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 interfolicular 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 Kambyssellis, 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 gerarium 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 gerarium as a newborn follicle.

The physical process of follicle formation in *Drosophila* has been supposed from extensive ultrastructural studies of the gerarium (reviewed by King, 1970, and Mahowald and Kambyssellis, 1980). Assembly of a follicle appears to begin when mesodermally derived prefollicle cells, originating near the surface of the roughly cylindrical gerarium, 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 gerarium, continued interleaving 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 (brm)* (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 *brm* appears to be required in the germline during follicle production. Since this germline requirement for *brm*+ seems to be linked with a somatic requirement for *top*+, *brm* 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 \( 
\text{brn} \) 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 \( \text{daughterless} \) (\( \text{da} \)) 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 \( \text{da} \) gene product participates in numerous developmental processes (reviewed by Cline, 1989). Most notably, maternally supplied \( \text{da} \) gene product is required for the activation of \( \text{Sex-lethal} \) (\( \text{Sxl} \)) 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 \( \text{da} \) 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, \( \text{da} \)'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 \( \text{da} \) during the late stages of oocyte development had been inferred from the weak \( \text{da}^+ \) mutant phenotype (Cline, 1976; Cronmiller and Cline, 1987). We have identified a much earlier \( \text{da}^+ \) oogenic function: egg chamber morphogenesis. We have characterized the expression pattern of \( \text{da} \) protein (\( \text{Da} \)) during oogenesis to demonstrate that \( \text{Da} \) is present in somatic cells in the ovary, and we have constructed several severe hypomorphic \( \text{da} \) mutant genotypes to show that \( \text{da}^+ \) is required in these cells for follicle formation. Furthermore, we have established genetically that \( \text{da} \) functions together with \( N \) and \( Dl \) in this process. Finally, we have discovered that another neurogenic gene, \( \text{mastermind} \) (\( \text{mam} \)), 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 \( \text{da}^{22} \) allele was isolated in a mutagenesis screen for dominant maternal enhancers of the \( \text{da}^1 \) female-specific maternal effect. The genetic scheme of the mutagenesis was based on the dominant mutant interactions displayed by \( \text{da}^- \) and \( \text{Sxl}^- \) mutations (Cline, 1986; Cronmiller and Cline, 1987). Males homozygous for a marked second chromosome \( \{ \text{brn} \ \text{top} \text{and} \text{cy} \} \) were fed EMS according to the method of Lewis and Bacher (1968) and mated to heterozygous \( \text{da}^1 \) \( \{ \text{brn} \ \text{top} \text{and} \text{cy} \} \) females. From the progeny of this mating, individual test females, carrying the \( \text{da} \) bearing chromosome in trans to a mutated homologue, were crossed to \( \text{Sxl} \) \( \{ \text{cm} \ \text{Sxl} \ \text{cy} \} \) males. Individual mothers with relative daughter viability <10% were identified as possible carriers of \( \text{Enhancers of daughterless} \). In addition to second site enhancers, six new \( \text{da} \) alleles were isolated in the screen, including \( \text{da}^{22} \).

The \( \text{da}^+ \) transgenic fly stock \( \{ \text{P} \{ \text{w} \ \text{hsp}70\text{-da}^+ \} \} \), A5, was obtained from A. Singson and J. Posakony. The transgene consists of a \( \text{da}^+ \) cDNA (PNBda: Van Doren et al., 1991) under the control of the \( \text{hsp70} \) 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 \( \text{X} \) chromosome, and the insertion is homozygous viable. \( \text{Da} \) protein produced from the transgene appears the same as wild-type \( \text{Da} \) 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 \( \text{da}^+ \) 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-\( \text{Da} \) antisemur (DAP 7555: Van Doreen et al., 1991) was used at 1:50 dilution; biotinylated secondary antibody (Vector Laboratories) was diluted 1:500. An ascites preparation was used at 1:500 dilution. Immunocytochemistry was performed at 1:100 dilution. Monoclonal antibodies 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 1x 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 from the youngest, in the germarium in 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 interleaving of somatic prefollicular cells that eventually com-
completely 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...
one or two stages later (Fig. 2A). Late expression of $du$ 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 $du$ continued to increase through late stage 10; however, in follicle cells the same low level of $du$ RNA was found throughout these later stages. The first signs of movement of $du$ 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 $du$ transcripts ended up in the oocyte, since unfertilized eggs contain large amounts of $du$ 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 prefolllicular 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 prefolllicular and follicular cells from the gerarium until stage 3. Overall, the earliest Da expression was found in the prefolllicular cells in region II of the gerarium, and in an irregularly shaped open cup of cells that closed at the posterior end of the gerarium (Fig. 3B). The most intense gerarium 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 gerarium (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 interfolllicular 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 gerarium. 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 transheterozygous combination with several $da$ mutant alleles, $da^{s22}$ showed reduced viability even with the weakest allele, $da^2$ (formerly $da^{P3}$) (Table 1, Cross D). Like $da^1$ and $da^2$, $da^{s22}$ was completely inviable in combination with a null allele, $da^0$ (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 $da^+$ 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 $da^{s22}/da^+$ females**

Heteroallelic $da^{s22}/da^+$ females were found to exhibit severely reduced fecundity, compared with their phenotypically wildtype (+/$da^+$) sibs (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; gerarium (g) at the left. Da protein staining is obvious in most somatic follicle cells from region II of the gerarium through stage 3. Thereafter, high levels of Da are present only in interfollicular stalk cells and follicular polar cells. The most intense gerarium staining appears as a thick band in the anterior portion of region III when a nascent follicle appears ready to pinch off from the gerarium (arrowhead). (B) Enlargement of the gerarium end of an ovariole. The arrow indicates the irregular cup-shaped pattern of Da expression in region III of the gerarium. (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.
To determine the precise nature of the daughterless mutant defect during oogenesis, we examined the ovarian morphology of da22/da7 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 gerarium, 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 gerarium. In ovaries of mature (>5 days old) mutant females, considerably more dramatic defects were observed. Generally, we found no morphological boundary between the gerarium and the vitellarium; instead, 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 interfused 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 gerarium, 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>
<thead>
<tr>
<th>Cross*</th>
<th>Genotype</th>
<th>Viability of da mutant flies relative to their da/+ siblings (%)†</th>
<th>Number of da/+ siblings recovered</th>
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<tr>
<td>A</td>
<td>da22/da22</td>
<td>0</td>
<td>1003</td>
</tr>
<tr>
<td>B</td>
<td>da22/da2</td>
<td>0</td>
<td>701</td>
</tr>
<tr>
<td>C</td>
<td>da22/da1</td>
<td>34</td>
<td>284</td>
</tr>
<tr>
<td>D</td>
<td>da22/da7</td>
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<td>931</td>
</tr>
<tr>
<td>E</td>
<td>hsp70-da+</td>
<td>3</td>
<td>389</td>
</tr>
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<td>hsp70-da+/hsp70-da+</td>
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<td>617</td>
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<tr>
<td>H</td>
<td>hsp70-da+/da7</td>
<td>32</td>
<td>447</td>
</tr>
</tbody>
</table>

Full genotypes of crosses:
A: cl da22 Int2LR/CyO, da+ pr cn2 females males.
B: cl da22 Int2LR/CyO, da+ pr cn2 females cl da2 Int2LR/CyO, da+ pr cn2 males.
C: cl da22 Int2LR/CyO, da+ pr cn2 females cl da1 Int2LR/CyO, da+ pr cn2 males.
D: cl da22 b pr cn Int2LR/CyO, da+ pr cn2 females cl da7 b pr cn cl da7 b pr cn males.
E: w P[w+] hsp70-da+ /+; cl da2 Int2LR/CyO, da+ pr cn2 females cl da7 b pr cn cl da7 b pr cn males.
F: w P[w+] hsp70-da+ /+; cl da2 b pr cn Int2LR/CyO, da+ pr cn2 females cl da7 b pr cn cl da7 b pr cn 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.

†Sibling females were generated from the following cross: cl da22 b pr cn Int2LR/CyO, da+ pr cn2 females cl da7 b pr cn/In(2LR)CyO, da+ pr cn2 males.

*±standard deviation of the mean.
†Number of test-mated females scored.

Table 2. Fecundity of da22/da7 females

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average no. eggs/female/day*</th>
<th>Average no. eggs/female/day †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental†</td>
<td>da22/da7</td>
<td>4.2±3.2 (n=36)</td>
</tr>
<tr>
<td>Control†</td>
<td>da+/da+</td>
<td>28.9±5.8 (n=58)</td>
</tr>
</tbody>
</table>

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.

Fig. 4. Da expression in da22/da22 embryos. Embryos from the balanced da22 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 da22 homozygote (a and d) shows significantly reduced levels of Da in comparison with the wild-type (da+/-) sibling partner (c and b, respectively). Embryos of the third genotypic class (da22/-/da22), which were also clearly detectable within the population, are not shown.

shown), the da22/-/da7 reduction in fecundity appears to be solely the consequence of reduced egg production.

daughterless is required for adult ovarian follicle formation

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first few that could be found in young females. Simply super-numerary 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 da22/da7 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 da2, hypomorphic da alleles are lethal; da7/da2 and da1/da2 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 da7/da2 females at a frequency similar to the survival of the das22/da7 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 da1/da2 escaper females. Ovaries of both rescued da− genotypes exhibited the same follicular defects observed in das22/da7 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).
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 da+/−; Dl+/− females and in N+/−; da+/− 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 gerarium 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 da+/−; Dl+/− females [or N+/−; da+/− 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 DI 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−; DI− 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 neutralized (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, DI 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*<sup>ts1/+; da<sup>2/+</sup> 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<sup>2/+; Dl<sup>9/+</sup>* 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<sup>2/+; +/+* female, recovered as a sibling to the *da<sup>2/+; Dl<sup>9/+</sup>* females. The only abnormality found in 25°C control ovarioles is a rare compound egg chamber (arrow). (E) Control ovariole from *N<sup>ts1/+; +/+* female exposed to 32°C for 2-3 days, recovered as a sibling to the *N<sup>ts1/+; da<sup>2/+* females. Abnormalities in singly heterozygous genotypes were never more severe than occasional compound egg chambers. (F) Ovariole from *N<sup>ts1/+; Dl<sup>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 vitellarian, Notch expression is highest in the somatic
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. 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 da2/mamIL113 female. Note the swollen and protracted germaria, as well as the abnormally contigured egg chambers. (B) Ovariole from da2/+ female, recovered as a sibling to the da2/mamIL113 female. No abnormalities were ever observed in da2 heterozygotes in this genetic background. Both panels are approximately the same magnification.

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 da22/da27 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 gerarium, 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 gerarium, 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, SxlM1, 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; Nothiger 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, DI and mam (reviewed by Artavanis-Tsakonas and Simpson, 1991). The dominant mutant interactions observed between da and N, DI, or mam during oogenesis suggest that these genes act in a common pathway leading to follicle formation: the functions of da*, N*, DI* 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 pronuclear gene, da, acts in the opposite regulatory direction from the neurogenics: da* is required to promote neural cell fates, but N*, DI* 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 DI (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 DI (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 DI: 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 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, prefolicular 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, da2, 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 da2 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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