Glue Secretion in the Drosophila Salivary Gland:
A Model for Steroid-Regulated Exocytosis

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Small hydrophobic hormones like steroids control many tissue-specific physiological responses in higher organisms. Hormone response is characterized by changes in gene expression, but the molecular details connecting target-gene transcription to the physiology of responding cells remain elusive. The salivary glands of Drosophila provide an ideal model system to investigate gaps in our knowledge, because exposure to the steroid 20-hydroxyecdysone (20E) leads to a robust regulated secretion of glue granules after a stereotypical pattern of puffs (activated 20E-regulated genes) forms on the polytene chromosomes. Here, we describe a convenient bioassay for glue secretion and use it to analyze mutants in components of the puffing hierarchy. We show that 20E mediates secretion through the EcR/USP receptor, and two early-gene products, the rbp1 function of BR-C and the Ca2+-binding protein E63-1, are involved. Furthermore, we demonstrate that 20E treatment of salivary glands leads to Ca2+ elevations by a genomic mechanism and that elevated Ca2+ levels are required for ectopically produced E63-1 to drive secretion. The results presented establish a connection between 20E exposure and changes in Ca2+ levels that are mediated by Ca2+-effector proteins, and thus establish a mechanistic framework for future studies. © 2001 Academic Press

Key Words: ecdysteroid response; glue secretion; green glue; genetic analysis; calcium changes; E63-1; steroid hormone; RNAi.

INTRODUCTION

Regulation of exocytosis by small hydrophobic hormones has been previously demonstrated in many vertebrate systems, including mediation of secretion by 1,25-dihydroxyvitamin D3 (Scharla et al., 1993; Shalita-Chesner et al., 1998), glucocorticoids (Thomas et al., 1996), thyroid hormone (Bonaterra et al., 1998; Hervas et al., 1975), estradiol (Kamel and Krey, 1982; Mahesh and Muldoon, 1987; Smith et al., 1984), and progesterone (Mahesh and Muldoon, 1987; Turgeon and Waring, 1990). These hormones exert their effects by binding to their cognate nuclear receptors to directly mediate changes in gene expression. In these systems, long-term hormone exposure (several minutes to hours) is required to stimulate exocytosis, implicating a genomic mechanism requiring de novo protein synthesis. In addition, changes in intracellular Ca2+ levels that accompany hormone exposure play an important role in modulating secretion (Ortmann et al., 1995; Thomas et al., 1996). Although changes in Ca2+ levels can be achieved within seconds by rapid nongenomic mechanisms, several studies have demonstrated that Ca2+ elevation depends on hormone-induced gene expression (Ritchie, 1993; Thomas et al., 1996; Tornquist and Tashjian, 1989). However, little is known about the molecular details connecting hormone exposure and the genomic responses leading to Ca2+ elevation and vesicle-mediated secretion.

In Drosophila melanogaster, a single steroid hormone, 20-hydroxyecdysone (20E), is capable of mediating major developmental changes such as the molting of larval cuticles and metamorphosis (reviewed in Riddiford, 1993). To date, many studies have focused on the pulse of 20E that occurs at the end of the third larval instar, 6 to 8 h before puparium formation (the premetamorphic pulse). This signal initiates metamorphosis and stimulates a stereotypic pattern of gene expression manifested as puffs on the giant
chromosomes of the salivary gland. A similar response can be recapitulated when glands are cultured in vitro with 20E (reviewed in Richards, 1997; Russell and Ashburner, 1996). Prior to hormone treatment, a few “intermolt” puffs that contain glue genes (encoding glycoproteins that adhere the animal to a substrate during metamorphosis) are activated. Within minutes of exposure these sites regress, and a small number (≤10) of “early” puffs containing primary-response genes begins to form. After several hours of hormone exposure, the early puffs regress and a large number (≥100) of “late” puffs containing secondary-response genes is induced. During the period between the maximum activity of the early and late puffs, another small set of loci, the “early-late” puffs, form (Ashburner and Richards, 1976).

The puffing hierarchy described above coincides with ecdysteroid-induced glue secretion (Boyd and Ashburner, 1977; Farkas and Sutakova, 1998). Furthermore, when glands are treated with a protein synthesis inhibitor, early puffs form but do not regress, early-late puffs are suboptimally induced, late puffs do not form at all, and secretion is completely blocked (Ashburner, 1974; Boyd and Ashburner, 1977). Thus, the emerging model suggests that one or more early or early-late proteins is needed to drive secretion either directly or indirectly through late-protein functions.

Our studies establish the link between 20E-stimulated gene expression and glue secretion. We functionally tested components of the puffing hierarchy by analyzing glue secretion in mutant backgrounds using flies expressing a glue-GFP vesicle content marker. Our results show that 20E acts through a genomic mechanism to regulate exocytosis and requires a functional ecdysteroid receptor consisting of EcR and USP. Also, after testing all the early and early-late genes that are thus far identified (BR-C, DHR3, E23, E63-1, E74, E75, E78), we find that the rpb4 mutation arising from the BR-C early locus delays secretion, suggesting that rpb4 is required for an optimal response. In addition, we can stimulate glue secretion independently of hormone, by ectopically inducing the E63-1 early protein (an EF hand family member), as long as the cytoplasmic levels of Ca²⁺ are elevated. This is consistent with our results showing that long-term culture of glands with 20E causes an elevation in Ca²⁺ levels prior to secretion. The work described here provides a framework for more extensive molecular dissections of this steroid-regulated, tissue-specific response.

**MATERIALS AND METHODS**

**Sgs-GFP Transgenic Flies**

The Sgs3 genomic clones aDm2023 and aDm2024 (Garfinkel et al., 1983) were used to make the SgsΔ3–GFP construct (Fig. 1). It contains 1.8 kb of the Sgs3’ regulatory information. The coding information for the carboxyl half of Sgs3 was replaced with an enhanced green fluorescent protein (GFP) gene from pGFP-N1 (Clontech, Palo Alto, CA), which was fused in frame with the addition of a linker. This insert (SgsΔ3–GFP) was cloned into the pCaSpeR-4 vector (Thummel and Pirrotta, 1992) and used to generate transformed flies as reported elsewhere (Andres and Cherbas, 1994; Robertson et al., 1988).

**Developmental Analysis of SgsΔ3–GFP Expression and Protein Localization**

Two cohorts of animals (w1118 and w1118, SgsGFP-2) were grown under identical conditions. Larvae were synchronized at hatching, and 100 animals of each genotype were seeded into vials containing 10 ml of hard agar molasses coated with a paste of baker’s yeast. Beginning at the second to third-instar molt (72 h after egg laying (AEL)), larvae were harvested at 2-h intervals until 50% pupariated (120 h AEL). At each time point, animals were examined under a fluorescence microscope and scored for SgsΔ3–GFP accumulation in the salivary glands. RNA was then isolated from the same animals and used for Northern analysis.

Prepupae were resynchronized at pupariation (0 h) and staged relative to this point. Because SgsΔ3–GFP is expelled at pupariation, its localization was not analyzed in prepupal samples.

Blots containing 10 μg of total RNA were prepared and hybridized as reported elsewhere (Andres et al., 1993). They were probed sequentially (after being stripped) for Sgs3, the GFP tag, and the rp49 loading control. DNA probes were labeled using Stratagene’s Prime-It II Kit (Stratagene, La Jolla, CA).

**Drosophila Mutant Analyses**

All flies were reared on standard cornmeal-molasses medium and were obtained from the Bloomington Stock Center, except the following: BR-C mutants from C. Bayer and L. Restifo; (I)ecd mutants from V. Henrich; EcR mutants from M. Bender; DHR3, DHR78, E74, and usp mutants from C. Thummel; and E23 and E75 mutants from D. Garcia. Transgenic flies carrying Apoaepoquin were obtained from J. Dow.

To monitor glue synthesis or secretion in mutants, animals carrying one copy of SgsΔ3–GFP and a mutant allele over either a deficiency or a strong loss-of-function allele of the locus, were assayed. usp and DHR3 early-lethal mutants were rescued to puparium formation using transgenes containing wild-type coding information under the control of an inducible [heat shock protein 70 (hsp70)] promoter as previously described (Hall and Thummel, 1998; Lam et al., 1999).

**Assaying Secretion in 20E-Treated Glands**

The physiological concentration of the premetamorphic pulse of ecdysteroids, as measured by radioimmunoassays (RIA) on carefully staged, wild-type larvae, is reported to be 890 ng of 20E equivalents per gram of fresh weight (−2 × 10⁻⁶ M) (Klose et al., 1980). A hormone concentration on that order of magnitude (5 × 10⁻⁶ M) will induce the maximal puffing response in cultured third-instar salivary glands (Ashburner, 1972), as well as induce secretion of glue in the gland (Farkas and Sutakova, 1998). Thus all 20E treatments in this study were performed at 5 × 10⁻⁶ M.

To inject larvae with exogenous 20E, a stock solution (5 × 10⁻⁵ M) of 20E (Sigma, St. Louis, MO) was prepared in sterile DPBS (Wünsch et al., 1993), and 0.1 μl was injected into larvae (the volume of a third-instar larva is estimated at 1 μl) using a previously reported procedure (Anderson et al., 1966).

To assay secretion in vitro, several pairs of glands (5–10) were dissected and transferred to depression slides containing 30 μl of
Schneider’s medium (Sigma) (adjusted to 1.0 mM CaCl₂, pH 7.2) with or without 20E. Incubations were carried out in dark humidified chambers at 22°C. After 4–6 h, secretion was scored on a fluorescence microscope. It should be noted that the physiological concentration of Ca²⁺ in larval hemolymph is reported to be approximately 1 mM (Schneider et al., 1996; Stewart et al., 1994). We estimated that the intracellular concentration of Ca²⁺ in unstimulated cells of the salivary gland is approximately 100 nM (A. Biyasheva and A. J. Andres, unpublished observation).

For confocal imaging, glands were fixed and mounted as reported previously (Vaskova et al., 2000). Z-sections were collected on a Zeiss Axiovert 100 M confocal microscope system (Zeiss, Thornwood, NY).

**RNA Interference (RNAi)**

Templates for RNAi were cloned into Bluescript (Stratagene) vectors, which have T3 and T7 promoters flanking the insert. For EcR-B1-specific RNAi, a 1.6-kb EcoRI/SacI fragment from pMK1 (Koele et al., 1991) was used. RNA common to all three EcR isoforms was generated from a 1.4-kb Accl/EcoRI fragment from the 3’ end of pMK1. For usp, a 1.4-kb BamHI/EcoRI fragment from the 3’ end of the cDNA clone pZ7-1 (Henrich et al., 1990) was used. An E63-1C (1.2-kb) insert was used as a negative control (Andres and Thummel, 1995). Templates for RNA synthesis were prepared by PCR using T3 and T7 DNA primers. RNA was synthesized (in separate reactions) using Stratagene's RNA Transcription Kit. RNA strands were annealed as previously described (Fire et al., 1998).

To load double-stranded RNA into cells by pinocytosis, dissected glands were placed in 10 µl of Influx Reagent (Molecular Probes, Eugene, OR) containing RNA (0.2 mg/ml). After 10 min, old medium was replaced with a hypotonic solution (6.4 dilution of Schneider’s medium;water) to lyse pinocytic vesicles, releasing RNA into the cytoplasm (Okada and Rechsteiner, 1982). After 2 min, glands were placed in fresh Schneider’s medium and incubated for 1 h before 20E treatment. Secretion was assayed 4–6 h later.

**Assaying Ca²⁺ Changes in Response to Hormone**

The photoprotein Aequorin was used to monitor qualitative changes in intracellular Ca²⁺ concentrations after 20E exposure. All salivary glands used in these experiments are from larvae raised on medium supplemented with bromphenol blue to aid in the selection of naive (those that have not yet been exposed to the endogenous metamorphic pulse of 20E) animals (Andres and Thummel, 1994; Maroni and Stamey, 1983). For luminescence measurements in single salivary glands, hs-Apoequorin larvae (w¹¹¹⁸; P[w’, UAS₅₉-aeq49A]/++; P[w’, hs-Gα₄]/+) (Brand et al., 1994; Rosay et al., 1997) were dissected. Glands were heat shocked (37°C, 1 h) and incubated in the dark (22°C, 1 h) in DPBS containing 2.5 µM Coelenterazine-n (Molecular Probes), under a saturating atmosphere of O₂, to reconstitute the active Aequorin complex. Each sample was treated with 20E and placed in a Monolight 2010 luminometer (Analytical Luminescence, San Diego, CA). Luminescence measurements were taken every 100–300 s for 5 h. At the end of the experiment, glands were lysed with 1% Triton X-100/100 mM CaCl₂, to measure the remaining active Aequorin. The amount of Aequorin consumed during each experiment was <2% of that reconstituted.

A modified Aequorin assay was used to compare functional complex remaining after 20E treatment. In these experiments, glands were dissected from SG-Apoequorin larvae (w¹¹¹⁸; P[w’, UAS₅₉-aeq49A]/P[w’, GawB]₃₄). The P[w’, GawB]₃₄ construct drives high-level expression of Gα₄ in the salivary glands and lower level expression in other tissues (A. Brand, unpublished observation). Ten pairs of glands were incubated with Coelenterazine as described above. Sister lobes were then separated into two lots to generate samples of identical physiological states. One lot was left untreated and the other was treated with 20E. Following a set incubation time, the samples were lysed and the relative rise in luminescence (ΔL = L₉₀ - L₀) was measured. An R value (ΔL/ΔL₀ = L₀) was calculated for each time point to normalize differences among pool samples resulting from variations in Aequorin expression, reconstitution efficiency, and gland size.
Ectopic Expression of E63-1

The E63-1 open reading frame (Andres and Thummel, 1995) was cloned into a pCaSpeR–hs-act vector (Thummel and Pirrotta, 1992), placing it under control of the hsp70 promoter (hsE63-1). This construct was used to generate a homozygous viable line with an insert on the second and third chromosomes (P[w<sup>1188</sup>, hsE63-1]; P[w<sup>1188</sup>, hsE63-1]). Salivary glands naive to ecdysteroids were dissected from SgsGFP-2/hsE63-1<sup>+/+</sup> and SgsGFP-2/+/+ control animals. Glands were heat shocked (37°C, 1 h) in Schneider’s medium, allowed to recover (22°C, 1.5 h), and transferred to medium containing the Ca<sup>2+</sup> ionophores A23187 (1 μM; Calbiochem, San Diego, CA) or ionomycin (1 μM; Calbiochem). Secretion was assayed after a 10- to 24-h incubation period.

RESULTS

Construction of Transgenic Flies That Produce “Green Glue”

The Sgs3 gene encodes one of a few (<10) glycoproteins that comprise the glue mix (reviewed in Lehmann, 1996; Meyerowitz et al., 1987). Previous work has shown that Sgs3 is expressed only in salivary glands of third-instar larvae, beginning at mid instar and continuing until pupariation, when transcript levels drop precipitously (Andres et al., 1993; Huet et al., 1993; Meyerowitz and Hogness, 1982). During the second half of the third instar, glue genes are
expressed at very high levels with Sgs3 comprising as much as 5–10% of the total protein of the gland (Korge, 1977). The regulatory elements necessary for the stage-specific, tissue-specific, and high-level expression pattern of Sgs3 were previously mapped (Crosby and Meyerowitz, 1986; Giangrande et al., 1987; Lehmann et al., 1997). We constructed a chimeric gene (Sgs\(\Delta 3\)-GFP) that contains this 5’ regulatory information, as well as half of the coding sequence for Sgs3 fused to an enhanced GFP tag (Cormack et al., 1996) (Fig. 1). Three homozygous viable lines, SgsGFP-1 (inserted on X), SgsGFP-2 (chromosome 2), and SgsGFP-3 (chromosome 3), were used in this work. All three lines are similar to wild-type animals in terms of development and fertility, and all three express Sgs\(\Delta 3\)-GFP at puparium formation (data not shown). The SgsGFP-2 line was analyzed in detail not only to ensure that the fusion construct is expressed in a pattern similar to the endogenous gene, but also to confirm that the chimeric protein can be used to monitor glue synthesis, secretion, and expectoration during development.

Consistent with previous reports (Andres et al., 1993; Huet et al., 1993), Northern analysis shows that the endogenous Sgs3 gene is expressed during the second half of the third instar, and mRNA levels are nearly undetectable after pupariation (Fig. 2A). The pattern of Sgs\(\Delta 3\)-GFP expression is identical to that of the endogenous gene. Furthermore, the chromophore is functional and can be used as a convenient marker for glue protein in living animals, allowing one to correlate the patterns of Sgs\(\Delta 3\)-GFP localization with third-instar larval development.

As illustrated in the representative drawings of the salivary glands (Fig. 2B), the polyplid cells of the tissue can be divided into three groups with respect to their ability to synthesize glue. The small, anterior-most cells that surround the ducts (Fig. 2B, d cells) do not produce glue. The transition cells (Fig. 2B, t cells) accumulate granules, but glue synthesis is delayed relative to the posterior corpus cells (Fig. 2B, c cells), which are the first to synthesize glue (reviewed in Berendes and Ashburner, 1978). These patterns are also observed when Sgs\(\Delta 3\)-GFP localization is analyzed in living larvae (Fig. 2B, photos of dissected salivary glands).

Thus, there appear to be five distinct patterns of Sgs\(\Delta 3\)-GFP accumulation in the salivary glands of developing larvae. Dissected glands from these different stages are depicted in Fig. 2B, with a drawing of the salivary gland and the percentage of animals representing each time point. In early-third-instar larvae (72–98 h AEL), animals have no detectable Sgs\(\Delta 3\)-GFP in their glands. From 102–112 h AEL, more than 50% of the animals display a pattern in which Sgs\(\Delta 3\)-GFP is detected predominantly in the posterior gland (corpus) cells. At 114–118 h AEL, most animals have glands in which both corpus and transition cells are filled with Sgs\(\Delta 3\)-GFP, but secretion has not occurred (Sgs\(\Delta 3\)-GFP not detected in lumen and ducts). Only animals a few hours away from pupariation (118–120 h AEL) contain Sgs\(\Delta 3\)-GFP in the ducts and lumen, indicating they have secreted glue (Fig. 2B). A fifth stage (Fig. 2B, bottom) is at puparium formation when Sgs\(\Delta 3\)-GFP is expelled (expectorated).

Three additional points about this analysis are noted: First, although Sgs\(\Delta 3\)-GFP is initially detected only in corpus cells, fluorescent proteins accumulate in the transition cells from undetectable levels to concentrations similar to those in corpus cells. Thus, the “half-gland” pattern (Fig. 2B, second row) is one in which the fluorescence intensity of Sgs\(\Delta 3\)-GFP in the transition cells is less than that detected in the corpus cells. Second, by monitoring individual larvae from molting to pupariation, we observe that the stages we have outlined are progressive (i.e., Sgs\(\Delta 3\)-GFP accumulates in corpus cells first, and eventually the transition cells accumulate similar levels of protein before secretion occurs). Finally, we propose that the high degree of variability in the data presented in Fig. 2B (e.g., 20% of the animals at 114 h AEL show no Sgs\(\Delta 3\)-GFP accumulation) is a reflection of the limitations of synchronizing larvae at hatching. We have found that selecting animals based on the pattern of Sgs\(\Delta 3\)-GFP accumulation in their salivary glands is a much more reliable method for choosing developmentally similar animals. For example, one can distinguish between early-third-instar larvae, in which the glue genes are not expressed, from older third-instar larvae, in which they are.

To ensure that the Sgs\(\Delta 3\)-GFP detected in the salivary glands is localized to secretory granules within cells, confocal images taken before and after secretion are shown (Fig. 3). Before secretion, optical sections at the surface (Fig. 3A) and through the middle (Fig. 3B) of the same gland show large granules containing Sgs\(\Delta 3\)-GFP evenly distributed throughout the cytoplasm. After secretion, surface (Fig. 3C) and midgland sections (Fig. 3D) illustrate a slight asynchrony of cells within the same tissue. One cell (n) still contains many granules, while an adjacent cell (s) has secreted most of them (Fig. 3C, arrows). However, it is evident that secretion has occurred in many cells when an optical section through the middle of the gland is examined, since Sgs\(\Delta 3\)-GFP is easily visible in the lumen as a large glue plug (Fig. 3D). It should be noted that by pupariation formation, all cells within a gland usually have secreted. These same ultrastructural observations (changes in the size and localization of granules, asynchrony of cells within secreting glands, granules remaining after secretion) were previously noted when electron micrographs of secreting glands were examined (Farkas and Sutakova, 1998; Lane and Carter, 1972; von Gaudecker and Schmale, 1974). In addition, one might expect the endoplasmic reticulum (ER) and Golgi to display fluorescence from Sgs\(\Delta 3\)-GFP routed through the secretory apparatus. Although this pattern is difficult to observe in wild-type cells because of the intense fluorescence of the large number of granules within a cell, it is clear in cells carrying a dominant-negative construct that antagonizes the function of an N-ethylmaleimide-sensitive fusion (DN SF-2) protein necessary for ER–Golgi traffic (Boulianne and Trimble, 1995). In these cells, Sgs\(\Delta 3\)-GFP is...
Glue Secretion in Drosophila

Glue Secretion Is Induced by 20E in Vivo and Requires a Functional EcR/USP Receptor Complex

To ensure that Sgs\(_\Delta3\)-GFP secretion is 20E-inducible, as was previously shown for endogenous glue proteins (Boyd and Ashburner, 1977; Farkas and Sutakova, 1998; Zhimulev and Kolesnikov, 1975), we dissected salivary glands from animals competent to respond to ecdysteroids (~110 h AEL) and treated them with a physiological concentration of 20E.

Bright-field (Fig. 4A) and fluorescence (Fig. 4B) images at the time of hormone addition show Sgs\(_\Delta3\)-GFP-containing granules within cells. The same gland was photographed 6 h later (Figs. 4C and 4D) when the fusion protein is detected in the lumen as secreted glue. Under these conditions, untreated glands do not secrete, as they display the same pattern of Sgs\(_\Delta3\)-GFP localization within cells (Fig. 4B), even after 20 h in culture. Also, these patterns are highly reproducible and were observed in over 200 dissected glands.

Next, we tested whether 20E is required for Sgs\(_\Delta3\)-GFP secretion in vivo by assaying secretion in I(3)ecd\(^2\) mutants. These animals carry a pleiotropic, temperature-sensitive mutation that severely compromises their ability to produce ecdysteroids (Garen et al., 1977; Henrich et al., 1987; Redfern and Bownes, 1983) and initiate hormone-signaling programs at the nonpermissive temperature (Clark et al., 1986; Lepesant et al., 1978; Murtha and Cavener, 1989; Sliter, 1989). When shifted to the nonpermissive temperature (29°C) after the second- to third-instar molt, I(3)ecd\(^2\) larvae remain as third instars for up to 15 days, eventually forming stationary pseudopupae. However, feeding or injecting them with 20E induces pupariation and partially rescues ecdysteroid signaling defects.

Analysis of I(3)ecd\(^2\) mutants shows that secretion of Sgs\(_\Delta3\)-GFP fails to occur at the nonpermissive temperature (Table 2, Fig. 5C). This observation is consistent with the in vitro data presented above and supports the hypothesis that 20E is necessary for glue secretion. To determine whether the failure to secrete is the result of ablation of the ecdysteroid signal, we attempted to rescue this defect with exogenous estrogen. As a control for exogenous hormone treatment, we injected 20E into wild-type animals displaying Sgs\(_\Delta3\)-GFP accumulation in transition and corpus cells (~110–118 h AEL). As shown in Table 1, 100% of 20E-injected wild-type animals (SgsGFP-2; +) secrete Sgs\(_\Delta3\)-GFP within 3 h of treatment, compared with only 22% for the saline-injected parental controls. Note that some saline-injected animals are expected to secrete in response to the endogenous hormone pulse. In contrast, when I(3)ecd\(^2\) larvae (raised at 29°C for 5 days after the molt to the third instar) are injected with 20E, 83% secrete glue by 8 h, compared to 0% of saline-injected controls. These results demonstrate that 20E can induce glue secretion in vivo.

After confirming that 20E can stimulate Sgs\(_\Delta3\)-GFP secretion, we investigated the possibility that it acts through the known 20E receptor to mediate the response. The only functional receptor thus far identified is a heterodimer consisting of EcR and the RXR family member USP (Koelle et al., 1991; Oro et al., 1992; Thomas et al., 1993; Yao et al., 1993). Three EcR protein isoforms have been identified (EcR-A, EcR-B1, and EcR-B2), with EcR-B1 the predominant form expressed in larval salivary glands (Talbot et al., 1993). usp gives rise to only one protein that is ubiquitously expressed (Henrich et al., 1994).

Loss-of-function mutations were generated for both receptor components. For EcR, three classes of mutations are reported (Bender et al., 1997; Schubiger et al., 1998), but only EcR-B1 mutants survive to the prepupal stage when glue secretion can be assayed. In our hands, animals lacking both EcR-B1 and EcR-B2 (Schubiger et al., 1998) die before glue secretion; however, a few animals live long enough to synthesize Sgs\(_\Delta3\)-GFP (see Discussion).

For usp, genetic null mutants die during early larval stages (Perrimon et al., 1985), but will survive until the prepupal period if a pulse of USP protein is provided (via a heat-shock transgene) during embryogenesis (Hall and Thummel, 1998; Oro et al., 1992).

We examined Sgs\(_\Delta3\)-GFP secretion in EcR and usp mutants, and we found that neither mutant extrudes the fusion protein at pupariation (Table 2). However, when mutant glands are dissected and analyzed, a small amount of Sgs\(_\Delta3\)-GFP is detected at the anterior end of the lumen (Figs. 5D and 5E, closed arrowheads, arrow). This may reflect constitutive, 20E-independent secretion. Alternatively, this secretion may result from the presence of other EcR isoforms or leaky hs-usp expression. We favor the latter explanation, as secretion is completely ablated in I(3)ecd\(^2\) mutants (Table 2, Fig. 5C).

To distinguish between these possibilities, we treated salivary glands dissected from wild-type animals with interfering double-stranded RNA (RNAi) directed against EcR and usp. RNAi was previously shown to selectively inhibit gene expression in C. elegans and Drosophila (Fire et al., 1998; Kennerdell and Carthew, 1998). Furthermore, we simplified the procedure by using a commercially available kit for introducing large water-soluble molecules into dissected tissues by pinocytosis.

When salivary glands are treated with RNAi directed against the 5’ end of EcR-B1, a pattern of secretion similar to that detected in EcR-B1 mutants is observed (Table 2, Fig. 5F). However, when RNAi against either the common region of EcR or the 3’ end of usp is performed, no Sgs\(_\Delta3\)-GFP secretion is observed after 20E treatment (Figs. 5G and 5H). Since mutants with an E63-1 deletion secrete glue normally (see below), wild-type glands were treated with double-stranded RNA against E63-1 as a control. As
FIG. 3. Confocal images of glue granules before and after SgsΔ3-GFP secretion. All images are at the same magnification; the white scale bar in (A) represents 50 μm. Optical sections at the surface (A) and center (B) of a gland that has not secreted (lumen is empty). Note the appearance of large granules evenly distributed throughout the cytoplasm. Nuclei are also indicated (red Ns). Optical sections at the surface (C) and center (D) of a secreting gland (lumen contains a large glue plug). Note the asynchronous secretion pattern with one nonsecreted cell (n) adjacent to one that has fully secreted (s), but still retaining some granules (red arrows). Also note the enlarged size of the granules in (n) as compared to those in (A) and (B). The larger size probably reflects granule fusion. These observations are in agreement with reports using fixed material in which periodic acid–Shiff base (PAS)-stained granules are assumed to contain glue (Berendes and Ashburner, 1978; von Gaudecker and Schmale, 1974).

FIG. 4. 20E stimulates SgsΔ3-GFP secretion in vitro. Salivary glands were dissected from naïve animals (not yet exposed to the endogenous premetamorphic pulse of hormone) and treated in vitro with 20E. The scale bar in (A) represents 200 μm. Presented is a bright-field (A) and fluorescence image (B) of a gland at the time of hormone addition. Note that SgsΔ3-GFP is intracellular and cytoplasmic (nuclei do not fluoresce). (C and D) Photographs of the same gland 6 h after adding 20E. Note the location of SgsΔ3-GFP within the glue plug of the lumen.
expected, these glands secrete normally after 20E treatment (data not shown). Thus, glue secretion is entirely dependent on a functional receptor consisting of EcR (one of three possible isoforms) and USP.

Mutations in Early-Gene Products Affect the Timing of Glue Secretion

It has been previously proposed that the products of the salivary gland ecdysteroid-responsive puffs regulate glue granule secretion (Boyd and Ashburner, 1977). If this hypothesis is correct, mutations in the early or early-late genes should prevent secretion because they either directly mediate the process or regulate late effector genes. This model (presented in Fig. 6) is tested in the experiments presented below.

To date, five early genes have been cloned and molecularly characterized (Fig. 6). As with all puff genes throughout this work, they are indicated by their more conventional names [FlyBase (http://flybase.bio.indiana.edu) names are indicated in parentheses]. They include the DNA-binding proteins of the BR-C (br) encoded by the br+, rbp+, and 2Bc functions (Bayer et al., 1997; Chao and Guild, 1986; DiBello et al., 1991); E74 (Eip74EF) encoding E74A and E74B (Burtis et al., 1990); and E75 (Eip75B), which gives rise to E75A, E75B, and E75C (Segraves, 1988; Segraves and Hogness, 1990). The other early genes are E23 (Eip23E), which encodes an ATP-binding cassette (ABC) transporter family member (Hock et al., 2000), and E63-1 (Eip63F-1), which encodes a high-affinity, calcium-binding protein (Andres and Thummel, 1995). In all cases, mRNAs from these genes are directly induced by 20E in the salivary gland, and nonlethal or late-lethal, loss-of-function mutations exist that allow glue secretion to be assayed (see Table 2 for a description of specific mutant alleles).

In addition, three early-late products were previously characterized at the molecular level (Fig. 6C). These include DHR3 (Hr46) (Koelle et al., 1992), E78 (Eip78C) (Stone

### TABLE 1

**20E Stimulates Sgs3–GFP Secretion in Vivo**

<table>
<thead>
<tr>
<th>Injection treatment</th>
<th>Genotype</th>
<th>Number secreting (%) after injection</th>
<th>Total injected</th>
</tr>
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<tbody>
<tr>
<td>Saline</td>
<td>SgsGFP-2; +</td>
<td>0 (0)  6 (22.2)  14 (51.9)  24 (88.9)</td>
<td>27</td>
</tr>
<tr>
<td>20E</td>
<td>SgsGFP-2; +</td>
<td>0 (0)  20 (100)  20 (100)  20 (100)</td>
<td>20</td>
</tr>
<tr>
<td>Saline</td>
<td>SgsGFP-2; l(3)ecd1</td>
<td>0 (0)  0 (0)  0 (0)  0 (0)</td>
<td>25</td>
</tr>
<tr>
<td>20E</td>
<td>SgsGFP-2; l(3)ecd1</td>
<td>0 (0)  5 (20.8)  16 (66.7) 20 (83.3)</td>
<td>24</td>
</tr>
</tbody>
</table>

Note. Animals were injected with either saline or 20E and scored for secretion (Sgs3–GFP in the lumen), by the times indicated.

![FIG. 5. Examples of Sgs3–GFP secretion in mutants of the 20E puffing hierarchy. Salivary glands were dissected at puparium formation (except in A, F, G, H, I) when glue should be secreted. Genotypes (see Table 2 for details) are indicated above each photo. Asterisks mark the outside border of the gland in samples where cells are not easily visualized because of the bright fluorescent plug of Sgs3–GFP. Closed arrowheads mark the position of lumens containing Sgs3–GFP (secretion), while open arrowheads mark the position of empty lumens (no secretion). All photos are at the same magnification; the white scale bar in (A) represents 50 μm. Note the variability in the size of glands from different mutants. Also note the reduction in the amount of Sgs3–GFP produced in the duct (arrow) and lumen. The pattern of secretion in wild-type transgene (Dhs-usp). Note the appearance of a small amount of Sgs3–GFP (secretion), while open arrowheads mark the position of lumens containing Sgs3–GFP in the lumen similar to the (Dhs-usp). Note the absence of Sgs3–GFP in the lumen after hormone treatment. (A) An rbp mutant gland. Although no Sgs3–GFP is detected in the lumen, granules do accumulate at the apical membranes (white arrowheads). These eventually become secreted during the prepupal period. (J) A dissected rbp mutant gland incubated with 20E in vitro. After 6 h of treatment, Sgs3–GFP is secreted into the lumen. (K) A br mutant gland. Secretion is complete. (L) A Bc mutant gland. Secretion is complete, but most animals do not extrude glue at pupariation (Table 2). (M) An E74A mutant gland. Secretion is complete, and animals expel glue normally. (N) An E75A mutant gland. Animals secrete and expel glue normally, despite the fact that many pupariate after the second-instar stage. (O) An E63-1 mutant gland. Secretion is complete.

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Glue Secretion in Drosophila

TABLE 2
Effects of Loss-of-Function Mutations on Glue Secretion

<table>
<thead>
<tr>
<th>Type of gene</th>
<th>Type of allele</th>
<th>Genotype assayed</th>
<th>SgsΔ3-GFP synthesized</th>
<th>SgsΔ3-GFP secreted</th>
<th>In vivo</th>
<th>In vitro</th>
<th>Genotype reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type parent</td>
<td>SgsGFP-1</td>
<td>y w SgsGFP-1; +/+</td>
<td>Yes</td>
<td>100 (25)</td>
<td>+</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>SgsGFP-2</td>
<td>w; SgsGFP-2; +/+</td>
<td>Yes</td>
<td>100 (25)</td>
<td>+</td>
<td>This work</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SgsGFP-3</td>
<td>w; +/SgsGFP-3</td>
<td>Yes</td>
<td>100 (25)</td>
<td>+</td>
<td>This work</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SgsGFP-2</td>
<td>y w; SgsGFP-2; +/+</td>
<td>Yes</td>
<td>100 (25)</td>
<td>+</td>
<td>This work</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20E receptor Ecd ts conditional</td>
<td>Yes</td>
<td>0 (25)</td>
<td>–</td>
<td>60 (10)</td>
<td>Henrich et al., 1987</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECR-B1</td>
<td>y w SgsGFP-2; +/y; EcR&lt;sup&gt;198&lt;/sup&gt;EcR&lt;sup&gt;R384M&lt;/sup&gt;</td>
<td>Yes</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Schlüter et al., 1998</td>
<td></td>
</tr>
<tr>
<td>usp conditional</td>
<td>usp&lt;sup&gt;ty2&lt;/sup&gt;; SgsGFP-2; +/; usp&lt;sup&gt;-3&lt;/sup&gt;-RNAi</td>
<td>Yes</td>
<td>0 (108)</td>
<td>±</td>
<td>67 (9)*</td>
<td>Hall and Thummel, 1998</td>
<td></td>
</tr>
<tr>
<td>compromised usp</td>
<td>w; SgsGFP-2; +/; usp&lt;sup&gt;-3&lt;/sup&gt;-RNAi</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0 (5)</td>
<td>Henrich et al., 1990</td>
<td></td>
</tr>
<tr>
<td>compromised Ecr-B1</td>
<td>w; SgsGFP-2; +/; Ecr-B1-RNAi</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>67 (3)*</td>
<td>Koelle et al., 1991</td>
<td></td>
</tr>
<tr>
<td>compromised Ecr</td>
<td>w; SgsGFP-2; +/; Ecr-common-RNAi</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0 (29)</td>
<td>Koelle et al., 1991</td>
<td></td>
</tr>
<tr>
<td>Orphan receptor DHR78</td>
<td>amorph</td>
<td>Yes</td>
<td>100 (25)</td>
<td>+</td>
<td>Fisk and Thummel, 1998</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early gene BR-C</td>
<td>amorph</td>
<td>y npr&lt;sup&gt;1/2&lt;/sup&gt;y; SgsGFP-2; +/+</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Kiss et al., 1988</td>
</tr>
<tr>
<td>rbp strong</td>
<td>y rbp&lt;sup&gt;1/2&lt;/sup&gt;y; SgsGFP-2; +/+</td>
<td>Yes</td>
<td>0 (48)</td>
<td>Delayed</td>
<td>100 (20)</td>
<td>Kiss et al., 1988</td>
<td></td>
</tr>
</tbody>
</table>

* Expectoration (glue extrusion from the gland) was used as a primary screen for secretion. Indicated are the percentage of animals that expelled SgsΔ3-GFP and the number that was assayed (parentheses).

* Animals defective for SgsΔ3-GFP expulsion were examined more carefully (salivary glands were dissected) for secretion defects in vivo: “+” = SgsΔ3-GFP fills lumen, “−” = no SgsΔ3-GFP in lumen, “±” = partial secretion of SgsΔ3-GFP into lumen and ducts. NA indicates that the sample could not be assayed because SgsΔ3-GFP was not expressed, mutants died before they secreted, or the sample was treated in vitro with RNAi after SgsΔ3-GFP was synthesized.

* Salivary glands were also dissected from animals that did not expel glue and were treated in vitro with 20E for 6 h. The percentage that secreted and the number assayed (parentheses) are presented. An asterisk ( *) indicates that the secretion was only partial.

Thummel, 1993), and E75B arising from the E75 transcription unit (Segraves and Hogness, 1990; White et al., 1997). The early-late genes are directly induced by the premetamorphic pulse of ecdysteroids in the salivary gland, but their maximal expression depends on de novo protein synthesis. Nonlethal or conditionally late-lethal mutations (where early lethality is rescued by a heat-shock transgene) in these genes also exist (Table 2).

To test whether the early and early-late genes are necessary for secretion, we examined larvae with an SgsΔ3-GFP transgene. Molecular summary of the 20E-regulated genetic hierarchy. (A) Summary of developmental hallmarks and predicted endogenous 20E peaks during third-instar and prepupal development. While the premetamorphic and prepupal hormone peaks have been well documented, the existence, number, and size of the mid-third-instar peak(s) are controversial (reviewed in Andres et al., 1993; Richards, 1981). (B) List of ecdysteroid-receptor components and early genes (BR-C, E74B) induced when glue genes, including SgsΔ3-GFP, are first expressed. Their role in SgsΔ3-GFP induction was tested in this study using loss-of-function mutants. (C) List of the genes directly regulated in the salivary gland by the premetamorphic ecdysteroid pulse. All were tested in this study by monitoring the effects of loss-of-function mutations on SgsΔ3-GFP secretion (see text for details).

FIG. 6. Molecular summary of the 20E-regulated genetic hierarchy. (A) Summary of developmental hallmarks and predicted endogenous 20E peaks during third-instar and prepupal development. While the premetamorphic and prepupal hormone peaks have been well documented, the existence, number, and size of the mid-third-instar peak(s) are controversial (reviewed in Andres et al., 1993; Richards, 1981). (B) List of ecdysteroid-receptor components and early genes (BR-C, E74B) induced when glue genes, including SgsΔ3-GFP, are first expressed. Their role in SgsΔ3-GFP induction was tested in this study using loss-of-function mutants. (C) List of the genes directly regulated in the salivary gland by the premetamorphic ecdysteroid pulse. All were tested in this study by monitoring the effects of loss-of-function mutations on SgsΔ3-GFP secretion (see text for details).
In Vitro Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Insc-E63-1 Transgene</th>
<th>Heat Shock</th>
<th>20E</th>
<th>Calcium</th>
<th>Secretion Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>99 (100)</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>0 (8)</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>0 (20)</td>
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<tr>
<td>4</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>100 (10)</td>
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<td>5</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>0 (10)</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>0 (8)</td>
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<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>100 (250)</td>
</tr>
</tbody>
</table>

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transgene in a background amorphic (null) or severely hypomorphic for each 20E-responsive gene described above. Animals were initially examined at puparium formation for expectoration, assuming that expelling Sgs3–GFP requires that it be properly secreted. Animals with Sgs3–GFP on the outside of the pupal case were scored as wild-type for secretion (Table 2). Animals defective in glue extrusion were more closely examined to ascertain whether defects were the result of an inability to synthesize, secrete, or expel Sgs3–GFP from the lumen. Although some mutants of 20E-responsive genes are clearly unable to expel Sgs3–GFP (Table 2), closer examination shows they secrete normally (E74B and 2Bc, Fig. 5L). Interestingly, secretion in rbp mutants appears to be delayed since glue granules accumulate at the apical membrane, but are not secreted at the time of pupariation (Table 2, Fig. 5I, white arrowheads). Secretion of granules does eventually occur, and Sgs3–GFP can be detected in the lumen of prepupal animals (data not shown). In addition, secretion is induced when rbp larval glands are treated with 20E in vitro (Table 2, Fig. 5J). Taken together, these observations suggest that, although rbp1 function may be involved in glue secretion, it is not essential, and at most plays a partially redundant role. Finally, npr1 mutants (missing all BR-C functions) fail to produce Sgs3–GFP. This result is consistent with previous reports that these mutants fail to express Sgs3 and other glue genes (Guay and Guild, 1991; Karim et al., 1993). However, because Sgs3–GFP is not expressed in these mutants, it is not possible to assay secretion in npr1 animals using the tools that are currently available.

Mutations in br, DHR3, E23, E63-1, E74A, E75A, E75B, E75C, and E78 do not appear to affect 20E-stimulated secretion (Table 2; Figs. 5K, 5M–5O).

Ectopic Expression of E63-1 Drives Glue Secretion
When Intracellular Levels of Ca\(^{2+}\) Are Elevated

Since the data with rbp mutants suggest that there may be functional redundancy among the early and early-late genes, it may be necessary to eliminate more than one component to affect secretion. Another way to test the function of these genes is to ask whether their ectopic expression is sufficient to drive secretion independently of hormone. E63-1 is the best candidate to begin these ectopic overexpression studies.

E63-1 is a high-affinity, calcium-binding protein which undergoes dynamic changes in subcellular localization during the secretion process (Vaskova et al., 2000). Since elevated cytoplasmic Ca\(^{2+}\) levels are involved in most secretory systems (Lang, 1999; Muallem and Lee, 1997), we hypothesized that E63-1 mediates Ca\(^{2+}\)-dependent events in exocytosis. To test this hypothesis, we generated transgenic flies that contain E63-1 under the control of a hs:Gal4 promoter (hsE63-1). Ectopic production of E63-1 in vitro induces 20E-independent Sgs3–GFP secretion, if intracellular Ca\(^{2+}\) levels are elevated using an ionophore (A23187 and ionomycin have the same effect) (Fig. 7A, Treatment 7; Fig. 7B). We do not observe 20E-independent secretion with heat shock (Fig. 7A, Treatment 2) or ionophore treatment alone (Fig. 7A, Treatment 6). Although these experiments demonstrate that E63-1 is sufficient for secretion, the process takes longer (10–20 h versus 6 h for 20E-dependent secretion), indicating that another 20E-regulated product(s) may be required for efficient secretion.

20E Treatment Leads to the Elevation of Ca\(^{2+}\) Levels

Since 20E is required for glue secretion, and our ectopic expression studies with E63-1 suggest that a Ca\(^{2+}\) signal is required, we asked whether hormone exposure modulates cytoplasmic Ca\(^{2+}\) levels. We used transgenic animals expressing the photoprotein Aequorin to measure changes in Ca\(^{2+}\) levels after 20E exposure. Aequorin is a Ca\(^{2+}\)-binding complex consisting of an Apoprotein and a luminophore, Coelenterazine (reviewed in Yoshimoto and Hiramoto, 1991). When the functional complex binds Ca\(^{2+}\), it is consumed and emits light (\(\lambda_{max} = 470\) nm) in a dose-dependent manner (Shimomura and Johnson, 1978). Fly stocks with Apoaquin under control of the UAS promoter (Rosay et al., 1997) make it possible to express Apoaquin either ubiquitously or selectively in the salivary gland (Brand and Perrimon, 1993). Ca\(^{2+}\) changes in tissues can be assessed by measuring luminescence from activated Aequorin. Using two different approaches, we assayed salivary glands for qualitative changes in intracellular Ca\(^{2+}\) levels after 20E treatment (Fig. 8).

In our first approach, we assayed 20E-regulated Ca\(^{2+}\) changes in single salivary glands from larvae expressing Apoaquin induced by a hs:Gal4 transgene. Because the amount of Apoaquin produced using this system is much higher than that produced by using the 5G:Gal4 driver (data not shown), we are able to measure luminescence from a single pair of glands. The results for three pairs of glands treated with 20E are presented (Fig. 8A, Traces 1–3). After 2 h of hormone treatment, cytoplasmic Ca\(^{2+}\) levels are elevated, and this elevation is sustained for several hours. No changes in luminescence were detected in glands not treated with 20E (Fig. 8A, Trace 4) and no specific luminescence is observed in larvae lacking an Apoaquin transgene (data not shown). Unfortunately, exact concentrations are difficult to measure using this system.

Because we observe wide variations in the intensity of luminescence among single glands driven by a heat-shock promoter, we performed a series of modified Aequorin experiments to normalize the data (Fig. 8B). In this second approach, we compare the amount of functional Aequorin remaining in two identical pools of glands after one is treated with 20E. As shown (Fig. 8B), treatment with the hormone results in a decrease in functional Aequorin levels and a corresponding increase in R value (see Materials and Methods). Our interpretation of these data is that the
steroid causes an elevation in cytoplasmic Ca\textsuperscript{2+} that binds to Aequorin and consumes the complex. In agreement with the results in Fig. 8A, a significant difference between control and 20E-treated samples does not occur until after 2 h of treatment.

**DISCUSSION**

*Sgs\textsubscript{D}3–GFP* Transgenes Can Be Used to Monitor Glue Secretion in Vivo and in Vitro, and Are Useful Tools for Developmental Staging

We have shown that *Sgs\textsubscript{D}3–GFP* is expressed in a pattern identical to that of the endogenous *Sgs3* gene, and that the fusion protein is properly sorted, secreted, and expelled from the salivary gland in a manner identical to that of the endogenous glue mix (Fig. 2). We found that the chromophore of this fusion protein is stable for several days and can be used to observe glue synthesis and secretion in living animals and dissected tissues. This allows us to easily perform genetic screens for mutations that affect exocytosis. Finally, the *Sgs\textsubscript{D}3–GFP* transgenes are important staging tools, allowing one to monitor development of third-instar larvae more precisely than current “blue food” methods (Andres and Thummel, 1994; Maroni and Stamey, 1983). This is possible because one can select individual larvae with specific patterns of “green glue” in their salivary glands resulting from a developmentally regulated physiological stimulus. We contend that this is a better method for staging for several reasons: It allows one to distinguish early-third-instar larvae from mid- to late-third-instar larvae during a period when *Sgs\textsubscript{D}3–GFP* is not expressed. Also, in our hands the “blue food” method is not reliable when animals are crowded (midguts look prematurely cleared), when the food is dry (animals do not leave the medium), or when mutants are used that extend the duration of third-instar development. Thus, introducing an *Sgs\textsubscript{D}3–GFP* transgene into the genetic background should allow for a more reliable method of selecting mass quantities of similarly aged animals for physiological and biochemical analyses.

**20E May Not Be Required for Glue Synthesis**

Although the majority of data presented here focuses on the secretion of glue granules by the premetamorphic pulse of ecdysteroids (Fig. 6C), there are reports which speculate that a small ecdysteroid pulse (Berreur et al., 1984; Schwartz et al., 1984) induces a series of developmental events, including glue gene induction during the mid-third instar (90–100 h AEL; Figs. 5A and 5B) (Andres et al., 1993; Furia et al., 1992; Georgel et al., 1991; Hansson and Lambertsson, 1989). However, the idea that 20E acts through the EcR/USP heterodimer to mediate these changes has been recently questioned (Fisk and Thummel, 1998; Hall and Thummel, 1998), and our results substantiate the
challenge. We show that I(3)ecd^1 mutants, severely compromised but probably not devoid of circulating ecdysteroids (Garen et al., 1977), synthesize SgsΔ3-GFP. In addition, animals depleted of USP, EcR-B1, or EcR-B2 also express the fusion gene (Table 2). However, since neither the EcR nor the usp mutants tested remove all protein (EcR-A remains intact; USP is truncated in usp^2 mutants, and some leaky expression might occur from the hs-usp construct), the caveat that glue gene induction requires only a small amount of ecdysteroid-EcR/USP signaling must be considered. We also note that the studies reporting that BR-C null mutants do not produce glue (see above), are consistent with our observation that an SgsΔ3-GFP transgene is not expressed in npr^1 animals.

Finally, it has been previously reported that the mid-instar developmental transitions fail to occur in DHR78 (Hr78) orphan nuclear receptor mutants (Fisk and Thummel, 1998). This observation has led to the speculation that a hormone signal, working through DHR78, is needed for these events. However, in our hands DHR78 mutants synthesize SgsΔ3-GFP and escapers that puateplicate secre te normally (Table 2), despite using two different DHR78 alleles and two different SgsGFP stocks (data not shown). One explanation for this observation is that genetic modifiers necessary for the mid-instar phenotype were lost or gained when the Sgs-GFP tester stocks were generated. However, we note that the lethal phase (mid-third instar) associated with the DHR78 parental stocks is extended to the late third-instar/early prepupal period after several generations on our media. This raises the interesting possibility that part of the reported DHR78 phenotype is environmentally or nutritionally influenced.

**Optimal Glue Secretion Requires 20E, an EcR/USP Receptor, and the rbp^1 Early-Gene Function**

Using classical genetic approaches and RNAi technology, we have demonstrated that the ecdysteroid receptor, consisting of EcR and USP, is required for glue secretion. Since limited secretion is detected in EcR-B1 and usp mutants, RNAi was used to establish their absolute requirement. This new technology is a rapid and simple approach to compromise candidate genes, to determine their requirement in secretion, especially when mutants are either not available or die early in development. Using osmotic shock to introduce double-stranded RNA into cultured glands greatly increases the utility of this method.

Our work and previous studies have shown that salivary glands cultured with 20E secrete glue, whereas glands cultured in the presence of both 20E and cycloheximide fail to secrete (Boyd and Ashburner, 1977; Farkas and Sutakova, 1998). This suggests that protein synthesis is required for glue secretion, but we have found that no single early or early-late product, thus far identified, is necessary. The rbp^1 function of the BR-C early locus, however, is required for secretion to occur in a timely manner. These mutants secrete SgsΔ3-GFP, but the process is delayed by at least 4 h. Although the rbp^1 lesion is expected to be an amorph (all BRC-Z1 proteins are truncated before the DNA-binding domain) (Emery et al., 1994), it does produce a small protein with some reported function (Bayer et al., 1997; Kiss et al., 1988).

Also of interest are the mutations that eliminate 2Bc and E74B. These result in animals that are unable to expel secreted glue onto the ventral body surface (Table 2). Since there must be a neural signal and pharyngeal muscle contractions to expel glue, these mutants may possess a neural and/or muscular defect. This would agree with previous studies that some BR-C functions are important for correct muscle attachment (Sandstrom et al., 1997), and that E74B is required for head eversion (Fletcher et al., 1995).

Because mutations in early and early-late genes fail to block secretion, we are left with two possible explanations: (1) either the early products we tested are functionally redundant or (2) additional hormone-responsive genes required for secretion have not yet been identified. We can now test these possibilities using double-mutant combinations, and ectopic expression studies like those conducted with E63-1.

**20E, Ca^{2+}, and E63-1: Framing Out the Secretion Pathway**

We have shown, for the first time, that calcium levels are elevated after 2 h of 20E treatment, and that a Ca^{2+} signal is necessary for secretion induced by ectopic expression of the E63-1 early protein. These results demonstrate that 20E acts genomically to upregulate at least two classes of proteins: those that increase cytoplasmic Ca^{2+} levels, and those (E63-1 and others) that mediate exocytosis in response to the Ca^{2+} signal.

At present, we do not know how 20E causes an increase in cytoplasmic Ca^{2+} concentrations, but we have found that, when Ca^{2+} entry into the gland is prevented using channel blockers (Ca^{2+}^- and Ca^{2+}^-free medium, 20E-stimulated secretion does not occur (A. Blyasheva and A. J. Andres, unpublished observation).

Finally, little is known about the proteins needed for organized transport of glue granules in the salivary gland. However, some SNARE proteins (synaptobrevin, SNAP-25, syntxin, etc.), shown to be involved in vesicle trafficking and exocytosis in nerve cells, are likely required (for a review of Drosophila SNARE homologs, see Littleton, 2000). We are now in a position to identify these molecules by screening mutant animals or tissues (mosaic screens) for secretion defects and by testing candidate molecules using RNAi technology.

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