# Developmental Arrest and Ecdysteroid Deficiency Resulting From Mutations at the *dre4* Locus of Drosophila

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ABSTRACT

Loss-of-function mutations of the *dre4* gene of *Drosophila melanogaster* caused stage-specific developmental arrest, the stages of arrest coinciding with periods of ecdysteroid (molting hormone) regulated development. Nonconditional mutations resulted in the arrest of larval development in the first instar; embryogenesis was not impaired, and mutant larvae were behaviorally normal and long-lived. At 31° the temperature-sensitive $dre4^{ts5}$ allele caused the arrest of larval development in the first or second instars. When upshifted to 31° at various times during development, $dre4^{ts5}$ mutants exhibited nonpupariation of third-instar larvae, failure of pupal head eversion, failure of adult differentiation, or noneclosion of pharate adults. Under some temperature regimens second-instar larvae pupariated precociously without entering the normally intervening third-instar. Nonpupariation and defects in metamorphosis were associated with the reduction or elimination of ecdysteroid peaks normally associated with late-larval, prepupal, pupal and pharate adult development. Ecdysteroid production by larval ring glands from $dre4^{ts5}$ hemizygous larvae was suppressed after 2 hr of incubation *in vitro* at 31°, indicating autonomous expression of the *dre4* gene in the ring gland. We postulate that the *dre4* gene is required for ecdysteroid production at multiple stages of Drosophila development and that the pathologies observed in *dre4* mutants reflect developmental consequences of ecdysteroid deficiency.

THE postembryonic growth and development of insects are regulated in part by changes in the circulating levels of ecdysteroids, a class of polyhydroxylated steroidal molt-eliciting hormones derived from either dietary cholesterol or phytosterols. Ecdysteroids act upon integumentary epidermal cells to control both the timing and, in conjunction with the juvenile hormones (JHs), the character of the molts; *i.e.*, whether the molts are for growth (larval-larval molts), or for metamorphosis (larval-pupal and pupal-adult molts) (STEEL and DAVEY 1985). Ecdysteroids play additional regulatory roles in a variety of developmental and reproductive processes, including the morphogenesis of imaginal discs (FRISTROM, LOGAN and MURPHY 1973; MARTIN and SHEARN 1980), glue protein secretion and polytene chromosome puffing in the larval salivary glands of Diptera (ASHBURNER *et al.* 1974; BOYD and ASHBURNER 1977), phenolic tanning of the larval cuticle at the time of puparium formation (pupariation) in the cyclorrhaphous Diptera (KRAMINSKY *et al.* 1980), commitment of the epidermis to larval or pupal development in Lepidoptera (RIDDIFORD 1978), the degeneration of larval tissues during metamorphosis (SCHWARTZ and TRUMAN 1984), and the control of reproduction in adult females (see HAGEDORN 1985). Ecdysteroids are produced by the larval prothoracic gland, which in the cyclorrhaphous Diptera is a component tissue of the ring gland (DAY 1943; AGGARWAL and KING 1969; DAI and GILBERT 1991). Developmental changes in ecdysteroid production are controlled in part by a cerebral neuropeptide, prothoracicotropic hormone (PTTH), that stimulates ecdysteroid production by the prothoracic gland via evolutionarily conserved signal transduction pathways utilizing both $Ca^{2+}$ and cAMP as second messengers in the regulation of PTTH-stimulated protein kinase activity (SMITH, GILBERT and BOLLENBACHER 1985; SMITH, COMBEST and GILBERT 1986; GILBERT *et al.* 1988).

In Drosophila, a number of larval lethal mutations have been described that prevent normal development by impairing ecdysteroid production, thereby resulting in ecdysteroid deficiency and developmental arrest. Typically, such mutations have been identified on the basis of their ability to block, or substantially delay, pupariation and thus extend larval life. Some ecdysteroid-deficient mutations appear to impair ecdysteroid production indirectly. For example, in the case of some temperature-sensitive (ts) cell-lethal mutations, nonpupariation following larval heat treatment appears to be a secondary consequence of regulative growth of imaginal discs following heat-induced cell death (SIMPSON, BERREUR and BERREUR-

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BONNENFANT 1980). However, some ecdysteroid-deficient mutations appear to act directly on the ability of the larval prothoracic gland to produce ecdysteroids. One such mutation is the ts-lethal mutation *lethal(3)ecdysoneless*$^{1}$ (*ec*$^{1}$). Nonpupariation of *ecd*$^{1}$ larvae at restrictive temperatures is associated with reduced whole-body ecdysteroid levels (GAREN, KAUVER and LEPESANT 1977). Reduced ecdysteroid production by ring glands of this mutant (REDFERN and BOWNES 1983) is an autonomous effect of the *ecd*$^{1}$ mutation on the biosynthetic capacity of the ring gland (HENRICH *et al.* 1987). The *ecd*$^{1}$ mutation also has autonomous effects on nonendocrine tissues such as the imaginal discs (SLITER 1989).

We are interested in the mutational analysis of ecdysteroid production in Drosophila as a means to investigate the physiology of the prothoracic gland and the genetics of animal steroidogenesis in general. Drosophila mutations that impair the ecdysteroid biosynthetic activity of the larval ring gland directly provide a powerful tool for understanding the subcellular mechanisms that underlie steroidogenesis and its neurohormonal regulation. The genes identified by such mutations may encode known or suspected components of the ecdysteroid biosynthetic pathway, or the PTTH signal transduction machinery (see GILBERT *et al.* 1988), or proteins of unanticipated function that might escape identification by traditional biochemical approaches due to low levels of expression or the absence of a known biochemical activity.

In this report we describe developmental and endocrine phenotypes associated with mutations of the *lethal(3)dre4* (*dre4*) gene of *Drosophila melanogaster*. The *dre4* gene was identified during the course of a cytogenetic analysis of the 62B–D region of the Drosophila third chromosome (SLITER *et al.* 1989). Preliminary studies indicated that a ts-allele of *dre4* (*dre4*$^{e55}$) caused nonpupariation in larvae transferred to a restrictive temperature early in the third larval instar, and that this developmental block was rescued by dietary 20-hydroxyecdysone (SLITER *et al.* 1989). Those results suggested that mutations of the *dre4* gene might interfere with the normal regulation of ecdysteroid levels at the time of pupariation. In the work presented here, we have undertaken a more detailed analysis of lethal stages and phenotypes caused both by the *dre4*$^{e55}$ mutation and by several nonconditionally expressed mutations of *dre4*. The data indicate that developmental arrest caused by *dre4* mutations is associated with reduced ecdysteroid levels at multiple stages of larval, pupal and pharate adult development, and that expression of the *dre4* gene in the prothoracic gland cells of the ring gland is required for normal ecdysteroid production during larval life.

## MATERIALS AND METHODS

**Genetic strains:** A full description of marker mutations and balancer chromosomes can be found in LINDSLEY and GRELL (1968). The isolation of *dre4* mutant alleles has been described previously (SLITER *et al.* 1989). The following *dre4* mutant chromosomes were used: *ru dre4*$^{e55}$ *st*, *ru dre4*$^{e105}$ *st e*$^{s}$, *ru dre4*$^{e108}$ *st e*$^{s}$, *ru dre4*$^{g11}$ *st e*$^{s}$, *ru dre4*$^{g12}$ *st e*$^{s}$, *ru dre4*$^{g23}$ *st e*$^{s}$, *ru dre4*$^{g36}$ *st e*$^{s}$, *ru dre4*$^{g40}$ *st e*$^{s}$. Two additional chromosomes were used: a control chromosome (*ru dre4*$^{+}$ *st e*$^{s}$) which was the parent chromosome that was mutagenized to produce the various *dre4* mutant alleles; and *Df(3L)R*$^{E}$, *ve dre4*$^{-}$, a chromosomal deficiency that deletes the *dre4* locus and several flanking genes (SLITER *et al.* 1989). Alleles of *dre4* whose superscript designations begin with "e" were induced with ethyl methanesulfonate (EMS); those alleles whose designations begin with "g" were induced by $\gamma$-irradiation. The *dre4*$^{e55}$ allele is a conditionally expressed, ts mutation; all other mutant alleles are nonconditionally expressed. The *dre4*$^{e55}$ mutation was coinduced with a second-site non-ts lethal mutation that mapped by recombination between the *st* and *e*$^{s}$ marker mutations. This second-site lethal mutation was subsequently removed by recombination with a lethal-free Canton S third chromosome, producing a *ru dre4*$^{e55}$ *st* chromosome that is homozygous viable at permissive temperatures. No effort was made to map or eliminate possible second-site lethal mutations from other *dre4* mutant chromosomes.

Mutant, control and deficiency chromosomes were maintained in heterozygous condition with either the *TM6B, Tb Hu* or the *TM3, Ser y*$^{+}$ balancer chromosomes. The dominant body shape marker mutation *Tubby* (*Tb*) carried on *TM6B, Tb Hu* was used to discriminate between balanced (Tb) and unbalanced (Tb$^{+}$) siblings in the second larval instar and later. In experiments that required identification of newly hatched larvae, the *TM3, Ser y*$^{+}$ balancer chromosome was used in conjunction with an *X* chromosome marked with *y w*. The unbalanced larvae (y) were therefore distinguishable from balanced larvae (y$^{+}$) on the basis of lightly pigmented *vs.* darkly pigmented mouth hooks.

**Rearing conditions:** Animals were reared on a standard artificial medium supplemented with live yeast, and methyl paraben and propionic acid to retard fungal growth. Experimental cultures were established either from 1-day-long egg collections or from 4–6-hr-long egg collections. Cultures were maintained in darkness, in constant temperature incubators (±0.5°). The chronological age of cultures was expressed as hours or days after egg laying (AEL). For some experiments, animals were resynchronized as newly formed white puparia and were then maintained in vented, 1.5-ml microcentrifuge tubes in a circulating water bath (±0.2°). Chronological age following resynchronization was expressed as hours postpupariation (PP).

**Developmental staging:** Animals were staged by visual inspection under a Wild binocular dissecting microscope using standard morphological criteria (ASHBURNER 1989). The three larval instars were distinguished by the size and morphology of the oral armature (mouth hook apparatus), and the morphology and pigmentation of the anterior and posterior respiratory spiracles. The stages of metamorphosis follow the morphological staging criteria of BAINBRIDGE and BOWNES (1981).

Due to the complexity of morphological changes at the onset of metamorphosis in cyclorrhaphous Diptera, the nomenclature used to describe the stages of early metamorphosis is not well standardized. For present purposes the following nomenclature based upon that of FRAENKEL and BHASKARAN (1973) is utilized. Following cessation of feeding

and migration from the food substratum and prior to formation of the definitive white puparium, animals are referred to as "wandering larvae." Following formation of the white puparium (stage P1 of BAINBRIDGE and BOWNES 1981) and prior to eversion of the pupal head sac [stage P4(ii)], animals are referred to as "prepupae." Following head eversion and prior to the first deposition of pigment in the developing eye (stage P7) which is roughly coincident with adult apolysis (FRAENKEL and BHASKARAN 1973; BAINBRIDGE and BOWNES 1981), animals are referred to as "pupae." Following eye pigment deposition and until the completion of adult eclosion (stage P15), animals are referred to as "pharate adults."

**Dissections and *in vitro* incubation of ring glands:** All dissections were performed in Grace's insect culture medium without insect hemolymph (GIBCO) adjusted to pH 6.9. To measure the production of ecdysteroids *in vitro*, brain-ring gland complexes or larval ring glands dissected free of their connections to the larval brain and imaginal discs were incubated in Grace's medium. Complexes were incubated individually in 10 μl medium/well. Isolated ring glands were incubated in groups of 5 in 100 μl, and triplicate aliquots of culture medium (10 μl) were then removed and subjected to quantitative ecdysteroid radioimmunoassay (RIA).

**Extraction and RIA of ecdysteroids:** Whole-body ecdysteroid levels were determined following methanol extraction of animals at various developmental stages. Briefly, individual animals or pooled groups were weighed and then extracted using a glass-on-glass homogenizer with 1 ml of 70% methanol. Following centrifugation to remove insoluble material, the methanolic extract was dried *in vacuo*, resuspended in Graces's medium and subjected to RIA.

Ecdysteroids in culture medium or methanolic extracts of whole animals were quantified by competitive ecdysteroid RIA as described previously (WARREN, SMITH and GILBERT 1984; WARREN and GILBERT 1986; PAK and GILBERT 1987) using [23,24-$^3$H]ecdysone (60 Ci/mmol; New England Nuclear) as the labeled ligand. Unlabeled ecdysone was used to generate the standard dilution curve. Therefore, concentrations of ecdysteroids are expressed in units of ecdysone equivalents. The H22 antibody used here recognizes a variety of ecdysteroids whose A and B ring structure is the same as that of ecdysone but may be modified on the side chain (WARREN and GILBERT 1986). These include 20-hydroxyecdysone, 20-deoxymakisterone A (24-methyl-ecdysone) and makisterone A (20-hydroxy-24-methyl-ecdysone), all of which are naturally occurring ecdysteroids in *D. melanogaster* (REDFERN 1984; PAK and GILBERT 1987). The limit of detection of the assay was 15 pg ecdysone.

## RESULTS

**Larval arrest phenotype of nonconditionally expressed *dre4* mutations:** Of the seven nonconditional alleles tested (Table 1), only $dre4^{g40}$ caused embryonic lethality in homozygotes. However, this was apparently the result of a second-site lethal mutation on the *ru* $dre4^{g40}$ *st* $e^s$ chromosome, since $dre4^{g40}$ hemizygotes hatched as first instar larvae at close to the expected frequency (0.33). The other alleles that were tested showed no embryonic lethality either as homozygotes or hemizygotes, indicating that normal zygotic expression of *dre4* was not required for embryogenesis. Following hatching, mutant first-instar larvae fed actively and showed no apparent behavioral or morphological abnormalities. The first evidence of abnormal development in mutant larvae became apparent at the time of the molt from the first to the second instar. Mutant larvae did not molt, and the first instar was prolonged substantially. Typically, surviving first instar larvae were found in cultures 2–4 days or longer after the expected time of molting. The severity of the mutant phenotype was as severe in homozygotes as in hemizgotes, suggesting that first instar developmental arrest represents the *dre4*-null zygotic phenotype.

**TABLE 1**

**Development of animals homozygous and hemizygous for nonconditional *dre4* mutations**

| Genotype[a] | Developmental stage[b] [relative frequency (*N*)] | |
|---|---|---|
| | 1st instar larva | 2nd instar larva |
| $dre4^+/dre4^+$ | 0.34 (225) | 0.31 (187) |
| $dre4^{e105}/dre4^{e105}$ | 0.32 (204) | 0.00 (95) |
| $dre4^{e108}/dre4^{e108}$ | 0.31 (192) | 0.00 (138) |
| $dre4^{g11}/dre4^{g11}$ | 0.31 (260) | 0.00 (101) |
| $dre4^{g12}/dre4^{g12}$ | 0.31 (320) | 0.00 (165) |
| $dre4^{g23}/dre4^{g23}$ | 0.29 (431) | 0.00 (152) |
| $dre4^{g36}/dre4^{g36}$ | 0.30 (248) | 0.00 (120) |
| $dre4^{g40}/dre4^{g40}$ | 0.00 (108) | ND |
| $dre4^+/Df(3L)R^E$ | 0.32 (109) | 0.28 (192) |
| $dre4^{e105}/Df(3L)R^E$ | 0.41 (209) | 0.00 (139) |
| $dre4^{e108}/Df(3L)R^E$ | 0.34 (201) | 0.00 (137) |
| $dre4^{g11}/Df(3L)R^E$ | 0.32 (279) | 0.00 (135) |
| $dre4^{g12}/Df(3L)R^E$ | 0.30 (216) | 0.00 (143) |
| $dre4^{g23}/Df(3L)R^E$ | 0.33 (165) | 0.00 (156) |
| $dre4^{g36}/Df(3L)R^E$ | 0.44 (199) | 0.00 (169) |
| $dre4^{g40}/Df(3L)R^E$ | 0.30 (107) | 0.00 (132) |

Cultures were established from 4–6-hr egg collections, and were maintained at 25°.

[a] Homozygotes: unbalanced (y) progeny of *y w;ru* $dre4^x$ *st* $e^s$/*TM3, Ser* $y^+$ males and females. Hemizygotes: unbalanced (y) progeny of *y w;Df(3L)*$R^E$*/TM3, Ser* $y^+$ females and *y w;ru* $dre4^x$ *st* $e^s$*/TM3, Ser* $y^+$ males.

[b] Relative frequency of unbalanced (y) animals. Expected frequency of unbalanced class = 0.33 (assuming no lethality of unbalanced or singly balanced classes and embryonic lethality of *TM3, Ser* $y^+$ homozygotes). ND, not determined.

**Temperature sensitivity of $dre4^{e55}$:** The effect of rearing temperature on expression of the $dre4^{e55}$ mutation was examined in $dre4^{e55}$ homozygotes and hemizygotes maintained continuously at temperatures from 22° to 31° (Tables 2 and 3). In control crosses the frequency of surviving unbalanced siblings (*ru* $dre4^+$ *st* $e^s$/*ru* $dre4^+$ *st* $e^s$ for the homozygote-producing cross; *ru* $dre4^+$ *st* $e^s$/*Df(3L)*$R^E$ for the hemizygote-producing cross) was close to the expected frequency of 0.33 at rearing temperatures up to 27°, and was somewhat greater than the expected frequency at 29° and 31° due to reduced viability of the *TM6B* balanced animals (Table 2). $dre4^{e55}$ homozygotes survived to adulthood at close to the expected frequency at rearing temperatures up to 27°, but invariably died

TABLE 2

**Survival of *dre4$^{e55}$* homozygotes and hemizygotes at various rearing temperatures**

| Genotype[a] | Developmental stage[b] [relative frequency (*N*)] Brown puparium (P2) | Eclosed adult (A3) |
|---|---|---|
| *dre4$^{+}$/dre4$^{+}$* | | |
| 22° | 0.33 (303) | 0.35 (367) |
| 25° | 0.35 (251) | 0.37 (349) |
| 27° | ND | 0.38 (246) |
| 29° | 0.44 (272) | 0.49 (237) |
| 31° | 0.45 (161) | 0.44 (278) |
| *dre4$^{e55}$/dre4$^{e55}$* | | |
| 22° | 0.32 (418) | 0.28 (694) |
| 25° | 0.35 (848) | 0.36 (474) |
| 27° | 0.31 (827) | 0.31 (483) |
| 29° | 0.26 (367) | 0.00 (425) |
| 31° | 0.003 (593) | ND |
| *dre4$^{+}$/Df(3L)R$^{E}$* | | |
| 22° | 0.35 (170) | 0.35 (423) |
| 25° | 0.38 (407) | 0.41 (341) |
| 27° | 0.34 (361) | 0.34 (689) |
| 29° | 0.33 (353) | 0.43 (243) |
| 31° | 0.56 (229) | 0.53 (163) |
| *dre4$^{e55}$/Df(3L)R$^{E}$* | | |
| 22° | 0.32 (191) | 0.34 (511) |
| 25° | 0.36 (265) | 0.36 (265) |
| 27° | 0.32 (168) | 0.00 (94) |
| 29° | 0.37 (228) | 0.00 (111) |
| 31° | 0.00 (159) | ND |

Cultures were established from 24-hr egg collections, and were maintained continuously at the indicated temperatures.

[a] Animals were derived from the following crosses. *dre4$^{+}$/dre4$^{+}$;ru dre4$^{+}$ st e$^{s}$/TM6B* × *ru dre4$^{+}$ st e$^{s}$/TM6B; dre4$^{e55}$/dre4$^{e55}$;ru dre4$^{e55}$ st/TM6B* × *ru dre4$^{e55}$ st/TM6B;dre4$^{+}$/Df(3L)R$^{E}$;ru dre4$^{+}$ st e$^{s}$/TM6B* × *Df(3L)R$^{E}$/TM6B;dre4$^{e55}$/Df(3L)R$^{E}$;ru dre4$^{e55}$ st/TM6B* × *Df(3L)R$^{E}$/TM6B.*

[b] Frequency of unbalanced (Tb$^{+}$Hu$^{+}$) progeny. Expected Mendelian frequency = 0.33 (assuming no lethality of unbalanced or singly balanced classes and embryonic lethality *TM6B* homozygotes). Stages after BAINBRIDGE and BOWNES (1981). ND, not determined.

prior to adult emergence at temperatures of 29° and higher (Table 2). Mutant hemizygotes displayed a lower critical temperature for lethality than did homozygotes. *dre4$^{e55}$* hemizygotes were fully viable when reared at temperatures up to 25°, but died at temperatures of 27° and higher (Table 2). At 27°, therefore, the *dre4$^{e55}$* allele retained sufficient wild-type function for viability in homozygotes but not in hemizygotes, and the expressivity of the *dre4$^{e55}$* mutation was increased by rearing temperatures above 27°

**Developmental pathologies of *dre4$^{e55}$* homozygotes and hemizygotes at constant temperatures:** To characterize the range of pathologies produced by the *dre4$^{e55}$* mutation, stages of lethality and lethal phenotypes were determined for *dre4$^{e55}$* homozygotes and hemizygotes at several rearing temperatures that were restrictive for overall viability to the adult stage. Several discrete lethal phases and pathologies were observed (Table 3).

*Defects in adult eclosion and differentiation (class I and class II phenotypes): dre4$^{e55}$* hemizygotes reared at 27° died during metamorphosis, predominantly at a late stage of adult differentiation (class I phenotype) while a minority failed to undergo differentiation of adult cuticular structures (class II phenotype). No appreciable lethality prior to metamorphosis was observed. Early metamorphosis appeared to be unaffected in mutant animals, *e.g.*, 100% of *dre4$^{e55}$* hemizygous puparia that formed at 27° displayed head eversion and expulsion of the oral armature, events which normally occur at about 12 hr PP at 25° (BAINBRIDGE and BOWNES 1981). A minority (7%) of hemizygous animals died following head eversion but prior to detectable deposition of eye pigment [yellow eye pigment stage; stage P7 of BAINBRIDGE and BOWNES (1981)], which begins about 40–45 hr PP at 25°. The majority of hemizygous animals at 27° (93%) underwent apparently normal metamorphosis to form fully differentiated adults which failed to eclose from the pupal case. Careful examination of uneclosed pharate adults failed to reveal any obvious external morphological abnormality that would account for the failure of pharate adults to emerge; *i.e.*, uneclosed animals exhibited all of the morphological changes characteristic of late metamorphosis, includ-

TABLE 3

**Survival of *dre4$^{e55}$* homozygotes and hemizygotes at various lethal rearing temperatures**

| Genotype[a] | *N* | Developmental stage[b] [relative frequency] Air bubble prepupa (P3) | Head everted pupa (P4ii) | Pigmented pharate adult (P15i) | Eclosed adult (A3) |
|---|---|---|---|---|---|
| *dre4$^{e55}$/dre4$^{e55}$* | | | | | |
| 29° | 56 | 1.00 | 0.00 | 0.00 | 0.00 |
| *dre4$^{e55}$/Df(3L)R$^{E}$* | | | | | |
| 27° | 90 | 1.00 | 1.00 | 0.93 | 0.00 |
| 29° | 35 | 1.00 | 0.00 | 0.00 | 0.00 |

Cultures were established from 24-h egg collections, and were maintained continuously at the indicated temperatures.

[a] For crosses, see footnotes Table 2.

[b] Survival to indicated stages of metamorphosis is expressed as the relative frequency of puparia (*N*) that formed at the indicated rearing temperature. Stages after BAINBRIDGE and BOWNES (1981).

ing full pigmentation of bristles, blackening of the wings, formation of the green meconium and tanning of the tergites (BAINBRIDGE and BOWNES 1981). The timing of these changes was not delayed when compared to control animals ($dre4^{+}/Df(3L)R^{E}$) reared at 27°. Furthermore, uneclosed pharate adults remained viable within the puparium for several days past the expected time of eclosion, as evidenced by the continued contraction of the dorsal aorta which was visible in intact animals. Despite their normal morphology and extended viability as pharate adults, mutant animals displayed none of the stereotypical behavior associated with the onset of adult eclosion, *i.e.*, leg twitching, rhythmic abdominal contractions and expansion of the ptilinum to open the operculum (BAINBRIDGE and BOWNES 1981).

*Prepupal defects (class III phenotypes):* Mutant hemizygotes and homozygotes maintained at 29° formed puparia at close to the expected frequency (Table 2), but failed to develop past the prepupal stage (Table 3). The development of hemizygotes appeared normal over the first few hours after pupariation, through formation of the gas bubble in the anterior abdomen which normally occurs by 3 hr after pupariation at 25° (BAINBRIDGE and BOWNES 1981). However, later prepupal events, notably posterior migration and expulsion of the air bubble, and eversion of the pupal head, did not occur. Animals ultimately died showing no evidence of the differentiation of adult cuticular structures.

*Defects in larval development (class IV, class V and class VI phenotypes):* In cultures maintained at 31°, larval lethality was characteristic of both $dre4^{e55}$ homozygotes and hemizygotes. Homozygotes died predominantly as nonpupariating third instar larvae (class IV phenotype) or second instar larvae, with a small minority of escaper animals (<1%) forming tanned puparia. In contrast, the majority (≈65%) of $dre4^{e55}$ hemizygotes failed to develop past the first larval instar (class VI phenotype), while ≈35% molted to form second instar larvae, but then failed to develop further (class V phenotype). Molt-arrested first or second instar larvae were relatively long-lived in these stages; *e.g.*, nonmolting second instar larvae survived for 4–5 days past the normal time of the molt to the third instar. Nonmolting second instar larvae showed no obvious behavioral abnormalities. Growth continued during the extended second instar, and mutant larvae achieved an average size (0.57 mg/larva; $n$ = 20) substantially greater than that of newly molted (0–4 hr) control third instar larvae (0.44 mg/larva, $n$ = 15).

In wild-type Drosophila larvae reared at 25°, molting is initiated by separation of the larval cuticle from the underlying epidermal cells (apolysis) approximately 12 hr prior to larval ecdysis (KAZNOWSKI, SCHNEIDERMAN and BRYANT 1985). Apolysis is coincident with mid-instar peaks in whole-body ecdysteroid levels (KRAMINSKY *et al.* 1980). In dissections of late second instar $dre4^{+}/Df(3L)R^{E}$ larvae, the apolysed cuticle was easily dissected from the underlying hypodermis. However, dissection of nonmolting second instar *dre4* hemizygotes showed no evidence of apolysis suggesting that the $dre4^{e55}$ mutation prevented the initiation of the larval molting. In overgrown, molt-arrested second instar larvae, the size and morphology of the internal organs were characteristic of the mid-third instar. In particular, wing imaginal discs grew beyond the size that was characteristic of newly molted third instar larvae, and began to develop the folding patterns that are characteristic of late-stage imaginal discs (Figure 1). These observations suggested that during larval life the $dre4^{e55}$ mutation preferentially blocked the molting process, and had a lesser or negligible effect upon the growth and development of larval and imaginal tissues.

**Developmental consequences of temperature-upshifts on $dre4^{e55}$ hemizygotes:** The results described above indicated that $dre4^{e55}$ mutant animals exhibited stage specific developmental arrest/lethality. The stage specificity of the mutation's lethal effect was dependent upon both rearing temperature and gene dosage, reflecting an incremental decrease in activity of the $dre4^{e55}$ gene product with increasing temperature. To investigate further the relationship between the stages of lethality and gene activity, the effects of temperature upshifts on $dre4^{e55}$ hemizygotes were examined at various times during postembryonic development.

*Upshift of early third-instar larvae:* Table 4 shows the results of experiments in which early third-instar larvae were upshifted from the permissive temperature of 22°. In control cultures, there was no observable effect of rearing temperatures up to 31° upon the survival of unbalanced animals to adult emergence. The lethal phenotypes of $dre4^{e55}$ hemizygotes upshifted to 27° or 29° were similar to those seen in hemizygotes reared continuously at these temperatures; mutant hemizygotes died predominantly as fully differentiated pharate adults when upshifted to 27° (class I), and as cryptocephalic prepupae when upshifted to 29° (class III). When upshifted to 31°, hemizygous third instar larvae survived for several days and died without pupariating (class IV). Neither larval behavior nor growth was affected appreciably by the upshift to 31°. At the time of wandering and pupariation of control larvae 2 days after transfer to 31°, $dre4^{e55}/Df(3L)R^{E}$ larvae had reached an average size (1.76 mg/larva, $n$ = 44) only slightly less that of control $dre4^{+}/Df(3L)R^{E}$ larvae (1.87 mg/larva, $n$ = 19). In each case this represented a 4–5-fold increase in fresh weight over the course of the third instar.

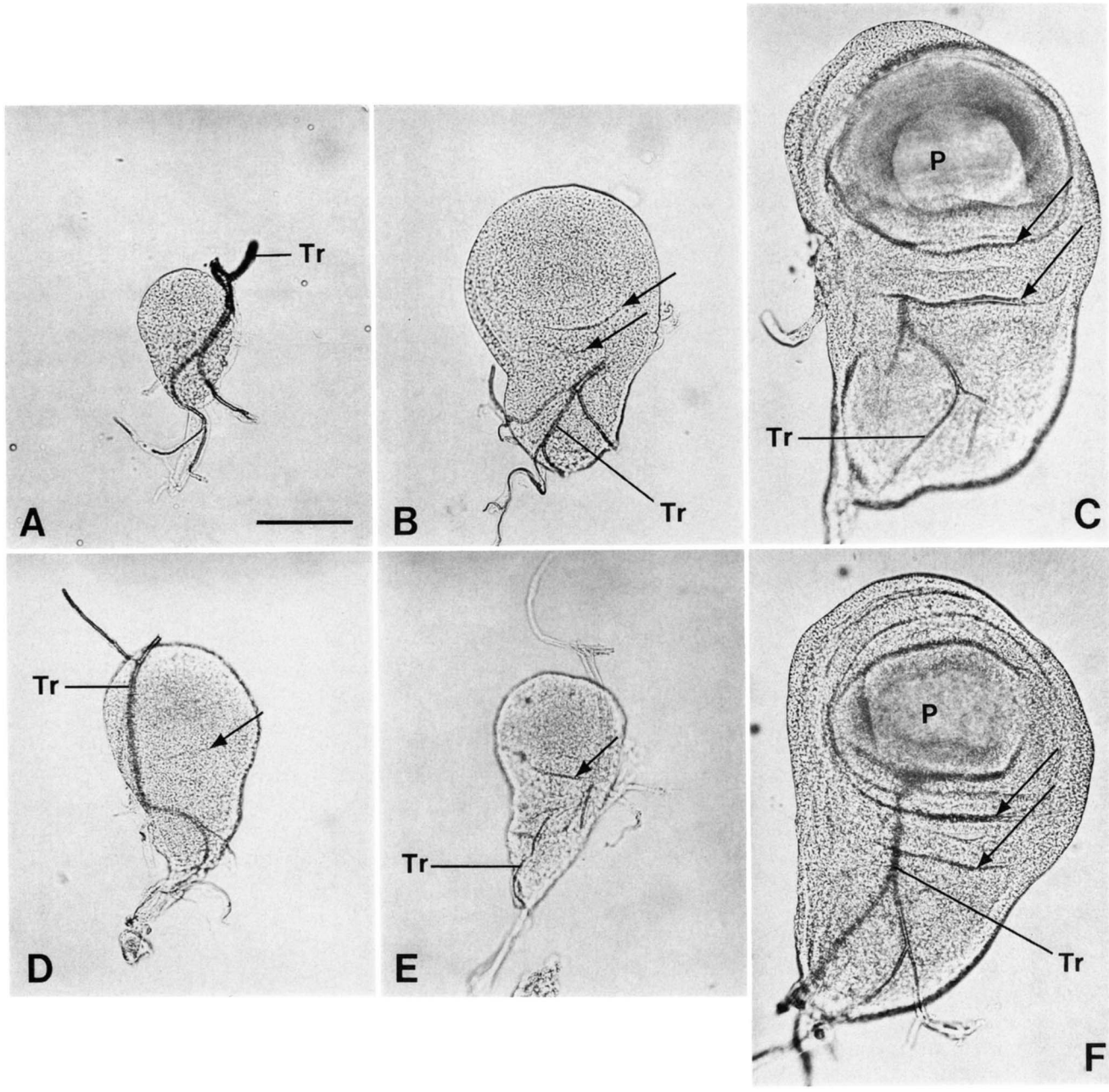


FIGURE 1.—Wing imaginal discs from control ($dre4^{+}/Df(3L)R^{E}$) and experimental ($dre4^{e55}/Df(3L)R^{E}$) larvae. (A–C) discs from control larvae reared at 31°: (A) early third instar; (B) mid-third instar; (C) late third-instar; (D and E) discs from molt-blocked second instar experimental larvae reared at 31° (2 days following the expected time of molting); (F) disc from nonpupariating late-third instar experimental larva, upshifted from 22° to 31° early in the third instar. Arrows, folds of the disc epithelium; P, wing pouch region (presumptive wing blade); Tr, trachea.

Moreover, no obvious morphological abnormalities of internal tissues were observed in nonpupariating mutant larvae. In particular, imaginal discs were of normal size and morphology, and showed no evidence of degeneration or abnormal regulative growth (Figure 1).

*Upshift of late third instar larvae:* The absence of detectable abnormalities in behavior or development prior to the time of pupariation suggested that the $dre4^{e55}$ mutation had a specific effect on late larval physiology directly related to pupariation and the onset of metamorphosis. To examine this possibility, $dre4^{e55}$ hemizygous larvae were reared at 22° until the onset of wandering behavior which begins approximately 6–8 hr prior to pupariation at 25°. Newly wandering larvae within 2 hr of leaving the food substrate were selected from the walls of culture bottles and transferred to 31°. The extent of pupariation was assessed 1 day later. In wild-type dipteran larvae, pupariation is manifested externally in two ways: (1) body retraction which is first evidenced by eversion of the anterior spiracles (anterior retraction) and later

**TABLE 4**

**Survival of $dre4^{e55}$ hemizygotes upshifted as early third instar larvae**

| Genotype[a] | Developmental stage [relative frequency (*N*)] | | | | |
|---|---|---|---|---|---|
| | White puparium (P1)[b] | Bubble prepupa (P3)[c] | Exocephalic pupa (P4ii)[c] | Late pharate adult (P15i)[c] | Eclosed adult (A3)[b] |
| $dre4^{+}/Df(3L)R^{E}$ | | | | | |
| 31° | ND | ND | ND | ND | 0.37 (301) |
| $dre4^{e55}/Df(3L)R^{E}$ | | | | | |
| 25° | ND | ND | ND | ND | 0.32 (231) |
| 27° | 0.31 (272) | 1.00 (120) | 1.00 (120) | 0.82 (120) | 0.00 (350) |
| 29° | 0.31 (310) | 1.00 (95) | 0.00 (95) | 0.00 (95) | 0.00 (236) |
| 31° | 0.00 (135) | ND | ND | ND | 0.00 (114) |

Cultures established from 4–6-hr egg collections were maintained at 22° for 4 days, then transferred to the indicated temperatures. Stages after BAINBRIDGE and BOWNES (1981). ND, not determined.

[a] For crosses, see footnotes Table 2.

[b] Survival of unbalanced ($Tb^{+}$ $Hu^{+}$) animals to indicated stages is expressed as the relative frequency of total animals (*N*). Expected Mendelian frequency = 0.33 (assuming no lethality of the unbalanced class and embryonic lethality of *TM6b* homozygotes).

[c] Survival of unbalanced ($Tb^{+}$ $Hu^{+}$) animals to indicated stages is expressed as the relative frequency of unbalanced puparia scored (*N*).

**TABLE 5**

**Tanning and retraction of $dre4^{e55}/Df(3L)R^{E}$ ($N = 130$) and $dre4^{+}/Df(3L)R^{E}$ ($N = 82$) animals transferred to 31° as early wandering larvae**

| Retraction index[a] | Tanning index[b] | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| 0 | 0.25 [0] | 0 [0] | 0 [0] | 0 [0] |
| 1 | 0.11 [0] | 0 [0] | 0 [0] | 0 [0] |
| 2 | 0 [0] | 0.34 [0] | 0.19 [0] | 0 [0] |
| 3 | 0 [0] | 0 [0] | 0.08 [0] | 0.03 [1.00] |

Larvae reared at 22° were transferred to 31° 0–2 hr after the onset of wandering behavior and scored one day later. The extent of tanning and body retraction is expressed as the relative frequency of treated animals (*N*). Unbracketed values, mutant animals. Bracketed values, control animals. For crosses, see footnotes Table 2.

[a] Body retraction index: 0, no retraction; 1, spiracles everted (anterior retraction); 2, spiracles everted and partial body retraction; 3, spiracle eversion and full body retraction. Mean retraction scores: control larvae = 3.0; mutant larvae = 1.49.

[b] Cuticle tanning index: 0, untanned (larval) cuticle; 1, pale brown cuticle; 2, light brown cuticle; 3, fully tanned cuticle. Mean tanning scores: control larvae = 3.0; mutant larvae = 0.97.

by posterior retraction to form the fully retracted, barrel-shaped puparium; and (2) hardening and tanning of the larval cuticle which begins following full retraction of the body (see ZDAREK 1985). In wild-type *D. melanogaster* at 25°, body retraction takes approximately 20 min, while tanning occurs over a period of 1–2 hr. For the purposes of assessing pupariation in mutant animals, we scored for the extent of both body retraction and cuticle tanning using a four-point scoring system (see Table 5).

When $dre4^{e55}$ hemizygous larvae were upshifted to 31° at the onset of wandering behavior, only 3% of the animals exhibited normal pupariation (Table 5). No detectable tanning or body retraction was observed in 25% of the treated mutant animals, while greater than 70% of the animals showed incomplete tanning or body retraction. In contrast to wild-type animals which tan their larval cuticle only after full retraction of the body, upshifted mutant animals commonly showed partial tanning in the absence of full retraction. Pupariation of control animals was unaffected by the upshift to 31° at the onset of wandering. These results indicated that the ability of Drosophila larvae to undergo successful pupariation was dependent upon normal *dre4* gene function late in larval development, during the period following the initiation of wandering behavior.

*Upshift at pupariation:* To examine the effects of the $dre4^{e55}$ mutation upon metamorphosis, mutant hemizygotes were upshifted from 22° to various temperatures as newly formed white puparia (Table 6). In control animals, upshifting to a temperature as high as 31° had no appreciable effect upon survival through adult eclosion. In contrast, mutant hemizygotes upshifted to 27° or 29° differentiated into pharate adults that failed to eclose (class I). When upshifted to 31° as white prepupae, 100% of mutant hemizygotes failed to undergo normal imaginal head eversion, and did not exhibit any differentiation of adult cuticular structures (class III phenotype).

**Reversibility of larval-arrest phenotypes.** When molt-arrested second instar larvae and nonpupariating third instar $dre4^{e55}$ hemizygous larvae were returned to the permissive temperature of 22° a substantial proportion of animals (>50%) exhibited full or partial recovery of normal development. Molt-arrested second instar mutant larvae returned to 22° 2–3 days following the upshift to 31° as newly laid eggs, subsequently molted to the third instar and ultimately formed morphologically normal adults that eclosed successfully. In contrast, when returned to 22° 4–5 days following egg collection, mutant animals did not molt to the third instar, but instead formed precocious

TABLE 6

**Survival of *dre4*$^{e55}$ hemizygotes upshifted as white puparia (P1)**

| Genotype[a] | *N* | Developmental stage[b] [relative frequency] | | | | |
|---|---|---|---|---|---|---|
| | | Brown puparium (P2) | Bubble prepupa (P3) | Exocephalic pupa (P4ii) | Late pharate adult (P15i) | Eclosed adult (A3) |
| *dre4*$^{+}$/*Df(3L)R*$^{E}$ | | | | | | |
| 25° | 41 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 27° | 19 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 29° | 48 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 31° | 45 | 1.00 | 1.00 | 1.00 | 0.98 | 0.98 |
| *dre4*$^{e55}$/*Df(3L)R*$^{E}$ | | | | | | |
| 25° | 17 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 27° | 21 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| 29° | 35 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| 31° | 30 | 1.00 | 1.00 | 0.00 | 0.00 | 0.00 |

Cultures were established from 1 day egg collections at 22°, and were maintained at 22°. Newly formed white puparia (P1) were collected and transferred to the indicated temperatures.

[a] For crosses, see footnotes Table 2.

[b] Survival to indicated stages is expressed as the relative frequency of treated puparia (*N*). Stages after BAINBRIDGE and BOWNES (1981).

TABLE 7

**Summary of *dre4* lethal stages**

| Phenotypic class | Lethal stage |
|---|---|
| I | Differentiated pharate adult (P15i) |
| II | Pupa (P5–P7) |
| III | Cryptocephalic prepupa (P4i) |
| IV | Third-instar larva |
| V | Second-instar larva |
| VI | First-instar larva |

miniature puparia which died subsequently without exhibiting the differentiation of adult structures.

**Summary of *dre4* phenotypes:** The analysis of lethal phenotypes of *dre4* mutants identified six lethal stages associated with progressive reduction of *dre4* gene activity (Table 7). Taken together, the results indicate that normal zygotic expression of the *dre4* gene is not required for embryogenesis. In larvae, *dre4* gene expression is required for larval molting (classes V and VI) and pupariation (class IV). The effects of *dre4* mutations upon larval viability and growth occur subsequent to the observed effects on molting and pupariation, and are likely to be secondary consequences of molt inhibition and pupariation inhibition. During metamorphosis, normal activity of the *dre4* gene is clearly required for early metamorphic events associated with the transformation of the cryptocephalic prepupa to the definitive phanaerocephalic pupa (class III), and late metamorphic events associated with adult eclosion (class I). There is also some indication of a role of *dre4* in the differentiation of adult structures (class II), although this phenotype was of low penetrance under the experimental conditions examined.

**Ecdysteroid levels in *dre4*$^{e55}$ hemizygotes:** The arrest of larval molting, pupariation, and metamorphosis in *dre4* mutants were consistent with an underlying defect in the developmental regulation of ecdysteroid levels. To determine if ecdysteroid deficiencies were associated with developmental arrest caused by *dre4* mutations, the effects of restrictive temperatures upon ecdysteroid levels in *dre4*$^{e55}$ hemizygotes were examined under three conditions: (1) upshift to 31° early in the third instar, which resulted in non-pupariation of larvae; (2) upshift to 28° at the white puparium stage, which resulted in failure of adult eclosion but did not prevent differentiation of adult cuticular structures; and (3) upshift to 31° at the white puparium stage which prevented both eversion of the imaginal head and adult differentiation.

*Effects on late larval ecdysteroid levels:* A number of previous studies have identified a late larval peak of whole-body ecdysteroids that is coincident with wandering and pupariation (KRAMINSKY *et al.* 1980). In control animals (*dre4*$^{+}$/*Df(3L)R*$^{E}$) raised at 22° or upshifted to 31° as early third instar larvae, ecdysteroid levels in wandering-stage animals were in the range of 50–70 pg/mg (Table 8) which is in good agreement with previous published values (see RICHARDS 1981). Comparable ecdysteroid levels were also found in experimental animals (*dre4*$^{e55}$/*Df(3L)R*$^{E}$) raised at 22°. However, ecdysteroids were present at less than 1/10 these levels in pupariation-blocked experimental animals at 31°. The pupariation block caused by *dre4*$^{e55}$ is therefore associated with reduced levels of RIA positive ecdysteroids at the end of larval life when ecdysteroids normally initiate metamorphosis.

*Effects on ecdysteroid levels during metamorphosis:* Reduced ecdysteroid levels were also observed during metamorphosis in *dre4*$^{e55}$ hemizygotes following transfer to restrictive temperatures at the white puparium stage (Figure 2). A major peak of RIA positive ecdy-

TABLE 8

**Ecdysteroid deficiency in nonpupariating *dre4*$^{e55}$ hemizygotes**

| Genotype[a] | Ecdysteroids (pg/mg ± SEM) |
|---|---|
| *dre4*$^{+}$/*Df(3L)R*$^{E}$ | |
| 22° | 69 ± 9[b] |
| 31° | 48 ± 9[b] |
| *dre4*$^{e55}$/*Df(3L)R*$^{E}$ | |
| 22° | 62 ± 7[b] |
| 31° (2 days) | 6.5 (35)[c] |
| 31° (2 days) | 4.3 (44)[c] |
| 31° (3 days) | 2.2 (26)[c] |

Cultures established from 4–6-hr egg collections at 22° were either maintained at 22°, or transferred to 31° after 4 days at which time the majority of animals were early third-instar larvae. Control (22° and 31°) and mutant animals (22°) were collected as wandering stage larvae. Nonpupariating mutant animals (31°) were collected from the food 2 or 3 days following transfer to 31°; wandering and pupariation of nonmutant siblings occurred 2 days following transfer to 31°.

[a] For crosses, see footnotes Table 2.

[b] Mean ecdysteroid content was determined from 15–20 larvae individually extracted and assayed.

[c] Because individual ecdysteroid content was below the detection limit of the assay (15 pg), ecdysteroid content was determined from groups of pooled larvae. Numbers in parentheses indicate the number of animals pooled.

steroids has been previously reported in metamorphosing *D. melanogaster* between 24 and 48 hr PP (RICHARDS 1981; PAK and GILBERT 1987). At 22°, there was essentially no difference in the peak level of ecdysteroids seen in mutant and control animals (Figure 2A). In control animals at 28° (Figure 2B), three distinct peaks in whole-body ecdysteroid levels were observed following the declining phase of the pupariation-associated ecdysteroid peak (0–6 hr PP): (1) a prepupal peak that began 6–9 hr PP and reached a level of 80–100 pg/mg, comparable to the pupariation peak seen at 0 hr PP; (2) a larger pharate adult peak at 30 hr PP that reached maximum levels of 270–300 pg/mg, comparable to the peak levels observed at 22°; and (3) a smaller pharate adult peak at 56 hr PP that reached a maximum level of 170 pg/mg. In *dre4*$^{e55}$ hemizygotes upshifted to 28° at pupariation (Figure 2B), the overall pattern and timing of ecdysteroid peaks were the same as in control animals. No effect was seen on either the declining phase of the pupariation peak or the prepupal peak. However, both the pupal and pharate adult ecdysteroid peaks were reduced by about 40% compared to control animals at 28°. In mutant animals transferred to 31° as white puparia (Figure 2C), the pupal and pharate adult peaks were eliminated, as was the prepupal peak. Therefore, associated with the developmental arrest during metamorphosis seen in *dre4*$^{e55}$ hemizygotes was a severe reduction in whole-body ecdysteroid levels. There appeared to be a quantitative relationship between rearing temperature and the extent of ecdysteroid deficiency during metamorphosis caused by

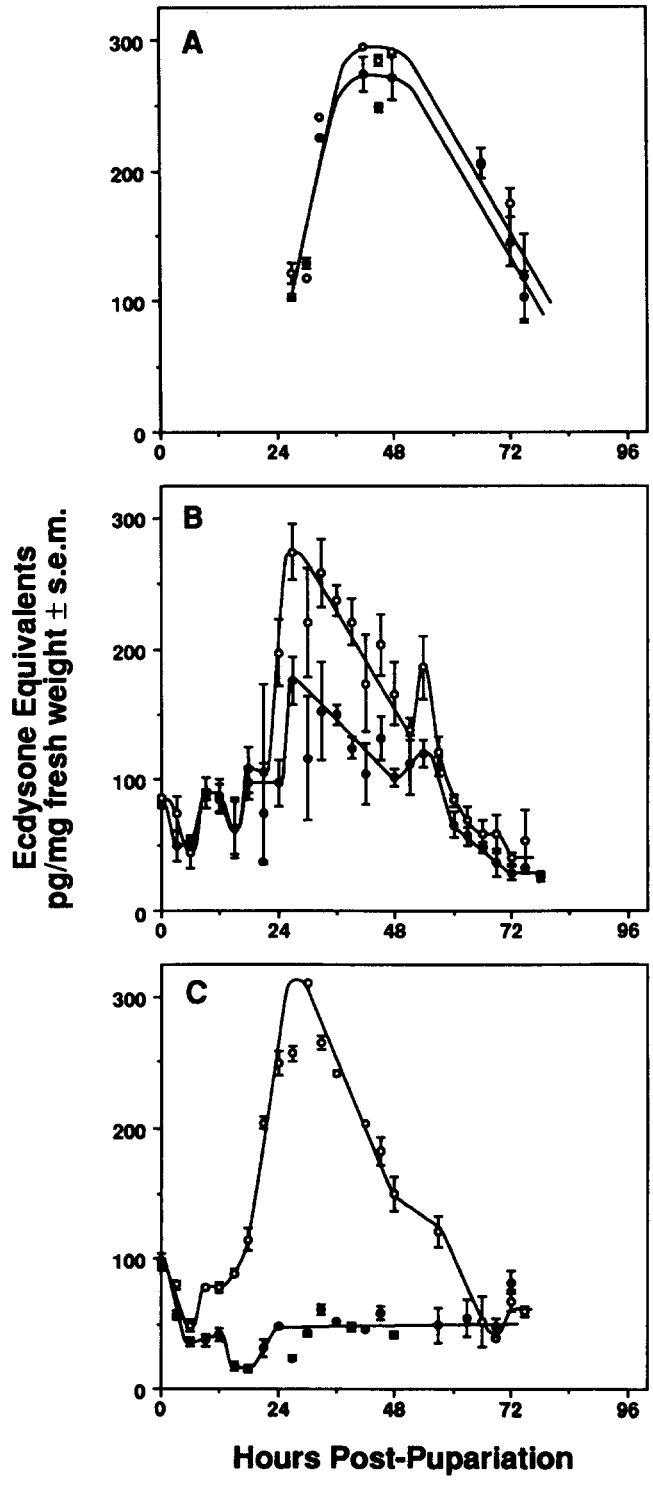


FIGURE 2.—Ecdysteroid deficiency during metamorphosis resulting from the *dre4*$^{e55}$ mutation. Animals raised at 22° were selected at the white puparium stage (0–2 hr PP) and maintained thereafter at various experimental temperatures: (A) 22°, a permissive temperature for development of *dre4*$^{e55}$ hemizygotes; (B) 28°, a semirestrictive temperature for *dre4*$^{e55}$ hemizygotes that permits differentiation of adult cuticular structures but prevents adult eclosion; and (C) 31°, a restrictive temperature that prevents development of *dre4*$^{e55}$ hemizygotes past the prepupal stage approximately 12–15 hr PP. Open circles: control animals (*ru dre4*$^{+}$ *st e*$^{s}$/*Df(3L)R*$^{E}$). Filled circles: experimental animals (*ru dre4*$^{e55}$ *st*/*Df(3L)R*$^{E}$).

*dre4*$^{e55}$. At the semirestrictive temperature of 28°, there was only a partial reduction in ecdysteroid levels, while at the more fully restrictive temperature of 31° all ecdysteroid peaks were eliminated.

**Effects of *dre4*$^{e55}$ on ecdysteroid production:** To determine if the observed ecdysteroid deficiency in pupariation-blocked *dre4*$^{e55}$ hemizygotes was associated with reduced production of ecdysteroids by the larval ring gland, control (*dre4*$^{+}$/*Df(3L)R*$^{E}$) and mutant (*dre4*$^{e55}$/*Df(3L)R*$^{E}$) larvae were raised at 22° until the beginning of the third instar and then transferred to 31°. Two days later, at the time control larvae were wandering and pupariating, brain-ring gland complexes were dissected from larvae, incubated *in vitro* at 25°, and the amount of ecdysteroid secreted into the medium was determined. Ring glands from wandering control larvae produced ecdysteroids *in vitro* at a rate of 76 ± 20 pg gland $^{-1}$ hr$^{-1}$; in contrast, complexes from nonpupariating mutant larvae did not produce detectable amounts of ecdysteroids (<15 pg gland $^{-1}$ hr$^{-1}$). Nonpupariation caused by *dre4*$^{e55}$ was therefore associated with reduced ecdysteroid synthesis by late third instar larvae.

Reduced ecdysteroid production by brain-ring gland complexes in *dre4*$^{e55}$/*Df(3L)R*$^{E}$ larvae transferred to 31° at the beginning of the third instar could reflect the autonomous expression of the mutation within cells of the prothoracic gland. Alternatively, it might reflect either direct or indirect suppression of prothoracicotropic hormone release from the central nervous system (Henrich *et al.* 1987). To determine if the *dre4*$^{e55}$ mutation suppressed the biosynthetic activity of the prothoracic gland directly, we examined ecdysteroid production by ring glands dissected from wandering stage control and mutant larvae reared at 22°. Ring glands were incubated for up to 4 hr *in vitro* at either 22° or 31° (Figure 3). The time course and absolute levels of ecdysteroid production were similar for ring glands from control larvae (*dre4*$^{+}$/*Df(3L)R*$^{E}$) at 22° and 31°, and experimental larvae (*dre4*$^{e55}$/*Df(3L)R*$^{E}$) at 22°. Ring glands from control larvae incubated *in vitro* at 22° produced approximately 210 pg of ecdysteroids during the first 2 hr of culture and approximately 25% less (160 pg) during the second 2 hr. Similar results were obtained with control glands at 31°; there was no increase in the absolute rate of ecdysteroid production at this higher temperature. Ring glands from experimental *dre4*$^{e55}$/*Df(3L)R*$^{E}$ larvae produced ecdysteroids at a slightly higher rate (270 pg/gland) than did control glands over the first 2 hr *in vitro* at 22°, but also showed slightly lower production during the second 2 hr of incubation. In contrast, ecdysteroid production by mutant glands at 31° was comparable to 22° production over the first 2 hr *in vitro*, but was reduced by 90% during the second 2-hr period. Therefore

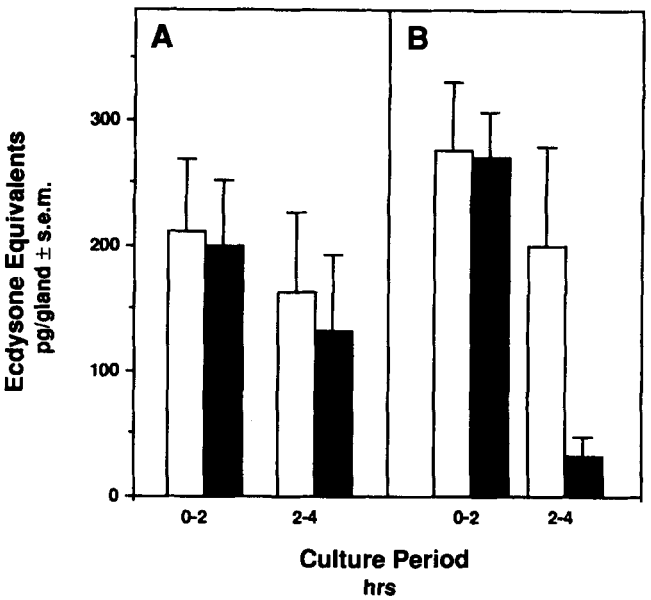


Figure 3.—Effect of *dre4*$^{e55}$ on ecdysteroid production *in vitro*. Ring glands dissected from wandering stage larvae reared at the permissive temperature of 22° were incubated *in vitro* for 4 hr at either 22° (open bars) or 31° (filled bars). At 2 hr and 4 hr, triplicate aliquots of medium were removed without replacement and subjected to ecdysteroid RIA. (A) Control glands from *ru dre4*$^{+}$ *st e*$^{s}$/*Df(3L)R*$^{E}$ larvae. (B) Experimental glands from *ru dre4*$^{e55}$ *st*/*Df(3L)R*$^{E}$ larvae.

ring glands from *dre4*$^{e55}$/*Df(3L)R*$^{E}$ larvae exhibited a temperature-dependent suppression of ecdysteroid synthesis *in vitro*. It therefore appeared that the *dre4*$^{e55}$ mutation was expressed autonomously in the ring gland, and that suppression of ecdysteroid production at the restrictive temperature represented a direct effect of the mutation on the biosynthetic activity of the prothoracic gland.

**ts period for prepupal arrest:** Since the *dre4*$^{e55}$ mutation exerted a direct effect on ecdysteroid production by larval rings glands, it was of interest to know if the effect of *dre4*$^{e55}$ upon the prepupal ecdysteroid peak in mutant animals upshifted to 31° at pupariation might be attributable to an effect on ecdysteroid production at these times. The principal developmental consequence of this treatment was the suppression of pupal head eversion. If suppression of prepupal ecdysteroid production and consequent ecdysteroid deficiency were responsible for the failure of mutant animals to undergo pupal head eversion at 31°, then the ts period (TSP) for suppression of head eversion in *dre4*$^{e55}$ hemizygotes should coincide with the rise in the ecdysteroid titer that occurs 6–9 hr PP. The TSP for suppression of head eversion was determined in a series of reciprocal temperature upshift and downshift experiments (Tarsoff and Suzuki 1970). Transferring animals from 22° to 31° at times up to 6 hr PP resulted in the complete suppression of head eversion (Figure 4). However, greater than 90% of the animals upshifted at 9 hr PP underwent normal

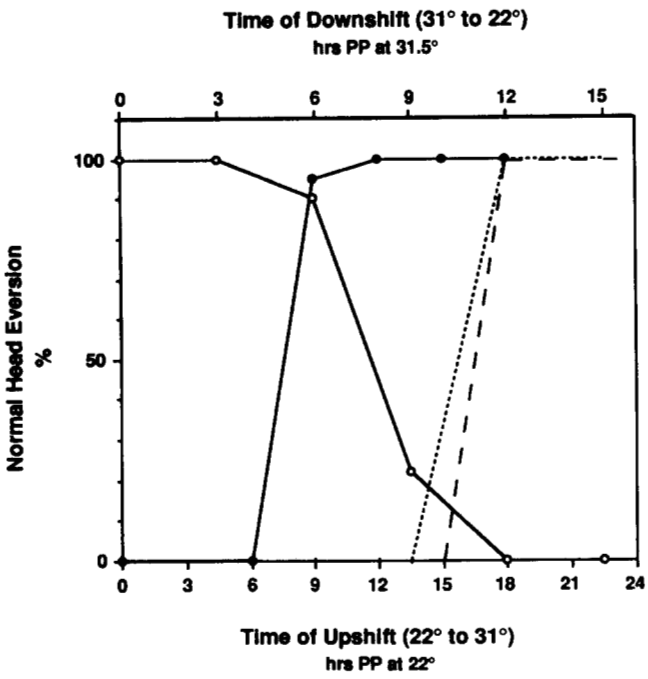


FIGURE 4.—TSP for suppression of pupal head eversion by $dre4^{e55}$. Animals (*ru* $dre4^{e55}$ *st*/$Df(3L)R^E$) were reared at 22° and synchronized as newly formed white puparia. In one experiment (filled circles) animals were initially maintained at 22° following synchronization, and then transferred to 31° at times thereafter. In a second experiment (open circles), animals were transferred to 31° immediately following synchronization, and then were returned to 22° at times thereafter. The cumulative frequency of animals displaying pupal head eversion is shown for *ru* $dre4^{e55}$ *st*/$Df(3L)R^E$ animals maintained at 22° (dashed line) and control animals (*ru* $dre4^{+}$ *st* $e^{s}$/$Df(3L)R^E$) maintained at 31° (dotted line) following pupariation.

head eversion, indicating that normal *dre4* gene function was not required after 9 hr PP. In downshift experiments, 80% of the animals exposed to 31° for the first 6 hr of prepupal development underwent normal imaginal head eversion, but 80% exposed to 31° for 9 hr after pupariation failed to undergo head eversion. The TSP for $dre4^{e55}$ suppression of head eversion therefore lay between approximately 6 and 9 hr PP, and corresponded closely to the time of the prepupal ecdysteroid peak (Figure 2B). These results suggest that normal function of the *dre4* gene is required for production of ecdysteroids coincident with the prepupal ecdysteroid peak, and that this peak drives the morphogenetic processes associated with imaginal head eversion during late prepupal development.

## DISCUSSION

Based upon the ability of dietary 20-hydroxyecdysone to stimulate tanning and hardening of the larval cuticle of nonpupariating $dre4^{e55}$ hemizygous larvae, it was suggested that an ecdysteroid deficiency might underlie the failure of this mutant to pupariate at a restrictive temperature (SLITER *et al.* 1989). The present results have demonstrated that the $dre4^{e55}$ mutation causes an ecdysteroid deficiency at the time of pupariation, and autonomously suppresses ecdysteroid production by larval ring glands *in vitro*. Additionally, the data reveal that during metamorphosis, stage-specific developmental arrest caused by the $dre4^{e55}$ mutation is associated with the reduction or elimination of one or more of the ecdysteroid peaks associated with prepupal, pupal and adult development. The time of *dre4* gene action during prepupal development, as indicated by the TSP for pupal head eversion, corresponds to the time of rising ecdysteroid levels. Ecdysteroid levels in animals exhibiting the arrested larval molting phenotype have not yet been determined, but the phenotype is consistent with a failure of ecdysteroids to trigger larval molting. Together, these results suggest that the *dre4* gene may play a direct role in regulating ecdysteroid production at most or all stages of postembryonic development.

**Interpretation of the $dre4^{e55}$ mutation:** Our views of the likely developmental roles of the *dre4* gene are based primarily upon the pathologies caused by the ts $dre4^{e55}$ mutation. In general, phenotypic analysis of a single ts mutant allele causing pleiotropic abnormalities can be problematic due to: (1) the inherent leakiness of ts mutations and the resulting difficulty in assessing the extent to which wild-type gene function is retained at any particular experimental temperature; and (2) the potential genetic complexity of the locus, and the unknown degree to which a single allele is representative of that complexity. The $dre4^{e55}$ allele clearly does retain partial wild-type gene function even at the highest rearing temperatures examined (31°), as indicated by the greater expressivity of $dre4^{e55}$ hemizygotes in comparison to homozygotes. However, two observations suggest that the pathologies of $dre4^{e55}$ hemizygotes at 31° represent strong mutant phenotypes. First, when reared at 31° from the time of egg laying, the majority of $dre4^{e55}$ hemizygotes exhibited a lethal phenotype that was as severe as the phenotype of animals carrying non-ts *dre4* mutations. The latter behave genetically as amorphs in gene dosage experiments. In both cases the extreme mutant phenotype was arrested larval molting with a prolongation of the first larval instar (class VI phenotype). Second, the ts loss of normal gene function appears to occur rapidly following transfer of animals to 31° and is not subject to long-term perdurance effects. Evidence for the rapid thermal inactivation of *dre4* gene function included both the inhibition of normal pupariation of $dre4^{e55}$ hemizygotes upshifted as early wandering larvae, and the virtually complete shut-down of ecdysteroid production by ring glands after two hours *in vitro*. These observations suggest that the $dre4^{e55}$ gene product becomes almost completely inactive within a maximum of several hours

after transfer to 31°. Furthermore, the results suggest that the *dre4* locus is not genetically complex. Neither interallelic complementation nor phenotypic pleiotropy has been observed among the many EMS- and γ-ray-induced mutants that have been characterized as to lethal stage (SLITER *et al.* 1989). We conclude that the pathologies observed at 31° in $dre4^{e55}$ hemizygotes probably represent a close approximation of the *dre4*-null phenotype.

**The regulation of Drosophila development by ecdysteroids:** The exposure of $dre4^{e55}$ hemizygotes to a high restrictive temperature may mimic the physiological effects of surgical extirpation of steroidogenic tissues at various developmental stages. For this reason the $dre4^{e55}$ mutation provides a potentially novel means for examining the developmental consequences of ecdysteroid deficiency during the Drosophila life cycle.

*Hormonal regulation of larval development:* The earliest overt indication of deranged development, the phenocritical period, in *dre4* mutant zygotes was the failure of first instar larvae to molt to the second instar. No indication was seen of impaired behavior, growth or viability of larvae within the normal time span of the first instar, and substantial prolongation of the first instar was observed in some mutant combinations. Similarly, $dre4^{e55}$ hemizygous escapers which successfully molted to the second instar in cultures reared continuously at 31° also exhibited normal behavior and growth for relatively prolonged periods but did not molt and showed no evidence of apolysis. These observations indicate that *dre4* mutations preferentially inhibit the transitions between larval instars, and may have little or no direct effect upon more general aspects of larval growth or behavior. The inhibition of molting in second instar larvae did not result in the arrest of imaginal disc growth at a corresponding stage. This is consistent with studies of imaginal disc growth following transplantation to adult hosts, which have shown that the growth of imaginal discs does not depend upon the discs experiencing the hormonal fluctuations associated with larval molting (HADORN and GARCIA-BELLIDO 1964).

The concept of a specific role for the *dre4* gene in the hormonal regulation of molting and metamorphosis is supported by the observation of the premature pupariation of molt-arrested second instar larvae when returned to permissive temperatures following several days at 31°. This is to our knowledge the only published report of precocious pupariation caused by a mutation of Drosophila, or, indeed, by any experimental manipulation of a cyclorrhaphous dipteran insect. In contrast, precocious metamorphosis can be elicited quite easily in most other insect species by the surgical extirpation of the JH-producing corpora allata from penultimate instar larvae (BOUNHIOL 1938). Removal of the corpora allata and the subsequent fall in JH levels leads to the premature reprogramming of epidermal cells in Lepidoptera and subsequent precocious metamorphosis (RIDDIFORD 1978). The precocious pupariation of second instar *dre4* mutant larvae represents an uncoupling of the normally closely linked processes of larval growth, larval molting and pupariation. It suggests that higher dipteran larvae like lepidopteran larvae exhibit developmental changes in the commitment of the epidermis to form larval cuticle or to tan in response to ecdysteroid stimulation. This is not genetically preprogrammed, but is probably under endocrine control, *e.g.*, changes in the JH titer (SLITER *et al.* 1987).

*Aspects of the hormonal regulation of metamorphosis:* Three peaks in the whole-body ecdysteroid titer during metamorphosis were observed, the appearance of which depended upon normal *dre4* gene function. A peak during prepupal development coincides with, and is probably responsible for, the appearance of a group of ecdysteroid-inducible polytene chromosome puffs in prepupal salivary glands, the late prepupal puffs (ASHBURNER *et al.* 1974). The role of ecdysteroids in regulating other aspects of Drosophila prepupal development are poorly understood. Our results suggest that the prepupal ecdysteroid peak may also be involved in regulating late prepupal morphogenetic movements involved with eversion of the imaginal head. Such a role may be analogous to the documented function of ecdysteroids in regulating morphogenetic changes at other times in development, most notably the eversion of imaginal discs following pupariation (FRISTROM, LOGAN and MURPHY 1973). Eversion of the pupal head is not a prerequisite for the later events of metamorphosis including adult differentiation, since the *cryptocephal* (*crc*) mutant of Drosophila fails to undergo head eversion but differentiates into otherwise morphologically normal adults (FRISTROM 1965).

The tissue source of ecdysteroids during pupal-adult development in Drosophila is unknown since the prothoracic gland degenerates and is inactive in pupae and pharate adults as determined by both physiological and ultrastructural criteria (DAI and GILBERT 1991). However, ring glands from Drosophila prepupae produce ecdysteroids *in vitro* (REDFERN 1983), and are probably the source of the peak of ecdysteroids detected 6–9 hr PP.

The pupal ecdysteroid peak is coincident with the initiation of adult differentiation. This peak has been described previously (RICHARDS 1981), and is known to be composed of a complex mixture of biological active ecdysteroids (20-hydroxyecdysone and makisterone A), and a variety of metabolites that may be biologically inactive but retain immunoreactivity with some anti-ecdysone antisera including the H22 anti-

serum used in our studies (PAK and GILBERT 1987). A role for this ecdysteroid peak in initiating adult differentiation has been assumed, but has never been demonstrated experimentally in Drosophila. Our results with $dre4^{e55}$ hemizygotes indicate that essentially normal differentiation of external adult cuticular structures can occur when the pupal-adult ecdysteroid peak is reduced by 40–50%. This same phenomenon has been observed in the lepidopteran *Manduca sexta* (SAKURAI, WARREN and GILBERT 1991), and suggests that ecdysteroid regulation of the metamorphosis of different cell types may be achieved in part through tissue-specific thresholds of ecdysteroid activation. In the case of epidermal cells, the threshold for initiation of adult development may be relatively low, as is the case at the time of pupariation when ecdysteroid levels reached a maximum level of 80–100 pg/mg. The peak level of ecdysteroids in pupae is 3–4 times this level, and may reflect a higher threshold requirement for ecdysteroids on the part of other, nonepidermal tissues.

A pharate adult ecdysteroid peak has been described previously only in morphologically staged animals (BAINBRIDGE and BOWNES 1988). Our ability to resolve this peak which has eluded several other investigators is probably due to a combination of measures undertaken to maximize the resolution of developmental changes. These measures included careful synchronization at white pupariation, maintaining animals in a constant temperature water baths which permitted greater reproducibility and constancy of rearing temperature, and assaying animals at close time intervals during development. The possible role of this pharate adult peak during metamorphosis remains unclear, but similar secondary peaks of ecdysteroids have also been reported in lepidopterans (WARREN and GILBERT 1986) and may regulate the terminal stages of adult differentiation. It may be possible to investigate the developmental consequences of elimination of this peak by transferring $dre4^{e55}$ hemizygotes to a high restrictive temperature just prior to the peak.

**Comparison of $dre4^{e55}$ with another Drosophila mutant affecting ecdysteroid production:** The $dre4^{e55}$ mutation is the second mutation of Drosophila shown to autonomously impair the biosynthesis of ecdysteroids by wandering-stage larval ring glands, the first being the ts lethal mutation $ecd^1$ (HENRICH *et al.* 1987) which like $dre4^{e55}$ causes nonpupariation at restrictive rearing temperatures. Despite these similarities, there are a number of differences in the detailed phenotypes of $dre4^{e55}$ and $ecd^1$ that indicate that the developmental roles of these two genes are not identical. (1) In larvae, the $ecd^1$ mutation causes marked cell death in imaginal discs, as evidenced by both histological criteria and by patterning abnormalities (deficiencies and duplications) in the adult derivatives of imaginal discs following short-term exposure (1–3 day) of larvae to a restrictive temperature (REDFERN and BOWNES 1983). In contrast, $dre4^{e55}$ had no discernible effect on larval imaginal discs at rearing temperatures that were restrictive for both larval molting and pupariation. (2) In prepupae, the $ecd^1$ mutation acts autonomously in imaginal discs to impair the development of adult sensory bristles, but does not prevent late prepupal events such as imaginal head eversion (SLITER 1989). However, $dre4^{e55}$ had no specific effect on bristle development, but did block head eversion. (3) During metamorphosis, $ecd^1$ does not prevent overall adult differentiation and has little or no effect on ecdysteroid levels during metamorphosis (MARSH and WRIGHT 1980). In contrast, $dre4^{e55}$ blocks the differentiation of adult cuticular structures, and depending upon rearing temperature, either severely reduces or completely eliminates the pupal-adult and pharate adult ecdysteroid peaks. Therefore, the $dre4^{e55}$ and $ecd^1$ mutations exhibit partially overlapping sets of developmental phenotypes. In contrast to $ecd^1$, $dre4^{e55}$ appears to be more endocrine-specific in that it has no apparent cell-lethal effects, but it causes ecdysteroid deficiency at developmental stages that are not affected by $ecd^1$.

**Possible roles of the *dre4* gene product:** Our results suggest strongly that the *dre4* gene product is expressed in cells of the larval prothoracic gland, and possibly other steroidogenic tissues of Drosophila that have yet to be identified, and that expression of the *dre4* gene is required for steroidogenesis. The ability of the loss-of-function $dre4^{e55}$ allele to prevent pupariation following a temperature upshift during the wandering period, and the autonomous suppression of ecdysteroid production by mutant ring glands cultured *in vitro* at a restrictive temperature, indicate that the *dre4* gene product plays a role in the physiology of the prothoracic gland directly related to ecdysteroid synthesis. Possible functions encoded by *dre4* would include an enzyme of the ecdysteroid biosynthetic pathway, a prothoracic gland-specific component of the PTTH signal transduction pathway, or some other structural or regulatory protein that is required to support the steroidogenic activities of the prothoracic gland.

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