Probing the function of *Drosophila melanogaster* accessory glands by directed cell ablation

(reproduction/diphtheria toxin/cell types/mating behavior/promoter fusions)

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ABSTRACT The female *Drosophila melanogaster* fly undergoes behavioral changes after mating, including an increase in egg laying and an avoidance of remating. Accessory-gland products elicit these changes transiently when introduced into unmated female flies. We report here the generation and phenotype of flies that lack functional accessory-gland main cells as a consequence of genetically directed delivery of diphtheria toxin subunit A to those cells. Only main-cell secretions are essential for the short-term inhibition to remating; no other products of the genital tract can replace their function. Long-term inhibition to remating depends only on the storage of sperm in the female. Both sperm and main-cell secretions have roles in the increase of egg laying by the mated female. In addition to full-strength diphtheria toxin, we used low-activity toxins to kill only those cells that express toxin at high levels. These transgenic strains that express diphtheria toxins of different strengths in accessory-gland main cells will be useful in further defining the role of these cells.

In insects, mating causes females to undergo physiological and behavioral changes that persist for several days (1, 2). After mating, females lay eggs at a high rate and they reject further male courtship and remating. They store and utilize sperm they receive. In *Drosophila melanogaster*, these behavioral changes are due in part to sperm and in part to seminal fluid (3–5). Seminal-fluid components have been shown to cause the initial decrease in receptivity, called the “copulation effect” (3), but the long-term decrease in receptivity, called the “sperm effect” (4, 5), is correlated with the presence of sperm in the female’s sperm-storage organs.

To determine which molecules are responsible for these behavioral changes, we need to know their source. Seminal-fluid effects on oviposition (egg laying) rate and receptivity were demonstrated in Hihara’s study (3) of mates of males that had depleted their seminal secretions due to multiple prior matings. Hihara (3) postulated that products of the accessory gland, a secretory tissue in the male reproductive tract, caused these behavioral changes. He reasoned this because the accessory glands of multiply mated males were shrunken, apparently due to depletion of their contents, and because transplantation of accessory glands (6, 7) or injection of their extracts (for review, see ref. 2) was known to cause increases in oviposition and decreases in receptivity. Chen *et al.* (8) and Aigaki *et al.* (9) later showed that a single accessory-gland peptide, the sex peptide, is able to induce oviposition and rejection when introduced into female flies. Another accessory-gland product, accessory-gland protein (Acp) 26Aa, has sequence similarity to egg-laying hormone of *Aplysia* (10). Although these data suggested that accessory-gland products could cause the behavioral effects, they did not prove that they are the products that work *in vivo* and,

most importantly, that only products of the accessory gland can elicit these effects. For example, some products of the ejaculatory duct, another male reproductive tract tissue, had been suggested to play a role in some of these processes (11, 12).

We wished to test directly whether the accessory gland is the only source of molecules essential for the increase in oviposition and short-term receptivity changes after mating and to examine other potential roles of accessory-gland secretions. To do this we have generated and analyzed flies whose accessory-gland function has been disrupted by production of an intracellular toxic protein in one of the two secretory-cell types of the accessory gland.

The *Drosophila* accessory gland contains two morphologically distinct secretory cell types, main cells (96% of the secretory cells) and secondary cells (13, 14). Each cell type expresses a unique set of genes (14–17). For example, Acp95EF and sex peptide are synthesized only by the main cells (15, 17).

To disrupt accessory-gland main-cell function genetically, we introduced diphtheria toxin subunit A (DTA) into those cells via the promoter of the *Acp95EF* gene. Intracellular production of DTA has been used to kill cells in a variety of systems by inhibiting translation (18–21). We fused sequences encoding DTA to sequences that promote expression beginning at or just before eclosion only in main cells (15). Females mated to transgenic flies carrying this fusion show reduced copulation effects on receptivity and oviposition, even in cases when they receive and store sperm. Our results suggest that accessory-gland main-cell secretions are the ejaculate secretions involved in the short-term block to receptivity and that both main-cell secretions and sperm are needed for the stimulation of egg laying.

MATERIALS AND METHODS

Generation of Transgenic Flies Carrying mc/DTA. The mc/DTA-E fusion encoding full-strength DTA was constructed by replacing the 3.1-kb *lacZ* fragment of the *Acp95EF-lacZ* (originally called *mst316-lacZ*) construct described in ref. 15 with a 700-bp *Bam*HI–*Bgl*II fragment containing the DTA gene from the DT-A cassette described in ref. 19. A *Hind*III fragment containing this fusion was cloned into the *Hpa*I site of the Carnegie 20 vector, which carries *ry*⁺ (22). This construct was introduced into *Adh*^{fr6} *cn*; *ry*⁵⁰² (*Acr*) flies by standard techniques (23). The constructs carrying less-active DTAs were generated by substituting a 400-bp *Acc*I–*Msc*I fragment containing the E148D mutation or the E148S mutation (24) for the same fragment of the DTA gene in the original mc/DTA-E fusion. These

Abbreviations: DTA, diphtheria toxin subunit A; Acp, accessory-gland protein.

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fusions, called mc/DTA-D and mc/DTA-S, respectively, were cloned into the w^+ -marked vector pW8 at its *Xba* I site (25). w^+ transformants were detected among the progeny of $z^1 w^{11E4}$ flies mated to w ; *ry Sb P* [ry^+ $\Delta 2-3$]/*TM6* flies that had been injected with this DNA as embryos (26). For each construct, at least two single-insert lines were analyzed. Confirmation that each line carried a unique nonrearranged insert was by Southern blot analysis. All nucleic acid manipulations were carried out essentially as described in ref. 27.

Receptivity and Egg Laying in the Mates of mc/DTA Males. To measure receptivity and egg laying in flies as isogenic as possible, the following flies were generated: For mc/DTA-E, control males were the *ry* progeny of a cross of ry^{506} to females heterozygous for mc/DTA-E; mc/DTA-E males were the ry^+ progeny of that cross; spermless males were generated by crossing *tud¹ bw sp* females (28) to ry^{506} males. For mc/DTA-D and mc/DTA-S, lines carrying X chromosome-linked inserts of the fusions were used. The control for those lines was the line pWgHL811.2, also a $z^1 w^{11E4}$ derivative, that carries a *P* element with w^+ and a *hsp26-fs(1)Ya* fusion (M. Park and M.F.W., unpublished data). To measure oviposition, 3-day-old males were crossed to 3- to 4-day-old virgin Oregon-R females. Females were removed for observation after a single mating. Egg production by individual females was measured by counting the number of eggs laid in a 24-h period. We present data from one representative experiment, though we have repeated it at least three more times for each line (except for the mc/DTA-S line, where we did only one egg count, but five adult progeny counts), and the results are fully reproducible. All ovipositions within one experiment were done on the same batch of yeast-glucose food. For receptivity experiments, 3- to 6-day-old virgin males were mated to 3- to 6-day-old virgin Oregon-R females or the *ry* female progeny of mc/DTA-E crosses; the males were removed soon after mating. Receptivity was measured by providing four of these mated females with six virgin males and observing whether or not matings occurred within 1 h. All receptivity assays were repeated at least three times, with a total sample size for each strain of ≥ 38 . The average of those results is presented.

Sperm Production and Transfer. Testes were examined by phase-contrast microscopy to observe the stages of spermatogenesis. Transfer of sperm and the number stored in ventral receptacles (seminal receptacles) were examined as in refs. 11 and 29. ANOVA tests on sperm-storage numbers were carried out as in ref. 30.

Measurement of Protein Production in mc/DTA Males. Amounts of male-specific proteins from male genital tracts were measured on Western blots, performed as in ref. 10 with minor modifications. Antibodies were used against Acp26Aa (formerly called msP355a or Mst26Aa; refs. 10 and 31), Acp26Ab (formerly called msP355b or Mst26Ab; refs. 10 and 31), Acp95EF (formerly called msP316 or Mst95E; refs. 15 and 31 and unpublished data), sex peptide (Acp70A; kindly provided by E. Kubli, Universität Zürich), and esterase 6 (ref. 32; kindly provided by R. C. Richmond, University of South Florida, Tampa). Even though it detects a main- and secondary-cell protein, we generally used anti-Acp26Aa, as it detects its antigen with far greater sensitivity than the others (or there is more of the antigen). To determine amounts of Acp95EF in mc/DTA males, the intensities of the Acp bands on the Western blots were compared to those in lanes carrying serial dilutions of Acp95EF from control w males. To detect transfer of esterase 6, we mated mc/DTA or control males to esterase 6 null females (kindly provided by R. C. Richmond).

To detect cell-type-specific protein synthesis *in situ*, we generated flies carrying one copy of the mc/DTA-E and one copy of an enhancer-trap or gene-fusion construct that pro-

duced β -galactosidase in specific accessory-gland cells. To examine main-cell gene expression, the reporter construct was *Acp95EF-lacZ* (15) or enhancer traps 69.4 or 72.1 (14); to examine secondary-cell expression, enhancer traps 23Z Δ 280.1.4 (14), 69.4, or 72.1 were used. Accessory glands of the progeny flies were stained for β -galactosidase as in ref. 15. 4',6-Diamidino-2-phenylindole (DAPI) staining of accessory glands from mc/DTA and control flies was done as in ref. 14, as was photography of stained tissue.

RESULTS AND DISCUSSION

Flies Whose Accessory-Gland Main Cells Are Nonfunctional. We fused 1.1 kb of upstream and transcribed sequences of *Acp95EF* to sequences encoding DTA (mc/DTA-E). Fusion of these *Acp95EF* sequences to *lacZ* (*Acp95EF-lacZ*) results in expression of β -galactosidase in accessory-gland main cells only; no β -galactosidase is detectable in any other cells (15). We were concerned that there might be very low levels of expression by this regulatory region, below the sensitivity of β -galactosidase assays. Such low-level expression might produce sufficient DTA to kill cells, as even one molecule of DTA has been shown to be lethal to cells where tested (33). We therefore made two constructs in which *Acp95EF* regulatory sequences drive the production of less-active mutant DTAs. The mc/DTA-D fusion results in a DTA of 100-fold lower activity than normal and the mc/DTA-S fusion produces DTA that is 300-fold less active than normal (24).

Male flies carrying mc/DTA fusions have abnormal accessory glands. Less than 1 h after eclosion, accessory glands from mc/DTA-E males are relatively normal in morphology, though they are slightly smaller and thinner than normal. This suggests that the tissue developed correctly but failed to fill with secretion. As mc/DTA-E flies age, their accessory glands do not grow (Fig. 1). Instead, their binucleate main cells become disorganized and eventually appear to degenerate (Fig. 1 E and F). This is consistent with what we expected for production of DTA under *Acp95EF* control. β -Galactosidase production from *Acp95EF-lacZ* is first seen in fully formed accessory glands around the time of eclosion (15). If DTA is produced in a similar manner, translation in main cells would be inhibited after the glands had formed but before substantial amounts of main-cell secretions were produced.

Flies simultaneously carrying one copy of the mc/DTA-E fusion and one copy of the *Acp95EF-lacZ* fusion (15) show no stainable β -galactosidase in their main cells (Fig. 1 A and B). However, main cells are the only somatic cells in the genital tract where gene expression is blocked by the presence of mc/DTA. mc/DTA-E flies carrying a reporter gene that normally produces β -galactosidase in secondary cells or in secondary cells and ejaculatory duct cells (14) show the same level and expression pattern of β -galactosidase as non-DTA controls at all ages (Fig. 1 C and D). Since the half-life of β -galactosidase in flies is 1 day (34), this result suggests that secondary cells and ejaculatory duct cells of mc/DTA flies are continually able to carry on protein synthesis. Esterase 6, an enzyme made in the ejaculatory duct (35), is found at normal levels in mc/DTA-E flies and is transferred from these males to females during mating (data not shown). Likewise, a normal-looking mating plug is seen in the mates of mc/DTA-E males. The mating plug, a waxy-appearing structure found in the uterus of mated females, is a product of the ejaculatory bulb (36). Flies carrying mc/DTA fusions show no alteration in viability or behavior.

The level of inhibition of main-cell protein synthesis is related to the activity of DTA in the constructs. Flies carrying mc/DTA-E show the most severe effect. For example, Acp26Aa, which is produced in main cells and secondary

FIG. 1. Accessory-gland morphology, cellular organization, and gene expression in mc/DTA-E males as compared to controls. (A, C, and E) Accessory glands from control males. (B, D, and F) Accessory glands from mc/DTA-E males. A–D show accessory glands from a 3-day-old fly stained for β -galactosidase activity. (Bar = 90 μ m.) A and B carry the main-cell-staining construct *Acp95EF-lacZ*. C and D have a secondary-cell enhancer-trap gene. E and F show 4',6-diamidino-2-phenylindole-stained accessory glands from a 7-day-old fly. (Bar = 12.5 μ m.) ag, Accessory gland; ed, ejaculatory duct; mc, main cell; sc, secondary cell.

cells (16), is undetectable (i.e., <1% of control) in mc/DTA-E flies, present at $\approx$ 1% of control levels in mc/DTA-D flies and at $\approx$ 3% of control amounts in mc/DTA-S flies (Fig. 2). Main-cell-specific products *Acp95EF* (15) and sex peptide (17) are also undetectable in mc/DTA-E flies, as is the main- and secondary-cell-specific product *Acp26Ab* (16) (data not shown). The lack of these proteins suggests that mc/DTA-E abolishes protein synthesis before detectable amounts of Acp95 can accumulate.

Male flies carrying one copy of mc/DTA-E do not produce sperm. The block to spermatogenesis is at the primary spermatocyte stage (ref. 37 and data not shown). We believe that the lack of sperm in these flies is due to a very few molecules of DTA produced in their germ cells due to "leakage" of the *Acp95EF* promoter. Three lines of evidence support this view. (i) Sperm are produced in testes transplanted from male larvae into females (38) and by *iab^{trh3}/Df(3R) C4* males, which lack accessory glands but cannot mate due to undeveloped external genitalia (data not shown and ref. 39). Thus, accessory-gland function is not required for spermatogenesis. (ii) The blockage to spermatogenesis in mc/DTA-E flies is at the stage when gene expression necessary for spermatogenesis occurs and thus is at the stage where one might expect a translational block to arrest (37). (iii) Males carrying less-active DTAs do not show this block, suggesting that insufficient DTA activity is present to stop spermatogenesis in those males.

FIG. 2. Western blot showing levels of *Acp26Aa*, which migrates as a doublet, in the mc/DTA lines indicated. Control lane (lane w) contains extracts of three accessory-gland pairs from 3-day-old w virgin males. DTA lanes (lanes S, D, and E) have the extracts of 10 accessory-gland pairs from 3-day-old virgin flies of the mc/DTA-S, -D, and -E lines, respectively. All lanes are from the same blot.

Because flies carrying mc/DTA-E do not produce sperm, experiments below that utilize those flies compare their phenotype to that of the spermless progeny of tudor (*tud^l*) females (28). In experiments where sperm are required, we used flies carrying mc/DTA-D that make sperm but nearly no accessory-gland secretions, detectably less than mc/DTA-S. We quantified the number of sperm in the mates of mc/DTA-D flies. Six hours after mating, a time at which the maximum number of sperm are reported to be in the ventral receptacle (11), mates of mc/DTA-D males had stored significantly fewer sperm than mates of controls (Fig. 3). Most of the mates of mc/DTA-D males had some sperm in their ventral receptacle, although fewer than in the controls. Only 17% had very few or no sperm. The upper end of the distribution of sperm numbers of the mates of mc/DTA-D

FIG. 3. Percentage of females storing the indicated number of sperm in their ventral receptacle 6 h after mating. Mates of control males (solid bars) stored an average of 286 sperm with a standard deviation of 65.0 ($n = 19$). Mates of mc/DTA-D males (hatched bars) stored an average of 157 sperm with a standard deviation of 83.2 ($n = 29$). Similar numbers are seen at 1 h after mating (data not shown). These numbers are lower than those reported by Gilbert (11) but may be explained by strain differences since he used Oregon-R males.

males overlaps the lower end of the distribution of mates of control males. Thirty-four percent of the mates of mc/DTA-D males stored more sperm than the lowest number of stored sperm in mates of control males.

We cannot distinguish between two possibilities for the lower number of sperm stored by mates of mc/DTA-D males. It is possible that those males make fewer sperm than normal, perhaps because some of their germ cells are killed by mc/DTA-D, but those sperm are transferred and stored with normal efficiency. This would be consistent with the report by Hihara (3) that seminal fluid is not required for sperm transfer and storage. Alternatively, mc/DTA-D males might make normal numbers of sperm but be unable to transfer or store them with full efficiency without main-cell secretions. This would be more consistent with the claim of Lefevre and Jonsson (29) that seminal fluid is completely necessary for sperm transfer and storage. In that case, our results would argue that main-cell secretions participate in, but are not the exclusive mediators of, sperm transfer or storage.

Effects of Accessory-Gland Secretions on Egg Laying in Mated Females. Though unmated females lay a few eggs (averaging <2.5 eggs per female daily), females mated a single time to normal males lay an average of 35.8 eggs on the first day after mating. They continue to lay eggs at an elevated, but decreasing, rate for at least 5 days after mating (Table 1). Oviposition by females who receive no sperm or main-cell secretions by mating with mc/DTA-E males are almost identical to that of unmated females (Table 1). This shows that secretions of cells other than main cells or mechanical effects of mating do not lead to increased egg laying and that sperm or main-cell secretions or both are required for the effects.

To determine whether the lack of stimulation of oviposition by mc/DTA-E males is due to their lack of main-cell secretions or to their lack of sperm, we examined egg laying by females whose mates lack sperm only. Females mated a single time to sons of *tud*¹ mothers (28) receive accessory-gland secretions (M. Bertram and M.F.W., unpublished data) but no sperm. These females show a partial stimulation of oviposition on day 1, laying an average of 26.1 eggs per female (Table 1). However, on subsequent days very little or no stimulation is seen. Thus, sperm are needed to get the full continued increase in egg laying, but main-cell secretions alone can give a partial short-term stimulation.

Similarly, mates of the mc/DTA-D and mc/DTA-S males, who receive sperm and no or very little main-cell secretions, show a short-term stimulation of egg laying. One day after mating, mates of these males lay an average of 24.0 or 16.9 eggs per female, respectively, and the average number decreases over the next 4 days (Table 1). However, there is scatter in the number of eggs laid per female on all days. About 25% of the females mated to mc/DTA-D males lay eggs at the elevated rate of mates of control males, 20% at the nonelevated rate of unmated females, and most of the remaining females with a partial stimulation on days 1 and 2 that disappears by the last days of the experiment.

These percentages correlate with the percentages of females mated to mc/DTA-D males that stored control levels of sperm or no sperm, respectively (Fig. 3). The 34% of the mates of mc/DTA-D that stored control levels of sperm parallels the 25% of females who laid eggs at an elevated rate throughout all the egg-laying experiment. Similarly, 17% stored few or no sperm, paralleling the 20% of females who laid eggs at the nonelevated rate in the egg-laying experiment. Therefore, we suggest females that store proper numbers of sperm lay eggs at the same rate as control females whereas those that store fewer than normal sperm show only limited stimulation of oviposition. Consistent with this suggestion, females who had stored very few or no sperm in the ventral receptacle prior to dissection had laid no eggs before dissection; whereas all females who stored control levels of sperm laid eggs during the 6 h before dissection (data not shown). The eggs laid by mates of mc/DTA-D and -S males also produced fewer adult progeny than controls, probably because of their storage of fewer sperm than normal (data not shown).

These results show that sperm and main-cell secretions are components of the ejaculate necessary for the stimulation of egg laying. Sperm participates in the short-term stimulation of oviposition, and its storage is correlated with continued stimulation of oviposition. Main-cell secretions are also involved in the short-term stimulation. We do not detect a role of main-cell products in the long-term effect. We cannot rule out an indirect role if these products affect sperm storage.

Effects of Accessory-Gland Secretions on Receptivity in Mated Females. One day after mating only 3% of females previously mated to control males mate again, whereas 95% of previously unmated females mate. Females previously mated to mc/DTA-E males behave much more like previously unmated controls; 86% of them remate. Therefore, accessory-gland main-cell secretions and/or sperm are required to inhibit remating, while the secreted products from other genital-tract tissues cannot alone perform these functions. Also, the change in receptivity is not a simple mechanical consequence of mating. To separate the effects of sperm and main-cell secretions, we examined the mates of spermless males (sons of *tud*¹ mothers). After 1 day, these females show a partial loss of receptivity to further mating (43% mated again). These data indicate that both main-cell secretions and sperm are needed for the full loss in sexual receptivity 1 day after the initial mating.

Two days after a mating, females mated to control males remain unreceptive to further mating—only 7% remated, compared to 98% of previously unmated females that mated. Females mated to mc/DTA-E males still behave more like unmated females—82% of them mate again. However, 90% of the females mated with spermless males mate again 2 days after the first mating. Very similar results are seen 7 days after the first mating (data not shown). The results show that sperm, rather than main-cell secretions, are responsible for the long-term inhibition to receptivity.

Conclusions. Our results show the importance of accessory-gland main-cell secretions in reproduction. Main-cell se-

Table 1. Effects of mc/DTA constructs on egg laying

Male mate	Average number of eggs laid by female mate each day after mating				
	Day 1	Day 2	Day 3	Day 4	Day 5
w control male (<i>n</i> = 16)	35.8 ± 10.2	23.6 ± 9.6	18.5 ± 7.9	14.5 ± 7.9	15.6 ± 4.6
mc/DTA-S male (<i>n</i> = 18)	16.9 ± 17.9	7.7 ± 9.7	3.3 ± 7.1	1.3 ± 3.3	0.7 ± 2.8
mc/DTA-D male (<i>n</i> = 20)	24.0 ± 18.8	9.2 ± 10.3	2.9 ± 6.8	4.9 ± 8.6	5.3 ± 8.6
mc/DTA-E male (<i>n</i> = 9)	0 ± 0	2.6 ± 5.4	1.1 ± 3.3	4.2 ± 10.9	1.4 ± 2.9
Son of a tudor mother (<i>n</i> = 20)	26.1 ± 17.5	6.2 ± 8.8	4.0 ± 8.6	2.1 ± 4.9	1.6 ± 3.8
None* (<i>n</i> = 20)	0 ± 0	1.7 ± 4.2	1.4 ± 5.4	6.8 ± 10.0	2.3 ± 6.8

*Unmated females occasionally "dump" the unfertilized eggs they have been holding.

cretions are the only genital tract secretions necessary for the short-term block to receptivity. The main-cell mediator of this block to receptivity is likely to include (or be) the sex peptide. Long-term blocks to receptivity depend on the presence of sperm in the female and do not show any direct influence of accessory-gland molecules. One model to explain the short- and long-term effects in the mated female is that it takes time to store sperm (11), so it is important to prevent the female from remating during that time. Accessory-gland molecules could provide the immediate response, but once sperm had been stored accessory-gland products would no longer be needed. Some products of other genital-tract tissues, such as the ejaculatory-duct enzyme esterase 6, have been suggested to affect receptivity (12). Our results would suggest that these effects are secondary to accessory-gland effects or somehow related to sperm storage. Both sperm and accessory-gland main-cell secretions are required for the full short-term peak in egg laying. Again, sperm storage is correlated with its persistence at elevated levels.

The flies we have generated here can be used to test for roles of accessory-gland products in processes not previously linked to the secretions of this tissue. For example, T. Prout and L. Harshman (personal communication) have used these flies to show that main-cell secretions participate in displacement of stored sperm during a second mating. Constructs producing DTA in other genital-tract cells can be used to determine the roles of their products in a manner analogous to what we have done here for main cells.

Finally, as a technical point, we suggest that we were able to make full-strength DTA transformants because the *Acp95EF* gene has a "tight" sex- and adult-specific promoter. We could circumvent the deleterious effects of low-level production of DTA in unintended tissues by using less-active DTAs.

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