SUCCESSFUL formation of a mature egg requires the integration of developmental, environmental, and nutritional cues. In Drosophila, the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) and juvenile hormone (JH) play critical regulatory roles in coordinating this process (Riddiford 1993). Drosophila oogenesis takes place within the ovariole, 1 of 16 parallel structures containing developing egg chambers that make up the adult ovary. Germline and somatic stem cells located at the anterior tip of each ovariole give rise to egg chambers that transit the ovariole during oogenesis. Egg chambers increase dramatically in size during maturation, in part as a result of yolk protein uptake by the oocyte that begins during pro-oogenesis (Mahowald and Kambsysellis 1980; Spradling 1993). At the time the adult female emerges from the pupal case, ovarioles contain only previtellogenic (stages 1–7) egg chambers (see King 1970; Spradling 1993 for staging criteria). Yolk proteins are produced by both ovarian and fat body tissue, and yolk protein uptake by the oocyte begins in stage 8 egg chambers. The first mature eggs (stage 14) may be fertilized and deposited 20–24 hr following eclosion (Mahowald and Kambsysellis 1980; Bownes 1986).

Hormone replacement experiments in Drosophila abdomens separated from the head and thorax (Handler and Postlethwait 1977, 1978; Postlethwait and Handler 1979; Jowett and Postlethwait 1980) showed that JH and ecdysone have important regulatory functions during vitellogenesis. JH release from the corpora allata following reception of a signal from the brain acts both to stimulate yolk protein production in ovarian follicle cells and to trigger yolk protein uptake by the oocyte (for reviews see Hagedorn 1985; Bownes 1989; Riddiford 1993; Kelly 1994). In contrast, ecdysone stimulates yolk protein production in fat body cells. It has also been suggested (Riddiford 1993) that ecdysone may have more direct effects on oogenesis, since ecdysone binding activity has been detected in the ovary (Handler and Maroy 1989), and ecd mutations that lower ecdysone titer result in oogenic defects (Audit-Lamour and Busson 1981; Redfern and Bownes 1983; Petavy 1990).

Ecdysone signaling in Drosophila is controlled by a heteromeric receptor composed of the EcR and USP nuclear receptor proteins (Koelle et al. 1991; Koelle 1992; Yao et al. 1992, 1993; Thomas et al. 1993). The ecdysone receptor (EcR) gene encodes three protein products (EcR-A, EcR-B1, and EcR-B2) that differ in their amino termini but share a common carboxy-terminal region containing DNA- and ligand-binding domains (Talbot et al. 1993). The ultraspiracle (usp) gene encodes a single protein product homologous to the vertebrate RXR receptor (Henrich et al. 1990; Oro et al. 1990; Shea et al. 1990). USP protein is expressed in ovarian follicle and nurse cells (Khoury Christianson et al. 1992), and genetic studies have shown that USP is required maternally for fertilization, eggshell formation, and embryonic development (Perrimon et al. 1985; Oro et al. 1992). A recent study has shown that USP and EcR are expressed in somatic cells of the ovary at the time of ovarian differentiation at metamorphosis.

Oogenesis in Drosophila is regulated by the steroid hormone ecdysone and the sesquiterpenoid juvenile hormone. Response to ecdysone is mediated by a heteromeric receptor composed of the EcR and USP proteins. We have identified a temperature-sensitive EcR mutation, EcR*483T, from a previously isolated collection of EcR mutations. EcR*483T is predicted to affect all EcR protein products (EcR-A, EcR-B1, and EcR-B2) since it maps to a common exon encoding the ligand-binding domain. In wild-type females, we find that both EcR-A and EcR-B1 are expressed in nurse cells and follicle cells throughout oogenesis. EcR mutant females raised at permissive temperature and then shifted to restrictive temperature exhibit severe reductions in fecundity. Oogenesis in EcR mutant females is defective, and the spectrum of oogenic defects includes the presence of abnormal egg chambers and loss of vitellogenic egg stages. Our results demonstrate a requirement for EcR during female reproduction and suggest that EcR is required for normal oogenesis.
and that usp is required for early events in ovarian differentiation (Hodin and Riddiford 1998). Mutational analysis of EcR has demonstrated EcR requirements during embryogenesis, larval molting, and metamorphosis (Bender et al. 1997; Schubiger et al. 1998). In addition, reduction of EcR dosage has been shown to result in defects in early ovarian differentiation (Hodin and Riddiford 1998).

In this article, we show that EcR-A and EcR-B1 proteins are expressed in both somatic and germline cells of the adult ovary and examine maternal requirements for EcR function. We have identified a temperature-sensitive mutation that is predicted to affect all EcR protein products because it maps to a common exon encoding the EcR ligand-binding domain. At restrictive temperature, EcR mutant females show severely reduced fecundity and defects in oogenesis, including the presence of abnormal egg chambers and loss of vitellogenic egg chambers. These genetic studies suggest that EcR is required for normal oogenesis and provide a foundation for detailed genetic dissection of ecdysone signaling during oogenesis.

**MATERIALS AND METHODS**

**Western analysis:** Protein extracts were prepared from a wild-type (Canton-S) strain by grinding whole pupae or adult female heads or ovaries in cracking buffer (0.125 M Tris-HCl, pH 8.8, 5% B-mercaptoethanol, 2% sodium dodecyl sulfate, 4 M urea). Samples equivalent to one pupa, five adult heads, or five adult ovary pairs per lane were loaded for polyacrylamide gel electrophoresis. Immunoblots were probed with a monoclonal antibody (15G1a) directed against EcR-A (Talbot et al. 1993), followed by incubation with a horseradish-peroxidase-conjugated secondary antibody (Promega, Madison, WI). Detection was performed with Super Signal Substrate (Pierce Chemical, Rockford, IL). Blots were stripped and reprobed with a monoclonal antibody (AD4.4) specific to EcR-B1 (Talbot et al. 1993).

For detection of yolk proteins, wild-type (Ore-R) and EcR mutant (EcR<sup>443T</sup> / EcR<sup>443T</sup>) females were raised at permissive temperature (22°) for 14 days, then shifted to 25° until eclosion. At the time of shift to 25°, most flies were at midpupal stages prior to darkening of wings (stage P12; Bainton and Bownes 1981). This protocol allowed a midpupal upshift and recovery of EcR mutant females after eclosion. Newly eclosed females were first collected from culture bottles after 3 days at 25° and were held at 29° after eclosion. Extracts were made at 0, 6, 12, 18, 24, and 30 hr following eclosion. Each sample consisted of three adult females, and the equivalent of one-half adult female was loaded for polyacrylamide gel electrophoresis. Coomassie Blue staining showed that EcR mutant lanes contained equivalent or slightly greater total protein compared to Ore-R samples. Immunoblots were probed with a polyclonal antiserum that recognizes yolk proteins 1, 2, and 3 (M. Bownes, personal communication). Quantitation of yolk protein signal was with a Stratagene (La Jolla, CA) Eagle Eye II densitometer.

**Immunostaining of ovaries:** Ovaries were dissected from wild-type (Canton-S) females in fixative [1× phosphate-buffered saline (PBS), pH 7.2, 4% paraformaldehyde, 0.02% sodium azide] and incubated in fixative for an additional 3-4 hr at room temperature. Ovaries were dehydrated in ethanol and blocked for 4 hr with 5% normal goat serum in PBT (1× PBS, 0.5% Triton X-100) at 4°. Primary antibody incubation was carried out at 4° overnight with 1:5 or 1:10 dilutions of 15G1a (anti-EcR-A) or AD4.4 (anti-EcR-B1) in PBT. Ovaries were washed three times in PBT for 3 hr, and incubated at 4° overnight with fluorescein-isothiocyanate-conjugated goat α-mouse IgG secondary antibody. Washes were performed as above. Ovaries were mounted in 50% glycerol and viewed using a Zeiss (Thornwood, NY) Axiopt microscope. We did not observe immunostaining of ovaries to which no primary antibody was added.

**Mutations used in this study:** The EcR<sup>443T</sup>, EcR<sup>344Q</sup>, and EcR<sup>559fs</sup> mutations were induced on a cn bw chromosome (Bender et al. 1997), while EcR<sup>554fs</sup> was induced on a Canton-S chromosome. EcR<sup>443T</sup> was isolated as a partial EcR noncomplementing (leaky) mutation at 25° and shown subsequently to map to EcR coding sequences. EcR<sup>554fs</sup> is a null mutation, and EcR<sup>344Q</sup> and EcR<sup>559fs</sup> are presumed null mutations based on molecular criteria and a similar lethal period to EcR<sup>554fs</sup> (Bender et al. 1997). Balancer chromosomes and marker mutations used are as described (Lindsley and Zimm 1992).

**Tests of conditional lethality:** Twenty EcR<sup>443T</sup> / SM 6b virgin females were mated en masse with 20 w;EcR<sup>554fs</sup>/CyO, EcR<sup>344Q</sup>/SM 6b, or EcR<sup>559fs</sup>/SM 6b males at 22°. After 3-5 days mated females were transferred to new bottles at 25°. Bottles were held until either 22° or 25° until eclosion, and heterozygous mutant progeny (lacking the SM 6b and CyO marker Cy) and siblings (carrying Cy) were counted. The number of heterozygous mutant progeny expected is one-half the number of Cy siblings, since SM 6b/CyO and SM 6b/SM 6b flies do not survive. Similar crosses were performed at 25° and 29° with EcR<sup>443T</sup> / SM 6b virgin females and Canton-S or w;EcR<sup>554fs</sup>/CyO males. Between 150 and 300 progeny were scored from each cross.

**Fecundity assays:** To generate EcR mutant females, EcR<sup>443T</sup> / SM 6b virgin females were mated to w;EcR<sup>554fs</sup>/CyO, EcR<sup>344Q</sup>/SM 6b, or EcR<sup>559fs</sup>/SM 6b males and were raised at 22°. A total of 20 F<sub>1</sub> virgin females heterozygous for EcR<sup>443T</sup> and either EcR<sup>554fs</sup>, EcR<sup>344Q</sup>, or EcR<sup>559fs</sup> were collected and mated en masse at 29° to 20 Canton-S males for 2 days. As a control, EcR<sup>554fs</sup>/CyO SM 6b virgin female siblings were collected and were mated to Canton-S males in the same way. Egg collections (6-hr) at 29° were made and were counted on each of three successive days. To examine effects on male fertility, F<sub>1</sub> heterozygous mutant or control (EcR<sup>554fs</sup>/SM 6b) males were crossed to Canton-S virgin females. These females laid similar numbers of eggs when mated to either heterozygous mutant or control males. Eggs from all four matings to mutant males were able to hatch to first instar larvae.

**Quantification of vitellogenic egg chambers:** Females heterozygous for EcR<sup>443T</sup> and either EcR<sup>554fs</sup>, EcR<sup>344Q</sup>, or EcR<sup>559fs</sup> that had eclosed at 22°, as well as control EcR<sup>554fs</sup>/SM 6b siblings and Canton-S females, were mated within 1 day of eclosion to Canton-S males at 29°. Ovaries were dissected 4-5 days later and fixed in 4% paraformaldehyde for 30 min, stained in 1 μg/ml rhodamine-phalloidin (Sigma, St. Louis) for 20 min, rinsed three times with PBS, stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 5 min, rinsed three times with PBS, and mounted in 50% glycerol in PBS. One ovary was dissected from four females of each genotype. Ovari- oles were viewed by differential interference contrast (DIC) microscopy or by epifluorescence using a Zeiss Axioskop microscope. For control EcR<sup>554fs</sup>/SM 6b females we examined a total of 66 ovarioles from four independent ovaries and counted the number of defective and nondefective egg chambers of each stage. The same procedure was performed for experimental animals: EcR<sup>443T</sup> / EcR<sup>554fs</sup> (70 ovarioles), EcR<sup>443T</sup> / EcR<sup>344Q</sup> (66 ovarioles), and EcR<sup>443T</sup> / EcR<sup>559fs</sup> (64 ovari-
RESULTS

EcR-A and EcR-B1 proteins are expressed in Drosophila ovaries: Western analysis of extracts prepared from whole pupae or adult female tissues showed that both EcR-A and EcR-B1 proteins are expressed in the ovary (Figure 1A, lanes 3 and 6). To determine the spatial pattern of expression in the ovary, we performed whole tissue immunohistochemistry with EcR-A- and EcR-B1-specific antibodies. EcR-A and EcR-B1 are each detected in both follicle and nurse cell nuclei. Figure 1, B–E, shows egg chambers stained with anti-EcR-A (B and C) or anti-EcR-B1 (D and E) antibodies. We observe nuclear EcR-A and EcR-B1 staining in the follicle cells that migrate during stage 9 to cover the oocyte (Figure 1, B and D, white arrows), as well as in nonmigratory follicle cells that remain associated with nurse cells in the anterior portion of the egg chamber (Figure 1, B and D, black arrows). EcR-A and EcR-B1 nuclear staining is also detected in nurse cells of developing egg chambers (Figure 1, B–E, arrowheads). EcR-A and EcR-B1 staining is observed in both follicle and nurse cells of very early egg chambers to stage 14 egg chambers (data not shown). Thus, both EcR-A and EcR-B1 proteins are expressed in follicle cells and nurse cells throughout egg chamber development.

EcR<sup>R<ins>4483T</ins></sup> is a conditional EcR mutation: Previously, we identified 16 mutations that map to EcR common exons (Bender et al. 1997). One of these, EcR<sup>R<ins>4483T</ins></sup>, partially complements an EcR null mutation for viability at 25°C. Table 1 shows that EcR<sup>R<ins>4483T</ins></sup> is temperature sensitive. Animals heterozygous for EcR<sup>R<ins>4483T</ins></sup> and the null mutation EcR<sup>R<ins>554fs</ins></sup> show strong survival when raised at either 22° or 25°C (Table 1). In contrast, EcR<sup>A4483T</sup>/EcR<sup>R<ins>554fs</ins></sup> heterozygotes do not survive when raised at 29°C (Table 1).

Similar results were obtained with heterozygotes for EcR<sup>A4483T</sup> and common exon alleles EcR<sup>R<ins>344Q</ins></sup> or EcR<sup>V559S</sup>, although the permissive temperature was lower than for EcR<sup>A4483T</sup>/EcR<sup>R<ins>554fs</ins></sup> heterozygotes. Adults heterozygous for EcR<sup>A4483T</sup> and EcR<sup>R<ins>344Q</ins></sup> or EcR<sup>V559S</sup> survive when reared at 22°, but not at 25°C (Table 1). Although EcR<sup>R<ins>554fs</ins></sup>, EcR<sup>R<ins>344Q</ins></sup>, and EcR<sup>V559S</sup> are all thought to be null mutations (Bender et al. 1997), a possible explanation for this temperature threshold difference is genetic background, since EcR<sup>R<ins>344Q</ins></sup> and EcR<sup>V559S</sup> share a common parental chromosome, distinct from that of EcR<sup>R<ins>554fs</ins></sup> (see materials and methods). Crosses with Canton-S animals at 25° or 29°C reveal only a slight decrease in progeny heterozygous for EcR<sup>A4483T</sup> and the wild-type chromosome at elevated temperature (Table 1). These results indicate that EcR<sup>A4483T</sup> is a conditional mutation of EcR, and should provide a useful means for removing EcR function from adult animals.

Maternal loss of EcR function results in reduced fecundity: To determine whether EcR functions in female

![Figure 1](image_url)

**Figure 1**—EcR-A and EcR-B1 proteins are expressed in adult ovaries. (A) Western blot probed with the EcR-A-specific monoclonal antibody 15G1a (lanes 1–3) or the EcR-B1-specific antibody AD4.4 (lanes 4–6). Lanes 1 and 4 contain pupal extracts (equivalent of one pupa). Lanes 2 and 5 contain adult female head extracts (equivalent of five heads). Lanes 3 and 6 contain adult ovary extracts (equivalent of five ovary pairs). (B–E) Immunolocalization of EcR in egg chambers with anti-EcR-A (B and C) or anti-EcR-B1 (D and E) antibodies. (B and D) Stage 10 egg chambers. Black arrows indicate stained nonmigratory follicle cell nuclei, white arrows indicate stained migratory follicle cell nuclei, and arrowheads indicate stained nurse cell nuclei. (C) Stage 8 egg chamber. Arrowhead indicates EcR-A nurse cell nuclear staining. (E) Ovary showing EcR-B1 nurse cell nuclear staining in stage 6 and 8 egg chambers (arrowheads).

**TABLE 1**

<table>
<thead>
<tr>
<th>Paternal allele</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22°</td>
</tr>
<tr>
<td>Canton-S</td>
<td>ND</td>
</tr>
<tr>
<td>EcR&lt;sup&gt;R&lt;ins&gt;554fs&lt;/ins&gt;&lt;/sup&gt;</td>
<td>97</td>
</tr>
<tr>
<td>EcR&lt;sup&gt;R&lt;ins&gt;344Q&lt;/ins&gt;&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td>EcR&lt;sup&gt;V559S&lt;/sup&gt;</td>
<td>43</td>
</tr>
</tbody>
</table>

EcR<sup>A4483T</sup>/SM 6b virgin females were mated to wild-type (Canton-S) males or to males carrying one of the three EcR mutations shown, and heterozygous mutant progeny were scored. Percentages shown represent the number of adult animals heterozygous for EcR<sup>A4483T</sup> and the indicated paternal allele divided by the expected number of progeny from this genotype calculated from the number of progeny of this genotype calculated from the number of eclosed sibling animals (see materials and methods). Between 150 and 300 progeny were scored for each cross, ND, not determined.
TABLE 2

Effects of maternal loss of EcR on fecundity

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Total</th>
<th>Average % reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcR&lt;sup&gt;RR544&lt;/sup&gt;/ SM 6b</td>
<td>237</td>
<td>272</td>
<td>303</td>
<td>812</td>
<td>0</td>
</tr>
<tr>
<td>EcR&lt;sup&gt;AA483T&lt;/sup&gt;/ EcR&lt;sup&gt;R554fs&lt;/sup&gt;</td>
<td>308</td>
<td>191</td>
<td>75</td>
<td>574</td>
<td>29</td>
</tr>
<tr>
<td>EcR&lt;sup&gt;AA483T&lt;/sup&gt;/ EcR&lt;sup&gt;R344Q&lt;/sup&gt;</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>EcR&lt;sup&gt;AA483T&lt;/sup&gt;/ EcR&lt;sup&gt;V559fs&lt;/sup&gt;</td>
<td>10</td>
<td>17</td>
<td>6</td>
<td>33</td>
<td>96</td>
</tr>
</tbody>
</table>

Twenty females of each genotype were mated to 20 wild-type (Canton-S) males at 29° for 2 days, and 6-hr egg collections were made and counted on each of the following 3 days. The average reduction is calculated by dividing the total number of eggs laid by EcR mutant females over the 3-day period by the total number of eggs laid by sibling females (EcR<sup>R554fs</sup>/ SM 6b) and subtracting this number from 100%.

reproduction, we tested females heterozygous for EcR<sup>AA483T</sup> and an EcR null mutation for egg-laying ability at nonpermissive temperature (29°). EcR<sup>AA483T</sup> / EcR<sup>R554fs</sup>, EcR<sup>AA483T</sup> / EcR<sup>R344Q</sup>, and EcR<sup>AA483T</sup> / EcR<sup>V559fs</sup> adult females were collected at 22°, and then shifted to 29° and mated to wild-type (Canton-S) males for 2 days. Egg collections (6-hr) were made on each of the following 3 days, and the number of eggs counted. Sibling EcR<sup>r554fs</sup>/ SM 6b females served as controls. Table 2 shows that EcR<sup>AA483T</sup> / EcR<sup>R344Q</sup> and EcR<sup>AA483T</sup> / EcR<sup>V559fs</sup> females exhibit a nearly 100% decrease in fecundity relative to sibling animals. EcR<sup>AA483T</sup> / EcR<sup>R554fs</sup> females are not as severely affected, but show a progressive reduction in fecundity over the 3-day period of egg collections (Table 2), culminating in a 75% reduction on the third day. Fecundity is affected to a greater degree (>90% reduction) in EcR<sup>AA483T</sup> / EcR<sup>r344Q</sup> animals shifted to 29° during the prepupal period and retained at 29° for mating (data not shown). Thus, all three EcR mutant genotypes examined exhibit strong reductions in fecundity at restrictive temperature.

In contrast to the effects of maternal loss of EcR function, we detect no obvious effects on male fertility in EcR mutant males. Heterozygous mutant males collected as described above and then mated at 29° to wild-type (Canton-S) virgin females were fertile since the females laid eggs that hatched to first instar larvae (data not shown).

EcR mutant females exhibit loss of vitellogenic egg chambers: To determine the basis for decreased fecundity of EcR mutant females, we examined ovaries from EcR mutants raised to eclosion at permissive temperature, and then shifted to restrictive temperature (29°) and mated to wild-type (Canton-S) males (see Materials and methods). We observed an excess of mature, stage 14 egg chambers and a decrease in the number of vitellogenic chambers between stages 7 and 14 in all three EcR mutant genotypes examined relative to the number present in EcR<sup>r554fs</sup>/ SM 6b sibling animals. Figure 2 shows a representative mutant ovariole dissected from an EcR<sup>AA483T</sup> / EcR<sup>r554fs</sup> female compared to a control ovariole from an EcR<sup>r554fs</sup>/ SM 6b female. The mutant ovariole lacks vitellogenic (stages 8–13) egg chambers and contains a second mature (stage 14) egg chamber (Figure 2B).

Quantitation of egg chambers in specific developmental stages in EcR mutant and control females is shown in Table 3. Ovaries from EcR<sup>AA483T</sup> / EcR<sup>r344Q</sup> and EcR<sup>AA483T</sup> / EcR<sup>V559fs</sup> females have severely reduced numbers of mid- (stage 10) and late (stages 11–13) vitellogenic

![Figure 2](image-url.png)

Figure 2.—EcR mutant females lack vitellogenic egg chambers. DAPI-stained ovarioles from control EcR<sup>r554fs</sup>/ SM 6b (A) or EcR<sup>AA483T</sup> / EcR<sup>r554fs</sup> (B) females are shown. Egg chambers between stages 7 and 14 are labeled. PV indicates earlier previtellogenic stages. The control ovariole (A) contains two intermediate stage 10 vitellogenic egg chambers, while the mutant ovariole (B) lacks vitellogenic stages. In addition, the mutant ovariole contains two stage 14 egg chambers (B).
TABLE 3

Quantitation of vitellogenic egg chambers and egg chamber defects

<table>
<thead>
<tr>
<th></th>
<th>EcR&lt;sup&gt;W554fs&lt;/sup&gt; / SM 6b (n = 66 ovarioles)</th>
<th>EcR&lt;sup&gt;RA483T&lt;/sup&gt; / EcR&lt;sup&gt;W554fs&lt;/sup&gt; (n = 70 ovarioles)</th>
<th>EcR&lt;sup&gt;RA483T&lt;/sup&gt; / EcR&lt;sup&gt;R344Q&lt;/sup&gt; (n = 66 ovarioles)</th>
<th>EcR&lt;sup&gt;RA483T&lt;/sup&gt; / EcR&lt;sup&gt;V559fs&lt;/sup&gt; (n = 64 ovarioles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stages 8 and 9 (early vitellogenic)</td>
<td>68</td>
<td>64</td>
<td>19</td>
<td>56</td>
</tr>
<tr>
<td>Stage 10 (midvitellogenic)</td>
<td>69</td>
<td>42</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Stages 11–13 (late vitellogenic)</td>
<td>33</td>
<td>18</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Stage 14 (mature)</td>
<td>20</td>
<td>48</td>
<td>87</td>
<td>82</td>
</tr>
<tr>
<td>Ovarioles with &gt;1 stage 14</td>
<td>0</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Defective egg chambers</td>
<td>2</td>
<td>10</td>
<td>58</td>
<td>51</td>
</tr>
</tbody>
</table>

Vitellogenic egg chambers (stages 8–14) from 4- to 5-day-old ovaries (one ovary each from four females per genotype) dissected from control sibling females (EcR<sup>W554fs</sup> / SM 6b) or EcR mutant females mated at 29°C were counted. Between 64 and 70 ovarioles per genotype were counted. All ovarioles contained several previtellogenic chambers. Defects associated with egg chambers are described in detail in the text.

<sup>a</sup> Two or three stage 14 chambers observed for each ovariole.

<sup>b</sup> Two stage 14 chambers observed per ovariole.

Egg chambers compared to control EcR<sup>W554fs</sup> / SM 6b siblings. EcR<sup>RA483T</sup> / EcR<sup>R344Q</sup> also exhibits a strong reduction in the number of early (stages 8 and 9) vitellogenic chambers. Consistent with earlier results (Tables 1 and 2), EcR<sup>RA483T</sup> / EcR<sup>W554fs</sup> females are less severely affected, although decreases in mid-(stage 10) and late (stages 11–13) vitellogenic stages are also detectable in this genotype. All three EcR mutant genotypes displayed an increase in the number of stage 14 (mature) egg chambers (Table 3), indicating that some chambers progress through oogenesis but are not laid. In mutant ovaries containing large numbers of stage 14 chambers, several ovarioles were observed that contained two or three stage 14 chambers in the most posterior positions of the ovariole (Figure 2B).

Ovaries from EcR mutant females contain abnormal egg chambers: Many mutant ovarioles also contained defective or degenerating egg chambers. Ovarioles from EcR<sup>RA483T</sup> / EcR<sup>R344Q</sup> and EcR<sup>RA483T</sup> / EcR<sup>V559fs</sup> females were most strongly affected, with >50 defective egg chambers observed in each genotype (Table 3). An increase in the number of defective egg chambers in EcR<sup>RA483T</sup> / EcR<sup>W554fs</sup> ovaries relative to control siblings was less pronounced but detectable (Table 3). Defects in follicle and nurse cell nuclear organization and appearance were assayed in DAPI-stained ovariole preparations. Some defective egg chambers (34%) contained very few follicle cells (data not shown), while others had defects that appeared limited to nurse cell nuclei. Nurse cell defects were of two types and accounted for 42% of defective chambers observed. One type of nurse cell defect was an apparent breakdown of the nuclei of stage 8 and 9 vitellogenic egg chambers (Figure 3E, arrow). In a second class of defect, nurse cell nuclei were dramatically smaller than normal (data not shown). This defect was found in both previtellogenic and early vitellogenic (stage 8 and 9) egg chambers. Of defective egg chambers, 12% had a generally degraded appearance with loss of both follicle and nurse cell nuclei (data not shown). Ovaries of all three EcR mutant genotypes exhibited defects described above.

Egg chambers were stained with rhodamine-phalloidin to test for chamber integrity and alteration in the Figure 3.—Loss of EcR function results in defective egg chambers. Ovaries were stained with DAPI (B and E) and rhodamine-phalloidin (C and F), and were viewed by DIC optics (A and D) or epifluorescence. (A–C) Egg chambers from control (EcR<sup>W554fs</sup> / SM 6b) sibling females. (D–F) Egg chambers from EcR<sup>RA483T</sup> / EcR<sup>V559fs</sup> females. E and F are magnified threefold relative to D to show defective chambers in more detail. The black arrow in A indicates accumulated yolk in a vitellogenic egg chamber. The arrowhead in B and white arrows in B and E indicate follicle and nurse cell nuclei, respectively. White arrows in C and F indicate ring canals.
structure of actin rings that separate nurse cells from each other. Wild-type egg chambers have 15 actin rings (Figure 3C, arrows) through which nurse cells provide the oocyte with nutrients. In most mutant chambers, actin rings were disorganized (Figure 3F, arrow) or absent (data not shown).

In addition to defects described above, we observed other classes of defects, each accounting for a small percentage (3–4%) of defective chambers (data not shown). These defects included chambers containing >15 nurse cell nuclei, some of which were likely to be compound chambers with two oocytes. Other chambers had <15 nurse cell nuclei or had incorrect polarity with the oocyte being located in the anterior rather than posterior region of the egg chamber.

**EcR mutant females exhibit reduced accumulation of yolk proteins:** To examine yolk protein accumulation in EcR mutant females, we developed a protocol that allowed an upshift at midpupal stages and recovery of EcR mutant females at eclosion (see materials and methods). Wild-type (Ore-R) and EcR mutant (EcR<sup>4483T</sup> / EcR<sup>3342Q</sup>) females were raised at permissive temperature (22°) until midpupal stages, shifted to 25° until eclosion, and finally held at 29° for 0, 6, 12, 18, 24, or 30 hr after eclosion. Western analysis of extracts revealed a temperature-sensitive mutation, EcR<sup>4483T</sup>. This mutation maps within a signature motif found in the ligand-binding domain of every member of the nuclear receptor family (Koelle et al. 1991; Wurz et al. 1996). Crystallography studies of the ligand-binding domains of five vertebrate nuclear receptors (Bourget et al. 1995; Renaud et al. 1995; Wagner et al. 1995; Brzozowski et al. 1997; Williams and Sigler 1998) reveal a conserved fold consisting of α-helical domains packed into an antiparallel α-helical sandwich that generates a hydrophobic ligand-binding pocket (Park and White 1996; Wurz et al. 1996). Sequence alignments of other nuclear receptor proteins suggest that all nuclear receptors share this structure (Wurz et al. 1996). With regard to this structure, EcR<sup>4483T</sup> is predicted to lie between helix 3 and helix 4 in the conserved core of the ligand-binding domain.

Mutants heterozygous for EcR<sup>4483T</sup> and an EcR null mutation are viable when raised at low temperature but do not survive to adulthood at elevated temperature (Table 1), suggesting that EcR function is lost or reduced at high temperature and allowing production of EcR mutant adult females through growth at permissive temperature. Subsequent shifts to restrictive temperature result in a reduction of fecundity and defects in oogenesis, including an excess of mature stage 14 egg chambers, loss of vitellogenic egg chambers, and presence of abnormal egg chambers. These results suggest that EcR is required for normal oogenesis and provide a foundation for detailed genetic dissection of ecdysone signaling during oogenesis.

**Loss of maternal EcR function results in reduced fecundity and defects in oogenesis:** At restrictive temperature, EcR mutant females exhibit reduced fecundity. Depending on the allelic combination and the time of shift of EcR females to restrictive temperature, this reduction can be nearly complete (see results and Table 2). Defects seen in ovaries dissected from EcR mutant females include an excess of mature stage 14 egg chambers and a loss of stage 10–13 egg chambers (Figure 2 and Table 3). The loss of egg chambers is dramatic in EcR<sup>4483T</sup> / EcR<sup>3342Q</sup> and EcR<sup>4483T</sup> / EcR<sup>5559R</sup> heterozygous combinations and is less striking but still detectable in the EcR<sup>4483T</sup> / EcR<sup>5546R</sup> combination (Table 3).
The heterozygous combination of EcR<sup>483T</sup> and EcR<sup>M554fs</sup> is consistently less sensitive to elevated temperature than combinations of EcR<sup>483T</sup> with either EcR<sup>R3440</sup> or EcR<sup>V559fs</sup> in assays for viability, fecundity, and defects in oogenesis and vitellogenesis (Tables 1–3). Although other interpretations are possible, the difference in sensitivity is most simply explained by differences in genetic background, since EcR<sup>483T</sup>, EcR<sup>R3440</sup>, and EcR<sup>V559fs</sup> share a common parental chromosome, distinct from that of EcR<sup>M554fs</sup>.

In addition to the defects mentioned above, ovaries from EcR females contain many defective or degenerating egg chambers (Figure 3 and Table 3). These defects include small nurse cell nuclei, breakdown of nurse cell nuclei in early vitellogenic egg chambers, and breakdown or loss of both follicle cell and nurse cell nuclei in some egg chambers. At low frequency, we also detect aberrations in normal nurse cell number or in egg chamber polarity.

Because of the intricate coordination of oogenesis and the presence of feedback controls that impact some aspects of the process (Spradling 1993), it is not possible to establish definitively from our data which of the observed defects are primary effects of the loss of receptor and which are secondary consequences of that loss. However, given the widespread expression of EcR in the ovary, the dramatic reduction in fecundity seen in EcR mutant females, and the spectrum of oogenic defects observed in mutant ovarioles, it is reasonable to speculate that EcR functions in controlling multiple aspects of oogenesis. Thus, the abnormal egg chambers observed in EcR mutant females may indicate a function for EcR in maintenance of egg chamber integrity or progression of egg chambers through oogenesis. In addition, the deviation from normal nurse cell number and egg chamber polarity defects seen at low frequency are reminiscent of those seen for mutations in the en core gene (Hawkins et al. 1996), suggesting a possible involvement of EcR in cytoskeleton divisions and oocyte determination.

It is also possible that EcR is involved in the control of oviposition, since EcR mutant females accumulate excess mature, stage 14 egg chambers. EcR mutants show reduced numbers of vitellogenic egg chambers (Table 3). Although earlier physiological experiments indicate a regulatory role for ecdysone in vitellogenesis (Postlethwait and Handler 1979; Jowett and Postlethwait 1980; Bownes et al. 1983), the existence of a feedback control that leads to loss of stage 8–13 egg chambers in females that are holding eggs (Spradling 1993) precludes a similar conclusion from our data. We do, however, detect small but reproducible decreases in yolk protein accumulation in EcR mutants (Figure 4).

The reproductive defects observed in EcR mutant females differ in some respects from defects seen in mutants lacking maternal usp function. For example, decreased fecundity was not reported among usp mutant females rescued to adulthood using a transgene expressing USP under heat-shock control (Oro et al. 1992). It is possible that basal expression of USP from the transgene in this system masked a fecundity defect. The fact that reducing usp gene dosage enhances fecundity defects seen in EcR heterozygotes (Hodin and Riddiford 1998) argues that EcR and usp are required together for normal fecundity. A second difference is that we do not observe defects in eggshell formation (data not shown) similar to those seen in eggs derived from rescued usp mutant mothers (Oro et al. 1992). This difference may indicate that usp carries out some functions independently of EcR, as has been recently suggested (Hall and Thummel 1998; Hodin and Riddiford 1998).

What are the sites of action of EcR during oogenesis?
We have shown that EcRA and EcR-B1 proteins are present in both germline and somatic cells of the adult ovary throughout oogenesis (Figure 1). The USP protein is also broadly expressed in both ovarian cell types during oogenesis (Khoury Christianson et al. 1992). These observations are consistent with the notion that the defective and degenerating egg chambers observed in EcR mutant females may result from action of EcR and USP heterodimers in ovarian germ cells and/or somatic cells. Recent analysis of expression and function of the ecdysone-dependent E75 early gene during oogenesis has led to the proposal of an ecdysone-dependent checkpoint in mid-oogenesis (Buszczak et al. 1999). In this study, removal of EcR through creation of EcR germline clones appeared to result in egg chamber arrest at stages 6–7, suggesting a requirement for EcR in the germline for progression through oogenesis. Thus, the reproductive defects in EcR mutant females described here are likely to result in part from loss of EcR function in germline cells.

Vitellogenesis begins with yolk protein synthesis in the fat body and ovarian follicle cells, and ecdysone signaling has been implicated in the control of fat body yolk protein synthesis (Postlethwait and Handler 1979; Jowett and Postlethwait 1980; Bownes et al. 1983). We presume that active ecdysone receptors are present in the fat body because of the detection of ecdysone binding activity in this tissue (Handler and Maroy 1989), although we have not examined the presence of EcR protein in the adult fat body directly. In experiments using a yolk protein polyclonal antiserum (kindly provided by M. Bownes), we detect small but reproducible decreases in yolk protein production in EcR mutant females compared to controls between 12 and 30 hr following eclosion (Figure 4). We speculate that the small reductions in yolk protein accumulation seen in EcR mutant females are due to a requirement for EcR in the fat body for yolk protein synthesis. If this is the case, methods that allow a more complete inactivation of EcR functions would be predicted to show more dramatic effects on yolk protein production.
The EcR<sup>1483T</sup> conditional mutation allows examination of EcR requirements in the whole animal, but it does not allow us to address directly the spatial requirements for EcR function during oogenesis. Thus, the defects that we observe may result from loss of EcR function in the ovary or in tissues outside of the ovary. In the future, transplantation of EcR mutant ovaries to wild-type females and wild-type ovaries to EcR mutant females will be useful in resolving this issue. The combination of EcR<sup>1483T</sup> with mutants that inactive specific EcR isoforms (Bender et al. 1997; Schubiger et al. 1998; M. Davis, G. E. Carney and M. Bender, unpublished data) should also prove useful in elucidating the functions of each EcR isoform during later development.

We thank Drs. Judy Willis, Mark Brown, Liz Gavis, and Michael Koelle for comments and helpful discussions during preparation of this manuscript and anonymous reviewers for valuable comments on the manuscript. Drs. Tien Hsu, Chun Cao, Sylvia Styhler, and Cindy Vied kindly provided ovary staining protocols. We thank Melissa Gilbert for initial tests for temperature sensitivity of the EcR<sup>1483T</sup> allele, Jennifer Keyes, and Anne Robertson for technical assistance, and Dave Brown for help with Photoshop documents. This work was supported by National Institutes of Health grant GM53681 (to M.B.) and National Institutes of Health Grant GM07103 (to G.E.C.).

LITERATURE CITED


Shea, M. J. D. King, M. J. Conboy, B. D. Mariani and F. C. Kafatos, 1990 Proteins that bind to Drosophila chorion cis-


Communicating editor: V. G. Finnerty