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The *daughterless* gene functions together with *Notch* and *Delta* in the control of ovarian follicle development in *Drosophila*

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SUMMARY

The *daughterless* (*da*) gene in *Drosophila* encodes a broadly expressed transcriptional regulator whose specific functions in the control of sex determination and neurogenesis have been extensively examined. We describe here a third major developmental role for this regulatory gene: follicle formation during oogenesis. A survey of *da* RNA and protein distribution during oogenesis reveals a multiphasic expression pattern that includes both germline and soma. Whereas the germline expression reflects *da*'s role in progeny sex determination, the somatic ovary expression of *da* correlates with the gene's role during egg chamber morphogenesis. Severe, but viable, hypomorphic *da* mutant genotypes exhibit dramatic defects during oogenesis, including aberrantly defined follicles and loss of interfollicular stalks. The follicular defects observed in *da* mutant ovaries are qualitatively very similar to those described in *Notch* (*N*) or *Delta* (*Dl*) mutant ovaries. Moreover, in the ovary da^{-} alleles exhibit dominant synergistic interactions with *N* or *Dl* mutations. We propose that all three of these genes function in the same regulatory pathway to control follicle formation.

Key words: *daughterless*, *Drosophila* oogenesis, follicle formation

INTRODUCTION

In most animals, including those as diverse as insects and mammals, developing oocytes in the gonad are associated with specialized somatic cells that provide physical and/or nutritional support. Such organized cell groupings constitute the ovarian follicle, and although follicle structure can vary dramatically from one species to the next, basic follicular form consists of a germline-derived oocyte surrounded by a somatically derived epithelium. The actual assembly of these sometimes complicated multicellular configurations is not at all understood; however, it is likely that processes such as intercellular communication, cell migration, and differential cell/tissue adhesion play important roles.

In the polytrophic ovary of *Drosophila melanogaster*, *each* follicle is composed of an oocyte, the 15 nurse cells that share a clonal germline origin with the oocyte, and an enclosing monolayer of somatic follicle cells (King, 1970; Mahowald and Kambysellis, 1980). During their passage through the vitellarium in the course of oocyte development, individual follicles are separated from each other by columns of somatic cells, called interfollicular stalks. Both the stalk cells and the cells of the follicles themselves (somatic as well as germline) are derived from stem cells that reside in the germarium of each ovariole. Founded by a stem cell division, each germline cyst becomes enveloped by proliferating somatic cells, and the resulting spherical structure leaves the germarium as a newborn follicle.

The physical process of follicle formation in *Drosophila* has been supposed from extensive ultrastructural studies of the germarium (reviewed by King, 1970, and Mahowald and Kambysellis, 1980). Assembly of a follicle appears to begin when mesodermally derived prefollicle cells, originating near the surface of the roughly cylindrical germarium, move inward along the surface of a germline cyst. Elongated mesodermal cells eventually surround the cyst completely, producing a lens-shaped nascent follicle. As the follicle moves through the germarium, continued interleafing of mesodermal cells at its anterior end ultimately gives rise to a stalk that will separate this follicle from the follicle forming behind it. The elaboration of the stalk fully defines the new follicle and marks its entrance into the vitellarium.

Although the biochemical and/or cell biological mechanisms that underlie the physical assembly of follicles remain totally unknown, analysis of this process by genetic means has begun to identify genes that function in its regulation. Most notably, functional roles in follicle formation have been described for a number of genes whose roles in developmental processes outside of oogenesis had been characterized previously. These include the neurogenic loci, *Notch* (*N*), *Delta* (*Dl*) and *brainiac* (*brn*) (Ruohola et al., 1991; Goode et al., 1992; Xu et al., 1992; Bender et al., 1993), as well as *torpedo* (*top*), which encodes the *Drosophila* homolog of the EGF receptor (DER) (Goode et al., 1992). Of these genes, only *brn* appears to be required in the germline during follicle production. Since this germline requirement for brn^{+} seems to be linked with a somatic requirement for top^{+}, *brn* and *top* might cooperate in an intercellular signaling process that sets up the pattern of follicle cell

migration in the germarium that is necessary for egg chamber individualization (Goode et al., 1992). With respect to the roles of *N* and *Dl* during follicle formation, one hypothesis suggests that these genes reiterate their shared developmental role during neurogenesis, namely lateral inhibition of cell fate choice (reviewed by Campos-Ortega, 1988, and Artavanis-Tsakonas and Simpson, 1991); in this context intercellular communication mediated by the *N* and *Dl* membrane proteins would be required to differentiate stalk cells, as opposed to follicle polar cells, to ensure that follicles are separated from each other (Ruohola et al., 1991). Alternatively, *N* and *Dl* could be important as follicle cell surface molecules helping to define egg chamber morphology through differential cell adhesion properties (Xu et al., 1992). Regardless of the precise mechanism(s) of the *N-Dl* involvement in follicle establishment and maintenance, any overlap, if it exists, between the *N* and *Dl* roles with those of *brn and top* remains to be determined. Clearly, the complexity of this process cannot be explained by the functions of these genes alone.

This study describes the role of the *daughterless* (*da*) gene in the regulation of ovarian follicle formation. A multifunctional transcription factor of the basic-helix-loop-helix (bHLH) variety (Murre et al., 1989), the *da* gene product participates in numerous developmental processes (reviewed by Cline, 1989). Most notably, maternally supplied *da* gene product is required for the activation of *Sex-lethal* (*Sxl*) expression in the initiation of female sex determination (Cline, 1980, 1983, 1984, 1988; Cronmiller and Cline, 1987; Keyes et al., 1992; reviewed by Cronmiller and Salz, 1993), while zygotic *da* function is essential for the differentiation of the peripheral nervous system (Caudy et al., 1988). Other suspected functions of the gene, including regulation of heterochromatic gene expression (Mange and Sandler, 1973; Sandler, 1975; Pimpinelli et al., 1985) and control of egg membrane synthesis/deposition (Cline, 1976; Cronmiller and Cline, 1987), have been less well characterized.

Previously, *da*'s involvement in the process of follicle formation escaped recognition as a result of the gene's vital function(s) during embryonic development, although a role for *da* during the late stages of oocyte development had been inferred from the weak da^1 mutant phenotype (Cline, 1976; Cronmiller and Cline, 1987). We have identified a much earlier da^+ oogenic function: egg chamber morphogenesis. We have characterized the expression pattern of *da* protein (Da) during oogenesis to demonstrate that Da is present in somatic cells in the ovary, and we have constructed several severe hypomorphic *da* mutant genotypes to show that da^+ is required in these cells for follicle formation. Furthermore, we have established genetically that *da* functions together with *N* and *Dl* in this process. Finally, we have discovered that another neurogenic gene, *mastermind* (*mam*), may function in the same pathway.

MATERIALS AND METHODS

Fly stocks

Flies were raised at approximately 25°C unless otherwise indicated. Mutations and chromosomes not listed in Lindsley and Zimm (1992) are described below.

The da^{s22} allele was isolated in a mutagenesis screen for dominant maternal enhancers of the da^1 female-specific maternal effect. The genetic scheme of the mutagenesis was based on the dominant mutant interactions displayed by da^- and Sxl^- mutations (Cline, 1986; Cronmiller and Cline, 1987). Males homozygous for a marked second chromosome *[cl b pr cn/cl b pr cn*] were fed EMS according to the method of Lewis and Bacher (1968) and mated to heterozygous da^1 [*cl* da^1 *cn bw/Cy(2L + 2R)*] females. From the progeny of this mating, individual test females, carrying the da^1-bearing chromosome in *trans* to a mutagenized homologue, were crossed to Sxl^{f1} [*cm* Sxl^{f1} *ct/Y*] males. Individual mothers with relative daughter viability <10% were identified as possible carriers of *Enhancers of daughterless.* In addition to second site enhancers, six new *da* alleles were isolated in the screen, including da^{s22}.

The da^+ transgenic fly stock [$P(w^+$ *hsp70-*da^+)], A5, was obtained from A. Singson and J. Posakony. The transgene consists of a da^+ cDNA (PNBda: Van Doren et al., 1991) under the control of the *hsp70* promoter. The transformation vector used to carry the transgene into the fly genome has been described previously (Bang and Posakony, 1992). The transgene in A5 is inserted into the X chromosome, and the insertion is homozygous viable. Da protein produced from the transgene appears the same as wild-type Da on western blots (data not shown).

Ovary in situ hybridization

Whole-mount in situ hybridization to ovaries was performed according to Cooley et al. (1992). A digoxigenin-incorporated probe was made from the da^+ cDNA, MN6 (Cronmiller and Cline, 1987) by random primer labelling. Labelling and detection utilized reagents from the Genius kit (Boehringer Mannheim).

Immunocytochemistry

Whole-mount antibody staining of ovaries or embryos was carried out as described previously (Cronmiller and Cummings, 1993). Affinity-purified polyclonal anti-Da antiserum (DAP 7555: Cronmiller and Cummings, 1993) was used at a 1:50 dilution; biotinylated secondary antibody (Vector Laboratories) was diluted 1:500. An ascites preparation of the monoclonal anti-Notch antibody, C17.9C6 (Xu et al., 1992), was used at a 1: 1000 dilution. A monoclonal supernatant (mAb 202) raised against a fusion protein containing amino acids 190-833 of the Delta protein (A. Parks and M. Muskavitch, personal communication) was used at a 1:20 dilution. Both monoclonals were detected with FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) diluted 1:100. Fluorescent images were obtained on a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope.

Ovary DAPI staining

Ovaries were stained with the nuclear dye, DAPI, to visualize morphology. Following fixation in 4% paraformaldehyde (in 1× PBS), ovaries were treated with DAPI as described previously (Cronmiller and Cummings, 1993).

RESULTS

Morphogenesis of oocyte development in *Drosophila* results in ovarioles that contain a sequence of egg chambers that range in maturity from the youngest, in the germarium at the anterior end, to the oldest, in the vitellarium nearest the oviduct (King, 1970; Mahowald and Kambysellis, 1980) (Fig. 1). At the tip of the germarium, a small number of germline stem cells undergo asymmetric divisions to generate cystoblasts, each of which then divides four times to yield a 16-cell germline cyst. Each growing egg chamber separates from region III of the germarium in a budding-off process that appears to succeed an interleafing of somatic prefollicular cells that eventually com-

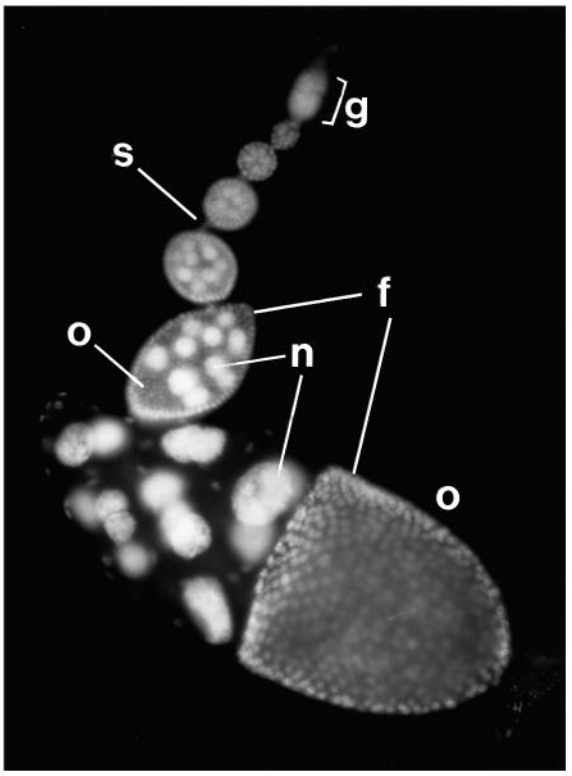

Fig. 1. Wild-type ovariole. Wild-type ovaries were stained with the nuclear dye, DAPI, and dissected into individual ovarioles to illustrate normal ovarian morphology. The anterior end, containing the least mature egg chambers, is located at the top. A stage 10 egg chamber is at the bottom. g, germarium; s, interfollicular stalk; f, follicle cell; n, nurse cell; o, oocyte.

pletely envelop the egg chamber At this point the fully enclosed egg chamber, composed of the germ cell cyst surrounded by its follicle cell epithelium, is tethered to the germarium by a single stalk of somatic cells. Repetition of this process generates a series of egg chambers attached to one another by interfollicular stalks, like larger and larger beads along a string. As each egg chamber grows, during its procession through the ovariole, the nurse cells undergo polyploidization, and the oocyte cytoplasmic volume increases. Late in oogenesis, the oocyte rapidly grows to its mature size, as its associated nurse cells deposit their cytoplasmic contents into the oocyte cytoplasm via intercellular bridges. Follicle cells that have aggregated around the oocyte synthesize the egg membranes and dorsal appendages, as oogenesis is completed.

Expression of *da* RNA during oogenesis

In situ hybridization was used to examine the distribution of *da* RNA in the ovary. Synthesis of *da* MRNA during oogenesis displays a dynamic pattern of regulation that is stage specific. Two temporally distinct phases of *da* mRNA synthesis were observed during oogenesis (Fig. 2). In the early phase, *da* RNA was detected at comparable levels in both the germline and the soma, with expression throughout the germarium and in all egg chambers until approximately stage 3 [staging according to King (1970)]; after stage 3 *da* RNA was undetectable in the germline and only marginally in the somatic follicle cells until

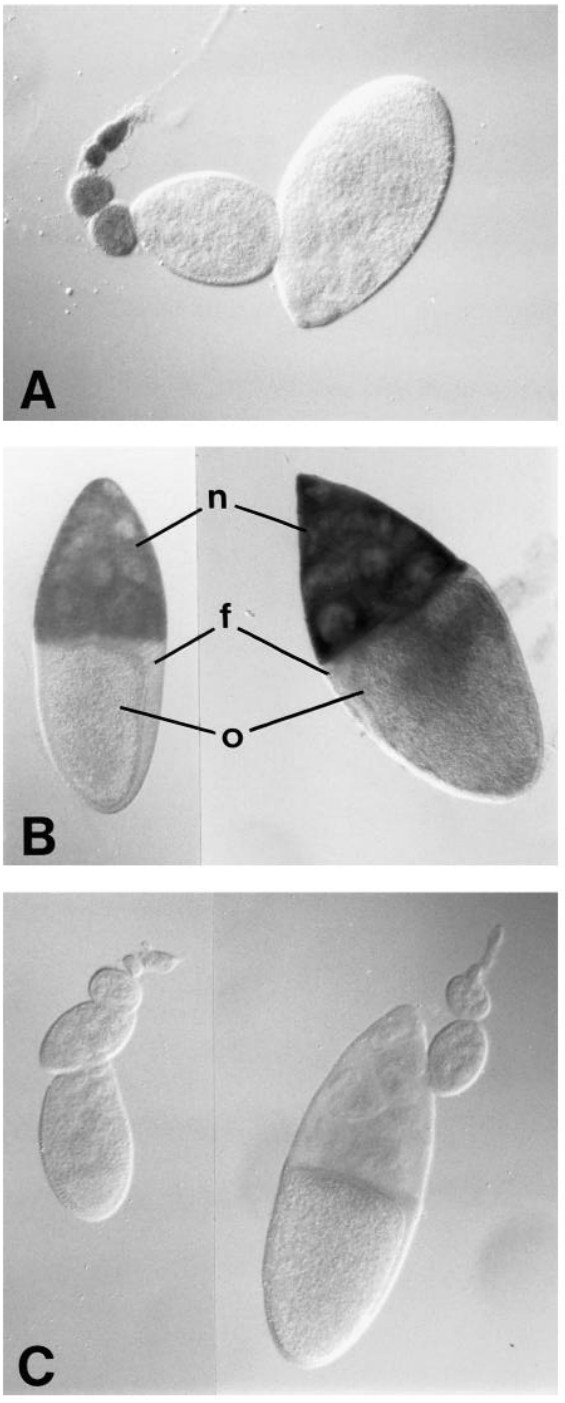

Fig. 2. Distribution of *da* mRNA during oogenesis. Wild-type ovaries were dissected and prepared for in situ hybridization. A full-length *da* cDNA, MN6, was used as a probe to visualize *da* RNA by nonradioactive detection. (A) Early oogenesis up to stage 7; germarium at the left. *da* RNA is present in both germline and soma through stage 3. (B) Stage 10 egg chambers: 10A on left, 10B on right. The low level of *da* RNA present in somatic follicle cells that cover the oocyte contrasts with the elevated expression in the germline nurse cells. The first signs of *da* mRNA transport to the developing oocyte can be seen in the stage 10B egg chamber. f, follicle cell: n, nurse cell; o, oocyte. (C) Control ovarioles that were processed without probe. All panels are approximately the same magnification.

one or two stages later (Fig. 2A). Late expression of *da* mRNA began at approximately stage 8 and was characterized by significantly higher levels in the germline (nurse cells only) than in the soma. Nurse cell expression of *da* continued to increase through late stage 10; however, in follicle cells the same low level of *da* RNA was found throughout these later stages. The first signs of movement of *da* RNA from the nurse cell cytoplasm to the oocyte were found at the end of stage 10 (Fig. 2B). Presumably all of the nurse cell-derived *da* transcripts end up in the oocyte, since unfertilized eggs contain large amounts of *da* RNA (C. Cronmiller, unpublished data), which is essential for the zygotic activation of *Sex-lethal* in female progeny.

Da protein is present at a low level in the female germline only where the gene is maximally transcribed

To examine the distribution of Da protein throughout oogenesis, we used a polyclonal antibody, DAP 7555 (Cronmiller and Cummings, 1993), to stain wild-type ovaries. Our previous studies had demonstrated that Da protein is not expressed at consistently detectable levels in the germline through stage 10 of oogenesis. It was possible, however, to detect low levels of Da in nurse cell nuclei of stage 9 and 10 egg chambers (Fig. 3A,D). Prior to these stages, staining of nurse cell nuclei did not exceed the background levels observed in concurrently stained negative control (no primary antibody) ovaries (data not shown). Even in stage 9 and later egg chambers, however, we never observed Da associated with the oocyte nucleus.

Da protein is present predominantly in somatic cells in the ovary

In contrast to the germline, in somatic cells of the ovary Da protein was found to be expressed in a temporal pattern that roughly mirrored the RNA expression pattern. Da was widely, though not uniformly, distributed throughout the somatic component of the ovary, including prefollicular somatic cells, as well as cells of the follicular epithelium (Fig. 3A).

Da protein expression in the somatic component of each ovariole can be described in three general stages. First, we found continuous expression in prefollicular and follicular cells from the germarium until stage 3. Overall, the earliest Da expression was found in the prefollicular cells in region II of the germarium, and in an irregularly shaped open cup of cells that closed at the posterior end of the germarium (Fig. 3B). The most intense germarium staining was observed as a thick band in the anterior portion of region III; however, this band of darkly staining cells was obvious only when a nascent follicle was judged to be fully formed, but not yet pinched off from the germarium (Fig. 3A). Subsequently, in egg chambers through stage 3, Da expression appeared to be maintained at an intermediate level in almost all of the nuclei of follicular epithelial cells. After stage 3, Da expression was found to diminish in the follicular epithelium; the protein level was significantly reduced in these cells by stage 6 and undetectable thereafter (Fig. 3C).

Second, we observed persistent expression in interfollicular stalk cells and interfollicular polar cells throughout egg chamber maturation. Among the follicular epithelial cells only the nuclei of the polar cells were found to express Da at the same high level as region III of the germarium. The stalk cells, which join adjacent follicles at their poles, were also found to express high levels of Da protein. The stalk and polar cells continued (permanently) to express Da, even after the protein disappeared from epithelial cell nuclei at stage 6. Finally, in stage 9 and later egg chambers, we detected uniform levels of Da protein in all follicle cells (Fig. 3D). Occasionally, the border cells could be distinguished as they migrated toward the oocyte. Both the squamous cells stretched over the nurse cell cluster and the columnar cells surrounding the oocyte expressed Da at moderate levels; this expression persisted throughout the period of egg membrane deposition (Fig. 3E).

Genetic characterization of the hypomorphic allele, da^{s22}

Based on the high temperature female sterile phenotype of the hypomorphic allele, da^{1}, Da expression in the membrane-secreting follicle cells that cover maturing oocytes was expected. Both the fragile egg phenotype associated with da^{1} sterility and the somatic cell origin of the phenotype had previously suggested a role for da^{+} in follicle cell function at the time of egg membrane deposition (Cline, 1976; Cronmiller and Cummings, 1987). Similarly, Da expression during the earlier stages of oogenesis implies a role for da^{+} at the beginning of egg development. However, alleles that are more severe than da^{1} are homozygous lethal and preclude a straightforward examination of the null phenotype in the somatic gonad. To determine the effects of drastically reduced da^{+} function on oogenesis, we utilized an EMS-induced allele that behaves genetically like an extreme hypomorph: da^{s22}.

Isolated as a dominant maternal enhancer of the da^{1} female-specific maternal effect, da^{s22} is a recessive lethal mutation that was identified as a *da* allele by several criteria. First, in trans-heterozygous combination with several *da* mutant alleles, da^{s22} showed reduced viability even with the weakest allele, da^{7} (formerly da^{Pa}) (Table 1, Cross D). Like da^{1} and da^{7}, da^{s22} was completely inviable in combination with a null allele, da^{2} (Table 1, Cross B). Second, the recessive lethality of da^{s22} as well as that of da^{s22}/da^{2}) could be rescued by a da^{+}-bearing transgene (data not shown). Third, da^{s22} mutant embryos were found to express reduced levels of Da protein, as detected by anti-Da antibody immunohistochemical staining (Fig. 4). Finally, da^{s22} was mapped by recombination to the *da* locus, using both da^{7} and a mutant allele of the nearby locus, *mfs48*, in separate analyses. The recombination frequency was 0.05% between da^{s22} and *mfs48* (22,469 progeny scored) and <0.03% between da^{s22} and da^{7} (0 recombinants/6,374 progeny). Taken together, these observations have identified the lesion in da^{s22} as a defect in *da* gene function.

Reduced fecundity of da^{s22}/da^{7} females

Heteroallelic da^{s22}/da^{7} females were found to exhibit severely reduced fecundity, compared with their phenotypically wild-type ($+/da^{7}$) siblings (Table 2). Although young mutant females (3-5 days old) deposited normal numbers of eggs in the first day of scoring, production dropped precipitously thereafter. By the third day of observation, most females (32/36) completely stopped laying eggs. Since the hatch rate for eggs laid by mutant females was not significantly different from that for eggs produced by control mothers (data not

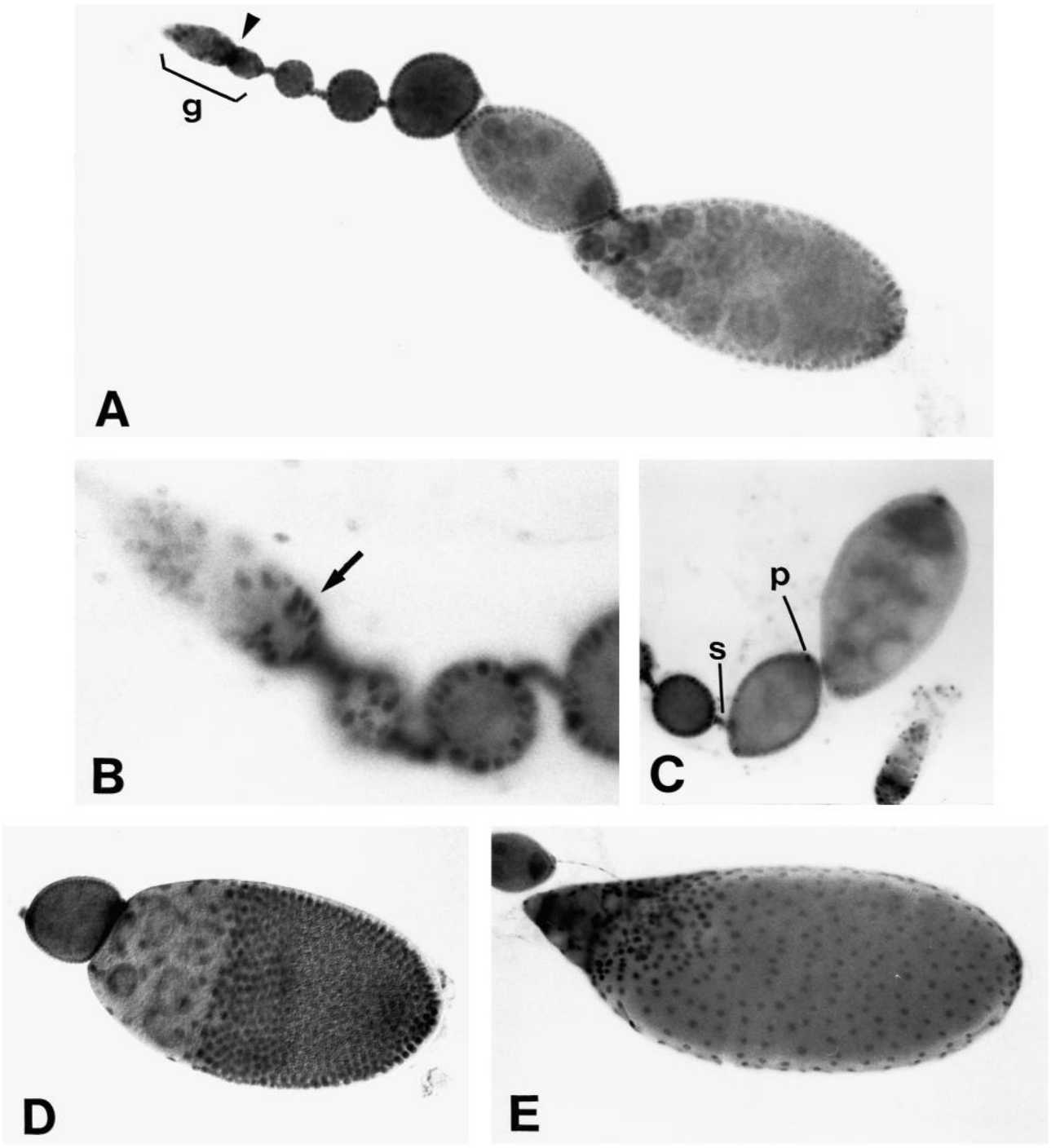

Fig. 3. Distribution of Da protein during oogenesis. Wild-type ovaries were dissected and prepared for immunohistochemical staining. A polyclonal antibody, DAP 7555, was used to visualize Da. (A) Oogenesis through stage 9; germarium (g) at the left. Da protein staining is obvious in most somatic follicle cells from region II of the germarium through stage 3. Thereafter, high levels of Da are present only in interfollicular stalk cells and follicular polar cells. The most intense germarium staining appears as a thick band in the anterior portion of region III when a nascent follicle appears ready to pinch off from the germarium (arrowhead). (B) Enlargement of the germarium end of an ovariole. The arrow indicates the irregular cup-shaped pattern of Da expression in region III of the germarium. (C) Stage 6 and stage 8 egg chambers, illustrating nearly complete disappearance of Da from cells of the follicular epithelium. Only stalk and polar cells still contain significant levels of Da. p, polar cells; s, interfollicular stalk. (D) Stage 9 egg chamber illustrating renewed Da expression in all follicle cells. Variable low levels of Da can also be detected in the nurse cells at this stage. (E) Stage 12 egg chamber showing general expression of Da in follicle cells. A, C and D are approximately the same magnification; the magnification of B is approximately a 3× enlargement; the magnification of E is approximately a 2× reduction.

Table 1. Relative viability of *da* mutant genotypes

Cross*	Genotype	Viability of *da* mutant flies relative to their da^+ siblings (%)†	Number of da^+ siblings recovered
A	da^{s22}/da^{s22}	0	1003
B	da^{s22}/da^{2}	0	701
C	da^{s22}/da^{1}	34	284
D	da^{s22}/da^{7}	28	931
E	*hsp70-da*$^+$/+ da^7/da^2	3	389
F	*hsp70-da*$^+$/*hsp70-da*$^+$ da^7/da^2	20	255
G	*hsp70-da*$^+$/+; da^1/da^2	10‡	617
H	*hsp70-da*$^+$/+; da^1/da^2 [37°C heat pulse]	32	447

Full genotypes of crosses:
A: *cl* da^{s22}/*In(2LR)CyO, da*$^+$ *pr* cn^2 females×males.
B: *cl* da^{s22}/*In(2LR)CyO, da*$^+$ *pr* cn^2 females×*cl* da^2/*In(2LR)CyO, da*$^+$ *pr* cn^2 males.
C: *cl* da^{s22}/*In(2LR)CyO, da*$^+$ *pr* cn^2 females×*cl* da^1 *cn bw/cl* da^1 *cn bw* males.
D: *cl* da^{s22} *b pr cn/In(2LR)CyO, da*$^+$ *pr* cn^2 females×*cl* da^7 *b pr cn/cl* da^7 *b pr cn* males.
E: *w P[w*$^+$ *hsp70-da*$^+$*]/w P[w*$^+$ *hsp70-da*$^+$*]; cl* da^2/*In(2LR)CyO, da*$^+$ *pr* cn^2 females×+/*Y; cl* da^7 *b pr cn/cl* da^7 *b pr cn* males.
F: *w P[w*$^+$ *hsp70-da*$^+$*]/+; cl* da^7 *b pr cn/In(2LR)CyO, da*$^+$ *pr* cn^2 females×*w P[w*$^+$ *hsp70-da*$^+$*]/Y; cl* da^2 *b pr cn/In(2LR)CyO, da*$^+$ *pr* cn^2 males.
G: *w P[w*$^+$ *hsp70-da*$^+$*]/w P[w*$^+$ *hsp70-da*$^+$*]; cl* da^1/*In(2LR)CyO, da*$^+$ *pr* cn^2 females×+/*Y; cl* da^2/*In(2LR)CyO, da*$^+$ *pr* cn^2 males.
H: Same cross as G. Progeny embryos were collected for 2 hours, subjected to 37°C for 1 hour and returned to 25°C to complete development.
*All crosses performed at 25°C.
†Relative viability calculated for females and males combined in crosses A, B, C and D, and for females alone in crosses E, F, G and H.
‡All escapers drop dead within 48 hours of eclosing prior to laying any eggs.

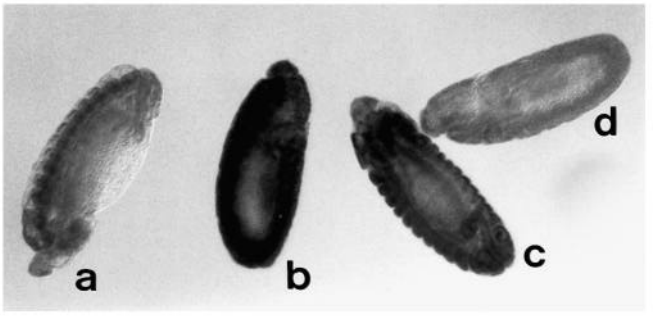

Fig. 4. Da expression in da^{s22}/da^{s22} embryos. Embryos from the balanced da^{s22} stock were prepared for immunohistochemical staining to visualize Da expression. Embryos a and c, and b and d provide pairs of similar developmental stages. In each pair the presumed da^{s22} homozygote (a and d) shows significantly reduced levels of Da in comparison with its wild-type (da^+/da^+) sibling partner (c and b, respectively). Embryos of the third genotypic class (da^{s22}/da^+), which were also clearly detectable within the population, are not shown.

shown), the da^{s22}/da^7 reduction in fecundity appears to be solely the consequence of reduced egg production.

daughterless is required for adult ovarian follicle formation

To determine the precise nature of the *da* mutant defect during oogenesis, we examined the ovarian morphology of da^{s22}/da^7 females. In very young (<3 days old) mutant females, ovaries were found to contain at least a few normal egg chambers and mature eggs in the posterior portion of most ovarioles. Such early follicles apparently produced the normal eggs that were recoverable from these females during fecundity tests. At and near the germarium, however, few normal follicles were ever observed. In the least defective ovarioles, germaria appeared swollen with multiple germline cysts in an extended region III (Fig. 5A). These nascent egg chambers were not separated by interfollicular stalks and frequently were not even completely segregated by the enveloping somatic epithelial layer; such egg chambers had presumably failed to bud off appropriately from the germarium. In ovaries of mature (>5 days old) mutant females, considerably more dramatic defects were observed. Generally, we found no morphological boundary between the germarium and the vitellarium; indeed, interfollicular stalks were missing throughout the ovariole. Demarcation of egg chambers was also defective: germline cysts were observed that were partially intersected by somatic follicle cell sheets (Fig, 5B, arrow). However, the converse, in which follicle cells interleafed only partially between germline cysts, was much more common. Consequently, egg chambers were often compound, containing two or more growing germline cysts. The most severe manifestation of this mutant phenotype was a continuum of several germline cysts surrounded by a single sheet of follicle cells (Fig. 5C). In the most mature ovarioles, such grossly compound follicles were usually found to undergo necrosis at the time the oldest oocyte should have been enlarging (Fig. 5D).

Oversized follicles in *da* mutant ovaries appear to result from improper follicle formation at the germarium, rather than from extra germline cystocyte divisions. First, within mutant compound egg chambers, individual germline cysts were recognizable as groups of 15 similarly sized nurse cells, and a single oocyte nucleus could usually be identified for each germline cyst. In these cases each oocyte was properly placed at the posterior end of each germline cyst, such that there was no disturbance of normal anterior-posterior polarity. Second, the absence of any organized stalks in mature mutant ovaries suggests that discrete egg chambers are never formed after the

Table 2. Fecundity of da^{s22}/da^7 females

Genotype	Average no. eggs/ female/day*	Average no. eggs/ female/day 1*
Experimental†		
da^{s22}/da^7	4.2±3.2 (*n*=36)	16.8±12.1 (*n*=56)
Control†		
da^7/da^+	28.9±5.8 (*n*=58)	16.9±8.6 (*n*=59)

Adult females were collected as virgins, aged 3-5 days and mated individually to wild-type males. Females were transferred to fresh vials daily for 6 days, and the number of eggs laid each day was recorded individually for each female. All manipulations were carried out at 25°C. Hatch rates of the eggs recovered from Experimental and Control females were not significantly different.
n=number of test-mated females scored.
*±standard deviation of the mean.
†Sibling females were generated from the following cross:
cl da^{s22} *b pr cn/In(2LR)CyO, da*$^+$ *pr* cn^2 females×*cl* da^7 *b pr cn/cl* da^7 *b pr cn* males.
Experimental full genotype: *cl* da^{s22} *b pr cn/cl* da^7 *b pr cn*.
Control full genotype: *cl* da^7 *b pr cn/In(2LR)CyO, da*$^+$ *pr* cn^2.

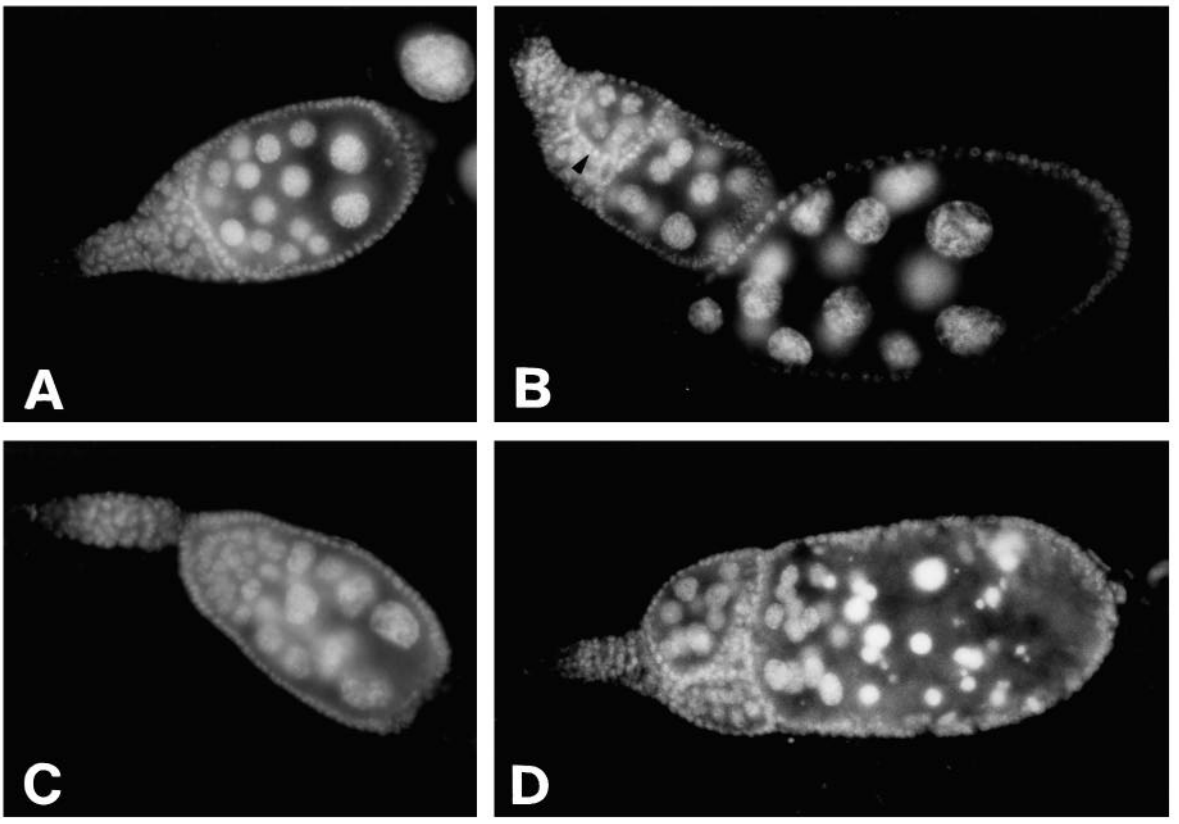

Fig. 5. The *da* mutant phenotype in the ovary. Ovaries from da^{s22}/da^{7} females were dissected and stained with the nuclear dye, DAPI, to examine ovarian morphology. Panels present different examples of da^{s22}/da^{7} mutant ovarioles. (A) Mutant ovariole with swollen, extended germarium and large compound egg chamber. (B) Mutant ovariole containing a normal, maturing egg chamber (right) and totally aberrant follicle formation at the germarium. The arrowhead indicates a germline cyst that has been partially intersected by an inappropriately interleafing follicle cell layer. (C) Mutant ovariole composed almost completely of one continuous compound egg chamber. (D) Mutant ovariole in which necrosis has spread throughout the oldest compound egg chamber. Germarium is to the left in all panels. All panels are approximately the same magnification.

first few that could be found in young females. Simply supernumerary divisions would be expected to result in excessively large egg chambers that are nevertheless appropriately formed, as in the case of *ovarian tumour* (*otu*) oncogenic alleles (Geyer et al., 1993). Finally, staining mutant ovaries with rhodamine-conjugated phalloidin to visualize filamentous actin showed that no cystocytes were connected to their neighbors by more than four ring canals (data not shown); therefore, we found no evidence for extra germline cell divisions.

The defects observed in da^{s22}/da^{7} mutant ovaries are not allele-specific and represent a da^{-} mutant phenotype. We generated two additional extreme hypomorphic *da* mutant genotypes by partially rescuing otherwise lethal da^{-} genotypes with an inducible da^{+} transgene; the severity of these mutant genotypes was estimated with reference to the gene's zygotic (somatic) function. In combination with a null allele, such as da^{2}, hypomorphic *da* alleles are lethal; da^{7}/da^{2} and da^{1}/da^{2} flies do not survive (Cronmiller and Cline, 1987; Cronmiller et al., 1988). Viability of both of these genotypes was partially rescued by adding copies of a transgene that carried a da^{+} cDNA under the control of the inducible *hsp70* promoter [$P(w^{+}$ *hsp70-*$da^{+})$ transgenic lines: A. Singson and J. Posakony, unpublished]. Basal expression (i.e. at 25°C) of a single copy of $P(w^{+}$ *hsp70-*$da^{+})$ provided minimal rescue of either lethal genotype (eg., Table 1, Crosses E and G). Higher frequencies of rescued flies were recovered either by increasing the number of transgene copies (Table, 1, Cross F) or by increasing the da^{+} expression of a single transgene (Table 1, Cross H). Uninduced expression of two copies of $P(w^{+}$ *hsp70-*$da^{+})$ yielded escaper da^{7}/da^{2} females at a frequency similar to the survival of the da^{s22}/da^{7} hypomorphic genotype, suggesting similar quantitative levels of da^{+} function in the soma. Likewise, induced expression (37°C/1 hour during the first 2 hours of embryonic development) of a single copy of $P(w^{+}$ *hsp70-*$da^{+})$ produced a comparable frequency of da^{1}/da^{2} escaper females. Ovaries of both rescued da^{-} genotypes exhibited the same follicular defects observed in da^{s22}/da^{7} ovaries, including compound egg chambers and absence of interfollicular stalks (Fig. 6). In addition, preliminary characterization of mosaic follicles containing *da* null somatic clones, generated by the FLP-FRT method (Golic, 1991; Chou and Perrimon, 1992), suggests that the defects in follicle formation described above may approximate the da^{-} phenotype for this ovarian function (Cummings and Cronmiller, unpublished data).

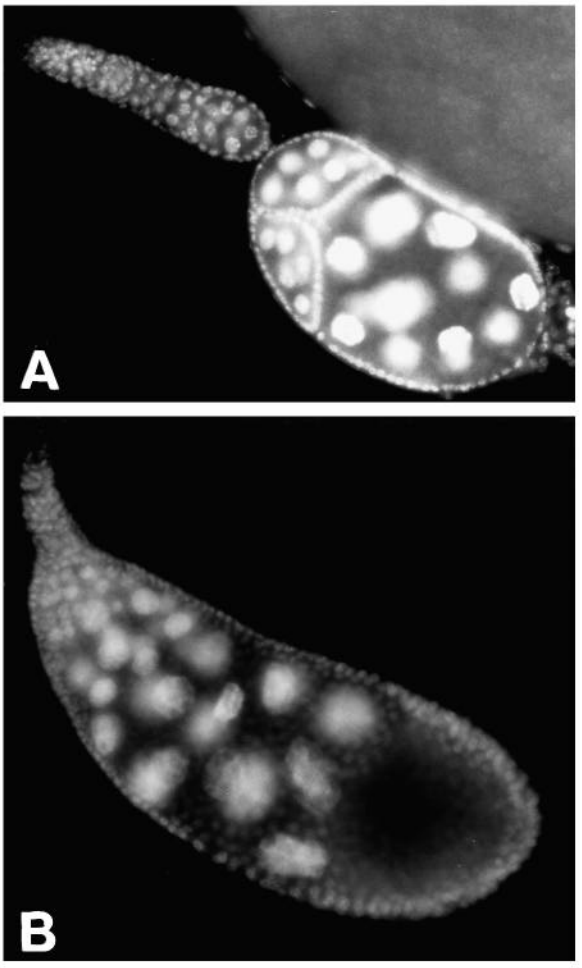

Fig. 6. The *da* mutant phenotype in the ovary. Ovaries from mutant females were dissected and stained with DAPI. (A) Ovariole from *P[w⁺ hsp70-da⁺]/+; da¹/da²* female, rescued by exposure for 1 hour to 37°C during preblastoderm development, but otherwise raised at 25°C. The swollen, protracted germarium and irregularly configured egg chambers are characteristic of the da⁻ mutant phenotype. (B) Ovariole from *P[w⁺ hsp70-da⁺]/P[w⁺ hsp70-da⁺]; da⁷/da²* female raised exclusively at 25°C. The ovariole is composed almost completely of one continuous compound egg chamber. Germarium is to the left in both panels. Both panels are approximately the same magnification.

Dominant interactions between *daughterless* and the neurogenic mutations, *Notch*, *Delta* and *mastermind*

The *daughterless* mutant phenotype in the ovary shares many common characteristics with the ovarian phenotypes described for loss-of-function mutations in the neurogenic genes, *Notch* and *Delta* (Ruohola et al., 1991; Xu et al., 1992; Bender et al., 1993). One possible explanation for such similarity in mutant phenotypes is that all three of these genes function in the same process during the early morphogenetic stages of oogenesis. If this is true, mutations in one gene might be expected to exhibit dominant interactions with, i.e. fail to complement, mutations in the others. We examined ovary morphology in females who were doubly heterozygous for *da⁻* and either *N⁻* or *Dl⁻*; both heterozygous combinations exhibited significant, often dramatic, ovarian defects. The abnormalities found in both heterozygous genotypes resembled those described for *da⁻* alone (Fig. 7). A moderately severe phenotype was observed in 3- to 5-day-old *da²/+*; *Dl⁹/+ f*emales and in *N^ts1/+*; *da²/+* females after 2-3 days at the restrictive temperature (Fig. 7A,B). The ovarioles in these ovaries were generally stalkless, contained multiple compound egg chambers, and usually displayed no real distinction between the germarium and vitellarium. Like the *da⁻* mutant ovary phenotype, this dominant interaction phenotype was found to worsen with the female's age. The ovaries of >5-day-old *da²/+*; *Dl⁹/+* females [or *N^ts1/+*; *da²/+* females after 5 days at the restrictive temperature] contained ovarioles filled largely with necrotic follicles (Fig. 7C). Control ovaries (from singly heterozygous sibling females) never contained more than occasional compound egg chambers (observed in <2% of ovarioles); however, the frequency of such follicles in control ovaries was always higher in genotypes that included balancer chromosomes (Fig. 7D) or in high temperature samples (Fig. 7E).

Since the *da* null mutation was found to interact with both *N⁻* and *Dl⁻* to produce a strong mutant phenotype in the ovary, we looked for a similar dominant effect between *N⁻* and *Dl⁻* themselves. Females doubly heterozygous for both *N⁻* and *Dl⁻* were found to exhibit only a weak mutant ovary phenotype (Fig. 7F). As in controls exposed to high temperature or containing balancer chromosomes, the *N⁻/+*; *Dl⁻/+* defects were limited usually to individual compound egg chambers or missing stalks separating adjacent egg chambers (compare Fig. 7D, 7E and 7F). In the experimental genotype, however, such weak defects were much more prevalent throughout ovarioles.

Genetic interactions have been used extensively to identify genes that function with *N* and *Dl* during neurogenesis and postembryonic development Such genetic interactions have been described previously between *N* alleles and alleles of another neurogenic locus, *mastermind* (*mam*; Brand and Campos-Ortega, 1990; Xu et al., 1990). Because of this functional relationship between *N* and *mam*, we looked for a mutant interaction between *da* and *mam* during follicle formation. We found that all females doubly heterozygous for strong *da* and *mam* alleles showed the same range of follicle defects that were observed in the *N* and *Dl* genotypes (Fig. 8A), including the absence of stalks, compound egg chambers and irregular follicle cell interleafing. Mutations in two other neurogenic genes that are expressed in the ovary (Ruohola et al., 1991), but that do not interact with *N* alleles earlier in development, namely *neuralized (neu)* and *big brain (bib)*, did not lead to mutant ovary phenotypes as heterozygotes in combination with da⁻/+ (data not shown); however, only single alleles of *neu* and *bib* were tested.

daughterless does not appear to regulate *Notch* or *Delta* expression during oogenesis

Since *da* encodes a bHLH-type transcription factor (Murre et al., 1989), the simplest molecular mechanism that might be proposed to account for the genetic interactions described here would be regulation by *da*, either direct or indirect, of *N*, *Dl* and/or *mam* expression. Indeed, Notch is expressed in a dynamic pattern that substantially overlaps that of Da (Xu et al., 1992); therefore, as the first step toward testing the above hypothesis, we used immunocytochemical staining to examine

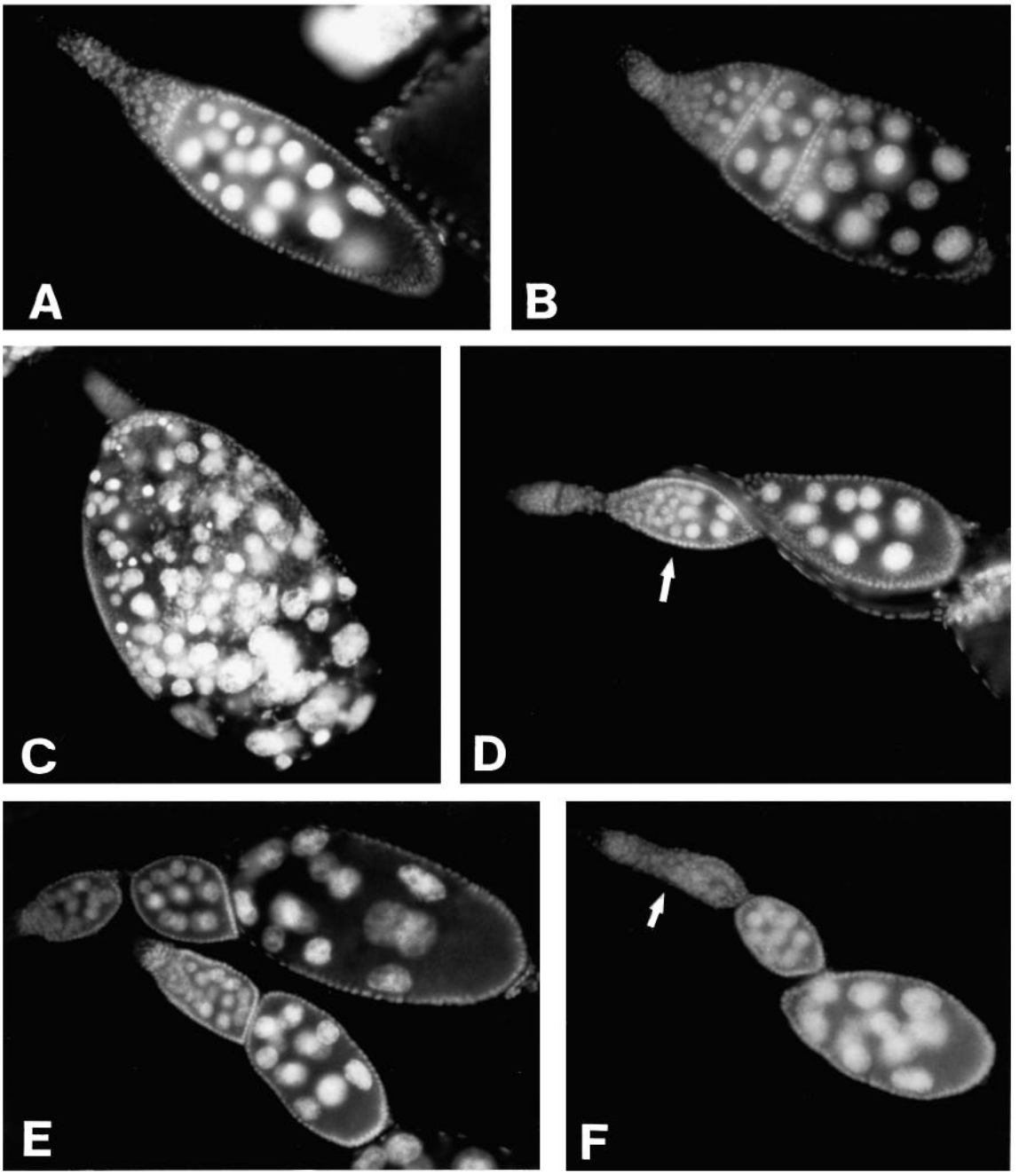

Fig. 7. Dominant synergistic interactions among *da*, *N* and *Dl* mutations in the ovary. Ovaries from mutant females were dissected and stained with DAPI. (A) Ovariole from N^{ts1}/+; da^{2}/+ female exposed to 32°C for 2-3 days. Note the absence of interfollicular stalks, swollen germarium and compound egg chamber. (B,C) Ovarioles from da^{2}/+; Dl^{9}/+ females, prepared at either 3-5 (B) or >5 (C) days after eclosion. Interfollicular stalks are missing in these ovarioles, and older ovarioles (C) become completely necrotic. (D) Control ovariole from da^{2}/+; +/+ female, recovered as a sibling to the da^{2}/+; Dl^{9}/+ females. The only abnormality found in 25°C control ovarioles is a rare compound egg chamber (arrow). (E) Control ovariole from N^{ts1}/+; +/+ female exposed to 32°C for 2-3 days, recovered as a sibling to the N^{ts1}/+; da^{2}/+ females. Abnormalities in singly heterozygous genotypes were never more severe than occasional compound egg chambers. (F) Ovariole from N^{ts1}/+; Dl^{9}/+ female exposed to 32°C for 2-3 days. The weak, but consistent, mutant synergism between *N* and *Dl* included swollen germaria (arrow) and compound egg chambers. Germarium is to the left in all panels. All panels are approximately the same magnification.

N (Notch) and *Dl* (Delta) protein expression in *da* mutant ovaries.

In wild-type ovaries, Notch protein can be found in the germarium, where the highest levels are present in the somatic cells in the boundary between regions II and III, as well as at the apical surface of follicle cells surrounding the nascent egg chamber in region III (Xu et al., 1992) (Fig. 9A). Through stage 5 in the vitellarium, Notch expression is highest in the somatic

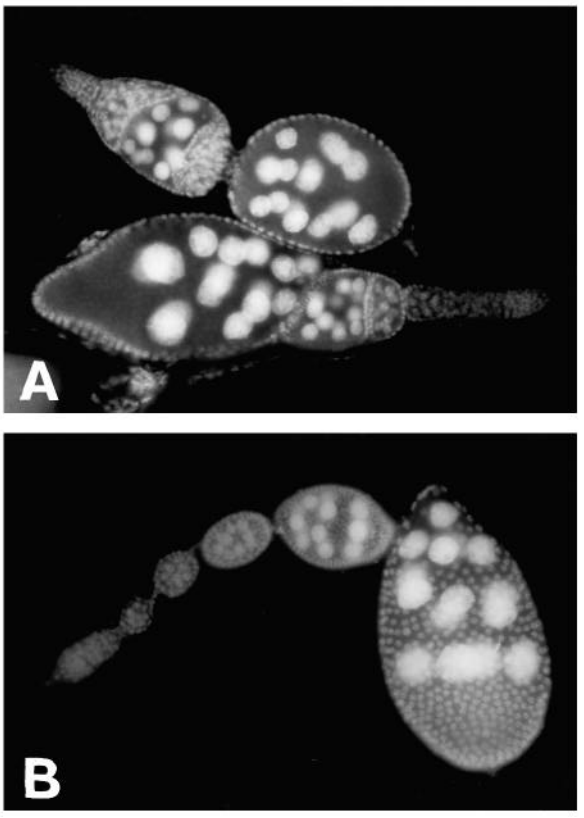

Fig. 8. Dominant synergistic interaction between *da* and *mam* mutations in the ovary. Ovaries from mutant females were dissected and stained with DAPI. (A) Ovarioles from da^2/mam^{IL113} female. Note the swollen and protracted germaria, as well as the aberrantly configured egg chambers. (B) Ovariole from $da^2/+$ female, recovered as a sibling to the da^2/mam^{IL113} female. No abnormalities were ever observed in da^2 heterozygotes in this genetic background. Both panels are approximately the same magnification.

cells of the follicular epithelia, again polarized toward the apical surface of the cell. Unlike Da expression, Notch levels are lower, rather than higher, in the interfollicular stalks.

In *da* mutant ovaries, Notch was found to be distributed in approximately the wild-type pattern (Fig. 9B,C). As in normal ovarioles, follicle cells in the germaria of mutant ovarioles expressed significant levels of Notch; however, the distribution of Notch in this region was more diffuse than in wild-type germaria. As in wild-type ovaries, very high levels of Notch were expressed by the cells of follicular epithelia surrounding growing egg chambers. through stage 5. In addition, Notch expression was still polarized toward the apical surface of follicle cells, even when contiguous egg chambers were not separated by stalks or when aberrant follicle formation had resulted in irregularly shaped, nonlinear arrays of egg chambers; however, the protein was not as tightly polarized as in wild-type ovaries. As in wild-type ovaries, follicular Notch expression in *da* mutant ovaries was downregulated after stage 5; reappearance of Notch in later (stage 9- 10) egg chambers could not be assessed, since older *da* mutant follicles generally became necrotic.

A similar comparison of Delta protein distribution in wild-type and *da* mutant ovaries demonstrated no obvious effect of

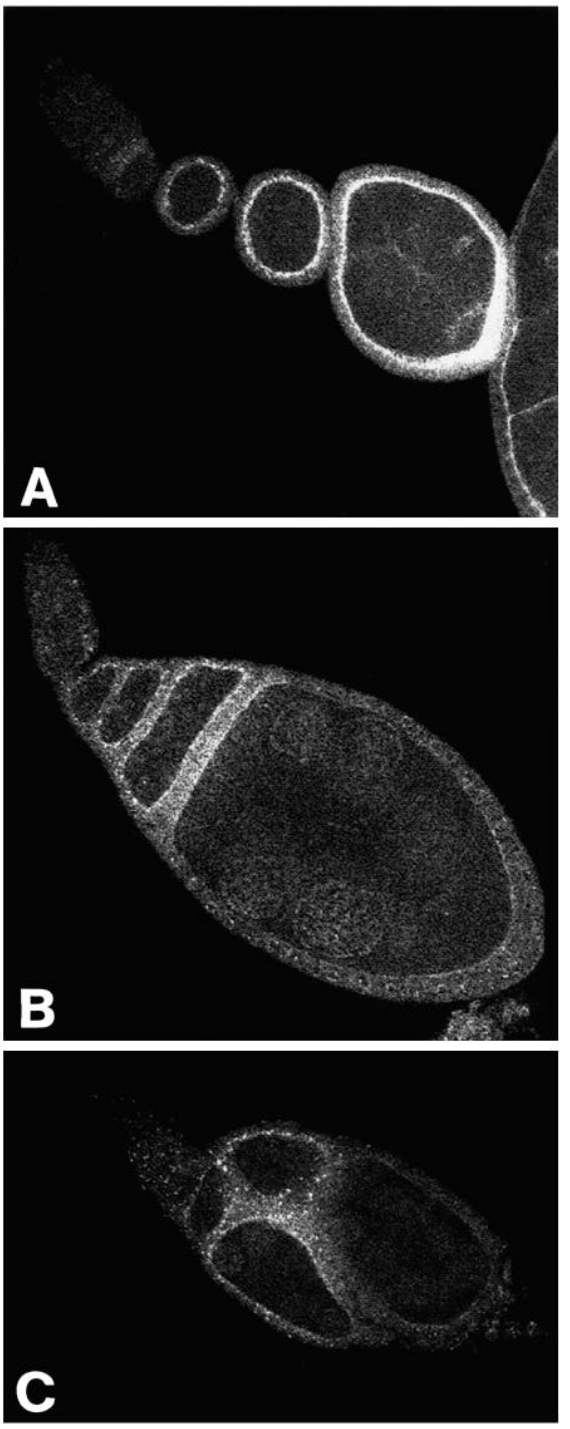

Fig. 9. Notch expression in *da* mutant ovaries. Ovaries were dissected, stained with anti-Notch (C17.9C6) antibody and visualized by confocal microscopy. (A) Wild-type ovariole illustrating Notch expression. Significant cytoplasmic staining is evident in the germarium. In the vitellarium, Notch is localized to the apical surface of follicular epithelial cells. (B,C) Ovarioles from da^{s22}/da^7 mutant females. The stage-specific distribution of Notch protein in these mutant ovarioles is the same as in wild-type. However, the localization within the germarium is more diffuse and the apical polarization of the membrane-associated protein in the vitellarium is less complete in mutant ovarioles. Germarium is to the left in all panels. All panels represent approximately the same magnification.

the mutant genotype on Delta expression. In wild-type ovaries, only low levels of Delta can be detected in the germarium, and diffuse cytoplasmic expression is obvious in stages 1-3 of the vitellarium. Thereafter, Delta protein can be observed associated with the nurse cell and oocyte membranes, as well as with those located between the nurse cells and follicle cells (Bender et al., 1993). In *da* mutant ovaries, we found no major disruptions of this expression pattern (data not shown). Because of the very low levels of Delta protein detected in the germarium by mAb 202, it is not possible to determine for certain that this expression was not perturbed at all in *da* mutant ovaries. However, the rest of the protein's pattern of distribution remained intact.

DISCUSSION

In addition to its essential functions in sex determination and neurogenesis during development, the *daughterless* gene provides a critical activity in the regulation of follicle formation during *Drosophila* oogenesis. Since da^+ function is not required in the germline for the production of functional eggs (Cronmiller and Cline, 1987), follicle morphogenesis must require the gene's activity in the somatic gonad. In the absence of sufficient somatic wild-type *da* function, adult ovaries contain aberrantly defined egg chambers in various stages of growth. The syndrome of defects associated with a nearly null phenotype includes an almost uniform absence of interfollicular stalks, compound egg chambers that contain multiple sets of germline cysts without intervening epithelial layers, and partially fragmented egg chambers in which epithelial cell layers have disrupted the integrity of individual cysts. All of these abnormalities probably derive from an earlier failure in the normal process of follicle formation in the germarium, whereby completed germline cysts must become (1) enveloped by a single cell layer of somatic epithelial cells and (2) separated from each other by interfollicular stalks prior to entering the vitellarium. Although it is not known precisely when da^+ is required for follicle establishment or maintenance, high levels of Da protein are present in the germarium, particularly in region III as the nascent follicle prepares to bud off to the vitellarium. However, since significant levels of Da are also present in later stage follicle cells, especially the stalk and polar cells, it is possible that *da*'s role in follicle formation is more complex and includes activities needed to preserve follicle structure, once constructed.

What constitutes the genetic regulatory pathway in which Da participates, probably as a transcription factor, during oogenesis? The transcriptionally regulated target of *da* activity in somatic sex determination is the gene, *Sex-lethal (Sxl)*, which is known also to direct germline sex determination (Schüpbach, 1985; Salz et al., 1987). Indeed, the mutant phenotype most often associated with loss of germline sex determination genes is the production of 'ovarian tumors' (Oliver et al., 1988, 1990; Pauli and Mahowald, 1990; Geyer et al., 1993; reviewed by Steinmann-Zwicky, 1992), a defect that could be viewed as a more severe manifestation of what we have described as compound egg chambers in the *da* mutant ovary phenotype. Moreover, germline sex determination in the female is known to depend upon an inductive signal from the somatic gonad (Steinmann-Zwicky et al., 1989). The da^- ovarian defects, however, do not result from a disruption of germline sex determination: a gain-of-function allele of *Sxl*, Sxl^{M1}, which suppresses the tumorous ovary phenotype of mutations in genes that lie genetically upstream in the regulation of *Sxl* and eliminates the normal dependence of germline sex determination on the somatic gonad (Steinmann-Zwicky, 1988; Nöthiger et al., 1989; Grandino et al., 1992; Salz, 1992), fails to suppress the da^- mutant phenotype in the ovary (Cronmiller and Cummings, unpublished data). Thus, the regulatory role performed by *da* during oogenesis must be independent of the sex determination pathway.

The follicular regulatory pathway that includes *da* appears to include several other genes that, like *da*, also function during neurogenesis, namely *N*, *Dl* and *mam* (reviewed by Artavanis-Tsakonas and Simpson, 1991). The dominant mutant interactions observed between *da* and *N*, *Dl*, or *mam* during oogenesis suggest that these genes act in a common pathway leading to follicle formation: the functions of da^+, N^+, Dl^+ and mam^+ all contribute in the same direction to the regulatory events that promote follicle formation, since da^- exacerbates the mutant effects of heterozygous *N*, *DI* or *mam* genotypes in the ovary. Such a relationship of similar function between *da* and this group of neurogenic genes during oogenesis is in contrast to the gene's role during neural development, whereby the proneural gene, *da*, acts in the opposite regulatory direction from the neurogenics: da^+ is required to promote neural cell fates, but N^+, Dl^+ and mam^+ are required to inhibit neural development in favor of epidermal cell differentiation. Indeed, in double mutant combinations, a *da* loss-of-function genotype can partially rescue the neural hyperplasia associated with neurogenic mutants, including *Dl* (Brand and Campos-Ortega, 1988). Thus, the genetic role of *da* during oogenesis is not simply a reiteration of the genetic role of *da* during neurogenesis; it seems unlikely that Da makes precisely the same biochemical association during both neurogenesis and oogenesis, even though many of the regulatory genes appear to be shared by these two developmental pathways. Nevertheless, it may be, as suggested by Ruohola et al. (1991), that the neurogenics, *N* and *Dl* (and *mam*?), do function as part of a regulatory 'cassette' that is utilized at multiple times during development.

A number of hypotheses can be proposed to account for the dominant mutant interactions described here. Perhaps the simplest is that Da, a transcription factor, regulates the expression of one or more of the other regulatory genes in the follicle morphogenesis pathway. It is clear that Da is not a direct transcriptional regulator of either *N* or *Dl*: by immunohistochemical staining, we find that the levels of expression of the Notch and Delta proteins in *da* mutant ovaries are not severely affected. Although the distribution pattern of Notch is more diffuse in da^- mutant versus wild-type germaria, this effect could result as a secondary consequence of the disrupted form of the swollen mutant germaria. Similarly, the incomplete apical polarization of Notch to the follicle cell membranes in da^- mutant ovaries could result as a secondary consequence of the disrupted geometry of the defective mutant ovarioles. If Notch apical localization normally requires cues from the germline cyst to distinguish between an egg chamber's 'inside' (i.e., germline side) and its 'outside' (i.e., ovarian lumen side), then follicle cells aberrantly wedged between two germline cysts might receive conflicting signals. In this way, mislocalization of Notch would not be attributable directly to defects

in regulation by Da. It is possible, however, that Da mediates Notch and/or Delta function indirectly by controlling the transcription of one or more intermediate regulatory genes. Mislocalization of Notch protein is also observed in N^{ts1} mutant ovaries (Xu et al., 1992). Since the molecular lesion of N^{ts1} lies in the EGF-like repeats of the protein's extracellular domain, Xu et al. (1992) have suggested that the mutant protein might be unable to bind a normally polarized ligand. If da^+ regulates such a ligand, wild-type Notch protein would be incompletely polarized in *da* mutant ovaries, thus mimicking the N^{ts1} defect. An alternative hypothesis places the regulation of *da* function downstream of the neurogenic genes: instead of *da* regulating N and/or *Dl*, *N* and/or *Dl* regulate *da*. The simplest version of this postulate can be ruled out also, since Da protein expression is unaffected in N^{ts1} mutant ovaries (Cummings and Cronmiller, unpublished data). However, it is possible that Notch and Delta mediate post-translational control of Da function via a signaling pathway, perhaps by activating required modifiers of Da protein function or by triggering some molecular event that is required for Da to find its specific gene target(s). For the present, our data are unable to distinguish among these hypotheses, and, because of the similarity of the mutant phenotypes, epistasis analysis cannot be used easily to determine the sequence of the genetic regulation.

Whatever the regulatory relationship between *da* and the neurogenics during ovarian follicle formation, it may not be a straightforward linear hierarchy. Although moderately defective *N* and *Dl* mutant ovaries are very similar to *da* mutant ovaries, they display phenotypic features that are different from and in addition to those associated with either the synergistic genotypes or *da* mutation alone (Ruohola et al., 1991; Xu et al., 1992; Bender et al., 1993). These differences could reflect wild-type functions for *N* and *Dl* during or after follicle formation that do not involve *da.* This idea seems plausible given the especially dynamic patterns of Notch and Delta protein expression during the early stages of oogenesis (Xu et al., 1992; Bender et al., 1993).

An important point in the elucidation of *da*'s regulatory role during follicle formation is whether the absence of interfollicular stalks in *da* mutant ovaries actually means the absence of stalk cell identity, per se. This question remains to be answered for *da* defective ovaries; however, experimental evidence from studies with cell-specific markers in *N* or *Dl* mutant ovaries suggests that loss of these gene functions results in hyperplasia of follicular polar cells at the expense of interfollicular stalk cells (Ruohola et al., 1991). Thus, the compound egg chamber phenotype associated with *N* and *Dl* mutations would derive from an initial failure to differentiate stalk cells and the consequent absence of assembled stalks to separate adjacent follicles. Ruohola et al. (1991) have argued that this result implicates *N* and *Dl* in a process of ovarian somatic cell fate choice that may be analogous to the lateral inhibition process in which these two genes participate during neurogenesis: repression of polar cell determination may be required to differentiate stalk cells in much the same way that neural cell fate must be inhibited to differentiate epidermis. As a transcription factor involved in cell fate choices elsewhere in development, Da would fit well with such a scheme, and we are currently using polar cell and stalk cell specific markers to examine *da* mutant ovaries.

In addition to cell fate regulation, control of cell migration is likely to play a critical role in defining new follicles (King, 1970; Mahowald and Kambysellis, 1980). In region II of the germarium, prefollicular cells of mesodermal origin invaginate between adjacent clusters of 16 cystocytes to delimit new egg chambers (Koch and King, 1966; King et al., 1968; Mahowald and Strassheim, 1970). Regulation of this migratory process probably includes (1) expression of differential cell surface properties and (2) some form of intercellular communication or recognition that mediate a dynamic cell sorting process. Considering the likely importance of cell surface characteristics for cell migration, it is intriguing that Notch and Delta have been shown to mediate cell aggregation via their extracellular domains (Fehon et al., 1990). Cell surface properties provided by Notch and Delta may be important for physical cell sorting during germline cyst enclosure in the germarium. For its part, Da may regulate the expression/function of these or other adhesion molecules that contribute to cell movement. Furthermore, regulation of cell surface characteristics could also underlie normal stalk formation, if adhesion differences cause neighboring cells to minimize their contact with each other (Steinberg and Poole, 1982). With respect to germline/soma intercellular signalling in the germarium, two likely components are the products of the *brainiac* and *Drosophila EGF receptor* (DER) loci. Goode et al. (1992) have shown that germline brn^+ function and somatic DER function are required for normal follicle cell migration in the germarium, and consequently for normal egg chamber individualization. Whether *da* participates in this communication system remains to be determined.

Although we are unable to describe precisely how follicle formation takes place, it is possible that egg chamber morphogenesis may proceed by somewhat different mechanisms in adult and pupal gonads and that the gene functions recognized here pertain mostly to the adult process. When dissected from young adults, *da* mutant ovaries, as well as mutant ovaries of synergistic genotypes, contain egg chambers that appear to be maturing normally; moreover, the average number of viable eggs produced for the first 1-2 days of egg laying by young adult females is normal. One explanation for this apparently leaky phenotype is that none of the genotypes examined represents a complete loss of the underlying wild-type function(s). Alternatively, these normal eggs may be the products of the first follicles formed during pupal oogenesis. If so, then the regulatory events that control pupal follicle formation might not be identical to those that control this process in the adult. As initially formed, ovarioles in the developing pupal ovary are devoid of discrete follicles (King et al., 1968). Although subsequent demarcation of distinct egg chambers is biased at first toward the posterior pole of each ovariole, several follicles seem to form concomitantly or in rapid succession, as though being molded during a reorganization of the ovariole, rather than being budded off from the germarium. In this way, a few complete egg chambers in each ovariole are established before eclosion of the adult, and these pupally derived follicles produce the first eggs laid by the adult. If da^+ is not required to shape follicles in the pupal gonad, *da* mutant ovaries would be capable of producing those few first eggs. In a preliminary examination of *da* mutant pupal ovaries, we found that interfollicular stalks do form in the posterior of the ovariole, connecting small numbers of apparently normal egg chambers. More anteriorly, however, stalkless follicles

were obvious, extending from the germarium (Cummings and Cronmiller, unpublished data).

Follicle formation is now the second somatic cell process in the adult ovary that has been found to require da^+. Characterization of the temperature-sensitive hypomorph, da^1, uncovered a putative role for da^+ during egg membrane synthesis and/or deposition; this allele's mutant phenotype includes high temperature female sterility that results from the production of flaccid eggs (Cline, 1976). Indeed, Da's temporally biphasic expression pattern in the somatic follicle cells is consistent with two separable gene functions during oogenesis, early for follicle formation and late for egg membrane construction. Because of the variable expression of the fragile egg aspect of the da^1 phenotype, the specific function provided by *da* in the final stages of egg assembly has not been identified. And, in the more extreme mutant genotypes used in this study, defects that result from da's early role in follicle formation terminate oocyte development prior to egg maturation or membrane deposition and are, therefore, epistatic to any later mutant effects. Consequently, clarification of *da*'s involvement in egg membrane construction may require the identification of the gene's regulatory targets in this process.

The coincidence of three presumably independent requirements for da^+ activity during oogenesis, two somatic and one germline, appears to be resolved by the regulation of the gene's expression in this tissue. Elsewhere during development *da* protein distribution is essentially ubiquitous (Cronmiller and Cummings, 1993). Our description and comparison of the *da* mRNA and protein distribution during oogenesis provide the first specific suggestions of regulation of the expression of the *da* gene itself. Although, detailed characterization of the genomic control regions of *da* will be required to understand the gene's regulation during oogenesis, the temporally biphasic nature of *da* RNA synthesis in the ovary could reflect the use of more than one transcriptional promoter, especially in view of the dramatically higher levels of RNA expressed in the germline cells during the late stages of oogenesis.

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