Structure and expression of a *Drosophila* male accessory gland gene whose product resembles a peptide pheromone precursor

Scott A. Monsma¹ and Mariana F. Wolfner^{2,3}

¹Section of Biochemistry, Molecular, and Cell Biology and ²Section of Genetics and Development, Cornell University, Ithaca, New York 14853 USA

The accessory gland of male insects is a genital tissue that secretes many components of the ejaculatory fluid, some of which affect the female's receptivity to courtship and her rate of oviposition. We have examined the structure and expression of two tightly linked genes that are expressed exclusively in the male accessory glands of adult *Drosophila melanogaster*. The two genes are transcribed from the same strand of DNA, and are separated by 20 bases. Both genes are regulated by the sex determination hierarchy and are expressed in the absence of germ cells. Immunological analysis reveals the protein products of at least one of these genes in the secretion of the accessory gland. The proteins are transferred to the female fly during copulation and are rapidly altered in the female genital tract. The predicted sequence of one protein has features of a peptide hormone precursor, and a region in which 11 of 17 amino acids are identical to egg-laying hormone (ELH) of the California sea hare, *Aplysia californica*.

[Key Words: *Drosophila*; accessory gland; *Aplysia* ELH; peptide pheromone; sex determination]

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In this study we describe genes that are expressed in the accessory gland, a male tissue whose secreted products mediate stereotyped behavioral and physiological changes in female *Drosophila melanogaster* after mating. Such behavioral changes include a decrease in female receptivity to courtship by males (evidenced by female avoidance of courting males, Manning 1962), and increased egg-laying behavior (oviposition) (Leopold 1976; Chen 1984). Physiological changes in the ovary are correlated with the increase in oviposition, such that the rate of egg production is doubled relative to unmated females (Fowler 1973; Chen 1984). Eggs laid by mated females are also larger than the sterile eggs laid by unmated females (Fowler 1973). These behavioral and physiological effects persist for long periods after mating: the decrease in receptivity lasts as long as 7–9 days (Manning 1962), while the enhancement of oviposition lasts about 14 days (Chen and Bühler 1970).

Some of these post-copulatory effects are induced by the secretions of the male accessory glands (paragonia), which are introduced into the female during mating (Chen 1984). Implantation of the male accessory gland (Garcia-Bellido 1964) or injection of its secretion into virgin females (Chen and Bühler 1970; Baumann 1974b) causes decreased receptivity to courtship and stimulation of egg production by the ovary. Accessory gland secretions may also be involved in sperm storage and utili-

zation (Fowler 1973). The accessory gland secretion is a complex mixture of free amino acids and amino acid derivatives, proteins, lipids, carbohydrates, and large (visible) aggregates and vesicles (Federer and Chen 1982). The complexity of the secretion and the difficulty of collecting sufficient material has hindered the purification and analysis of specific components that might be involved in eliciting these behavioral and physiological effects.

Several studies have examined the protein components of the accessory gland secretion using one- or two-dimensional gel electrophoresis (Ingman-Baker and Candido 1980; Stumm-Zollinger and Chen 1985; Whalen and Wilson 1986); considerable protein heterogeneity is seen between species (Chen et al. 1985). In *D. melanogaster*, certain polymorphic protein bands have been correlated with specific chromosomal regions (Whalen and Wilson 1986); however, the functions of these proteins are unknown.

Two components have been purified from the male accessory gland secretion of *Drosophila funebris* (Baumann 1974a). PS-1, a peptide of 27 amino acids, causes a decrease in receptivity of unmated females to male courtship. PS-2, a glycine-carbohydrate derivative, causes an increase in oviposition when injected into unmated females (Baumann 1974b). Neither isolated component elicits the effect as strongly as unfractionated secretion, nor does either component elicit a detectable effect when injected into female *D. melanogaster*.

³Corresponding author.

(Baumann 1974b). The genes that are involved in the production of these two components are unknown, although the amino acid sequence of the peptide has been determined (Baumann et al. 1975).

We have taken a molecular genetic approach to accessory gland function: cloning genes that are expressed specifically in the accessory gland, to examine their structure and expression with the goal of defining the function of their products. Here, we report the structure and expression of two genes that encode secretory proteins of the male accessory gland of *D. melanogaster*.

Results

mst 355a and *mst 355b*: male-specific transcripts

We initially identified a restriction fragment encoding male-specific transcripts in screens of a genomic walk (MacIntyre and Davis 1987) of the 26A cytological region of chromosome arm 2L. Southern blots were made with restriction-cut DNA from overlapping phage clones in the walk. Duplicate blots were probed with ^{32}P -labeled cDNA synthesized from adult male or female poly(A)⁺ RNA; one restriction fragment from phage m5.5 was found to hybridize exclusively with the male probe. This 5.2-kb *Eco*RI restriction fragment was subcloned; a restriction map of this fragment is shown in Figure 1A. Strand-specific RNA probes were generated from this fragment and hybridized to Northern blots of adult male and female poly(A)⁺ RNA (Fig. 1B). Two adult male-specific transcripts of approximately 0.9-kb and 0.5-kb lengths were detected only with the T3 polymerase-generated probe (antisense probe, Fig. 1B, lane 2). A non-sex-specific transcript of 1.6 kb was also detected occasionally with this probe, and will not be discussed

further in this paper (Fig. 1A, dotted line; Fig. 1B, lane 2). The probe from the opposite strand did not hybridize to any detectable transcripts, and no hybridizing bands were detected in poly(A)⁻ RNA fractions with either strand-specific probe. The two male-specific transcripts hereafter will be referred to as *mst 355a* (0.9 kb) and *mst 355b* (0.5 kb), for male-specific transcript, in accordance with the established terminology (Schäfer 1986; DiBenedetto et al. 1987; Chapman and Wolfner 1988). This 5.2-kb genomic fragment contains unique sequences, as only the expected bands were seen when Southern blots of genomic DNA were probed (data not shown).

mst 355a and *mst 355b* are expressed exclusively in adult male accessory glands

To determine the tissue(s) in which the male-specific transcripts are expressed, 30 adult Canton S males were dissected into four tissue fractions: (1) accessory gland, (2) testis plus vas, (3) ejaculatory duct plus sperm pump, and (4) the remaining carcass. A Northern blot was made with total RNA extracted from each tissue fraction, and hybridized with the antisense RNA probe. The results are shown in Figure 2A: Both *mst 355a* and *mst 355b* are detected exclusively in the accessory gland fraction (lane 1).

To examine the temporal patterns of expression of these transcripts, a Northern blot was made with poly(A)⁺ RNA isolated from both male and female third instar larvae, mid-pupae (24 hr after puparium formation), and adults (Fig. 2B). Both *mst 355a* and *mst 355b* are expressed exclusively in the adult male (lane 6). In a more detailed analysis of the temporal pattern of expression of these transcripts, Chapman and Wolfner (1988)

Figure 1. Map of *mst 355* genomic region and transcripts. (A) Restriction map of the *mst 355* genomic region. This 5.2-kb *Eco*RI restriction fragment encodes three transcripts of approximately 1.6 kb (dotted arrow), and 0.9 and 0.5 kb (bold arrows); all three transcripts are encoded by the same strand of DNA. The 1.6-kb transcript has not been localized precisely, but it does not overlap with *mst 355a* or *mst 355b*. Restriction sites are abbreviated: (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (R) *Rsa*I; (Xb) *Xba*I; (Xh) *Xho*I. (B) Northern blot of ~5 μg poly(A)⁺ RNA from adult females and males, probed with a strand-specific RNA probe covering the entire 5.2-kb restriction fragment shown in A. The *Eco*RI restriction fragment was subcloned in the plasmid vector BlueScribe; a ^{32}P -labeled antisense RNA probe was generated in vitro by transcription from the T3 promoter. (Lane 1) Female RNA; (lane 2) male RNA. The antisense probe detects three transcripts: the 1.6-kb transcript is found variably in both male and female adult flies, and the 0.9-kb (*mst 355a*) and 0.5-kb (*mst 355b*) transcripts are found only in adult male flies. No transcripts were detected with sense strand RNA probes in either poly(A)⁺ or poly(A)⁻ fractions (data not shown).

Figure 2. Tissue and temporal patterns of *mst* expression. (A) Northern blot of total RNA extracted from male tissue fractions. Forty adult male Canton S flies were dissected into four tissue fractions and RNA was extracted. (Lane 1) Accessory gland (AG); (lane 2) testis plus vas (T); (lane 3) ejaculatory duct plus sperm pump (EjD); (lane 4) 10 of the remaining carcasses (C). The probe was a primer-extended genomic DNA fragment containing only *mst 355a* and *mst 355b*. (B) Northern blot of ~5 µg of poly(A)+ RNA extracted from unisex animals (the progeny of *cin y w* females) at three developmental stages. (Lanes 1 and 4) Third instar larvae (L); (lanes 2 and 5) mid-pupae 24 hr after puparium formation (P); (lanes 3 and 6) 2-day-old adults (A). The probe used was antisense RNA as in Fig. 1B.

have found that *mst 355a* and *mst 355b* first become detectable during the late pupal stage P13 [Bainbridge and Bownes 1981], about 12 hr prior to eclosion at 25°C; maximal levels of RNA accumulation are reached within 24 hr after eclosion.

Expression of mst 355a and mst 355b does not require spermatogenesis, and is independent of the state of the tra2 locus in adults

The products of the accessory gland are involved in male fertility, thus the expression of accessory gland products may require normal development of the male germ line. To determine if the expression of *mst 355a* and *mst 355b* depends upon spermatogenesis, we examined the adult male progeny of tudor (*tud¹*) female flies for the presence or absence of the transcripts. The *tud¹* mutation is a maternal-effect that blocks the formation of pole cells in all progeny of a *tud¹/tud¹* mother [Boswell and Mahowald 1985]. Since the pole cells generate the germ line of the adult fly, progeny of *tud¹* mothers lack germ cells and those somatic functions that require the presence of the germ line for their own development. Both *mst 355a* and *mst 355b* were found in adult male progeny of *tud¹/tud¹* mothers (Fig. 3A, lane 2), demonstrating that normal expression of these two transcripts is not dependent upon spermatogenesis.

The male accessory gland is an adult somatic tissue. To see whether the expression of the accessory gland transcripts is affected by genes known to regulate somatic sexual development, we looked for the transcripts in X/X flies lacking activity of the *tra2^{ts2}* gene product. The *tra2^{ts2}* gene product is required for normal female somatic development [Belote and Baker 1982]. X/X flies

lacking *tra2^{ts2}* product throughout development are somatic males, and as adults have accessory glands and other male genital tissues derived from the genital disk. The results (Fig. 3B, lane 5) show that both *mst 355a* and *mst 355b* are expressed in such somatically transformed X/X adult flies. Restoration of *tra2^{ts2}* activity in adulthood does not abolish the expression of *mst 355a* or *mst 355b* (Fig. 3B, lanes 7–10); thus we conclude that these accessory gland genes are no longer regulated by the somatic sex determination gene *tra2^{ts2}* at the time of their expression.

Sequence organization of the *mst 355a* and *mst 355b* genes

The genomic DNA sequence and the organization of the two genes was determined by sequence analysis of genomic and cDNA clones; the results are summarized in a map of the genes (Fig. 4A) and the genomic sequence of the region (Fig. 4B). The 5' ends of both transcripts were determined by extension of synthetic oligonucleotide primers complementary to the mature mRNAs; one major start site and one minor start site were seen for the upstream transcript (*mst 355a*), while the downstream transcript (*mst 355b*) showed a single position for the 5' end (data not shown; see Methods for details). We have assigned the major start site of *mst 355a* as +1; unless noted otherwise, all positions are indicated relative to this nucleotide. The minor start of *mst 355a* occurs at position +2, and the 5' end of *mst 355b* occurs at position +1031.

To isolate cDNA clones of *mst 355a* and *mst 355b*, we constructed a cDNA library in the vector λgt10 from poly(A)+ RNA isolated from adult male flies. From this

Figure 3. Genetic regulation of *mst 355a* and *mst 355b* expression. (A) Northern blot of ~5 µg poly(A)⁺ RNA extracted from germ-lineless adult progeny of *bw sp tud1/bw sp tud1* mothers. (Lane 1) Female progeny; (lane 2) male progeny. The probe used was the antisense RNA as in Fig. 1B. (B) Effect of *tra2* on *mst 355a* and *mst 355b* expression. Northern blot of total RNA extracted from 50 adult flies for each lane. Genotypes of 2-day-old adults, raised and kept at 16°C: (lane 1) X/X, *tra2^{ts2} bw/CyO*; (lane 2) X/X, *tra2^{ts2} bw/tra2^{ts2} bw*; (lane 3) X/Y, *B^s; tra2^{ts2} bw/tra2^{ts2} bw*. Genotypes of 2-day-old adults, raised and kept at 29°C: (lane 4) X/X, *tra2^{ts2} bw/CyO*; (lane 5) X/X, *tra2^{ts2} bw/tra2^{ts2} bw*; (lane 6) X/Y, *B^s; tra2^{ts2} bw/tra2^{ts2} bw*. (Lanes 7–10) X/X, *tra2^{ts2} bw/tra2^{ts2} bw* adults raised to adulthood at 29°C, then downshifted to 16°C for the indicated period of days before RNA extraction. (Lane 7) 7 days after downshift; (lane 8) 5 days; (lane 9) 3 days; (lane 10) 2 days. The intensity of *mst 355a* on Northern blots is usually greater than that of *mst 355b* (see A), although *mst 355a* occasionally gives a weaker signal, as on this panel. This may reflect a strain-specific variation.

library we isolated four cDNA clones of *mst 355a* and two cDNA clones of *mst 355b*. All of the cDNA clones were sequenced completely on at least one strand. None of the cDNA clones extended to the 5' ends predicted by the primer extension experiments: of the *mst 355a* clones, one began at nucleotide +20, two began at nucleotide +151, and one began at nucleotide +18, preceded by five bases not found anywhere in the genomic sequence. We searched the genomic sequence for two kilobases upstream of *mst 355a* for this five-base sequence, but found no matches. Since the fusion point of this pentamer to the genomic sequence bears no resemblance to consensus splice junctions, we regard the extra five bases as an artifact of the cDNA library construction. Of the *mst 355b* clones, one began at nucleotide +1045, or nucleotide +16 of *mst 355b*; the other cDNA clone began at nucleotide +1050, or nucleotide +21 of *mst 355b*. Except for the unidentified five bases, all of the cDNA sequences matched the genomic sequences exactly. Reasonable matches to established consensus sequences (Mount 1982) were found at each intron boundary.

Two sequence similarities are observed in comparisons of the nucleotide sequences upstream of each transcript. First, at a position 450 bases upstream of *mst 355a* and 250 bases upstream of *mst 355b*, there is an identity of 18 out of a stretch of 20 nucleotides (small stippled box in Fig. 4A). Second, at a position about 100 bases upstream of both transcripts, there is an identity of 9 out of a stretch of 10 nucleotides (small open box in Fig. 4A). Upstream of *mst 355a* is a reasonable match to the Goldberg–Hogness consensus sequence, beginning at nucleotide -28: TATATATAA (bold letters in Fig. 4B). Interestingly, upstream of *mst 355b* there is no match to the Goldberg–Hogness consensus sequence at the appropriate position. The closest match is the polyadenylation signal of *mst 355a* (TAATAAA), but this begins 48 nucleotides upstream of the first base of *mst 355b*.

Two of the *mst 355a* cDNA clones terminate with long poly(A) tracts at +1009 (vertical arrow, Fig. 4B); the other two *mst 355a* cDNA clones terminate without poly(A) at positions +1002 and +1004. The two *mst 355b* cDNA clones are derived from RNAs with distinct

Figure 4. Map and genomic sequence of the *mst 355* region. (A) Detailed map *mst 355a* and *mst 355b*. The boxes under the solid line represent the exons of *mst 355a* and *mst 355b*. The filled regions of the boxes represent the open reading frames; open regions represent 5' and 3' nontranslated sequences. Sites where addition of poly(A) has been found in cDNA clones are indicated by appended A_n. The small square superimposed on the solid line represent sequence identities; the stippled boxes outline sequence elements having 18 out of 20 identical nucleotides (numbered as in B, positions -221 to -202: GAGGCCAGAA AAATGTATAT and +541 to +560: GAGGCCAGAA AAACGGATAT); the open boxes outline sequence elements having 9 out of 10 nucleotide sequence identity (positions -115 to -106: ATTGCAATAC and +939 to +948: ATTGCAAGAC). Restriction sites are abbreviated: (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (R) *Rsa*I; (Xb) *Xba*I; (Xh) *Xho*I. (B) Genomic sequence of the *mst 355* region, from -300 relative to the start site of *mst 355a* to +1736, the end of the restriction fragment. The TATA element at -28 is shown in bold letters, splice junction sequences are underlined once, and poly(A) addition signals (Birnstiel et al. 1985) are double underlined. The first nucleotide of *mst 355b* corresponds to nucleotide +1031 of the genomic sequence. Vertical arrows denote poly(A) addition sites, except the last arrow at position +1594 which denotes the 3' end of one cDNA clone which did not have a poly(A) tail.

Figure 4. (See facing page for legend.)

3' ends (vertical arrows, Fig. 4B): one contains 19 A residues beginning at nucleotide +1539 while the longer clone terminates at nucleotide +1594 without a poly(A) tract. The significance of this 3' heterogeneity is unknown, as the only long open reading frame of *mst 355b* terminates before the first polyadenylation site. Our Northern blots show a diffuse band for *mst 355b*, probably due to variability in the length of the poly(A) tails; thus the relative contribution from species utilizing either poly(A) site cannot be distinguished. The predicted sizes of the mature transcripts, excluding the poly(A) tail, are 957 nucleotides for *mst 355a*, and 448 and 504 nucleotides for *mst 355b*.

Finally, each transcript contains only one long (>50 amino acid) open reading frame beginning with ATG. The ORF of *mst 355a* encodes a basic protein of 264 amino acids, hereafter called *msP 355a*. The ORF of *mst 355b* encodes an acidic protein of 90 amino acids, hereafter termed *msP 355b*.

Both *msP 355a* and *msP 355b* have features of secreted proteins

As the male accessory gland is a secretory tissue, proteins encoded by accessory gland RNAs would be expected to have hydrophobic amino-terminal signal sequences (Harwood 1980). As seen in hydrophilicity profiles of the predicted amino acid sequences (Fig. 5A,B), both *msP 355a* and *msP 355b* exhibit hydrophobic amino termini. Examination of these hydrophobic segments for potential signal peptide cleavage sites using the methods of von Heijne (1983) predicts positions with a high probability of cleavage, after amino acid 21 of the predicted *msP 355a*, and after amino acid 18 of the predicted *msP 355b* (arrows, Fig. 5C). Thus, both *msP 355a* and *msP 355b* are potentially secreted proteins.

We searched the amino acid sequence of *msP 355a* for other signals commonly found on secreted proteins. The sequence Asn-X-Ser/Thr is a signal for N-linked

Figure 5. Features of the predicted proteins. (A) Hopp-Woods hydrophilicity profile of the predicted *msP 355a* amino acid sequence. This profile was determined with an averaging length of 6 amino acids (Hopp and Woods 1981), using the DNA Inspector II+ sequence analysis program (Gross 1986). The abscissa represents fractional length of the protein, which is a total of 264 amino acids long. (B) Hopp-Woods hydrophilicity profile of the predicted *msP 355b* amino acid sequence. This profile was determined as in A; the length of this protein is 90 amino acids. (C) Maps of *msP 355a* and *msP 355b*. Hatched segments represent hydrophobic stretches at the amino terminus of each sequence. Vertical arrows represent signal peptide cleavage sites predicted by the methods of von Heijne (1983). Small circles below *msP 355a* mark N-linked glycosylation signals (Asn-X-Ser/Thr; Kornfeld and Kornfeld 1985). Small solid bars represent possible peptidase cleavage sites. The stippled area represents region of identity between *msP 355a* and ELH (aligned sequences are shown in Fig. 7). Small numbers above each diagram indicate distance between adjacent cleavage sites in amino acids. The predicted *msP 355a* would have a net charge of +5 at pH 7.0, and the predicted *msP 355b* would have a net charge of -4 at pH 7.0.

glycosylation of secretory proteins (Kornfeld and Kornfeld 1985). The predicted *msP 355a* sequence contains five such *N*-linked glycosylation signals, four of which are clustered near the central region of the protein, and the fifth of which lies near the carboxyl terminus (circles in Fig. 5C). No such signals are found in the predicted sequence of *msP 355b*.

Peptide hormones and peptide pheromones, such as yeast mating factor (Kurjan and Herskowitz 1982), usually are synthesized in the form of larger precursor proteins, which are later cleaved at specific positions to release the bioactive peptides. Pairs of basic amino acids (Lys or Arg) are frequently the sites of such specific peptidase cleavages (Giesow and Smyth 1980). The predicted *msP 355a* sequence contains five such pairs of basic amino acids, at amino acid positions 67, 157, 180, 194, and 201 (solid crossbars in Fig. 5C). Thus, *msP 355a* encodes a secreted protein (see below) which may also be glycosylated and cleaved into smaller peptides, in the manner of known precursors to peptide pheromones and hormones.

msP 355a is secreted and transferred to the female during mating

To follow the protein encoded by *mst 355a*, antibodies against a β -galactosidase fusion protein were raised in rabbits. The antiserum was used to probe Western blots of proteins extracted from various *Drosophila* tissues. Extracts were prepared from male accessory glands and from the internal genitalia (excluding the ovaries) of unmated (virgin) female flies. Anti-*msP 355a* recognizes three male accessory gland-specific bands of M_r 41, 37, and 36 kD (Fig. 6, lane 1), of which the 37-kD band is the

most intensely stained. These bands are not present in the unmated female flies (Fig. 6, lane 2), and they migrate slower than would be expected from the amino acid sequence alone, which predicts an M_r of 29 kD (including the putative signal peptide). We consider these three bands to be alternate forms of the protein encoded by *mst 355a*, and in this paper we will refer to the set collectively as *msP 355a*.

During *Drosophila* mating, ejaculation normally occurs between 5–10 min after the beginning of copulation, which normally lasts for about 20 min (Fowler 1973). Mating couples were interrupted either after 10 min of copulation or allowed to complete copulation, and protein extracts were prepared from the internal genitalia (excluding ovaries) of the females. *msP 355a* is transferred to the female fly during the first 10 min of copulation (Fig. 6, lane 3). At the end of mating, 10–15 min after ejaculation, *msP 355a* has been altered; the major band now appears at 29 kD (Fig. 6, lane 4).

Preliminary results using antibodies raised against an *msP 355b-lacZ* fusion protein indicate that *msP 355b* also encodes an accessory gland protein that is transferred to the female genital tract during mating. The accessory gland protein which is recognized by anti-*msP 355b* migrates at 11–14 kD, which is close to the 10-kD size (including the putative signal peptide) predicted from the sequence of *msP 355b*.

msP 355a has amino acid sequence similarity to *Aplysia* egg-laying hormone

The National Biomedical Research Foundation's protein sequence database was searched for any matches to the predicted *msP 355a* and *msP 355b* amino acid sequences, using MicroGenie software (Beckman). No significant sequence matches were found for *msP 355b*. One match was found for the *msP 355a* amino acid sequence, with egg-laying hormone (ELH) of the mollusc *Aplysia californica* (the California seahare). ELH is a 36-amino-acid peptide neurohormone which is involved in eliciting stereotyped postmating behavior in *Aplysia* (Chiu et al. 1979). The similarity lies in the stretch from amino acids 121 to 137 of *msP 355a*, in which 11 out of 17 amino acids are identical to those in the stretch from amino acid 2 to 18 of ELH, allowing for a single amino acid gap in each sequence (Fig. 7).

Discussion

The male accessory gland of *Drosophila* produces a complex secretion of amino acids, proteins, peptides, lipids, and carbohydrates (Federer and Chen 1982; Chen 1984). Certain components of the accessory gland secretion elicit specific behavioral and physiological responses in the female fly after mating (Chen 1984); others have been suggested to play a role in sperm storage and nutrition (Fowler 1973). While the accessory gland secretion of *D. melanogaster* has been shown to increase oviposition and decrease receptivity, little progress has been made in the analysis of specific secretion

Figure 6. Immunological detection of *msP 355a*. Western blot probed with anti-*msP 355a*. Proteins were extracted from dissected tissues of 10 flies and run on 12.5% polyacrylamide-SDS gels. (Lane 1) Male accessory glands; (lane 2) internal genital ducts (excluding ovaries) of virgin female flies; (lane 3) internal genital ducts (excluding ovaries) of mated female flies, dissected 10 min after the beginning of copulation; (lane 4) internal genital ducts (excluding ovaries) of mated female flies, dissected at end of mating, 20 min after beginning of copulation. The three accessory gland bands detected with anti-*msP 355a* at 41 kD, 37 kD, and 36 kD are indicated; in lane 4 the main band has shifted down and now migrates at 29 kD.

Figure 7. Amino acid alignment of *msP 355a* with ELH. The regions of *msP 355a* sequence identity with ELH are outlined; the full sequence of ELH (Chiu et al. 1979) is presented for clarity. Allowing one gap in each sequence, 11 of 17 amino acids are identical (boxed); if no gaps are allowed, 9 of 17 amino acids are identical. Amino acid 155 of *msP 355a* is followed by the possible cleavage signal Arg-Lys; amino acid 36 of ELH is followed by Gly-Lys-Arg. The probability of finding 9 identities in a stretch of 17 amino acids is 3.1×10^{-6} as calculated by the method of Fitch (1966), using the amino acid frequencies in the ELH precursor (271 amino acids; Nambu and Scheller 1986) and *msP 355a* (264 amino acids). We feel that this match has biological significance, because the probability of such a random match is so low, and because there are only 6.3×10^4 possible 17-amino-acid comparisons possible between the ELH precursor and *msP 355a* (Fitch 1966).

components. Two components of the *D. funebris* secretion have been purified and linked to specific aspects of the female response to mating (Baumann 1974b); however, the *D. funebris* components do not function in *D. melanogaster* (Baumann 1974b). We have taken a molecular genetic approach to analyze the functions of accessory gland products by examining the structure and expression of genes that encode components of the accessory gland secretion. This information will allow a more detailed analysis of the roles of the protein products of these genes in reproduction.

Expression of *mst 355a* and *mst 355b*

We report here that the accumulation of *mst 355a* and *mst 355b* RNAs is not controlled by the somatic sex determination hierarchy in adults. A similar pattern of regulation is observed for another male accessory gland specific transcript, *mst 316* (DiBenedetto et al. 1987). Chapman and Wolfner (1988) have shown that adult expression of *mst 355a*, *mst 355b*, and *mst 316* is specified by the sex determination loci during the late third larval instar. At this time, some of the cells of the genital imaginal disk become determined to produce accessory glands (Chapman and Wolfner 1988). During the third larval instar, alteration of the expression of *tra2^{ts2}* by temperature shift can affect subsequent adult expression of *mst 355a* and *mst 355b*. As shown here, alteration of *tra2^{ts2}* expression in the adult can no longer affect adult expression of *mst 355a* and *mst 355b*. We have also shown that expression of the male accessory gland transcripts *mst 355a* and *mst 355b* does not depend upon a functional germ line. Male progeny of *tud¹* mothers, without germ lines, express both *mst 355a* and *mst 355b* at essentially wild-type levels, and these animals have normal appearing accessory glands.

Taken together, these analyses of the expression of *mst 355a* and *mst 355b* suggest that the regulation of these genes by the somatic sex determination genes (such as *tra2*) occurs at the level of tissue specificity. The somatic sex determination hierarchy establishes whether any male accessory gland tissue will develop from the genital disk; as a consequence of accessory gland differentiation, both *mst 355a* and *mst 355b* tran-

scripts are expressed. Once the genital disk cells have become determined to produce accessory glands, alteration of *tra2* expression does not affect *mst 355a* or *mst 355b* expression (at the level of RNA accumulation).

Gene structure

The organization of the genes encoding *mst 355a* and *mst 355b* is unusual, in that the first nucleotide of *mst 355b* occurs only 20 nucleotides downstream of the *mst 355a* polyadenylation site. Although it is formally possible that both transcripts are alternate processing products from a single RNA precursor, we have detected no such precursor in primer extension or S1 nuclease experiments. Other preliminary evidence indicates that *mst 355a* and *mst 355b* are the products of independent transcription units (S.A. Monsma and M.F. Wolfner, in prep.). An *mst 355b-lacZ* fusion gene which lacks all sequences 5' to the intron of *mst 355a* is transcribed with the same sex and tissue specificity as the endogenous *mst 355b* gene. Therefore, transcription of *mst 355b* requires neither sequences upstream of *mst 355a* nor the initiation site (+1) of *mst 355a*.

The *mst 355a* gene has a good TATA homology at -28, a typical position. In contrast, the only TATA homology upstream of *mst 355b* is the polyadenylation signal of *mst 355a*, which, at -48 relative to the start of *mst 355b*, lies further upstream than is typical for this type of regulatory element (Breathnach and Chambon 1981). Thus, the *mst 355b* transcription unit may not have a functional TATA element. This is not uncommon in *Drosophila*, as several other *Drosophila* genes lacking TATA homologies have been described (e.g., Mlodzik and Gehring 1987; Scholnick et al. 1987; Bowtell et al. 1988; Reinke et al. 1988; Sun et al. 1988).

Towards the function of *msP 355a* and *msP 355b*

The male accessory gland produces secreted products that play a role in the postmating response of female flies. We have shown that the antiserum raised against the protein encoded by *mst 355a* recognizes a set of accessory gland protein bands, referred to as *msP 355a*. We have also demonstrated transfer of *msP 355a* from the

male to the female fly during copulation, and have shown that modification of *msP 355a* occurs in the female genital tract within 10–15 min after ejaculation. Transfer of *msP 355a* to the female fly may imply a role in the female postmating response.

The protein encoded by *mst 355a* has many features associated with peptide pheromone and hormone precursors. Aside from the putative signal peptide, the sequence of *msP 355a* also exhibits five N-linked glycosylation signals. Modification of *msP 355a* by addition of carbohydrate does occur, and accounts for some of the difference between the observed M_r of 36–41 kD and the predicted M_r of 29 kD (S.A. Monsma, unpubl.). *msP 355a* also contains five pairs of basic amino acids, commonly used as cleavage signals in polyprotein precursors. The observed shift in *msP 355a* mobility by the end of mating may reflect proteolytic or glycolytic cleavage in the female genital tract. Such cleavage might serve to liberate physiologically active components from *msP 355a*.

The predicted amino acid sequence of *msP 355a* has no similarity to the reported amino acid sequence of the *D. funebris* peptide PS-1, which affects the receptivity of female *D. funebris* (Baumann et al. 1975). However, *msP 355a* has an intriguing characteristic—its region of amino acid sequence similarity with ELH of *A. californica*. ELH is a 36-amino acid peptide hormone that is released from the bag cells of the abdominal ganglion as a result of mating (Kaldany et al. 1985). ELH stimulates specific neurons of the abdominal, pedal, and buccal ganglia, thus contributing to the production of several stereotyped egg-laying behavior patterns (Scheller et al. 1983). ELH also circulates in the bloodstream and stimulates contraction of the ovotestis, contributing to release of the egg mass. Thus, ELH functions both as a neurotransmitter and as a peptide hormone. ELH is contained within a 271-amino-acid precursor protein (Nambu and Scheller 1986), and is released from this precursor by cleavage at paired basic amino acids. In this light, it is intriguing that *msP 355a* contains a pair of basic amino acids at the same position, relative to the region of ELH identity, as the authentic cleavage site at the carboxyl terminus of ELH (see Fig. 7 legend). Cleavage of *msP 355a* at this site and at the nearest pair of basic amino acids to the amino terminus would result in a 90-amino-acid peptide, with the similarity to ELH occurring between amino acids 65 and 80 of the *msP 355a* peptide.

One other *Drosophila* protein, the product of the *dunce* gene (*dnc*⁺), has been found to have sequence similarity to the ELH precursor (Chen et al. 1986). However, the region of ELH with similarity to the *dnc*⁺ product is distinct from the region with similarity to *msP 355a*. In addition to the learning disability of *dnc* mutants, Chen et al. note that *dnc* females are sterile, partly due to their failure to lay eggs. This female sterility phenotype may be genetically suppressed independently of the learning defect, suggesting that the *dnc*⁺ product may have independent functions in both learning and in fertility.

At present, the amino acid sequence similarity be-

tween *msP 355a* and ELH allows only speculation as to the function of *msP 355a*. Both ELH and the secretions of the accessory gland are involved in the production of post mating behavioral and physiological responses. If the amino acid sequence similarity we observed reflects a functional relationship, it would suggest that *msP 355a* or its cleavage products might be involved in the increased egg-production rate of the mated female's ovary, or in eliciting oviposition behavior. These hypotheses can be tested by application of *msP 355a* to isolated ovaries or by injection into virgin female flies, and by using the antibodies to follow *msP 355a* in mated female flies.

Methods

Fly handling and rearing

Drosophila were raised on yeast–glucose medium at 25°C unless otherwise noted. For preparation of RNA or protein, flies were collected within 8 hr of eclosion and aged for 2 days at 25°C in the absence of the opposite sex. All protein experiments were carried out with Canton S flies; unless noted otherwise, RNA experiments were carried out with Canton S flies or with unisex cultures derived from the cross of *cin y w* as described in DiBenedetto et al. (1987). Where tested, the same results were obtained with Canton S as with *cin* progeny.

For experiments to determine the genetic regulation of the accessory gland transcripts, X/X *tra2^{ts2}* homozygotes were obtained from the *B^sY; tra2^{ts2} bw/CyO* stock, and used as in DiBenedetto et al. (1987). Flies with no germ line were generated by crossing *bw sp tud¹* virgin females to Canton S males as described in DiBenedetto et al. (1987).

Tissues for RNA or protein preparation were obtained by dissection in 0.7% wt/vol NaCl, and were extracted immediately as described below. Fly matings for protein experiments were performed by adding unanesthetized 2-day-old virgin female Canton S flies to vials containing excess unanesthetized 2-day-old virgin male Canton S flies. Couples were removed to empty vials within 5 min of the beginning of copulation. Couples were either anesthetized with CO₂ at 10 min after the beginning of copulation and vortexed to separate the flies, or allowed to complete copulation and anesthetized at 20 min after the beginning of mating. The internal genitalia (excluding the ovaries) of 10 mated female flies from each time point were dissected immediately and extracted as described below.

Nucleic Acids

Cloning and routine nucleic acid manipulations (Southern blots, plaque filter screening) were performed as described in Maniatis et al. (1982). RNA extractions and Northern blots were performed as in DiBenedetto et al. (1987). Nested deletions of the genomic subclones for sequencing were obtained using exonuclease III (Henikoff 1984) and mung bean nuclease (Komalski et al. 1976). Sequencing was performed by the dideoxy chain-termination method (Sanger et al. 1977), with the addition of deoxy-7-deaza-GTP (Mizusawa et al. 1986) to resolve compression artifacts. Oligonucleotide primers were synthesized by the Cornell Biotechnology Facility. The primer synthesized for *mst 355a* was complementary to exonic sequences to either side of the intron, and had the sequence 5'-AAAAG-CAGCAGTAAAATTGG-3'. The primer synthesized for *mst 355b* was complementary to positions +1192 to +1211 of the

genomic sequence (Fig. 4) and had the sequence 5'-TTGTGAAGTAGACTGAACGC-3'.

The cDNA library was constructed from 5 µg of poly(A)⁺ RNA extracted from 2-day-old adult male progeny of the *cin y w* cross described in DiBenedetto et al. (1987). Double-stranded cDNA was synthesized and inserted into λgt10 arms using commercial kits and protocols (Amersham). Size-fractionation steps were omitted, except for removal of excess linkers on a gel filtration column. Independent recombinant phage 2.2×10^5 were obtained; a total of 1×10^5 phage were screened to obtain cDNA clones.

Production of fusion proteins and immunological techniques

The *lacZ*-*msP 335a* fusion was constructed using the pWR590-1 bacterial expression vector (Guo et al. 1984). This vector contains the *lac* regulatory sequences and about 600 amino acids of the amino terminal of *lacZ*, followed by a multiple cloning site into which a cDNA clone of *mst 355a* was inserted. Fusion protein expression was induced in *Escherichia coli* strain JM101 by addition of 3 mM IPTG to the culture medium; a fusion protein of 94 kD was purified from 10% acrylamide-SDS gels by electroelution from gel slices. One hundred micrograms of the fusion protein was emulsified with Freund's complete adjuvant (Sigma), and injected into giant chinchilla rabbits at multiple subdermal sites. Two weeks after the initial immunization, the rabbits were boosted with 50 µg of fusion protein emulsified with Freund's incomplete adjuvant (Sigma). Beginning 2 weeks after the boost, the rabbits were bled twice weekly and boosted as necessary.

Drosophila protein extracts were prepared by grinding whole flies or dissected tissues in lysis buffer [1.5% SDS, 2.5% β-mercaptoethanol, 25 mM Tris-HCl (pH 7.5), 0.05% bromophenol blue, 5% glycerol] and heating at 100°C for 5 min. Just prior to electrophoresis, the samples were spun in a microfuge for 1 min, and the supernatants reheated briefly (100°C for 1 min). SDS gels were 12.5% polyacrylamide (36.5 parts acrylamide : 1.0 part bis-acrylamide; Laemmli 1970). Electrophoretic transfer to nitrocellulose was performed as described in Towbin et al. (1979), with the addition of 0.1% SDS to the transfer buffer. Western blots were blocked by incubation in TBS (500 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 3% gelatin for 45 min. The blots were incubated for 3–16 hr with rabbit anti-serum diluted 1 : 500 in TBS containing 1% gelatin and 0.5% Tween 20. The blots were washed 3 × for 5 min in TBS with 0.5% Tween-20, then incubated for 1 hr with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) diluted 1 : 3000 in TBS containing 1% gelatin. After washing 2 × for 5 min in TBS with 0.5% Tween-20 and once for 5 min in TBS, color development was carried out with 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) and nitro blue tetrazolium (Sigma).

Note

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries under accession number Y00219.

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Structure and expression of a *Drosophila* male accessory gland gene whose product resembles a peptide pheromone precursor.

S A Monsma and M F Wolfner

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