start site for *Ubi-p* (Lee *et al.* 1988) and within the protein coding information for *dSc-2* (Figure 4).

The *l(3)05634* insert fails to complement the lethality associated with the two alleles of *l(3)63Eb*, *l(3)63Eb*^{A4} and *l(3)63Eb*^{E15}, identified in a previous screen (Harrison *et al.* 1995). These data suggest that the *l(3)05634* insert and the EMS-induced mutations of *l(3)63Eb* affect the *dSc-2* gene. Consistent with this proposal is the fact that all three mutants appear to display the same lethal phase. Hemizygous animals complete embryogenesis and hatch on schedule, but they die during the larval period (Table 1) as first or second instars. No animals are ever detected that reach the third-instar larval stage (data not shown).

DISCUSSION

The *E63* genes are dispensable for Drosophila development: Previously, we have shown that the 63F early puff contains two genes that are induced by the steroid hormone ecdysone in the larval salivary gland. *E63-1* encodes an EF-hand family member (Andres and Thummel 1995). Furthermore, we have observed that it binds calcium with high affinity (S. Mirzoeva, D. M. Watterson and A. J. Andres, unpublished observation). We have shown by antibody staining that E63-1 protein is localized to many larval tissues, including the midgut/gastric caeca, brain, muscle/pharynx, and salivary gland. In addition, we have shown that E63-1 displays a dynamic pattern of protein localization in the salivary gland during ecdysone-stimulated secretion. However, complete loss-of-function *E63-1* mutations do not significantly compromise the animal's ability to develop normally or secrete glue. One possible interpretation of this result is that E63-1 performs a redundant function in the fly.

Examination of the E63-1 protein sequence leads to several predictions regarding its function. The E63-1 protein has two conserved domains. The N-terminal domain is ~45% identical (depending on species) to various myosin light chains, while the Ca^{2+}-binding domains are ~40% identical to those of calmodulin (CaM). Since CaM has been shown to function as a light chain for unconventional myosin motors (Cheney *et al.* 1993; Hasson and Mooseker 1994; Stoffler and Bahler 1998), it is tempting to speculate that E63-1 functions in a similar capacity with myosins in the brain, gut, muscle, and salivary gland, where it might aid in the movement of cargoes along a cytoskeletal network. Such a mechanism must be in place to direct the secretion of glue granules in the salivary gland in response to ecdysone. Consistent with this model, we have recently shown that salivary gland secretion can occur independently of hormones by ectopically expressing E63-1 (T.-V. Do, A. Biyasheva, Y. Lu, M. Vaskova and A. J. Andres, unpublished results). We have also shown that E63-1 coimmunoprecipitates with an unconventional myosin in the salivary gland (T.-V. Do and A. J. Andres, unpublished observation). Furthermore, loss of *E63-1* function may not lead to lethality or secretion defects since CaM or another calcium-binding protein may be able to act in the same capacity. However, by compromising the functions of both light chains a phenotype might be observed. We are currently testing this possibility by examining the fate of salivary glands that are mutant for more than one Ca^{2+}-binding protein.

The *E63-2* gene was identified at the same time as *E63-1* (Andres and Thummel 1995). These two genes are divergently transcribed and separated by 1.1 kb of 5′ sequence (Figure 4). *E63-2* is clearly induced in the salivary gland by ecdysone, but as yet its sequence has provided no clues regarding possible functions during development. In this study, we have shown that the removal of *E63-2* by a small deficiency causes no significant developmental or morphological defects. This absence of a mutant phenotype, together with the observation that *E63-2* does not contain a clear AUG-initiated ORF, suggests that it is nonfunctional and that it may be induced by ecdysone due to its proximity to *E63-1*.

***maggie* encodes a putative Tom22 protein that is necessary for viability:** The *mge* gene resides immediately 3′ (within 400 nucleotides) of *E63-1*. Animals containing genetic null mutations in *mge* can survive as first-instar larvae for as long as 9 days before they die. During this extended period of larval life, the animals do not attain the size normally observed in mid-first-instar wild-type animals (data not shown). Morphologically, *mge* mutants look like wild type and appear to feed normally, but they display a slower rate of locomotion.

It is not uncommon for mutations in essential genes to display a first-instar larval lethal phenotype. In many cases the mutant animals survive the embryonic period on a supply of maternally provided products, but mutant larvae eventually die as these maternal stores are depleted and newly synthesized zygotic products cannot be made (reviewed in Spradling 1993). In support of this possibility, we have observed high levels of *mge* mRNA during the early stages of embryogenesis (Figure 7A). One way to test this hypothesis would be to make germline clones to determine whether *mge* is a maternal effect gene and to uncover possible early lethal phenotypes.

From the results presented here, the most likely cause of the *mge* mutant phenotype is an inability to effectively import proteins into the mitochondria. We have shown that an epitope-tagged Mge protein is localized to mitochondria, and Mge is similar to Tom22 proteins of fungi; therefore, we propose that the first-instar lethal pheno-

type occurs after the gradual depletion of functional mitochondria that are maternally supplied.

The *t*ranslocase of the mitochondrial *o*uter *m*embrane (TOM) is responsible for the recognition and translocation of nuclear-encoded proteins into the mitochondria. At least nine Tom components have been identified in fungi, and these can be divided into two categories: receptor components and general insertion pore (GIP) components (Neupert 1997; Pfanner *et al.* 1997; Ryan *et al.* 2000). Tom22 is a multifunctional protein required for recognition and transfer of preproteins from the receptors to the GIP and for the higher-level organization of the TOM machinery (Ahting *et al.* 1999; van Wilpe *et al.* 1999). Tom22 is anchored in the outer membrane by a single transmembrane domain. Its negatively charged N terminus is exposed to the cytosol, and its C terminus is exposed to the intermembrane space (Figure 6B). The N-terminal domain is essential for both targeting of Tom22 into mitochondria and for binding of preprotein sequences (reviewed in Neupert 1997; Pfanner *et al.* 1997; Rodriguez-Cousino *et al.* 1998). In yeast, it has been demonstrated that Tom22 is needed for vegetative growth on nonfermentable carbon sources (yeast can survive without mitochondria using an anaerobic pathway), since *tom22*$^{-}$ spores germinate, but arrest after 10–12 divisions (Lithgow *et al.* 1994; Nakai and Endo 1995). However, it has been shown recently that ~20% of cells can survive the loss of Tom22, although they grow very slowly and are compromised in their ability to import proteins into the mitochondria (van Wilpe *et al.* 1999). In contrast, a sheltered disruption of *tom22* in *N. crassa* indicates that the gene is essential for viability in this organism (Nargang *et al.* 1995). These mutant phenotypes are reminiscent of the *mge* null phenotype (growth arrest), suggesting that the function of this family of proteins may be conserved from fungi to Drosophila.

We have molecularly characterized two mutations in *mge*. *mge*^{b10} is a genetic null that results in a truncation of the Mge protein at the beginning of the transmembrane domain (Figures 5 and 6B). This would effectively prevent insertion of Mge into the mitochondrial outer membrane. The transmembrane domain is also required for the higher-level organization of the large GIP complex (consisting of Tom5, Tom6, Tom7, Tom22, and Tom40, reviewed in Neupert 1997; Pfanner *et al.* 1997; Ahting *et al.* 1999) that must be formed properly for efficient import (van Wilpe *et al.* 1999). Thus, we predict that in the absence of anchored Mge, the GIP complex cannot associate into its proper higher order conformation, hindering the import of nuclear-encoded proteins into the mitochondria.

We have also characterized the molecular defect in the *mge*^{d13} hypomorphic mutant. This mutation causes a single amino acid change in the C terminus, substituting a positively charged lysine for a negatively charged glutamic acid residue (Figures 5 and 6B). This mutation is predicted to affect the overall charge of the C-terminal domain projecting into the intermembrane space. However, functional studies of this C-terminal domain are conflicting. In *N. crassa*, this domain is dispensable for assembly of Tom22 into the TOM complex and is not essential for the binding of preproteins, although it may enhance the efficiency of transferring preproteins to the translocation machinery of the inner membrane (Court *et al.* 1996). On the other hand, experiments in yeast have led to the conclusion that the negatively charged C terminus of Tom22 represents a binding site for imported preproteins with cleavable targeting sequences (Moczko *et al.* 1997; Komiya *et al.* 1998). Thus it is reasonable to hypothesize that the *mge*^{d13} mutation results in a decreased efficiency of protein import into the mitochondria, which is manifested as the delayed growth and late-stage lethal phenotype we observe (Table 1).

While Tom22 has been well characterized and studied functionally in fungi, nothing is known about this protein in higher organisms. National Center for Biotechnology Information database searches have revealed additional putative Tom22 proteins, including a *Caenorhabditis elegans* sequence (accession no. AAB71053) that has 28% identity to Mge and a human sequence (accession no. Z92540) that is 41% identical over an 81-amino-acid overlap. However, as far as we can discern, this is the first study in which a putative Tom22 mutant phenotype has been described for a higher eukaryote.

Characterization of the 63F3-7 region: We have completed the genetic and molecular analysis of the genomic region between *E63-1* and *Ubi-p* and have correlated two mutant phenotypes with the molecular disruption of two genes. The *l(3)63Eb* complementation group represents mutations in the previously uncharacterized *dSc-2* gene. Here we describe the lethal phases for three alleles, including a *P*-element insertion that maps immediately downstream of the start of dSC-2 translation. We show that all three alleles display the same phenotype of early larval lethality. However, since we have not yet characterized the molecular defects of the EMS-generated alleles or attempted to mobilize the *P[ry*$^{+}$*, PZ]* element to restore viability, we leave open the possibility that these mutations do not affect the *dSc-2* transcription unit. Nevertheless, the high degree of similarity with the mammalian SC-2 protein (42% identity, 53% similarity) suggests that these mutants might be useful reagents in understanding the function of SC-2 both in flies and other higher organisms.

Also in this study we report the initial characterization of the *ida* gene, reported as *l(3)63Fb* in a previous study (Harrison *et al.* 1995). We show by deficiency mapping and sequence analysis that this gene is located between *l(3)63Eb*/*dSc-2* and *mge* and that null mutations in this gene lead to prepupal lethality with no effects on earlier developmental stages (Table 1). The molecular characterization of this gene and a more detailed phenotypic

analysis will be reported elsewhere (A. M. Bentley, B. Williams, M. Goldberg and A. J. Andres, unpublished results).

Two final notes about the *Ubi-p* locus are worth mentioning here. First, we have compared the BDGP/Celera Genomics sequence to our restriction map of the 63F walk. In both cases only four ubiquitin repeats were identified (Figure 4, box marked with question mark). This is in marked contrast to the molecular characterization of the *Ubi-p* transcription unit in which 15 to 18 ubiquitin repeats are reported (Izquierdo *et al.* 1984; Lee *et al.* 1988). One explanation for this discrepancy may be cloning or sequencing artifacts resulting from recombination among the repeats. The second note concerns the *l(3)63Fa* complementation group. Since we have mapped this gene to the 63F3-7 region, it should be further investigated as a possible mutation in the *Ubi-p* locus. The characterization of the one allele isolated [$l(3)63Fa^{D9}$] (Harrison *et al.* 1995) is recorded in Table 1.

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