

Ecdysone response genes govern egg chamber development during mid-oogenesis in *Drosophila*

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SUMMARY

The steroid hormone ecdysone regulates larval development and metamorphosis in *Drosophila melanogaster* through a complex genetic hierarchy that begins with a small set of early response genes. Here, we present data indicating that the ecdysone response hierarchy also mediates egg chamber maturation during mid-oogenesis. *E75*, *E74* and *BR-C* are expressed in a stage-specific manner while *EcR* expression is ubiquitous throughout oogenesis. Decreasing or increasing the ovarian ecdysone titer using a temperature-sensitive mutation or exogenous ecdysone results in corresponding changes in early gene expression. The stage 10 follicle cell expression of *E75* in wild-type, *K10* and *EGF receptor (Egfr)* mutant egg chambers reveals regulation of *E75* by both the *Egfr* and ecdysone signaling pathways. Genetic analysis indicates a germline requirement for ecdysone-responsive gene expression. Germline clones of *E75* mutations arrest

and degenerate during mid-oogenesis and *EcR* germline clones exhibit a similar phenotype, demonstrating a functional requirement for ecdysone responsiveness during the vitellogenic phase of oogenesis. Finally, the expression of *Drosophila* Adrenodoxin Reductase increases during mid-oogenesis and clonal analysis confirms that this steroidogenic enzyme is required in the germline for egg chamber development. Together these data suggest that the temporal expression profile of *E75*, *E74* and *BR-C* may be a functional reflection of ecdysone levels and that ecdysone provides temporal signals regulating the progression of oogenesis and proper specification of dorsal follicle cell fates.

Key words: *Drosophila*, Ecdysone, Oogenesis, *E75*, *E74*, *BR-C*, Germline, Follicle cell, Dorsoventral patterning

INTRODUCTION

Changes in the titer of the steroid hormone 20-hydroxyecdysone (ecdysone) coordinate gene expression during the development of *Drosophila melanogaster*. Experiments examining changes in the puffing patterns of the larval polytene chromosomes in response to ecdysone stimulation led to the proposal of a hierarchical model whereby ecdysone directly promotes the appearance of early puffs (Ashburner, 1974; Ashburner et al., 1974; Ashburner and Richards, 1976; Thummel, 1996). The model further predicted that the early puff genes encode proteins responsible for the induction of late puffs and the simultaneous attenuation of early puff activity.

Molecular characterization of early puff loci has confirmed this hierarchical model. The 2B5, 74EF and 75B early puffs contain the *Broad-Complex (BR-C)*, *E74* and *E75* genes, respectively. The *BR-C* encodes a family of related transcription factors sharing a common core region and containing one of four possible pairs of zinc finger domains (Bayer et al., 1996; DiBello et al., 1992). The *E74* gene

encodes two isoforms, designated *E74A* and *E74B*, that contain an identical ETS DNA-binding domain (Burtis et al., 1990). The *E75A*, *E75B* and *E75C* isoforms encoded by the *E75* gene are orphan receptors in the nuclear hormone receptor superfamily (Feigl et al., 1989; Segraves and Hogness, 1990). Analysis of expression patterns and the effects of mutations in these early genes suggests that these transcription factors are involved in controlling parallel genetic pathways mediating ecdysone response in a variety of tissues and developmental stages (Thummel, 1996).

Ecdysone activates transcription of early response genes by binding to a heterodimeric receptor composed of *EcR* and *USP* (Yao et al., 1992, 1993). The *EcR* gene is a member of the nuclear hormone receptor superfamily and encodes three isoforms that differ in their N termini (Koelle et al., 1991; Talbot et al., 1993). The *usp* gene encodes the *Drosophila* homologue of the mammalian RXR molecule (Oro et al., 1990; Shea et al., 1990). Genetic and molecular analysis of both of these genes suggests that these molecules are essential for normal development and act, as predicted, at the top of the

ecdysone response hierarchy (Bender et al., 1997; Oro et al., 1992). However, subtle phenotypic differences between *usp* and *EcR* mutants suggest the possibility that receptors other than the EcR-USP heterodimer could be involved in mediating response to ecdysteroids (Buszczak and Segraves, 1998).

While ecdysone response has been characterized most extensively in larval development and metamorphosis, significant levels of ecdysone can be found in adult females, within which the ovary is the major steroidogenic tissue. *Drosophila* have meroistic ovaries made up of 15-20 ovarioles that each hold 7-8 sequentially more mature egg chambers (Spradling, 1993). Individual egg chambers are composed of one oocyte directly connected to fifteen supporting nurse cells. These germline cells are surrounded by a layer of somatically derived follicle cells. An egg chamber proceeds through fourteen morphologically distinguishable stages of development (Spradling, 1993). Studies performed on other species suggest that ecdysone regulates several control points during insect oogenesis. This is best understood in the yellow fever mosquito *Aedes aegypti*, in which the transition into the vitellogenic phase of oogenesis depends critically on the increase in ecdysone titer following a blood meal (Dhadialla and Raikhel, 1994; Hagedorn, 1983; Hagedorn et al., 1985; Raikhel, 1992).

While the potential role of ecdysone in *Drosophila* oogenesis has not been extensively studied, several findings suggest that this steroid hormone may be required for oogenesis. Ecdysone promotes the production of yolk proteins by the fat body and follicle cells (Bownes, 1986; Hagedorn, 1989). Furthermore, characterization of the temperature-sensitive mutants *ecdysoneless¹* (*l(3)ecd¹*) and *l(3)3^{DTS}*, which as adults have low ovarian steroid hormone titers at the restrictive temperature, suggest that ecdysone may be required for egg chamber progression past stage 8 (Audit-Lamour and Busson, 1981; Walker et al., 1987). Although transplantation experiments show that the temperature-sensitive defects in *l(3)ecd¹* egg chambers are autonomous to the ovary (Garen et al., 1977), lack of evidence showing that steroid response genes are affected by this mutation have precluded the interpretation that ecdysone response hierarchies function during oogenesis. Moreover, *l(3)ecd¹* exhibits pleiotropic effects in some tissues not thought to be dependent upon ecdysone (Redfern and Bownes, 1983; Sliter, 1989).

In this study, we provide new evidence that components at several levels of the ecdysone response pathway, steroidogenic enzymes, ecdysone receptors and ecdysone response genes, are each functionally required in the ovary. The stage-specific expression patterns of *E75*, *E74* and *BR-C* suggest that these early ecdysone-responsive genes may be regulated by a common signal, possibly ecdysone. Experiments using the *l(3)ecd¹* mutant and exogenous ecdysone reveal that the expression of early response genes decrease or increase in response to changes in the ovarian ecdysone titer, further suggesting that ecdysone drives the expression of these genes in the ovary. In follicle cells, the *Egfr* signaling pathway spatially regulates *E75* expression. In conjunction with recent findings that describe *BR-C* regulation by the dorsoventral signaling pathways (Deng and Bownes, 1997), these data suggest that expression of early response genes in follicle cells is refined by the integration of temporal and spatial cues required for proper follicle cell patterning. Several experiments reveal a germline requirement for ecdysone response genes

during the vitellogenic phase of oogenesis. Germline clones of *E75* mutations result in degeneration of egg chambers after stage 8, with a phenotype similar to that of *l(3)ecd¹*. In addition, clonal analysis using an *EcR* null mutation indicates that the ecdysone receptor functions during mid-oogenesis. Lastly, expression and genetic analysis of a newly cloned steroidogenic enzyme, *Drosophila* Adrenodoxin Reductase, further confirms a functional requirement for ecdysone synthesis in the egg chamber. Taken together, these data suggest that ecdysone, produced by nurse cells, is required in an autocrine manner by the germline for egg chamber maturation during mid-oogenesis.

MATERIALS AND METHODS

Fly stocks

All flies were cultured on standard cornmeal medium at room temperature. The following *Drosophila melanogaster* strains were used: Canton-S, *l(3)ecd¹* (Garen et al., 1977), *EcR^{M554fs}* and *EcR^{A483T}* (Bender et al., 1997), *fs(1)K10¹* (Wieschaus, 1978), *Egfr⁴¹* (*top^{2Y1}*; Schüpbach, 1987), *dare³⁴* (Freeman et al., 1999), *E75^{e213}* (W. S. et al., unpublished data). 29°C was used as the restrictive temperature in temperature-shift experiments.

Egg chamber staining procedures

Ovaries were dissected in IMADS (Cooley et al., 1992). Immunochemical analysis of whole ovaries was performed as described in Robinson and Cooley (1997). For antibody staining, ovaries were stained with EcR common region antibody hybridoma supernatants, AG10.2 or DDA2.7 (1:1) (Talbot et al., 1993), BR-C common region antibody hybridoma supernatant 25E9 (Emery et al., 1994) (1:1) or lamin rabbit polyclonal antibodies (Fisher and Smith, 1988). For actin visualization, ovaries were stained with rhodamine-conjugated phalloidin (Molecular Probes). For nuclear visualization, ovaries were mounted in SPIF (Lundell and Hirsh, 1994). Immunolocalizations were visualized using a laser scanning confocal microscope (BioRad MRC 600).

Digoxigenin-labeled antisense RNA probes were made using the DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. For the *E74* probe, a 6.0 kb fragment that included both common and A-specific regions was used. For the *E75* probe, 3.0 kb of the common region was used as a template. For in situ hybridization, ovaries were dissected in IMADS and fixed for 30 minutes with fresh 4% paraformaldehyde in PBS and 10% DMSO. The ovaries were washed with PBST (PBS; 0.1% Tween-20), treated with 50 µg/ml Proteinase K for 30 minutes and refixed for 20 minutes. After washing with PBST, the ovaries were prehybridized in HB buffer (50% formamide; 5× SSC; 100 µg/ml salmon sperm DNA; 50 µg/ml heparin; 0.1% Tween-20) at 60°C for 2 hours. The prehybridization buffer was replaced with a solution containing DIG-labeled probe diluted in HB buffer and incubated overnight at 60°C. The ovaries were washed in HB buffer at 60°C for 6 hours. They were then washed with PBST. The staining reaction was carried out according to the manufacturer's instructions (Boehringer Mannheim). For experiments using in situ hybridization to compare mRNA levels of different samples, the ovaries were all processed and stained exactly the same way. Staining was allowed to proceed until at least one sample appeared dark, then staining of all samples was stopped at the same time using PBST. In each case, sense strand control probes resulted in no detectable staining. Microscopy was carried out on a Zeiss Axiophot.

Ovary culture

Ovaries were dissected in IMADS and then placed in 250 µl of Hyclone Insect Tissue Culture Media (Hyclone) with (0-0.4 mg/ml)

20-hydroxyecdysone (Sigma). Ovaries were constantly agitated at room temperature during the incubation period. For RNase protection and in situ hybridization experiments, the ovaries were cultured for 4 hours.

RNase protection

For the *E75* probe, a 436 bp *XhoI-StuI* fragment was cloned into pBluescript-SK (Stratagene) and the RNA probe was transcribed using T3 RNA polymerase. Total RNA was isolated from the samples using Trizol (GibcoBRL). RNA probes and the RNase protection assay were done using an Ambion RNase Protection Kit (Ambion) according to the manufacturer's protocol.

Germline clones

To generate *EcR* germline clones, the *EcR* null allele, *EcR^{M554fs}*, was placed in *trans* to P[FRT, *mini w⁺*]^{2R-G13}, P[*mini w⁺*; *ovoD1*]. Clones were induced in first to second instar larvae using 1000 rads of X rays. Resulting adult females were assayed for egg laying and ovaries were dissected 5 days after eclosion and stained with anti-*EcR* antibodies as described above.

To obtain FLP-FRT mosaics, recombinants were made between either *dare³⁴* and P[FRT, *mini w⁺*]^{2R-G13}, L/CyO or between *E75^{e213}* and *w*; *D*, P[FRT, *mini w⁺*]/TM3, *Sb* to produce P[FRT, *mini w⁺*]^{2R-G13}, *dare³⁴*/CyO or *E75*, P[FRT, *mini w⁺*]/TM3, *Sb*. These lines were crossed to either *w*, *hsFLP*; P[FRT, *mini w⁺*]^{2R-G13}, P[*mini w⁺*; *ovoD1*]^{2R} or *w*, *hsFLP*; P[*mini w⁺*; *ovoD1*]^{3L-2X48}P[FRT; *mini w⁺*] males and third instar larval progeny were heat shocked in a 37°C waterbath for 2 hours on 2 consecutive days.

RESULTS

Expression of early ecdysone-responsive genes during oogenesis

In order to investigate the role of ecdysone-responsive gene expression in the ovary, we looked at the expression of three classical early ecdysone-responsive genes, *E75*, *E74* and *BR-C*. In situ hybridization revealed that the *E75* and *E74* genes were transcribed in remarkably similar patterns during oogenesis. Both *E75* and *E74* transcripts were first detected in region 2b of the germarium (Fig. 1A-D). Expression decreased during stages 2-4 and low levels of *E75* and *E74* mRNA were again detected in stage 5-7 egg chambers. Transcription of *E75* and *E74* appeared to be upregulated during stage 8 in both the germline and soma. This expression continued to increase until stage 10B when transcription of both genes peaked in the follicle cells and the nurse cells.

Next, immunofluorescent staining revealed the presence of *BR-C* protein in the follicle cell nuclei beginning between stages 5 and 6 of oogenesis (Fig. 1E). In most of the egg chambers examined, *BR-C* appeared to be completely absent from the germline. However, in rare cases, low levels of expression could be detected in the nurse cell nuclei. These observations are consistent with a recent report that describes follicle cell expression of *BR-C* RNA (Deng and Bownes, 1997).

The expression of *E75*, *E74* and *BR-C* in egg chambers suggested that they were co-regulated by a common signal. If these early response genes were being regulated by ecdysone, one would expect a

dependence on the ecdysone receptor. To determine whether the ecdysone receptor was present in the ovary, egg chambers from Canton-S females were stained using anti-*EcR* antibodies. Antibody staining revealed that germline and somatic cells expressed *EcR* protein in their nuclei. (Fig. 1F). This expression was first detected in the germarium, appeared to be slightly upregulated during stage 4 and persisted until the late stages of oogenesis. Additionally, border cells strongly expressed *EcR* during their migration through the nurse cell cluster (data not shown). USP has also been detected in all cells within the ovary (Khoury-Christianson et al., 1992). Thus, both components of the functional ecdysone receptor are present in the germline and soma during all stages of oogenesis.

Expression of early response genes is sensitive to changes in the ovarian ecdysone titer

To test the dependence of early response gene expression on ecdysone, we studied the effects in ovaries of the *l(3)ecd^l* mutation. Females homozygous for the temperature-sensitive mutation *l(3)ecd^l* had previously been shown to lose the ability to lay eggs after just 2 days at the restrictive temperature and to have 13% of the wild-type ovarian ecdysone titer when shifted to the restrictive temperature for 4 days (Garen et al., 1977). *E75* transcript levels were compared in wild-type and *l(3)ecd^l*

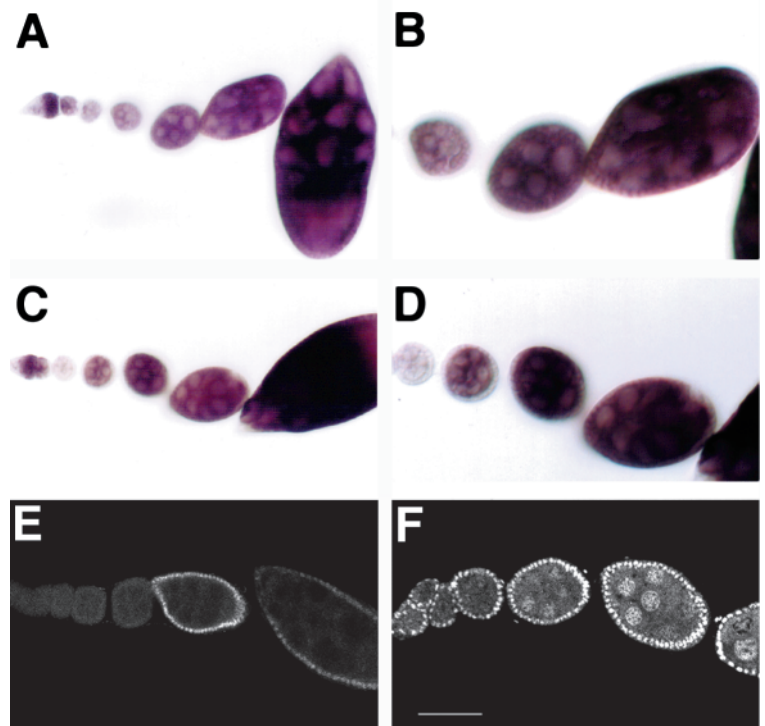


Fig. 1. Expression of *E75*, *E74*, *BR-C* and *EcR* during oogenesis. (A-D) In situ hybridizations performed on wild-type ovaries using an *E75* RNA probe (A,B) or an *E74* RNA probe (C,D). Expression of both *E75* and *E74* is first detectable in region 2B of the germarium. This expression rapidly decreases. Low levels of expression are again detectable in stage 5 egg chambers. Transcription of both genes increases dramatically between stages 8 and 9 of oogenesis. (E) Anti-*BR-C* antibody staining on wild-type egg chambers. *BR-C* expression is not detectable before stage 5 of oogenesis after which the protein is readily detectable in the follicle cells. (F) Anti-*EcR* antibody staining on wild-type ovaries reveals that germline and somatic cells express *EcR* during all stages of oogenesis.

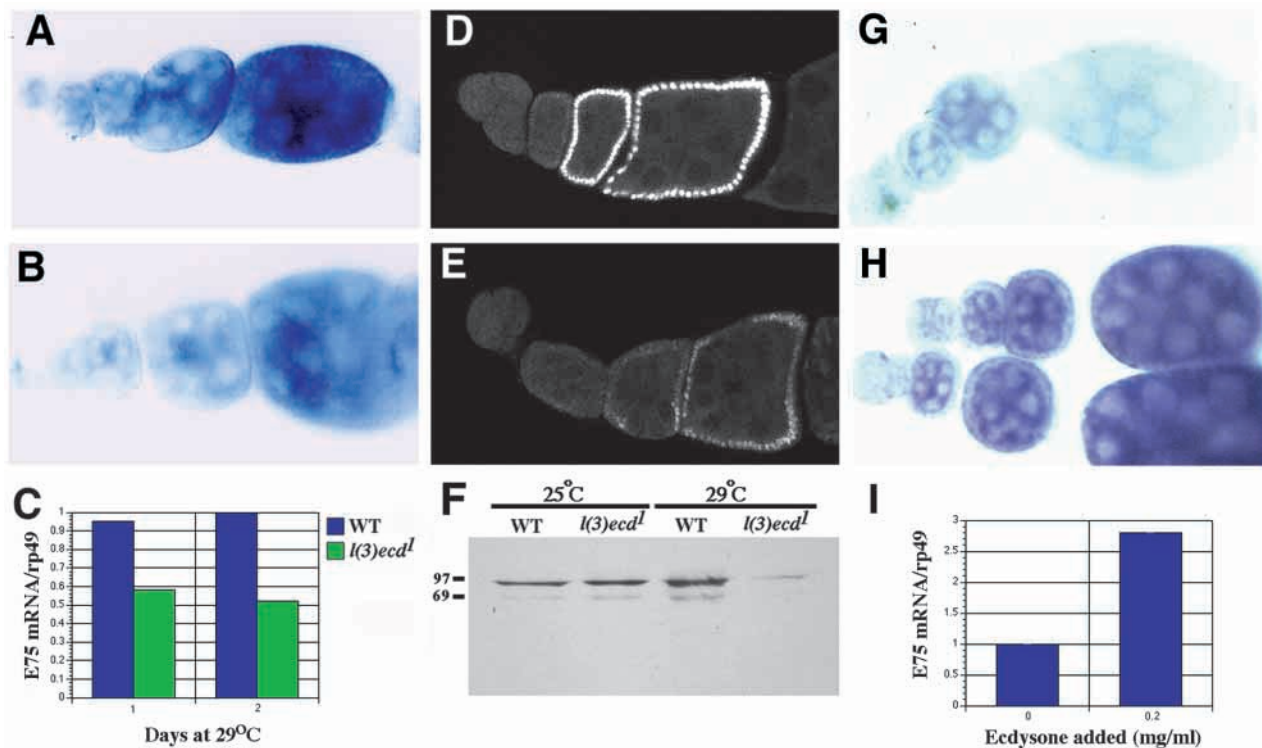


Fig. 2. Ovarian expression of *E75* and *BR-C* changes in response to changes in the ecdysone titer. (A,B) In situ hybridization using an *E75* probe on egg chambers from (A) wild-type and (B) *l(3)ecd¹* females shifted to 29°C for 2 days. (C) Quantitation of a typical RNase protection assay comparing *E75* mRNA levels in ovaries from wild-type and *l(3)ecd¹* females shifted to 29°C for 1 and 2 days. This analysis shows that ovaries from *l(3)ecd¹* flies have approximately half the *E75* mRNA of wild-type ovaries relative to rp49 control RNA. (D,E) Anti-*BR-C* antibody staining reveals a reduction of *BR-C* expression in ovaries from (E) *l(3)ecd¹* adults relative to (D) wild-type controls shifted to 29°C for 2 days. (F) Western blot analysis also reveals a significant reduction of *BR-C* expression in *l(3)ecd¹* mutants. (G–I) Culturing wild-type ovaries in the presence of ecdysone results in increased *E75* expression. In situ hybridization on ovaries cultured without (G) and with ecdysone (H) reveals an increase of *E75* expression in both germline and follicle cells. (I) A RNase Protection assay confirms increased *E75* expression in ovaries cultured with ecdysone relative to controls. Paired panels (A,B) and (G,H) represent separate experiments and are not directly comparable.

females shifted to the restrictive temperature for different lengths of time. Using in situ hybridization, no difference in *E75* mRNA levels could be detected between ovaries taken from wild-type and *l(3)ecd¹* females maintained at 25°C (data not shown). However, there was a reproducible reduction of *E75* mRNA in *l(3)ecd¹* ovaries relative to wild-type controls shifted to the restrictive temperature for 2 days (Fig. 2A,B). An RNase protection assay was used to quantitate the difference in *E75* transcription in *l(3)ecd¹* and wild-type ovaries. This analysis revealed that *l(3)ecd¹* ovaries contained approximately half the *E75* mRNA of wild-type ovaries when subjected to restrictive conditions (Fig. 2C). *BR-C* expression in wild-type and *l(3)ecd¹* ovaries was also assayed. Immunofluorescent staining showed that *BR-C* protein levels appeared to be reduced in *l(3)ecd¹* ovaries relative to wild-type controls (Fig. 2D,E). A reduction of *BR-C* expression in ovaries from mutants shifted to 29°C was also detected on western blots (Fig. 2F).

The presence of EcR protein and previous work showing the presence of USP in ovarian cells of all stages suggested that these cells are competent to respond to ecdysone (Khoury-Christianson et al., 1992). We tested whether an increase in the ecdysone titer could induce *E75* expression in the ovary. Ovaries were cultured in the presence or absence of 20-hydroxyecdysone. In situ hybridization showed that *E75*

transcription increased in early egg chambers in response to ecdysone and that the increase of expression occurred in both the follicle cells and germline (Fig. 2G,H). An RNase protection assay was used to quantitate the induction of *E75* transcription by exogenous ecdysone. This analysis demonstrated that increasing amounts of ecdysone in the culture media led to increased expression of *E75* (Fig. 2I).

The *Egfr* signaling pathway spatially modulates *E75* expression in the follicle cells

During stage 10, the follicle cell expression of *E75* became enriched in the dorsal anterior cells (Fig. 3A). This suggested that inputs in addition to ecdysone were needed to refine *E75* expression. Previous work has shown that follicle cell polarity is established during mid to late oogenesis and depends on the interaction between *gurken* (*grk*) and the *Drosophila* homologue of the mammalian EGF receptor (*Egfr*) (Neuman-Silberberg and Schüpbach, 1993; Roth and Schüpbach, 1994; Schüpbach, 1987). To determine whether *E75* expression was under control of the dorsoventral signaling pathway, we examined ovarian *E75* RNA distribution in dorsalized and ventralized mutant backgrounds. In *fs(1)K10* mutants, mislocalization of GRK protein results in activation of EGFR in all anterior follicle cells surrounding the oocyte (Roth and

Schüpbach, 1994). In situ hybridization showed that, in *fs(1)K10* mutant egg chambers, *E75* expression expanded to a ring of anterior follicle cells surrounding the oocyte (Fig. 3B). Mutations in *Egfr* prevent signal transduction by the receptor and lead to the ventralization of the eggshell and embryo (Schüpbach, 1987). In situ analysis indicated that stage 10 follicle cells overlying the oocyte in *Egfr* mutants no longer expressed *E75* (Fig. 3C). However, *E75* expression in the nurse cells was unaffected (Fig. 3C). These experiments showed that the *Egfr* signaling pathway regulated *E75* expression in the dorsal follicle cells but not in the germline.

Functional requirement for the ecdysone response pathway during mid-oogenesis

To investigate the functional role of *E75* in the germline, we generated germline clones of a strong *E75* allele using the FLP/FRT system of mitotic recombination and the *ovo^{D1}* dominant female sterile transgene (Chou et al., 1993; Chou and Perrimon, 1996). While heterozygotes carrying a control chromosome over *ovo^{D1}* laid eggs following clone induction, females carrying the *E75^{e213}* mutation in *trans* to the *ovo^{D1}* chromosome did not lay eggs after clone induction. Upon dissection, *E75^{e213}* germline clones appeared to arrest and degenerate at stages 8-9 (Fig. 4A,B). Some yolk could be seen in the oocyte but the follicle cells rarely (<3%, *n*=126 clonal egg chambers) completed their migration to the oocyte (Fig. 4C). While the germline in these clonal egg chambers degenerated, the follicle cell layer remained remarkably intact (Fig. 4B). Oocytes in *E75^{e213}* germline clones were small and sometimes displaced to one side of the posterior end of the egg chamber (data not shown). Although we did not use markers

to determine whether follicle clones were also present in the clonal egg chambers, the frequency of the observed phenotypes strongly suggested that *E75* function was required in the germline to progress beyond early vitellogenesis.

If *E75* expression depends upon ecdysone response, similar phenotypes should result from ecdysone receptor mutations. To test whether *EcR* is required for the completion of oogenesis, germline clones of the *EcR* null mutation, *EcR^{M554fs}* (Bender et al., 1997) were analyzed. A small number of females (4/202) laid eggs 2-4 days after eclosion. Although few in number (2-3/female/day), these eggs appeared to be normal in shape and size. However, egg laying ceased after 4-5 days. Upon dissection, ovaries from several females (15/202) appeared to have clonal egg chambers many of which were arrested at stage 6 or 7. These egg chambers were easily distinguished from non-clonal *ovo^{D1}* egg chambers by the lack of EcR antibody staining in nurse cell nuclei (compare Fig. 5A and C).

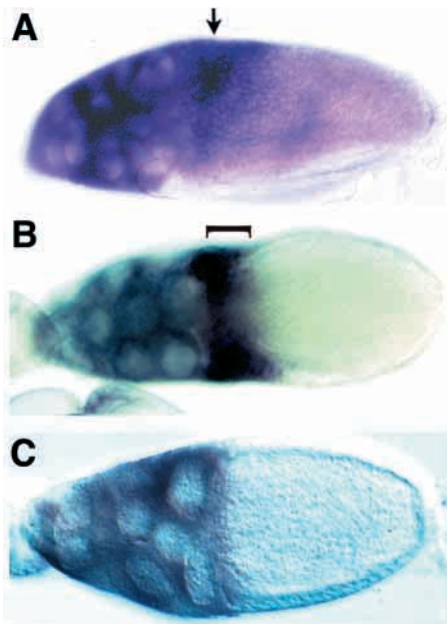


Fig. 3. The *EGFR* pathway regulates *E75* expression in the follicle cells. (A-C) In situ hybridization in wild-type and mutant ovaries. (A) *E75* expression in wild-type stage 10 egg chambers is enriched in the dorsal follicle cells (arrow). (B) In *fs(1)K10* egg chambers, *E75* expression expands in a band (bracket) around the entire egg chamber. (C) *Egfr^{rl}* mutant egg chambers show loss of *E75* expression in stage 10 follicle cells.

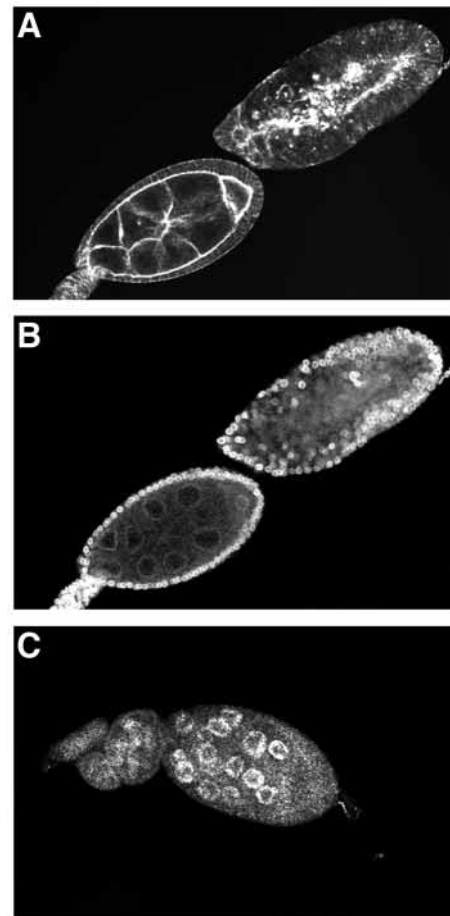


Fig. 4. *E75* null mutation germline clones arrest during mid-oogenesis. (A-C) *E75^{e213}* germline clones at various stages of degeneration. *E75^{e213}* clones are readily distinguishable from egg chambers carrying the chromosome 3L *ovo^{D1}* transgene, which results in earlier degeneration (data not shown). (A) Rhodamine-phalloidin staining of *E75^{e213}* germline clones reveals that the actin cytoskeleton remains intact just prior to egg chamber degeneration. (B) Anti-lamin antibody staining of the nuclear envelope shows that although the germline cells degenerate, the follicle cell layer remains remarkably intact. (C) SPIF staining reveals DNA condensation at the nuclear envelope just prior to degeneration.

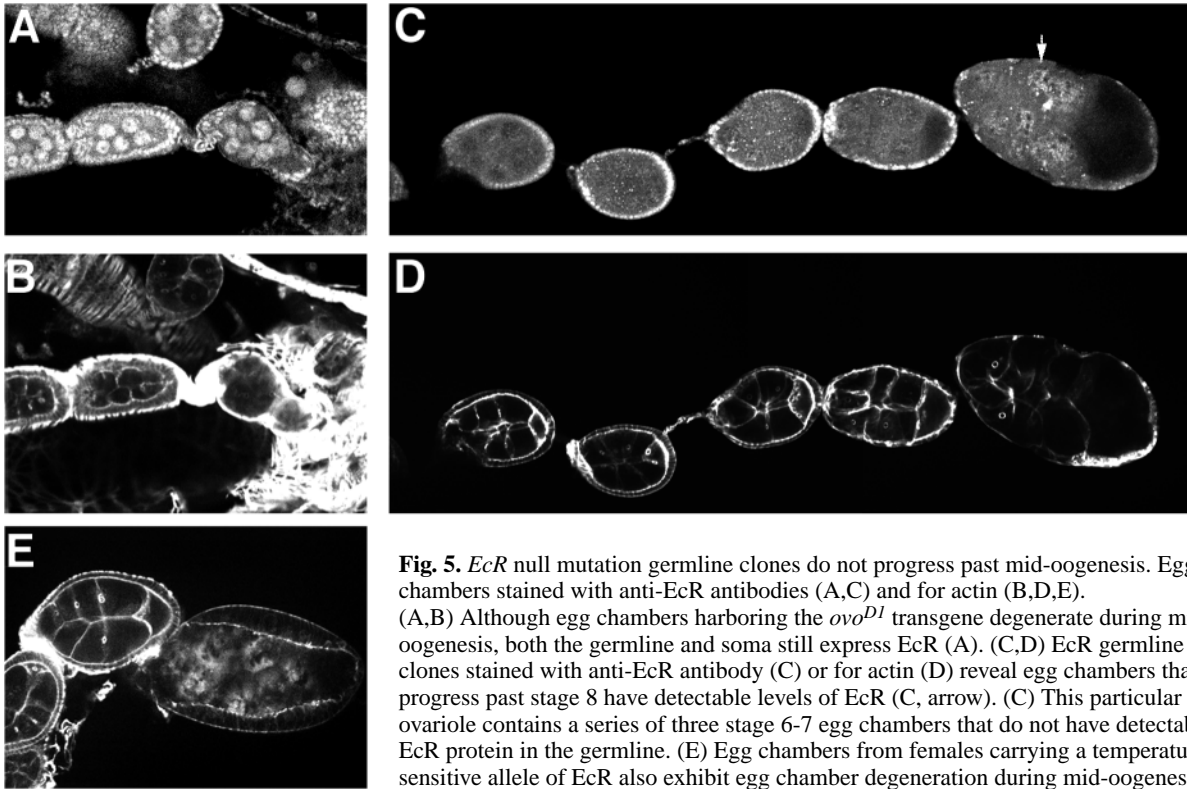


Fig. 5. *EcR* null mutation germline clones do not progress past mid-oogenesis. Egg chambers stained with anti-*EcR* antibodies (A,C) and for actin (B,D,E). (A,B) Although egg chambers harboring the *ovo^{D1}* transgene degenerate during mid-oogenesis, both the germline and soma still express *EcR* (A). (C,D) *EcR* germline clones stained with anti-*EcR* antibody (C) or for actin (D) reveal egg chambers that progress past stage 8 have detectable levels of *EcR* (C, arrow). (C) This particular ovariole contains a series of three stage 6-7 egg chambers that do not have detectable *EcR* protein in the germline. (E) Egg chambers from females carrying a temperature-sensitive allele of *EcR* also exhibit egg chamber degeneration during mid-oogenesis.

Therefore, it appeared as though *EcR* was required for egg chamber progression beyond mid-oogenesis. A few egg chambers had developed beyond stage 8 ($n=32$); however, all of these had detectable levels of nuclear *EcR* protein (Fig. 5C, arrow). A possible explanation for the presence of late stage egg chambers is that wild-type *EcR* protein perdures after clone induction but is gradually depleted with ensuing mitotic divisions (Jordan and Karess, 1997). This interpretation was supported both by the antibody staining in egg chambers older than stage 7 and by the loss of egg laying ability as females aged. A similar range of phenotypes was obtained using a temperature-sensitive mutation of *EcR* (*EcR^{A483T}*), which exhibited egg chamber degeneration during mid-oogenesis when shifted to the restrictive temperature (Fig. 5E; G. Carney and M. Bender, unpublished data).

Adrenodoxin reductase is necessary for normal egg chamber development

In order to address more directly the timing and autonomy of steroid hormone signaling in the *Drosophila* ovary, we analyzed the expression pattern and germline requirements for the recently described *dare* gene (Freeman et al., 1999). *dare* encodes the *Drosophila* homologue of adrenodoxin reductase, an enzyme required for the synthesis of steroid hormones in vertebrates (Miller, 1988). Biochemical studies from insects, and genetic analysis of the *dare* locus in *Drosophila*, strongly suggest that insect steroid hormone production is also critically dependent upon adrenodoxin reductase, and null alleles of *dare* appear to effectively block the synthesis of ecdysteroids (Freeman et al., 1999).

In situ hybridization revealed that low levels of *dare* RNA could be detected in the germline beginning at stage 6 of

oogenesis (Fig. 6). Expression of *dare* remained relatively low until stage 10 when egg chambers exhibited a dramatic increase of *dare* transcription (Fig. 6).

To further assess the requirements for *dare* during oogenesis, germline clones using the *dare³⁴* allele (Freeman et al., 1999) were induced. While all of the control females (100%; $n=25$) laid eggs, very few (25%; $n=30$) females with presumptive *dare³⁴* clones laid eggs, and these females lost the ability to lay eggs after 1-2 days. Upon dissection, *dare* germline clones could not be phenotypically distinguished from the surrounding tissue carrying the second chromosome *ovo^{D1}* mutation, which results in arrest at stage 8-9. These findings indicate that *dare* has a critical germline function, and are consistent with the possibility that *dare* clones arrest at a point similar to that seen for other components of the ecdysone response pathway. Interestingly, significant *dare* expression was not seen in the germarium; while lower levels of *dare* could potentially be



Fig. 6. The steroidogenic enzyme gene *dare* is expressed during mid-oogenesis. In situ hybridization using a *dare*-specific RNA probe reveals that *dare* transcription is first detectable between stages 6 and 7. This expression continues to increase until stage 10.

sufficient to allow some ecdysone synthesis, this raises the possibility that signals other than ecdysone could contribute to germarial expression of early response genes.

DISCUSSION

An ecdysone-dependent checkpoint during mid-oogenesis

Although a great deal is now known about the regulation of egg chamber and oocyte patterning in *Drosophila*, the mechanisms through which environmental conditions influence the overall rate of oogenesis and egg laying are not well understood. Factors including day/night cycle, humidity and nutrition appear to play some role in female egg laying behavior. Under unfavorable environmental conditions, few eggs are laid and the progression of egg chambers into the vitellogenic stages is largely blocked. Previous work shows that egg chambers exhibit the greatest frequency of degeneration at stage 8, suggesting that defective egg chambers are eliminated during mid-oogenesis (Giorgi and Deri, 1976). Given these and our own data, we propose that egg chambers must pass through an ecdysone-responsive genetic checkpoint before they can progress past stage 8 and commence vitellogenesis.

The expression of several genes in the steroid response hierarchy indicates that ecdysone may regulate *Drosophila* oogenesis. EcR and USP, the two components of the functional ecdysone receptor, are expressed in both the soma and germline throughout oogenesis. Intriguingly, the three classic early ecdysone response genes, *BR-C*, *E74* and *E75*, are expressed in a stage-specific manner. The observed reduction in ovarian expression of *BR-C* and *E75* in *l(3)ecd¹* females supports the hypothesis that ecdysone regulates the expression of these early genes in the ovary as it does during larval development.

Previously, it has been observed that *l(3)ecd¹* egg chambers arrest in mid-oogenesis prior to degeneration (Audit-Lamour and Busson, 1981). However, the proposed cell lethality of *l(3)ecd¹* (Sliter, 1989) and the inability to determine the stage at which ecdysone was acting precluded the interpretation that ecdysone was required specifically at the time of this transition. Furthermore, the presumption that ecdysone would act as a diffusible endocrine factor made this hormone less attractive as a putative stage-specific signal in ovaries that sustain asynchronous egg chamber development.

Genetic data presented in this paper now strongly suggest that the ecdysone response pathway may indeed function in a stage-specific manner during oogenesis. The inability of *EcR* germline clones to progress beyond mid-oogenesis suggests that ecdysone signaling mediates egg chamber maturation. Moreover, germline clonal analysis of *E75* shows that this early response gene is needed by the germline for the completion of oogenesis. We obtained similar results from germline clones of *E74* mutations (unpublished data). Together, the correlation between *l(3)ecd¹* arrest, early gene expression and the similar germline clone phenotypes of *EcR*, *E75* and *E74* shows that induction of ecdysone-regulated genes is a stage-specific signal required for progression beyond the early vitellogenic stages of oogenesis. Whether the earlier expression of *E74* and *E75* reflects an additional role for ecdysone response in the germline remains to be determined; experiments

investigating potential roles of ecdysone in other stages of oogenesis are in progress.

Previous clonal analysis of *usp* mutants suggested that this component of the ecdysone receptor may not be strictly required for the progression of oogenesis (Oro et al., 1992; Perrimon et al., 1985). *usp* mutant germline clones exhibited some loss of fertility associated with chorion defects (Oro et al., 1992), but a mid-oogenesis phenotype of *usp* germline clones was not reported. Two possibilities could explain the discrepancy between the *EcR* and *usp* phenotypes. When *EcR* clones were induced, we observed apparent perdurance of wild-type protein in clonal egg chambers, in association with the transient progression of a small number of egg chambers beyond the mid-oogenesis checkpoint. Similar perdurance of wild-type USP could potentially explain the production of mature eggs in *usp* clones. Alternatively, it may be possible that *EcR* has another binding partner within the germline. Recent work on ecdysone response during late larval development and pupariation raises the possibility that other molecules, besides the canonical *EcR*-USP ecdysone receptor, may mediate ecdysone response in certain tissues or stages of development (Buszczak and Segraves, 1998).

Possible autocrine mechanism for germline expression of ecdysone response genes

In other insects, including the mosquito *Aedes aegypti*, the transition from the previtellogenic to the vitellogenic state is governed by ecdysone-regulated hierarchies (Dhadialla and Raikhel, 1994; Hagedorn, 1983, 1985; Raikhel, 1992). *Aedes* egg chambers develop synchronously and remain arrested in a previtellogenic state until the female takes a blood meal. This triggers production of the ecdysiotropic neuropeptide (EDNH) that stimulates ovarian synthesis of ecdysone. The resulting increase in the ecdysone titer leads to the expression of *E75* and controls induction and progression of vitellogenesis and further egg development (Pierceall et al., 1999).

Our findings suggest the previously unexpected possibility that ecdysone could be regulating egg chamber progression past stage 8 in *Drosophila* in a similar fashion. Thus, the stage-specific expression of ecdysone response hierarchies and their control over egg chamber development may represent an evolutionarily conserved mechanism for coordinating the developmental processes that occur during insect oogenesis.

While the synchronous development of a cohort of eggs under endocrine control, as seen in the mosquito, would not be unexpected, the asynchronous progression of *Drosophila* oogenesis under similar hormonal control raises an interesting and important question. How can a presumed endocrine factor, such as ecdysone, regulate the sequential, asynchronous induction of these genetic regulatory programs during *Drosophila* oogenesis? Stage specificity of ecdysone-responsive gene expression could, in principle, be controlled either at the level of competence to respond to ecdysone or at the level of the production or availability of the hormone itself. Our results suggest the latter possibility. While analysis of *EcR* germline clones indicates that the receptor is required during mid-oogenesis, the ecdysone receptor is present throughout oogenesis. The expression of the ecdysone response genes *BR-C*, *E74* and *E75*, in contrast, is stage-specific, and varies in conjunction with experimental manipulation of hormone titer. These findings suggest that individual egg chambers are

exposed to different amounts of hormone as they progress through oogenesis.

The stage-specific expression of the steroidogenic enzyme *dare* suggests a mechanism through which this localized hormone exposure could arise. As the expression of *dare*, and perhaps other steroidogenic enzymes, increases during mid-oogenesis, the rate of ecdysone synthesis could be expected to increase. The requirement for *dare* expression within the germline and the observed correlations between early gene expression and *dare* expression support the hypothesis that ecdysone synthesis within the egg chamber is required for controlling the progression of oogenesis, and that induction of ecdysone response programs may occur via an autocrine mechanism in association with the stage-specific increase in ecdysone synthesis.

Ecdysone is, however, a lipophilic molecule that might be expected to diffuse from the egg chambers where it is produced into the surrounding tissue. Therefore, the modulation of ecdysone availability or turnover would be expected to play an important role in restricting premature hormonal stimulation of adjacent early egg chambers. Current evidence indicates that ecdysteroids are esterified into inactive apolar conjugates and loaded into the egg for use during embryogenesis (Bownes et al., 1988), and we propose that this conjugation may serve to limit escape into the surrounding tissue. It is also known that 20-hydroxyecdysone, the biologically active hormone, can be rapidly converted to other, relatively inactive ecdysteroids (Williams et al., 1997), and these other metabolic pathways may play a similar role.

Ecdysone and *Egfr* signaling regulate follicle cell development

In young egg chambers, all follicle cells express early response genes, possibly as a result of a paracrine influence by ecdysone produced in nurse cells. The follicle cell expression patterns for *BR-C* and *E75* are refined during stage 10 so that the highest levels of expression are in the dorsal-anterior cells. This resolution of early response gene expression requires input from the *Egfr* signaling pathway (this paper; Deng and Bownes, 1997). Genetic studies of *BR-C* (Deng and Bownes, 1997) and *E75* (M. Buszczak, L. C. and W. S., unpublished data) show that early response genes are required for dorsal appendage formation. These findings suggest that the ecdysone and *Egfr* signaling pathways converge to regulate early response genes in follicle cells.

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