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Investigating the function of follicular subpopulations during *Drosophila* oogenesis through hormone-dependent enhancer-targeted cell ablation

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SUMMARY

Although it is known that the establishment of polarity during *Drosophila* oogenesis is initiated by signalling from the oocyte to the overlying follicle cells, much less is understood about the role of specific follicular subpopulations. One powerful approach for addressing this question, toxigenic cell ablation of specific subpopulations, has not previously been applicable to studying follicular subpopulations because many of the genes and Gal4 enhancer trap insertions that are expressed in the ovary are also expressed at earlier times in development. To overcome this problem, we have utilized a fusion protein between Gal4 and the human estrogen receptor to achieve hormone-dependent, tissue-specific gene expression of UAS-linked transgenes in flies. We used this system to study the role of the polar subpopulations of follicle cells during oogenesis by expressing within them a modified form of diphtheria toxin that causes cell death. Our results confirmed previous functions ascribed to these cells, and also demonstrated a previously undescribed role for the border cells in facilitating the migration of the anterior Fasciclin III-expressing polar pair cells to the edge of the oocyte.

Key words: Inducible, ablation, GalER, diphtheria toxin, border cells, polarity, oogenesis, *Drosophila melanogaster*

INTRODUCTION

Pattern formation during *Drosophila* embryogenesis is initiated by localized spatial cues that are deposited into the developing egg by the germline-derived nurse cells and the somatically derived follicular epithelium. Proper positioning of these cues, which determine the orientation of the anterior-posterior and dorsal-ventral axes of the embryo, is dependent upon the prior establishment of these axes within the follicle cell layer. The development of follicular polarity is mediated by localized activation of the somatically expressed *Drosophila* Epidermal Growth Factor Receptor (DER) by Gurken (Grk) (reviewed by Ray and Schüpbach, 1996), a Transforming Growth Factor-α (TGF-α) homolog (Neuman-Silberberg and Schüpbach, 1993) that is expressed by the oocyte nucleus (Saunders and Cohen, 1999). Although a great deal of progress has been made recently in our understanding of the role of oocyte/follicle cell communication in the development of follicular and embryonic polarity, much less is known about the mechanisms that regulate the developmental potential of the specific subpopulations of follicle cells involved in these processes. Elucidation of the function of specific follicular subpopulations using genetic methods alone has been difficult, because many of the genes involved in follicular patterning are also required at earlier times during development. Consequently, it has been necessary in many cases to utilize hypomorphic alleles or to generate follicles that are mosaic for the mutation of interest.

As an alternative approach, we set out to examine the consequences of eliminating specific subpopulations of follicle cells. Cell ablation has proved to be a highly effective technique for identifying the developmental functions of individual cells and cell types in organisms ranging from *C. elegans* (Sulston, 1988) and *Drosophila* (Doe and Goodman, 1985) to mouse (Evans, 1989). In the *Drosophila* ovary, ablation has been used previously to demonstrate that the oocyte nucleus plays a role in the development of the dorsal-ventral polarity of the eggshell (Montell et al., 1991), and to show that terminal filament cells negatively regulate stem cell division in the germline (Lin and Spradling, 1993). However, these studies were carried out using laser ablation, which involves the follicles being removed from donor females and then transplanted back into host females following laser treatment. Although oogenesis is not disrupted, the transplanted follicles are not connected to the oviduct of the host females. Consequently, the eggs that develop from the treated follicles do not become fertilized, thus preventing the observation of the effects of ablation on embryogensis.

To avoid this limitation, we elected to ablate cells in situ in living females by expressing toxic gene products in them. This technique has been successful in ablating cells in other tissues of the fly (Bellen et al., 1992; Moffat et al., 1992; Kalb et al., 1993). Bellen et al. (1992) generated several temperature-sensitive mutations in the A chain of diphtheria toxin (DTA) which, when linked to an eye-specific promoter, exhibited temperature-dependent cell killing (Bellen et al., 1992), whereas Moffat et al. (1992) utilized a cold-sensitive ricin

mutant, RAcs2. However, when we used the Gal4/UAS system (Brand and Perrimon, 1993) to express these mutant toxins in the ovary, we found that the M and R DTA mutants, as well as the ricin mutant RAcs2, were ineffective in killing cells. Conversely, the activity of the I DTA mutant (DTI) did not appear to be sufficiently temperature-sensitive, as we were not able to obtain adult flies carrying both the Gal4 enhancer trap and the UAS-linked DTI (UASDTI) insertions at any temperature. As many Gal4 enhancer trap insertions with ovarian expression patterns are also expressed at earlier times during development (Schüpbach and Wieschaus, 1998), it is likely that premature expression of the toxin leads to the death of cells required for the viability of the organism.

To overcome this problem, we have modified the Gal4/UAS system to achieve inducible tissue-specific gene expression. Fusion of the ligand-binding domain of the human estrogen receptor (ER) to the DNA-binding domain of Gal4 confers hormone dependence upon the fusion protein without changing its DNA-binding specificity (Webster et al., 1988). In this report, we demonstrate that GalER fusion proteins exhibit cell-specific and hormone-dependent activity in flies. Further, we show that when GalER fusion protein is expressed in individuals that also carry a UAS-linked toxin gene, administration of hormone leads to ablation of the GalER-expressing cells. In the absence of hormone treatment, however, the GalER-expressing cells, as well as individual flies, are unaffected. We used a cloned enhancer from the *torso-like* (*tsl*) gene, as well as enhancer trap insertions, to drive GalER expression in a polar pattern in the ovary and to ablate GalER-expressing border cells as well as a posterior polar subpopulation of follicle cells. Our results suggest that the failure of micropyle pore formation in follicles that lack border cells is not due directly to the lack of border cells, but rather from the failure of the polar pair cells to undergo migration to the anterior edge of the oocyte in the absence of the border cells. Ablation of the posterior polar subpopulation during stage 7 of oogenesis appeared to lead to defects in the anterior-posterior polarity of the oocyte, consistent with proposals that the posterior follicle cells are required for the establishment of the anterior-posterior axis in the oocyte (Lane and Kalderon, 1994; González-Reyes et al., 1995; Roth et al., 1995; see also Ray and Schüpbach, 1996). These results indicate that the GalER/UAS system will provide a powerful method to achieve temporal as well as spatial control of gene expression in *Drosophila*.

MATERIALS AND METHODS

Fly stocks

All stocks were maintained and eggs collected according to standard procedures (Roberts, 1986; Wieschaus and Nüsslein-Volhard, 1986). The enhancer trap lines Q21a (Fasano and Kerridge, 1988) and 5A7 (Roth et al., 1995) have been described previously, as have the lines carrying the insertions UAS-lacZ (Brand and Perrimon, 1993) and khc:lacZ (Clark et al., 1994). Unless noted, all of the experiments reported here were carried out at room temperature (approx. 22°C).

Plasmids and transformation

tsl5′lacZ

The mutant phenotype of embryos derived from *tsl* mutant mothers can be rescued when the mothers are transformed with a 10 kb *Eco*RI fragment of genomic DNA containing the *tsl* locus (Savant-Bhonsale and Montell, 1993; D. Beuchle and L. S., unpublished data). To identify upstream regulatory elements, a 3.5 kb fragment was isolated that contained sequences from the 5′ *Eco*RI site to a *Hin*dIII site present in the 5′ untranslated region of the *tsl* cDNA (Savant-Bhonsale and Montell, 1993; Martin et al., 1994). *Hin*dIII-*Eco*RI adaptors (Stratagene) were ligated to the *Hin*dIII site, and this fragment was then digested with *Eco*RI and cloned into the *Eco*RI site of pCaSpeR-AUG-β-gal, a reporter plasmid that can be used to examine the activity of heterologous promoters (Thummel et al., 1988).

hsGalER(147/251)

The full length Gal4 was first cloned into pSPBP4, which contains a modified *Xenopus* β-globin leader to enhance translation (Driever et al., 1990). The ligand binding domain of the ER (aa 282-595) was then excised from the plasmid HE14 (Kumar et al., 1986) and cloned in frame with the C terminus of Gal4. The entire Gal4-ER coding region plus the β-globin leader was isolated by partially digesting with *Hin*dIII and *Eco*RI. This fragment was made blunt ended, and then cloned into hsCaSpeR (Bang and Posakony, 1992) cut with *Xba*I and also made blunt ended. To create hsGalER(147/251), a *Cla*I-*Eco*RI fragment from the full length GalER in hsCaSpeR was replaced with the corresponding *Cla*I-*Eco*RI fragment from the Gal-ER (147/251) plasmid, which contains a Gal4 protein that is truncated at amino acid 147, and fused to the C terminus of the ER (beginning at amino acid 251) (Webster et al., 1988).

tslGalER

To place GalER (147/251) under the control of the 5′ regulatory region from the *tsl* gene, a 3.5 kb fragment spanning the 5′ *Eco*RI site to an *Xba*I site present in the 5′ untranslated region of *tsl* (Savant-Bhonsale and Montell, 1993; Martin et al., 1994) was isolated from the *tsl* genomic DNA clone. This fragment was cloned into pCaSpeR-AUG-β-gal (Thummel et al., 1988) that had been cut with *Eco*RI and *Xba*I, resulting in a replacement of the *lacZ* coding sequences with the *tsl* fragment. The GalER (147/251) fusion, including the β-globin leader, was then excised from hsGalER (147/251) following partial *Xba*I digestion and was cloned behind the *tsl* sequences at the *Xba*I site, producing the plasmid tslGalER.

UASDTI

The sequences encoding the DTI mutant form of DTA (Bellen et al., 1992) were amplified using PCR with high fidelity Vent polymerase (New England Biolabs) and cloned into pSPBP4 using *Nco*I and *Xba*I sites. This plasmid was then cut with *Hin*dIII, made blunt-ended, ligated to *Bcl*I linkers, and a *Bcl*I-*Xba*I fragment containing the β-globin leader plus DTI was then excised. pUASt (Brand and Perrimon, 1993) was cut with *Eco*RI, made blunt-ended, ligated to *Bcl*I linkers, and then cut with *Bcl*I and *Xba*I. The BP4DTI fragment was then ligated to the pUASt vector.

All plasmids were transformed into flies using standard techniques (Spradling, 1986).

Hormone treatment

β-estradiol (Sigma) was dissolved in DMSO at a concentration of 260 mg/ml and mixed 1:3 (vol/vol) with wet yeast paste. Diethylstilbestrol (DES; Sigma) was dissolved in acetone at a concentration of 125 mg/ml and mixed 1:3 (vol/vol) with wet yeast paste. Females were maintained on apple juice/agar plates with a drop of fresh yeast (with or without hormone) daily. To feed larvae, eggs were transferred to plates containing 50-100 ul β-estradiol/yeast paste, which was replenished daily.

GalER enhancer trap screen

A third chromosomal insertion of hsGalER(147/251), GalER#7, was crossed to TMS, Sb Δ2-3-carrying flies (Robertson et al., 1988).

GalER#7/TMS, Δ2-3 males were mated to w^- females. Males carrying new insertions of hsGalER were identifed as w^+; Sb^- and mated to w^- females. Male progeny carrying a new insertion of hsGalER but lacking Δ2-3 were identified as w^+; Sb^+. To identify the expression pattern of the new hsGalER insertion in the ovary, these males were individually mated to females carrying the UASlacZ reporter gene. The ovaries of female progeny with both insertions were dissected and stained for β-gal activity.

β-galactosidase staining

Staining for β-galactosidase (β-gal) activity was carried out according to the method of Fasano and Kerridge (1988), with slight modifications. Ovaries were dissected in PBS, fixed in 2.5% glutaraldehyde in PBS for 10 to 15 minutes, then washed several times with PBS and stained in staining solution [150 mM NaCl, 10 mM sodium phosphate pH 7.2, 1 mM $MgCl_2$, 3.1 mM potassium ferrocyanide, 3.1 mM potassium ferricyanide, 0.3% Triton X-100] with 0.2% X-gal for several hours or overnight at either 37˚C or room temperature. After staining they were washed several times in PBS before mounting in 80% glycerol in PBS. To detect larval expression of GalER insertions, hormone-fed larvae were dissected at 3rd instar and individual tissues stained as described above.

Acridine orange staining and TUNEL assay

Acridine orange staining was carried out exactly as described by Masucci et al. (1990). The TUNEL assay was done using the following protocol (from P. Tran and R. Nagoshi, personal communication), with slight modifications. The ovaries were dissected in PBT, fixed for 2 hours in 4% paraformaldehyde/heptane (1:1), and permeablized in 0.3% Triton X-100 overnight. The following day they were washed and incubated in 0.2 mg/ml proteinase K at room temperature for 15 minutes, washed and incubated in 2% H_2O_2 in methanol for 15 minutes, then incubated briefly in TdT buffer (30 mM Tris pH 7.2, 0.024% $CoCl_2$, 140 mM sodium cacodyle) at room temperature before incubating for 1 hour at 37°C in TdT reaction mixture (TdT buffer with 15 μM dUTP, 7.5 μM Biotin-dUTP, and terminal transferase; Boehringer). The ovaries were then incubated in TB [300 mM NaCl, 30 mM sodium citrate] for 15 minutes at room temperature, blocked in PBS plus 10 mg/ml BSA, incubated for 1 hour in 1:50 horseradish peroxidase (HRP)-streptavidin (Boehringer) in PBS plus 1 mg/ml BSA, then rinsed and developed in 0.2 mg/ml di-amino benzidine (DAB) and 0.006% H_2O_2.

Fasciclin III antibody staining

Ovaries were dissected and fixed in 4% paraformaldehyde in PBS plus 10% DMSO mixed 1:3 in volume with heptane, rinsed in methanol, and incubated in 3% H_2O_2 in methanol for 15 minutes. They were then washed in PBS^{++} (PBS + 1% BSA + 0.1% Tween) and blocked in PBS^{++} with 10% normal goat serum before incubating overnight at 4°C in PBS^{++} with anti-Fascicein III antibodies (Patel et al., 1987; Developmental Studies Hybridoma Bank) at a dilution of 1:100. The ovaries were again washed and blocked before incubating either overnight at 4°C or for 4 hours at room temperature in pre-absorbed biotin-labeled anti-mouse secondary antibodies at a dilution of 1:500 (Jackson Immunoresearch). The ovaries were then washed and incubated in AB solution (Vector Labs) for 1 hour before developing with DAB.

Preparation of embryonic cuticles

Embryos were collected overnight from females of the appropriate genotype. To view the micropyle, unhatched eggs were fixed in 4% paraformaldehyde in PBS for one hour before mounting in Hoyers/lactic acid (Wieschaus and Nüsslein-Volhard, 1986). To view the aeropyle, unhatched eggs were placed in Hoyers/lactic acid and then fine needles were used to tear the main body of the chorion so that the aeropyle would lie flat after the cover slip was applied.

RESULTS

Development of an inducible system for gene expression

To add temporal specificity to the activity of the Gal4 transcription factor in flies, we utilized a fusion of Gal4 to the human estrogen receptor (ER), which had previously been shown to exhibit hormone-dependence but unchanged DNA binding specificity in tissue culture cells (Webster et al., 1988). To determine whether the GalER fusion protein would exhibit similar properties in flies, we decided to express it in a defined pattern in the ovary by using regulatory sequences from the *tsl* gene, which is expressed in a polar pattern in the follicle (Savant-Bhonsale and Montell, 1993; Martin et al., 1994). At the anterior, *tsl* is expressed by the border cells, which originate at the anterior pole of the follicle and during stage 9 migrate through the nurse cells to the anterior edge of the oocyte, and by the centripetal cells, which migrate between the nurse cells and the oocyte during stage 10B. At the posterior, *tsl* is expressed by a group of approximately 20-30 cells at the posterior pole of the oocyte. When a 3.5 kb fragment from the 5′ end of the *tsl* locus was used to drive *lacZ* expression in flies, β-galactosidase activity was detected in the border cells and in the posterior-most follicle cells in stage 8 and older follicles (Fig. 1A). It should be noted, however, that these *tsl* regulatory sequences do not recapitulate the complete expression pattern described for the endogenous gene (Savant-Bhonsale and Montell, 1993; Martin et al., 1994), which is also expressed in the centripetal cell population, and is reported to be present as early as stage 3 of oogenesis.

We generated a construct, tslGalER, in which GalER was cloned under the control of the 3.5 kb *tsl* 5′ fragment, and isolated transformant flies following embryo injection. To characterize different tslGalER lines, we generated adult female flies carrying a tslGalER construct as well as an insertion of the UASlacZ reporter construct (Brand and Perrimon, 1993). Adult females were fed for several days with yeast mixed with either β-estradiol or DES, and then their ovaries were dissected and assayed for β-gal activity. Although not every follicle expressed detectable levels of *lacZ*, the overall pattern of expression was very similar to that seen in females carrying the tsl5′lacZ insertion: β-gal activity was present in the border cells and the posterior polar follicle cells in stages 8 and older (Fig. 1B). The intensity of β-gal staining, and the relative strengths of anterior versus posterior *lacZ* expression varied considerably in females carrying the 28 independent insertions of tslGalER that we examined. Except where noted, the experiments reported below were performed using tslGalER28, which produced a strong and consistent *lacZ* expression pattern. Importantly, β-gal activity was never detected in the follicles of females that were not fed ER agonists (Fig. 2A), nor was expression detected in non-polar follicle cells in treated females (Fig. 2B).

When we examined the time course of the induction of *lacZ* expression, we found that β-gal activity could be detected within 12 hours of exposing females to 125 mg/ml DES mixed 1:3 with yeast (not shown), and appeared to reach maximal levels within 2.5 days of constant exposure (Fig. 2B). Induction of *lacZ* expression by β-estradiol occurred a few hours later than DES-induced expression and reached a comparable level after 3 days of exposure (not shown). Within 3 days of

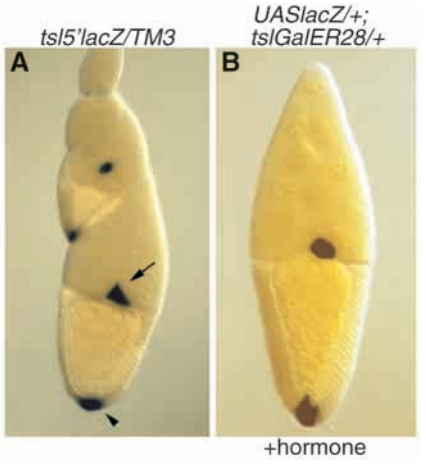

Fig. 1. *tsl* 5′ sequences direct polar expression pattern in the follicle. Follicles stained for β-gal activity. Genotypes are shown at top. Anterior is up. (A) In the follicles of females carrying the tsl5′lacZ insertion, *lacZ* is expressed in the border cells at the anterior (arrow) and a group of cells at the posterior pole of the oocyte (arrowhead). The two follicles in which β-gal staining can be detected are at stage 10 (bottom) and stage 9 (directly above) of oogenesis. (B) Stage 10 follicle from hormone-treated female carrying tslGalER and UASlacZ, showing β-gal activity in the border cells and posterior follicle cells.

removing females from β-estradiol following 5 days of treatment, β-gal activity was undetectable except in older stages (Fig. 2C), which likely reflects perdurance of the β-gal protein.

Inducible expression of DTI resulted in cell-specific ablation of the polar subpopulations

Our demonstration that the GalER fusion protein was both active and hormone-dependent in flies suggested that it could be used to express toxic gene products in a temporally as well as spatially specific manner. To test this possibility, we used GalER produced from tslGalER to drive toxin expression from the UASDTI insertion. When we crossed flies carrying tslGalER to UASDTI-carrying flies, adult females carrying both insertions were obtained at expected frequencies. This was in constrast to the Gal4 enhancer trap insertions we had tested previously, indicating that the simultaneous presence of both tslGalER and UASDTI was not deleterious to the fly itself.

To follow the fate of the toxin-expressing cells, we crossed into the tslGalER/UASDTI background an enhancer trap insertion, Q21a, that is expressed in the border cells and the centripetal cells at the anterior, and in the posterior-most follicle cells (Fig. 3A; Fasano and Kerridge, 1988). Females carrying all three insertions were fed for 3-5 days with either β-estradiol or DES, which produced similar results. 96% of stage 10 follicles (52/54 follicles from 9 females) lacked border

Fig. 2. GalER activity in the ovary is hormone-dependent and reversible. Follicles of females carrying tslGalER and UASlacZ stained for β-gal activity. Genotype is shown at the top, hormone treatment below. Anterior is pointing up. (A) In the absence of hormone, there is no detectable expression of *lacZ* in the ovary. (B) After 2-3 days of exposure to hormone, *lacZ* expression is observed in the border cells and in a small number of cells at the posterior pole of the follicle. Some stage 8 and older follicles do not express detectable levels of *lacZ*, whereas others exhibit β-gal activity at only one pole of the follicle (*). (C) Following removal of the hormone, *lacZ* expression declines.

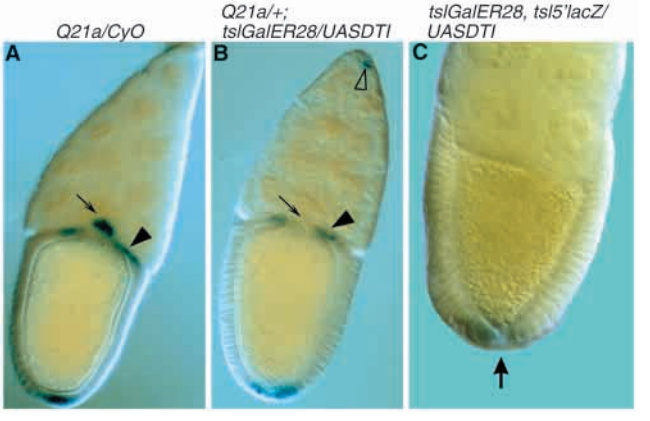

Fig. 3. Hormone-dependent expression of DTI leads to border cell and posterior polar follicle cell defects. All females were treated with hormone for 3.5 days prior to ovary dissection and staining for β-gal activity. Genotypes are shown at the top. Anterior is up. (A) Stage 10 follicle from female carrying the Q21a enhancer trap marker, which is expressed in the border (arrow) and centripetal (arrowhead) cells at the anterior, and in a group of follicle cells at the posterior pole. (B) In a stage 10 follicle from a toxin-expressing female, Q21a-driven β-gal activity is detected in the centripetal cells (filled arrowhead), but no staining is present where the border cells would normally lie (arrow), and the staining at the posterior pole is discontinuous. β-gal activity is also detected at the anterior pole, where the border cells originate (empty arrowhead). (C) A higher magnification view of the disrupted posterior follicular epithelium (arrow) of a stage 10 follicle from a toxin-expressing female. *lacZ* expression is driven by the tsl5′lacZ insertion.

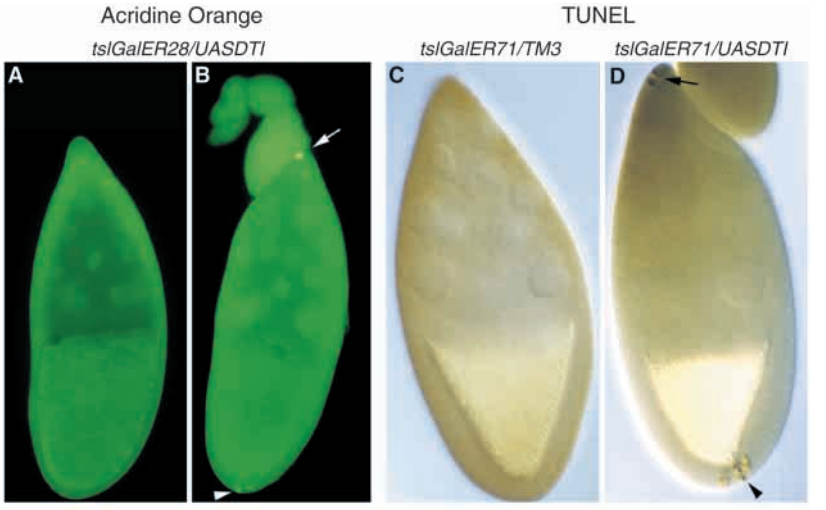

Fig. 4. Hormone-dependent expression of DTI causes cell death. Cell death was detected using acridine orange staining (A,B) and the TUNEL technique (C,D). Genotypes are shown at top. Anterior is up. (A) Stage 10 follicle from untreated female. No Acridine orange staining is detected in the follicle cell layer. (B-D) Females treated with hormone. (B) Stage 10 follicle from toxin-expressing female exhibits Acridine orange fluorescence at both the anterior (arrow) and posterior (arrowhead) poles. (C) Stage 9 follicle from hormone-treated female lacking the toxin insertion. No staining is visible. (D) Stage 9 follicle from toxin-expressing female shows punctate TUNEL staining at the anterior (arrow) and posterior (arrowhead) poles.

Table 1. Frequency of border cell defects in the follicles of hormone-treated *tslGalER/UASDTI* and *tslGalER/TM3* females

Genotype	Number of females	Stages of oogenesis	Total number of follicles	Number of follicles with border cell defects	Number of follicles with normal border cells	Percent of follicles with border cell defects
tslGalER28, 5A7/UASDTI	21	9	23	23	0	100%
		10	68	62	6	91%
		11	7	7	0	100%
		12	20	17	3	85%
		13	23	22	1	96%
		All stages combined	141	131	10	93%
tslGalER28, 5A7/TM3	13	9	14	1	13	7%
		10	56	1	55	2%
		11	3	0	3	0%
		12	15	0	15	0%
		13	22	0	22	0%
		All stages combined	110	2	108	2%

Numbers represent the combined results of females treated with either β-estradiol or DES, which gave comparable results. Border cells were visualized by their expression of the enhancer trap insertion 5A7. Follicles were scored as having border cell defects if the border cells either did not migrate at all, or if their distance from the anterior pole was less than expected compared to wild-type follicles at the same stage of oogenesis. The morphology of the migration-defective border cells also appeared abnormal.

cells at the anterior edge of the oocyte, whereas the centripetal cells, in which the *tsl* regulatory sequences used in this construct are not expressed, were unaffected (Fig. 3B). The posterior follicular epithelium appeared discontinuous (Fig. 3B,C), suggesting that cells had died and been eliminated.

Some stage 9 and older follicles, however, did exhibit some β-gal staining at the anterior pole (Fig. 3B), the site from which the border cells migrate. This finding raised the possibility that rather than eliminating the border cells, the expression of DTI was simply interfering with their ability to migrate. As cell death induced by DTA has been reported to occur through apoptosis (Kochi and Collier, 1993), we examined the follicles for programmed cell death markers using Acridine orange staining (Masucci et al., 1990) and the TUNEL assay (Gavrieli et al., 1992). Both methods revealed the presence of dead or

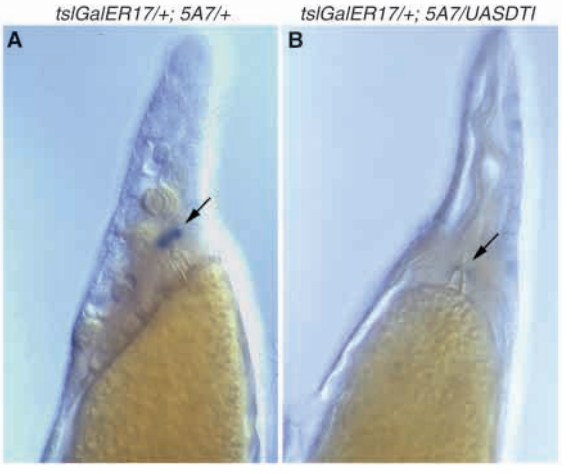

Fig. 5. Border cells are ablated in toxin-expressing females. Staining for β-gal activity produced by the border cell-specific marker 5A7 in the follicles of hormone-treated females. Genotypes shown at top. Anterior is up. (A) Stage 12 follicle from a female lacking UASDTI. *lacZ*-expressing cells (arrow) are observed at the micropyle. (B) Stage 13-14 follicle from a toxin-expressing female. No β-gal-positive cells are detected near the micropyle (arrow).

dying cells at the two poles of the follicle (Fig. 4), indicating that the hormone treatment was effective in killing border cells as well as posterior follicle cells. This result suggests that the persistence of β-gal activity at the anterior pole may have been due, at least in part, to perdurance of the β-gal protein in the remnants of dead cells.

To further address the question of whether some border cells were delayed in their migration but did survive toxin treatment, we used the border cell-specific marker 5A7 (Roth et al., 1995) to follow the fate of the border cells in older follicles. We examined 141 follicles between stages 9 and 13 in 21 females fed either DES or β-estradiol for 3.5 days (Table 1). Consistent with our previous findings, in this experiment the border cells in 93% (85/91) of stages 9 and 10 follicles from hormone-treated females either failed to initiate migration or exhibited migratory delays. Further, no border cell staining was detected at the micropyle in 92% (46/50) of stages 11-13 follicles (Fig. 5; Table 1). This result, together with our evidence for the induction of cell death, implies that rather than simply delaying border cell migration, toxin expression was effective in eliminating the border cell population.

Significantly, border cells were never absent from follicles of control females of the same genotype that were not exposed to hormone, nor were defects detected in the posterior follicular epithelium of these females. Follicles from females that were fed hormone but that lacked either the tslGalER or UASDTI insertion also appeared unaffected: less than 2% (2/110) of stages 9-13 follicles from 13 hormone-treated control females showed border cell migration defects (Table 1). Thus, the GalER fusion protein was not detectably active in the absence of hormone, and the effects seen were dependent upon the presence of both GalER and UASDTI.

Based on the following two calculations, we estimate that cell death was induced by DTI within approximately 6 hours. First, experiments using *lacZ* as a reporter gene indicated that tslGalER-mediated expression was initiated during stage 8 of oogenesis. We assume that like *lacZ*, DTI expression was activated during stage 8. When tslGalER was used to drive DTI expression, we found that clear defects in the posterior follicular epithelium could be detected in the majority of egg chambers by late stage 8. Since follicles are reported to require 6 hours to pass through stage 8 of oogenesis (Spradling, 1993), this suggests that cell death was induced in 6 hours or less following the onset of DTI expression. Second, we observed that follicles from females carrying tslGalER and UASlacZ first exhibited β-gal activity 16-20 hours after the females were exposed to β-estradiol, while Acridine orange-positive cells were first seen in follicles from females carrying tslGalER and UASDTI after 24 hours of hormone treatment. Again assuming that DTI, like *lacZ*, was first expressed after 16-20 hours of hormone treatment, this would suggest that cell death occurred within 4-8 hours of the activation of DTI expression.

Ablation of the polar subpopulations resulted in chorionic defects

The results described above provided histological evidence for the ablation of the polar subpopulations. To confirm these findings, we determined whether any of the known functions of the polar cells were disrupted in treated females. The posterior polar subpopulation that expresses *tsl* has been correlated with the synthesis of the aeropyle (Stevens et al., 1990), a distinctive structure at the posterior pole of the eggshell that is postulated to act in respiration (Margaritis et al., 1980). The aeropyle consists of the imprints of approximately 6-15 small central cells surrounded by the imprints of 10-15 peripheral cells, both of which can be distinguished from the larger imprints of the main body follicle cells (Fig. 6C; Margaritis et al., 1980). At the posterior pole of many of the eggs laid by treated females, these distinctive imprints were either reduced in number or completely absent (Fig. 6D). This suggests that in some follicles, 16 or more posterior polar cells were ablated. It is possible, however, that in some cases the failure of aeropyle formation resulted from the absence of certain key cells, rather than ablation of the entire population.

It has previously been shown that ablation, or delayed migration, of the border cells results in failure to form the pore in the micropyle through which sperm travels on its way to fertilize the egg (Montell et al., 1992). Examination of the micropyles of eggs from hormone-treated females revealed the presence of material blocking the micropyle pore (Fig. 6B). Consistent with this result is our finding that 92% (191/207) of eggs laid by 21 tslGalER/UASDTI females between 60-84 hours of hormone treatment were unfertilized. In contrast, less than 2% (2/115) of the eggs produced by 13 control females fed DES or β-estradiol were unfertilized. Thus, the eggshells produced by treated females displayed functional defects that corresponded with our observations that 92-93% of stage 9-13 follicles lacked border cells at the nurse cell/oocyte border and that many stage 8 and older follicles from hormone-fed females exhibited disruptions of the posterior follicular epithelium.

Ablation of the border cells disrupted the migration of the anterior polar pair cells

In addition to the polar cells described above, there is a specialized pair of follicle cells, referred to here as the polar pair cells, that are present at each pole of the oocyte and that differ from the rest of the epithelium in their distinct size and shape (Brower et al., 1981), early exit from the cell cycle (Calvez, 1980; Margolis and Spradling, 1995), and expression of particular genes (Ruohola et al., 1991). The anterior polar pair migrates to the anterior edge of the oocyte surrounded by a phalanx of border cells. Using GFP-Moesin as a marker, Edwards et al. (1997) recently reported that it is the polar pair cells, rather than the border cells themselves, that extend processes into the forming micropyle to create the micropylar pore. This suggests that the blocked micropyles that we observed in follicles with ablated border cells might have arisen as a result of defects in the polar pair cells, rather than the border cells themselves. However, we did not expect these cells to be directly affected by toxin expression, as double staining experiments indicated that the polar pair cells do not express the *tsl* regulatory sequences that were used to drive GalER expression (data not shown). To address this question, we examined the follicles of *tslGalER/UASDTI* females with an antibody against Fasciclin III (Patel et al., 1987), a cell adhesion protein that is strongly expressed in the polar pairs (Brower et al., 1981; Ruohola et al., 1991; Fig. 7). In the follicles of hormone-treated females, the posterior polar pair appeared intact even in follicles with obvious disruption to the posterior follicular epithelium (Fig. 7B). Although the anterior polar pair cells also appeared quite normal in morphology, they

nevertheless failed to migrate to the edge of the oocyte (Fig. 7B). This suggests that the movement of the anterior polar pair through the nurse cells may require contact with the migrating border cells. To investigate this possibility further, we used Fasciclin III antibodies to stain the follicles of females homozygous mutant for *slow border cells (slbo)*, in which the border cells exhibit delayed migration (Montell et al., 1992). We found that the migration of the anterior polar pair was also delayed in this mutant background (data not shown). These results are consistent with the idea that the movement of the anterior polar pair to the edge of the oocyte is dependent upon the migrating border cells and lend further support to the proposed role for the anterior polar pair in micropyle formation (Edwards et al., 1997).

Temporal regulation of GalER activity

Consistent with the report that most Gal4 enhancer trap lines with ovarian expression are also expressed at earlier times during the fly life cycle (Schüpbach and Wieschaus, 1998), our initial experiments with the Gal4/UAS system indicated that Gal4-driven toxin expression is likely to lead to death prior to adulthood in individuals carrying both Gal4 enhancer trap and UASDTI insertions. Thus, one important criterion for the system we intended to develop was that the activity of GalER be tightly regulated throughout development as well as in the adult ovary. To examine this question, newly hatched larvae carrying tslGalER and UASlacZ were fed yeast containing β-estradiol until they reached the third larval instar, when they were dissected and stained for β-gal activity. Weak *lacZ* expression was detected in the brain (data not shown), suggesting that the tslGalER insertion was expressed during the larval instars. To determine whether hormone treatment would activate UAS-linked DTI expression, we fed hormone-containing yeast to the larval progeny of tslGalER28, UASDTI/TM3 flies. (In this stock, the only flies that do not carry both insertions are those that are homozygous for the TM3 balancer chromosome, which itself is homozygous lethal). Although several hundred larvae were treated, very few pupated and no flies eclosed, indicating that tslGalER-driven DTI expression was lethal to the organism. Thus, our ability to readily generate adult flies carrying both tslGalER and UASDTI in the absence of hormone indicates that the activity of larvally expressed GalER was hormone dependent.

Although we had shown that the level of tslGalER activity during the larval period was high enough to transcribe lethal levels of DTI, because tslGalER expression was relatively weak the possibility remained that higher levels of GalER expression might not be sufficiently repressed in the absence of hormone. To determine whether the GalER/UAS system could be combined with stronger and perhaps less spatially specific transcriptional drivers such as enhancer trap insertions, we carried out a preliminary screen for enhancer trap insertions of an enhancerless GalER vector. Of 252 new insertions, 23 exhibited specific ovarian expression patterns and were retained.

The larval expression patterns of five of these lines were monitored using UASlacZ and found to be hormone dependent (Fig. 8 and data not shown). We assayed the effect of larval hormone treatment on the recovery of adult flies carrying the enhancer trap insertion and UASDTI, and although flies carrying either GalER or UASDTI were obtained, in no case did we recover adult flies carrying both insertions (Table 2). In the absence of hormone, however, adult flies carrying both GalER and UASDTI insertions were obtained at expected frequencies for all five lines tested.

Table 2. Activation of larvally expressed GalER by β-estradiol is lethal to individuals that also carry UASDTI

Enhancer trap line	Number of flies carrying these insertions				Total number of flies that eclosed
	UASDTI	GalER	Neither	Both	
GalER54	8	16	8	0	32
GalER115	12	28	19	0	59
GalER156	19	14	11	0	44
GalER168	12	62	10	0	84
GalER212	9	32	4	0	45

Numbers represent the combination of results from two separate experiments, one at room temperature and one at 26.5°C, which produced comparable results. Eggs derived from the cross *GalER/CyO* × *UASDTI/TM3* were collected and placed on apple juice/agar plates with hormone-containing yeast paste. Pupae were transferred to vials at room temperature and following eclosion were scored for the presence of the w^+-marked GalER and UASDTI insertions and the absence of the dominantly marked *CyO* and *TM3* balancer chromosomes. No adult flies of the genotype *GalER/+; UASDTI/+* were recovered.

We then tested the ability of the GalER enhancer trap lines to carry out cell-specific ablation in the ovary using three lines with differing patterns of polar expression (Fig. 9). GalER54 is expressed by follicle cells at both poles of the follicle (Fig. 9A), while GalER212 expression is detected primarily in cells at the most anterior tip of the follicle (Fig. 9D). Both of these enhancer trap lines are expressed in the migrating border cells. When females carrying both UASDTI and either GalER54 or GalER212 were fed hormone, staining for the border cell-specific marker 5A7 was either undetectable (Fig. 9F) or remained at the anterior pole of the follicle (Fig. 9C), consistent with ablation of the border cells soon after their differentiation. Follicles from hormone-treated GalER54 females carrying UASDTI also exhibited defects in the posterior epithelium (Fig. 9C).

GalER168 is expressed by a variable number of follicle cells at the posterior pole of the oocyte beginning at stage 7 (Fig. 9G). During stages 6-7 the posterior follicle cells are thought to transmit to the oocyte a signal that triggers a reorganization of its cytoskeleton such that the oocyte cytoplasm acquires anterior-posterior polarity (Lane and Kalderon, 1994; González-Reyes et al., 1995; Roth et al., 1995; see also Ray and Schüpbach, 1996). We monitored the effect of posterior cell ablation on oocyte polarity using a Kinesin-β-gal (kin:βgal) fusion protein that is localized to the posterior pole of wild-type stage 9 oocytes (Clark et al., 1994; Fig. 9H), and is displaced to the center of the oocyte in mutants in which the anterior-posterior polarity of the oocyte is disrupted (Clark et al., 1994; Lane and Kalderon, 1994; González-Reyes et al., 1995; Roth et al., 1995; Larkin et al., 1996). kin:βgal staining was detected in the middle of the oocyte in many stage 9 follicles from hormone-fed females carrying both GalER168 and UASDTI (Fig. 9I). In contrast, when we examined kin:βgal localization in the follicles of hormone-treated females carrying UASDTI and tslGalER, which doesn't initiate expression until stage 8 of oogenesis, no changes were detected

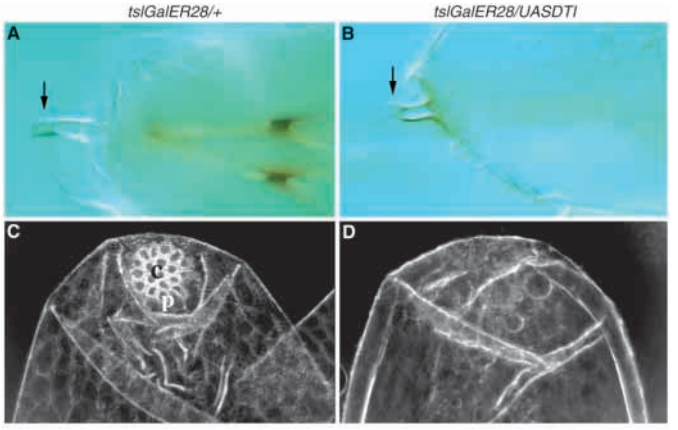

Fig. 6. Polar cell ablation produces chorionic defects. The chorions of eggs laid by hormone-treated control (A,C) and toxin-expressing females (B,D). Maternal genotypes are shown at top.
(A,B) Micropyles visualized using Nomarski optics; anterior is to the left. (A) In this normal micropyle, a pore can be observed at the tip (arrow). (B) In this egg from a toxin-expressing female, no pore can be seen and the micropyle appears to be blocked (arrow).
(C,D) Dark-field images of the aeropyle at the posterior pole of the eggshell. Posterior is up. (C) In this normal aeropyle, the bright imprints of the small central cells (c) and the surrounding peripheral cells (p) can be detected. (D) In this egg from a toxin-expressing female, the distinctive cell imprints that form the aeropyle are not present.

Fig. 7. Border cell ablation prevents the migration of the anterior polar pair. Fasciclin III staining of polar pair cells in stage 10 follicles from hormone-treated females. Genotypes are shown at top. Anterior is pointing up. (A) In this control follicle, the anterior polar pair is detected near the anterior border of the oocyte (arrow). (B) In this follicle from a hormone-treated female in which the border cells were presumed to be ablated, the anterior polar pair remains at the anterior pole of the follicle (arrow).

(data not shown). Thus, our results are consistent with the proposed role for the posterior follicle cells in the establishment of the anterior-posterior polarity of the oocyte and also imply that the posterior follicle cells are not required for maintenance of this polarity subsequent to stage 8 of oogenesis.

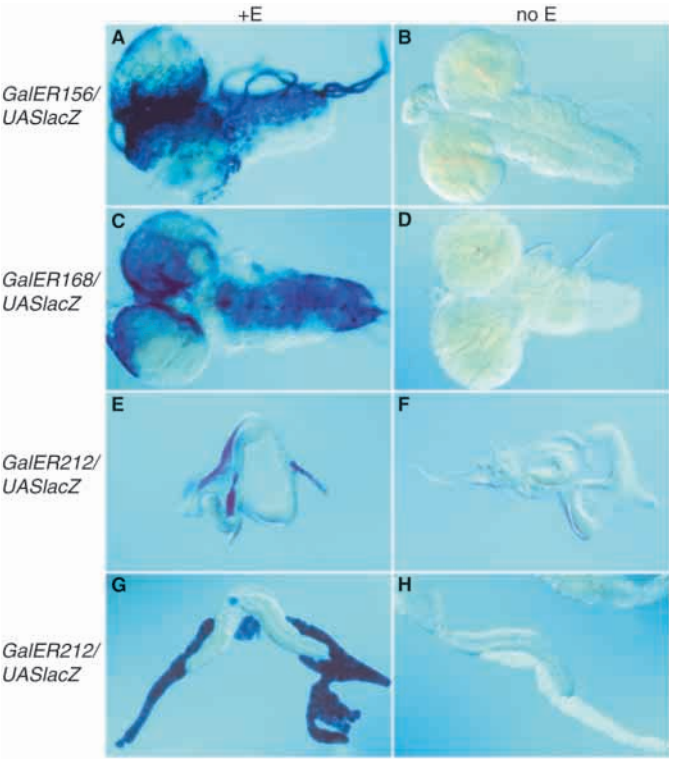

Fig. 8. Larval expression patterns of GalER enhancer trap lines are hormone-dependent. Larval genotypes are shown on the left. Tissues were dissected from 3rd instar larvae that were either fed yeast plus β-estradiol (left column) or yeast alone (right column) and stained for β-gal activity. Following hormone treatment, *lacZ* expression is activated in the central nervous system of larvae carrying GalER156 (A) and GalER168 (C). No staining is detected in larvae from the same crosses that were not exposed to hormone (B,D). (E) Eye-antennal imaginal disc from 3rd instar hormone-fed larva carrying GalER212 shows β-gal activity in Bolwig's nerve. (G) Strong *lacZ* expression in the fat body of GalER212-carrying larva (pictured here with the non-expressing salivary glands). (F,H) No β-gal activity is detected in these tissues in non-treated progeny from the same cross.

DISCUSSION

In addition to signalling between the germline and follicle cells, the establishment of polarity in the follicle and the future embryo requires the differentiation of, and communication between, a number of different follicular subpopulations (Ray and Schüpbach, 1996). Although its complexity has made the system a difficult one to study, it also provides an excellent opportunity to investigate the factors that regulate the differentiation of specific subgroups within a population of presumably equipotent cells. An additional factor complicating studies of oogenesis is that it occurs late in the life cycle of the fly. Because many of Gal4 enhancer trap lines that are expressed in the *Drosophila* ovary are also expressed earlier in development (Schüpbach and Wieschaus, 1998), they cannot be used to express toxic genes specifically during oogenesis because death of the individuals occurs prior to adulthood. To overcome this lack of temporal regulation, we have developed a method for inducible, tissue-specific gene expression in

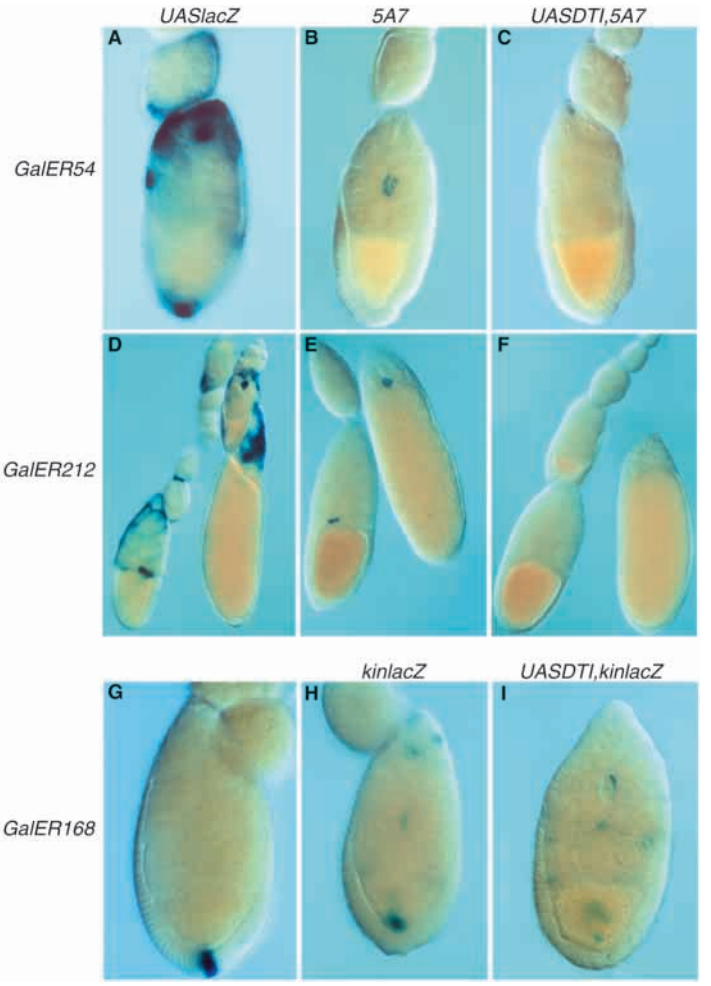

Fig. 9. Toxin expression driven by GalER enhancer trap lines leads to cell-specific defects and alterations in oocyte polarity. GalER enhancer trap lines are shown on the left. Additional insertions present in the females are shown at top. Females were treated with hormone in all cases. Anterior is up. (A) GalER54 drives expression in follicle cells at both poles beginning at stage 7, and is also expressed in the migrating border cells. (B) β-gal activity in the border cells of control females carrying GalER54 and the border cell marker insertion 5A7 but not UASDTI. (C) In females carrying all three insertions, the number of cells expressing 5A7 is reduced and they remain at the anterior pole of the follicle. (D) GalER212 is primarily expressed from stage 7 on by cells at the anterior pole of the follicle, including the border cells. (E) 5A7 expression in the border cells of control females carrying only GalER212 and 5A7. (F) No 5A7 expression is detected in the follicles of females carrying all three insertions, implying that the border cells have been ablated. (G) GalER168 expression is initiated at stage 7 in a variable number of posterior follicle cells. (H) In control females carrying only GalER168 and the kinlacZ reporter gene, β-gal activity is localized to the posterior pole of the oocyte in stage 9 follicles. (I) In stage 9 follicles from females carrying all three insertions, β-gal activity is displaced from the posterior pole and weak staining is detected in the middle of the oocyte.

Drosophila that utilizes a fusion protein between the Gal4 transcriptional activator from yeast and the human ER. By using this system to express a toxic gene product, DTI, we have shown that in the absence of exogenously administered hormone the GalER protein is not detectably active, while in the presence of hormone it transcribes lethal levels of DTI. We anticipate that this method, which provides temporal as well as spatial regulation of gene expression, will prove to be extremely useful to express gene products that would be lethal to the organism if expressed prematurely or nonspecifically.

Regulation of the activity of vertebrate hormone receptors is dependent not just upon the presence of ligand, but also on a number of other molecules that are required to maintain the receptor in an inactive state in the absence of ligand, and to cooperate with the ligand-bound receptor in activating and repressing gene transcription (reviewed by Tsai and O'Malley, 1994; Beato et al., 1995; Freedman, 1999). Previous work has suggested that these molecules are highly conserved throughout eukaryotes, as the human ER has been shown to be active and hormone dependent in yeast (Metzger et al., 1988), as is the rat glucocorticoid receptor in yeast (Schena and Yamamoto, 1988), cultured *Drosophila* Schneider cells (Yoshinaga and Yamamoto, 1991) and flies (D. H. and L. S., unpublished results). Our work extends these findings and demonstrates that the regulation of vertebrate steroid receptor activity can be tightly controlled in living flies. Christopherson et al. (1992) developed a powerful method utilizing insect hormones and receptors to achieve inducible expression of heterologous genes in mammalian cells. The results presented here suggest that vertebrate hormone receptors can be correspondingly valuable in the *Drosophila* system. The temporal regulation that this inducible system adds to cell-specific gene expression will be useful for expressing many gene products, and essential for those that are deleterious to the organism.

The use of toxigenic cell ablation to identify the function of specific cell types in the adult fly has until now been restricted to populations for which enhancers can be isolated that exhibit extremely specific spatial and temporal control (Bellen et al., 1992; Moffat et al., 1992; Kalb et al., 1993; McNabb et al., 1997). Even the *tsl* regulatory sequences that were used in our experiments, which direct expression in a highly specific pattern in the adult ovary, drive high enough expression in larvae to cause lethality when combined with the UASDTI insertion. Other methods are limited to cells that are identifiable and accessible to laser ablation (Montell et al., 1991; Montell et al., 1992; Lin and Spradling, 1993), or whose unique mitotic patterns allow them to be specifically eliminated through agents that kill dividing cells (de Belle and Heisenberg, 1994). More generally applicable techniques, such as the Gal4/UAS system, have been restricted to embryogenesis (Hidalgo et al., 1995; Lin et al., 1995), or

mosaic adults (Smith et al., 1996), because of the difficulty in recovering adult flies carrying both the Gal4 insertion and a UAS-linked toxin gene.

Another method that may also prove useful for the expression of deleterious gene products in *Drosophila* was recently described by Bello et al. (1998), who used a tetracycline-dependent transactivator system (Gossen and Bujard, 1992) to bring about spatially and temporally specific gene expression. When this system was used to mis-express the homeotic protein Antennapedia, Antennapedia-induced larval defects were only observed following the removal of tetracycline (Bello et al., 1998), indicating that there is no detectable expression of the Antennapedia transgene in the presence of tetracycline-mediated repression. A potential disadvantage of this technique, however, is that tetracycline must be present throughout development to maintain repression. Although repression can be sustained during the embryonic, larval and early pupal periods by feeding tetracyline to the mothers and then to the larvae themselves, during the course of pupation the concentration of tetracycline declines (Bello et al., 1998), which may lead to premature expression of the transgene. In contrast, the inducible system described here, in which transcription is not activated unless the individuals are fed hormone, is particularly well-suited for expression targeted to adults.

Our results indicate that the activity of GalER itself is tightly regulated in flies. However, there is 'leaky' expression of UAS-linked genes that may prove problematic for expressing even more toxic proteins in flies. For example, the Gal4/UAS system has been used to drive expression of the wild-type DTA in embryos, but the embryos were mosaic for functional UASDTA constructs (Lin et al., 1995). In contrast, even in the absence of activation by Gal4, we were not able to obtain lines of adult flies carrying the wild-type DTA linked to UAS. Given the potency of the wild-type toxin, which is reported to be lethal at a concentration of one molecule per cell (Yamaizumi et al., 1978), it is not surprising that any non-specific expression would be lethal to the organism.

It has been reported that wild-type ricin is more effective in cell killing than attenuated forms of DTA, such as the DTI used in our experiments (Hidalgo et al., 1995). When we tested wild-type ricin in our inducible system, however, flies carrying UAS-ricin and tslGalER appeared to exhibit lower levels of cell ablation (data not shown). One possible explanation for the difference between our results and those of Hidalgo et al. (1995) is that in our UASDTI construct, the DTI gene is preceded by the β-globin leader, a translational enhancer (Driever et al., 1990) that may have resulted in higher levels of DTI protein expression.

In our experiments, flies tolerated well the presence of the attentuated toxin gene, DTI, linked to UAS, and our results indicate that the GalER/UAS system provided temporal as well as spatial regulation of UAS-linked gene expression. By using the highly specific *tsl* enhancer to drive GalER expression in the follicle, we were able to demonstrate the efficient ablation of the border cells and the posterior polar subpopulation and the corresponding defects in the eggshell structures to which these cells contribute. We also isolated a number of GalER enhancer trap lines with expression both in the ovary and at earlier times during development and showed that in the absence of hormone it is possible to obtain adult flies carrying both GalER and UASDTI. When adult females were then fed hormone, cell-specific ablation of the GalER-expressing populations in the ovary was readily detected. Particularly exciting was our preliminary finding that ablation of the posterior polar subpopulation at stage 7 disrupted the anterior-posterior polarity of the oocyte as monitored by localization of the kin:βgal reporter protein. This result provides independent support for the hypothesis that the establishment of the anterior-posterior axis of the oocyte is dependent upon a signal from the posterior follicle cells. Further, it emphasizes the potential utility of the GalER system in studies of the roles of specific cell types in oogenesis as well as in the development and function of other complex tissues in the adult fly.

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