FUNCTIONS AND REGULATION OF RNAs TRANSCRIBED FROM THE *DROSOPHILA MELANOGASTER* 68C PUFF

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ABSTRACT

Puffs appear and disappear on *Drosophila* salivary gland polytene chromosomes at specific developmental timepoints or in response to external stimuli. This thesis is an analysis of the functions and regulation of three RNAs transcribed from the puff at 68C on the left arm of the third chromosome.

The nucleotide sequence of the DNA encoding these RNAs was used to predict the physical and chemical properties expected of their protein products. Analysis of radiolabeled salivary glands revealed polypeptides having the characteristics predicted for the products of the 68C RNAs. Amino acid sequencing of these proteins showed that they are in fact encoded by the 68C RNAs. All three polypeptides were found to be part of the salivary gland glue: one is the previously described sgs-3, the others the newly identified glue proteins sgs-7 and sgs-8.

The effect of the steroid hormone ecdysterone on the 68C RNAs was examined by culturing salivary glands *in vitro* in the presence or absence of the hormone. The presence of the steroid caused the RNAs to disappear more rapidly than they would in its absence. Pulse-labeling experiments demonstrated that the effect of ecdysterone is on an early step in RNA production, probably transcription. The effect on the 68C RNAs is very rapid, more rapid than puff regression. The three RNAs appear to be coordinately regulated.

The expression of glue protein genes in a non-pupariating mutant strain of *Drosophila* has been studied. Although a puff is present at position 68C on the third chromosome in the mutants, the *Sgs-3*, *Sgs-7* and *Sgs-8* genes are not expressed. Pulse-labeling experiments indicate that the mutation affects transcription of these genes. Other researchers have mapped the non-pupariating mutation to position 2B on the X chromosome. It appears that a product of a gene

at 2B or a product whose synthesis is induced by a gene at 2B is necessary for transcription of the 68C glue protein genes. This *trans*-acting regulatory element produces its effect by interacting with DNA sequences within or very close to the glue genes at 68C. These results along with transcription autoradiograms show that the puff at 68C is not caused by transcription of *Sgs-3*, *Sgs-7*, *Sgs-8* or any other genes located within the puff region.

TABLE OF CONTENTS

CHAPTER 1

Introduction

Polytene Chromosome Puffs in the *Diptera*

Certain tissues in dipteran larvae contain polytene chromosomes. These chromosomes can consist of more than a thousand strands of DNA bundled together and when stained and viewed through a light microscope they appear as long tubular structures. Dark bands of varying thickness are visible running perpendicular to the long axis of the chromosomes. These bands are interspersed with lightly stained regions thus creating a pattern on each chromosome which is distinct from that found on the other chromosomes. In a given organism, the same banding pattern is observed in all tissues which contain polytene chromosomes. These bands provide a means by which the various chromosomes can be identified and also provide a cytological map which can be correlated with a gene linkage map (Slizynsky, 1950; Beermann, 1952; Pavan and Breuer, 1952).

A number of small segments of polytene chromosomes are wider and have a more diffuse banding pattern than the rest of the chromosome. These expanded regions are known as Balbiani rings or puffs. The size and location of puffs changes during development in a tissue-specific manner. About thirty years ago it was postulated that chromosomal puffs are an indication of gene activity at a particular locus (Beermann, 1952; Breuer and Pavan, 1955). Support for this hypothesis comes from several lines of evidence. Observation of *Chironomus tentans* polytene chromosomes stained with toluidine blue reveals that there is a much higher concentration of RNA in puffed regions than in unpuffed regions (Pelling, 1964). Autoradiographs of *Chironomus* and *Drosophila* chromosomes pulse-labeled with ^{3}H-uridine shows that puff sites are synthesizing RNA at a faster rate than other parts of the chromosomes (Pelling, 1964; Berendes, 1968; Ellgaard and Clever, 1971; Tissières *et al.*, 1974; Zhimulev and Belyaeva, 1975; Belyaeva and Zhimulev, 1976; Bonner and Pardue, 1977; Mitchell *et al.*, 1978). Since the RNA in a puff is undoubtedly transcribed from the puff DNA the staining and labeling results imply that genes within a puff are more

active than genes located elsewhere. Also, there are several examples in *Drosophila melanogaster* of proteins which are only synthesized when the chromosomal regions containing their genes are puffed (Korge, 1977; Akam *et al.*, 1978; Mirault *et al.*, 1978; Moran *et al.*, 1978; Velissariou and Ashburner, 1980 and 1981; Guild, 1984).

The steroid hormone ecdysterone regulates many of the puffs which appear on polytene chromosomes in *Drosophila melanogaster* salivary glands. These puffs can be divided into three groups based on the developmental time course of their appearance. The intermolt puffs are visible at the earliest time at which such structures can be seen on polytene chromosomes, the middle of the third larval instar, and then regress at the end of this stage as a result of an increase in the concentration of ecdysterone in the larval hemolymph. The early puffs are induced almost immediately by the increase in hormone concentration while the late puffs appear several hours later, approximately the time of puparium formation. This has been proven by following the change in the puffing pattern on the salivary chromosomes during development *in vivo* and by examining the effect of ecdysterone on the puffs in salivary glands cultured *in vitro* (Ashburner, 1967; Ashburner, 1972; Ashburner, 1973; Ashburner *et al.*, 1973).

Indirect immunofluorescence experiments have shown that ecdysterone binds to one of the intermolt puffs, that located at position 68C on the third chromosome, at the time it is regressing. Presumably the steroid reacts with a receptor protein in the cytoplasm and the steroid-receptor complex then translocates to the nucleus and binds to the 68C puff causing it to contract (Maroy *et al.*, 1978; Gronemeyer and Pongs, 1980).

The main activity in *Drosophila* salivary glands during the third larval instar is the synthesis of salivary gland secretion (sgs) proteins (Zhimulev and

Kolesnikov, 1975). These polypeptides are retained in the gland cells until the end of the third instar when they are secreted into the lumen of the gland. As the larval cuticle hardens the sgs polypeptides are expelled through the salivary duct as part of the mucoprotein glue which then hardens and causes the newly formed puparium to adhere to the surface upon which it rests (Fraenkel and Brookes, 1953; Korge, 1975; Beckendorf and Kafatos, 1976; Korge, 1977). The intermolt puffs are present on the salivary chromosomes at the same time the glue proteins are being synthesized. Genetic mapping experiments have revealed that five of the intermolt puffs each contain a gene which codes for one of the sgs proteins. The puff at 68C harbors the structural gene for the glue polypeptide known as sgs-3 (Korge, 1977; Akam *et al.*, 1978; Velissariou and Ashburner, 1980 and 1981; Guild, 1984).

Molecular analysis of the 68C locus has shown that three abundant salivary gland RNAs are transcribed from the puff. The 68C transcripts found in the cytoplasm of salivary gland cells are polyadenylated and are associated with polyribosomes. They are not found in other tissues of third instar larvae (Meyerowitz and Hogness, 1982). The RNAs are approximately 320, 360 and 1120 bases long and the genes from which they are transcribed are clustered within 4500 base pairs of DNA (Garfinkel *et al.*, 1983). Thus the 68C puff is regulated by a steroid hormone, contains a glue protein gene, and produces three abundant, salivary gland-specific RNAs which have the characteristics of messenger RNAs.

I have sought to gain an understanding of the functions and regulation of the 68C RNAs. More specifically, I have tried to determine if the RNAs code for proteins and if they do, what the function of the proteins is. I have attempted to find out if ecdysterone regulates the RNAs as it does the puff, and if so, which process in RNA metabolism is affected by the steroid and how much time is required for the effect. Also, I have been interested in the relationship between

puffing and transcription at 68C, and in regulation of puff formation and glue gene expression at 68C by proteins coded elsewhere in the *Drosophila* genome.

Chapter 2 of this thesis describes experiments which prove that the three RNAs transcribed from the 68C puff are messengers for glue polypeptides. The longest RNA codes for sgs-3, while the 320 and 360 nucleotide RNAs code for the newly identified sgs-7 and sgs-8 glue proteins respectively. In Chapter 3 evidence is presented that the presence of the steroid hormone ecdysterone in salivary glands reduces the levels of the 68C RNAs by affecting an early step in RNA production, probably transcription. The hormone acts more rapidly on the RNAs than it does on the puff. The results discussed in Chapter 4 show that a mutation at position 2B on the X chromosome inhibits transcription of the 68C glue genes without preventing puff formation. This indicates that the puff at 68C is not caused by synthesis of the glue RNAs and that a product of a gene at 2B or a product induced by the 2B gene is necessary for activation of transcription at 68C.

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CHAPTER 2

The Structural Genes for Three *Drosophila* Glue Proteins Reside at a Single Polytene Chromosome Puff Locus

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The Structural Genes for Three *Drosophila* Glue Proteins Reside at a Single Polytene Chromosome Puff Locus

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The polytene chromosome puff at 68C on the *Drosophila melanogaster* third chromosome is thought from genetic experiments to contain the structural gene for one of the secreted salivary gland glue polypeptides, sgs-3. Previous work has demonstrated that the DNA included in this puff contains sequences that are transcribed to give three different polyadenylated RNAs that are abundant in third-larval-instar salivary glands. These have been called the group II, group III, and group IV RNAs. In the experiments reported here, we used the nucleotide sequence of the DNA coding for these RNAs to predict some of the physical and chemical properties expected of their protein products, including molecular weight, amino acid composition, and amino acid sequence. Salivary gland polypeptides with molecular weights similar to those expected for the 68C RNA translation products, and with the expected degree of incorporation of different radioactive amino acids, were purified. These proteins were shown by amino acid sequencing to correspond to the protein products of the 68C RNAs. It was further shown that each of these proteins is a part of the secreted salivary gland glue: the group IV RNA codes for the previously described sgs-3, whereas the group II and III RNAs code for the newly identified glue polypeptides sgs-8 and sgs-7.

Drosophila melanogaster begins life as a fertilized egg, which after a day of embryonic development hatches as a wormlike first-instar larva. After another day this larva molts and becomes a larger, second-instar larva; one further day leads to a second molt and the last stage of larval life, the third larval instar. This lasts two days, at the end of which the larval cuticle hardens to form a pupal case. After an immobile prepupal period, pupation and metamorphosis occur, resulting in an adult fly. The major synthetic activity of the salivary gland cells of the third-instar larva is the production of a set of about 10 polypeptides that are secreted into the lumen of the gland after synthesis and that at the end of larval life are expelled through the duct of the salivary glands and deposited on the surface upon which the larva rests. This protein secretion serves as a glue that causes the prepupa, and subsequently the pupa, to adhere to its substrate for the duration of the pupal period (5, 8, 15, 16).

At the time of glue protein synthesis there are visible a number of prominent puffs, or actively transcribing sites, on the salivary gland polytene chromosomes. These are called the intermolt puffs (3). Genetic experiments have identified four of these puffs as the probable loci of structural genes coding for four different glue polypeptides (1, 16, 30, 31). One of the largest of these puffs is at position 68C on the third chromosome; it has been correlated with the structural gene, *Sgs-3*, of the secreted glue polypeptide sgs-3, a glycoprotein and a major glue component (fraction 3 [15, 16]; protein P1 [5]). Puffing at 68C correlates with transcription of the puff DNA (19), and regression of the puff is the direct result of an interaction of the puffed chromosomal region with the steroid hormone ecdysone (4, 10). Molecular cloning of the puff DNA has shown that it contains the coding sequences for three different polyadenylated RNA species, all abundantly present on polyribosomes in third-larval-instar salivary glands. These RNAs have been designated the group II, group III, and group IV RNAs. They measure 360, 320, and 1,100 nucleotides, respectively, are all coded in a single 5,000-base-pair region of the puffed DNA, and appear and disappear from the salivary glands coordinately (19). To understand the regulation of the steroid-repressed 68C gene cluster, it is necessary to know the function of the three RNAs coded by the 68C puff. Circumstantial evidence has implied that the group IV RNA is the sgs-3 messenger; two

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possibilities for the roles played by the two small RNAs have been proposed (19). One is that their translation products serve a regulatory function in the coordination of expression of the various unlinked glue structural genes; the other is that they translate to produce glue proteins. In this paper we demonstrate that the group IV RNA does indeed translate to produce sgs-3. In addition, we show that the group II and group III RNAs are mRNAs coding for two additional secreted glue proteins, here named sgs-8 and sgs-7. This brings to six the number of glue polypeptides whose structural genes have been located in previously described intermolt puffs and presents the first case in which more than one glue protein derives from a single puff.

MATERIALS AND METHODS

Materials. Aminophenylthioether paper was prepared according to the procedure of Seed (26). Oligodeoxythymidylic acid-cellulose was purchased from Boehringer Mannheim Biochemicals. RNase A (bovine pancreatic) was purchased from Sigma Chemical Co., DNase I was from Worthington Diagnostics, and ampholines were from LKB Instruments Inc. Cyanogen bromide was obtained from Eastman Kodak, and formic acid was from Fisher Chemical Co. Polaroid Coaterless Land Pack Film 667 was used for photography of stained gels. *Escherichia coli* RNA polymerase I was a gift from C. Parker. The cyanogen bromide cleavage products of myoglobin were provided by J. R. Bell. Phosphorylase A, bovine serum albumin, egg albumin, carboxypeptidase A, trypsinogen, β-lactoglobulin, and lysozyme were purchased from Sigma Chemical Co. The isoelectric point markers, which were prepared by British Drug House Chemicals, Ltd., were purchased from Gallard-Schlesinger. Dithiothreitol was obtained from Calbiochem. Iodoacetamide was purchased from Sigma and was recrystallized twice before use. Acetonitrile (UV) was obtained from Burdick and Jackson Laboratories. The Oregon R strain of flies contained the homozygous third chromosome of strain OR16f (19). The Formosa strain of flies was kindly provided by S. K. Beckendorf, Department of Molecular Biology, University of California, Berkeley.

Preparation of DNA filters. Plasmid DNAs were purified by CsCl equilibrium centrifugation as described by Meyerowitz et al. (18), sheared by heating to 100°C for 15 min in 0.3 N NaOH (30 to 75 μg of DNA per ml), neutralized, and ethanol precipitated. The fragmented DNA was then dissolved at 0.4 to 1.0 mg/ml in 80% dimethyl sulfoxide–13 mM citric acid–17 mM sodium phosphate (pH 4.0) and transferred to disks of aminophenylthioether paper (1.1-cm diameter) that had been freshly converted to diazophenylthioether paper (2). A 10-μl sample of the DNA solution was spotted on each filter. After 20 h of incubation at 24°C, the filters were washed in water at 24°C, in 0.4 N NaOH at 37°C, and again in water at 24°C and stored in 50% formamide–1 M NaCl–20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8)–0.02% sodium dodecyl sulfate (SDS)–1 mM EDTA at 4°C.

Purification of RNA. Late-third-instar larvae were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The tissue was solubilized in 20 mM NaCl–20 mM Tris-hydrochloride (pH 7.5)–1% SDS–40 mM EDTA and extracted with equal volumes of phenol and chloroform-isoamyl alcohol (100:1). The aqueous phase was extracted again with phenol plus chloroform and then with chloroform alone. The nucleic acid was ethanol precipitated and passed through an oligodeoxythymidylic acid-cellulose column in 0.5 M NaCl–10 mM Tris-hydrochloride (pH 7.4)–0.2% SDS–1 mM EDTA. The column was washed with four volumes of the same buffer to remove unbound material, and the bound polyadenylated RNA was then eluted with 10 mM Tris-hydrochloride (pH 7.4)–0.2% SDS–1 mM EDTA.

Hybrid selection of RNA and translation in vitro. Polyadenylated larval RNA (3 μg) was incubated with a 1.1-cm-diameter filter disk containing sheared DNA in 75 μl of hybridization buffer (50% formamide, 1 M NaCl, 20 mM PIPES [pH 6.8], 0.2% SDS, 1 mM EDTA) at 42°C with gentle agitation for 20 h. Unhybridized RNA was removed, and the filters were washed twice in 75 μl of hybridization buffer at 42°C for 20 min and then three times in 1 ml of 0.2 M NaCl–20 mM PIPES (pH 6.8)–0.2% SDS–1 mM EDTA at 42°C for 5 min. The hybridized RNA was then eluted by shaking the filters in 75 μl of 99% formamide at 56°C for 20 min. The elution was repeated two more times, and the elutions were pooled (G. Guild, personal communication). After addition of 2 μg of yeast tRNA as carrier, the eluted RNA was ethanol precipitated, dissolved in 2 μl of water, and translated in a micrococcal nuclease-treated rabbit reticulocyte lysate (New England Nuclear Corp.), prepared as described by Pelham and Jackson (25). The total reaction volume was 6.5 μl. [^{35}S]cysteine or [^{35}S]methionine (400 to 1,100 Ci/mmol; New England Nuclear) was used as the label in the translations, and the products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Labeling of proteins in vivo. Salivary gland proteins were labeled by injecting [^{35}S]cysteine into larvae or by culturing dissected salivary gland lobes in the presence of a radiolabeled amino acid. For injection, [^{35}S]cysteine was dissolved at 83 μCi/μl in culture medium (10 mM morpholinepropanesulfonic acid [pH 7.0], 80 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$), and 0.1 μl was injected into each larva (20, 29). The larvae were then incubated on filter paper moistened with 10% sucrose at 22 to 24°C for 30 min. Salivary gland lobes were then dissected from the larvae, and proteins were extracted as described below. For labeling in culture, dissected salivary gland lobes were incubated in a 5- to 10-μl drop of culture medium containing 10 μCi of a radiolabeled amino acid per μl (21, 29). The specific activities (curies per millimole) of the amino acids (all from New England Nuclear) were as follows: [^{35}S]cysteine and [^{35}S]methionine, 400 to 1,100; [^{3}H]proline, 102 to 139; [^{3}H]isoleucine, 83; [^{3}H]lysine, 68; [^{3}H]glycine, 53; and [^{3}H]threonine, 5.3. After 30 min at 22 to 24°C the glands were removed from the labeling medium, and proteins were extracted from salivary gland lobes by one of the following four procedures. For one-dimensional SDS-PAGE the glands were heated for 5 min at 100°C in 0.5% SDS–1% 2-mercaptoethanol (1 to 2 μl per lobe). The solubilized proteins were then diluted with 10 volumes of 50 mM Tris-hydrochloride (pH

6.8)–0.5% SDS–1% 2-mercaptoethanol–10% (vol/vol) glycerol. For two-dimensional isoelectric focusing (IEF)–SDS-PAGE the glands were heated for 5 min at 100°C in 0.5% SDS–1% 2-mercaptoethanol (1 to 2 μl per lobe). Urea was then added to give a final concentration of 8 M, and Nonidet P-40 (NP-40) was added to give a final concentration of 2%. For two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE)–SDS-PAGE the glands were sonicated, and treated with RNase A and then with DNase I as described by O'Farrell (23). The proteins were then solubilized in 8 M urea–5% 2-mercapthethanol–1% NP-40–1% ampholines. For one-dimensional acid-urea-PAGE the glands were sonicated and nuclease treated as above, and the proteins were reduced and alkylated as described by Korge (16), except that 6% iodoacetamide was used instead of 2.5% ethylenimine (12).

Labeled glue proteins were isolated by the following procedure. Larvae were injected with [^{35}S]cysteine as described above or incubated for 3 h on filter paper moistened with 10% sucrose containing a labeled amino acid. [^{3}H]threonine was spread on the filter paper at 0.43 μCi/cm^2, and [^{35}S]cysteine was spread at 3.4 μCi/cm^2. Larvae were then transferred to fresh filter paper and left there until pupation. The pupae were then removed, and pieces of the filter paper containing the secreted glue were excised and pooled (B. McGinnis and S. Beckendorf, Drosophila Information Service, in press). Glue proteins were then eluted from the paper by one of four methods. For SDS-PAGE or IEF–SDS-PAGE the paper was heated for 10 min at 100°C in 50 mM Tris-hydrochloride (pH 6.8)–0.5% SDS–1% 2-mercaptoethanol–10% (vol/vol) glycerol. For NEPHGE–SDS-PAGE the proteins were eluted from the paper by either heating for 10 min at 100°C in 0.05% SDS–5% 2-mercaptoethanol or by sonicating in 5% 2-mercaptoethanol, adding NP-40 to a final concentration of 0.05%, and then heating for 10 min at 100°C. For one-dimensional acid-urea-PAGE the paper was suspended in 5% 2-mercaptoethanol, sonicated, and then heated for 10 min at 100°C. This solution was then removed, and the paper shreds were washed in 8 M urea–10 mM Tris-hydrochloride (pH 8.0)–5% 2-mercaptoethanol–1 mM EDTA for 18 h at 24°C with gentle agitation. At this point the liquid was separated from the paper and dialyzed against 1 mM Tris-hydrochloride (pH 8.0) for 18 h at 2°C. The dialyzed protein solution was lyophilized to dryness, and the proteins were reduced and alkylated as described above.

Isolation of unlabeled glue proteins. Bloated salivary glands from late-third-instar larvae were transferred to 95% ethanol, causing the glue to form a solid plug. The hardened glue was then dissected free of salivary gland cells with tungsten needles (14). The isolated glue proteins were then reduced and alkylated as described above.

PAGE. One-dimensional SDS-PAGE was carried out by the method of Laemmli (17), two-dimensional IEF–SDS-PAGE was carried out by the method of O'Farrell (23), and two-dimensional NEPHGE–SDS-PAGE was carried out by the method of O'Farrell et al. (24). IEF gels contained a mixture of pH 4 to 6, pH 5 to 7, and pH 3.5 to 10 ampholines. NEPHGE gels contained pH 3.5 to 10 ampholines. For preparative two-dimensional PAGE the SDS stacking gels were overlaid with SDS sample buffer (63 mM Tris-hydrochloride [pH 6.8], 2.3% SDS, 10% [wt/vol] glycerol, 1% agarose) containing 10 mM sodium thioglycolate. The thioglycolate was included to scavenge free radicals or oxidants in the gels (M. W. Hunkapiller, E. Lujan, F. Ostrander, and L. E. Hood, Methods Enzymol., in press). One-dimensional acid-urea-PAGE was carried out on 8 M urea–7% polyacrylamide gels as described by Korge (16).

Analytical gels of radiolabeled proteins were fixed in 50% methanol–10% acetic acid, treated with En^{3}Hance (New England Nuclear), dried under vacuum, and exposed to Kodak X-Omat AR5 film. Analytical gels of unlabeled proteins were fixed and stained in 0.25% Coomassie brilliant blue R–50% methanol–10% acetic acid and destained in 25% methanol–10% acetic acid.

Determination of isoelectric points. Nondenaturing isoelectric focusing gels consisted of 5% polyacrylamide, 12.5% sucrose, and 2.4% pH 3.5 to 10 ampholines. The electrode solutions were 1 M orthophosphoric acid and 1 M sodium hydroxide. Proteins were loaded onto the anodes (acidic ends) of these gels, and the gels were run at 1,000 V for 1.5 h. The marker proteins (British Drug House Chemicals) used were all highly colored and hence visible in the focusing gels without staining.

[^{35}S]cysteine-labeled salivary gland proteins were mixed with 235 μg of marker proteins and focused as described above. The focusing gel was then loaded onto an SDS gel, and second-dimension electrophoresis was carried out as described above with a 15% polyacrylamide separating gel. The slab gel was then fixed, stained with PAGE blue 83 (British Drug House), destained, treated with En^{3}Hance, and vacuum dried. The positions of the stained markers in the dried slab gel relative to the anode of the focusing gel were then determined. Radiolabeled salivary gland proteins were located by fluorography.

[^{3}H]threonine-labeled salivary gland proteins were mixed with 235 μg of marker proteins and focused as described above. After focusing, the distance of each marker protein from the anode was measured, and the gel was loaded onto an SDS gel. Second-dimension electrophoresis was performed as described above with a 10% acrylamide separating gel. The location of radiolabeled sgs-3 was determined by fluorography.

Isolation of labeled proteins from polyacrylamide gels. Salivary gland proteins were labeled in cultured glands and separated by preparative IEF–SDS-PAGE or NEPHGE–SDS-PAGE (see above). For isolation of ^{3}H-labeled proteins, a small amount of [^{35}S]cysteine-labeled protein was added to the sample before electrophoresis. After electrophoresis, gels were washed briefly in water, transferred to Whatman 3MM paper, and covered with Saran Wrap. The gels were dried under vacuum at 80°C for 1.5 to 2.0 h, and proteins were located by autoradiography. Spots containing proteins of interest were cut out of the gels and rehydrated. Proteins were electroeluted from the gel slices as described by Hunkapiller et al. (in press).

Amino-terminal sequence analysis of labeled proteins. ^{3}H- or ^{35}S-labeled proteins were reduced in 0.2 M Tris-hydrochloride (pH 8.4)–20 mM dithiothreitol under nitrogen in the dark at 37°C for 2 h. The proteins were then alkylated by adding solid iodoacetamide to a final concentration of 50 mM and incubating at 24°C in the

dark for 15 min. The Tris-hydrochloride and excess iodoacetamide were then removed by electrodialysis, and the proteins were subjected to automated Edman degradation on the spinning cup sequenator described by Hunkapiller and Hood (13). The fractions released by the sequenator were dried under vacuum, and each was resuspended in 0.5 ml of acetonitrile. The radioactivity in each fraction was then quantitated by scintillation counting using 5 ml of Aquasol 2 (New England Nuclear) per fraction.

RESULTS

Three 68C RNAs are mRNAs. The nucleotide sequence of the DNA coding for the three 68C RNAs is known, and from this sequence the amino acid sequences of the primary products expected from translation of these RNAs has been determined (Garfinkel, Pruitt, and Meyerowitz, submitted for publication). The expected properties of the three predicted peptides are as follows. Protein II, the primary translation product of the group II RNA, would be 75 amino acids long and would have a molecular weight of 7,704. It would contain 13 mol% cysteine residues and methionine residues at positions 1, 13, and 56. Protein III, the primary product of translation of the group III RNA, would be expected to be 74 amino acids long and 7,849 in molecular weight, to contain 12% cysteine residues, and to have methionines at positions 1 and 73. Protein IV, the unprocessed product of the group IV RNA, should contain 307 amino acids and measure 32,080 in molecular weight. It is not expected to be as high in cysteine content as the other two proteins, only 6%, but it should contain 15% proline and 42% threonine residues. Protein IV has only one methionine residue, at position 1. All three of the proteins expected from translation of the 68C RNAs are therefore quite unusual: proteins II and III are small, cysteine rich, and relatively methionine poor; protein IV is unusually rich in proline and threonine.

To be certain that RNAs II, III, and IV are indeed mRNAs, and therefore that the predicted proteins II, III, and IV might be found in larval salivary glands, the RNAs were purified and translated in vitro. Cloned DNA representing sequences from each of the RNA groups was denatured and covalently bound to diazotized paper. The clones used were the cDNA clone adm109F4 for the group II RNA, the cDNA clone adm127C8 for group III RNA, and the genomic clone aDm2023, a *Sal*I subclone which contains the entire coding sequence of the large RNA on a 2.4-kilobase fragment originally derived from λbDm2002, for the group IV RNA. These clones are described in detail by Meyerowitz and Hogness (19). Polyadenylated whole third-instar-larval RNA was hybridized with the DNA on these filters, nonspecifically bound RNA was washed away, and the bound RNA was eluted. This eluted RNA was added to a micrococcal nuclease-treated rabbit reticulocyte lysate and translated in the presence of either [^{35}S]methionine or [^{35}S]cysteine. Translation of group II RNA in the presence of [^{35}S]methionine gave no detectably labeled protein, as compared with a control translation without added RNA. Translation with [^{35}S]cysteine yielded a single protein species with an apparent molecular weight of 7,200, as measured by SDS-PAGE. [^{35}S]methionine also failed to detectably label the translation product of RNA III. [^{35}S]cysteine was incorporated into an RNA III-directed translation product of apparent molecular weight 7,700. Figure 1 shows the results of electrophoresis of these proteins. The group IV RNA also failed to cause detectable incorporation of methionine into protein; with [^{35}S]cysteine label radioactivity was converted into trichloroacetic acid-insoluble form, but the labeled protein did not enter SDS-polyacrylamide gels in electrophoresis experiments, as if it were insoluble in aqueous buffer. Thus, all three 68C RNAs are mRNAs, and they translate in vitro to polypeptides with some of the characteristics predicted from the RNA nucleotide sequences.

Third-instar salivary glands synthesize proteins similar to those translated in vitro from the 68C RNAs. The next step was to determine whether the predicted proteins II, III, and IV, or proteins deriving from post-translational modification of these unprocessed peptides, could be detected in third-instar salivary glands. Salivary glands were dissected from larvae and incubated in medium containing [^{35}S]cysteine for 20 min at room temperature, after which proteins were extracted from the labeled glands and subjected to electrophoresis in an SDS–15% polyacrylamide gel. A number of bands appeared in autoradiographs of the gel; the two most heavily labeled bands migrated at apparent molecular weights of 5,000 (5K) and 5.5K. When an equal number of salivary glands were incubated in an equivalent experiment using [^{35}S]methionine, these two bands also appeared in gel autoradiographs, but with manyfold-reduced intensity as compared with the [^{35}S]cysteine-labeled bands (data not shown). These two proteins are thus candidates for processed peptides coded by the group II and group III RNAs.

In a parallel experiment, dissected salivary glands were incubated with [^{3}H]threonine, and the resulting labeled proteins were separated in an SDS–10% polyacrylamide gel. The gel fluorograph showed only two very strongly labeled protein bands, at apparent molecular weights of 162K and 112K. The strong threonine labeling indicates that one or both of these proteins may be the product of the group IV RNA, whereas

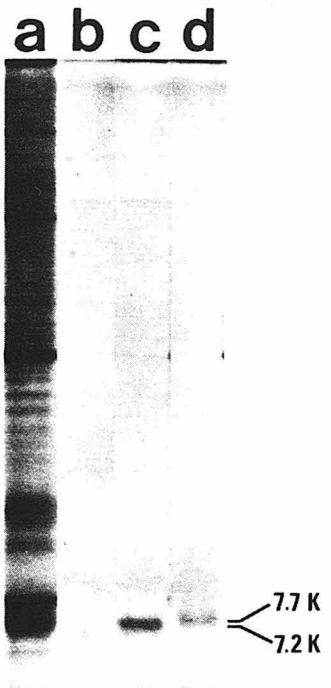

FIG. 1. In vitro translation of hybrid-selected RNAs. Polyadenylated RNA from late-third-instar larvae was hybridized to single-stranded cDNA clone DNA covalently bound to diazophenylthioether paper (see text). Bound RNA was eluted and translated in a rabbit reticulocyte in vitro translation extract with [^{35}S]cysteine. The translation products were heated for 5 min at 50°C in 50 mM Tris-hydrochloride (pH 6.8)–0.5% SDS–1% 2-mercaptoethanol–10% glycerol, separated by electrophoresis through a 15% polyacrylamide-SDS gel, and visualized by fluorography. Lanes: translation of total larval polyadenylated RNA (a) and translation with no added RNA (b), translation of RNA bound by adm109F4, the group II cDNA clone (c), and translation of RNA bound to adm127C8, the group III cDNA clone (d). The molecular weights of the proteins shown in lanes c and d were determined by comparison with the migration of protein standards (see footnote *a* of Table 1).

the molecular weight implies that if this protein were translated from RNA IV, it must have undergone some post-translational modification, such as glycosylation, that increases apparent molecular weight in SDS gels (27).

To better characterize these 68C protein candidates, the migration of labeled salivary gland proteins in two-dimensional gel electrophoresis was examined. Two types of gels were used: in one type the first dimension was NEPHGE and the second dimension was SDS-PAGE (NEPHGE–SDS-PAGE [24]); in the second gel type the first dimension was an IEF gel and the second dimension was an SDS-polyacrylamide gel (IEF–SDS-PAGE [23]). Proteins from dissected salivary glands labeled in vivo with [^{35}S]cysteine were loaded on both types of gel, and the resulting gel fluorographs and autoradiographs are shown in Fig. 2A and B. These show that the low-molecular-weight, cysteine-rich proteins that may be products of RNAs II and III are a group of three separable peptides: a 5.5K peptide with a basic isoelectric point and two 5K peptides with more acidic isoelectric points. The similar isoelectric points, molecular weights, and cysteine contents of the two 5K proteins indicate that they may be encoded by the same mRNA and differ due to a post-translational modification.

[^{3}H]threonine-labeled salivary gland proteins were separated by NEPHGE–SDS-PAGE and showed only the two proteins observed in one-dimensional SDS-polyacrylamide gels: the 162K and 112K proteins that migrated in the NEPHGE dimension, indicating a basic isoelectric point (Fig. 2C). The identical migration of these proteins in the first dimension of the two-dimensional gel and their heavy threonine labeling may indicate that they, too, are translation products of the same mRNA and differ due to a post-translational modification.

One further type of gel analysis of labeled salivary gland proteins was performed: IEF in the absence of the urea and NP-40 included in the first dimension of the IEF–SDS-PAGE gels. The reason for these nondenaturing IEF gels was to obtain an accurate measurement of isoelectric point for all of the putative 68C proteins; consequently, labeled salivary gland proteins were mixed with a series of unlabeled proteins of known pI, and IEF was performed on the mixture. The results of the pI measurements were as follows: the cysteine-rich 5.5K protein had a pI of 10.3, the cysteine-rich 5K proteins ran as one on these nondenaturing gels at a pI of 7.0, and the threonine-rich proteins migrated to a position slightly more basic than the most basic (pI 10.6) marker protein, indicating a pI of approximately 10.8.

Partial amino acid sequence of the proteins confirms their 68C origin. There were thus five proteins that on the basis of molecular weight or amino acid composition appeared to be likely candidates for the products of the three 68C mRNAs. To determine whether any of these proteins was indeed derived from the cloned 68C puff, partial determinations of the amino acid sequences of all five proteins were made. The first step in this determination was to establish the approximate positions of the methionine residues in the three low-molecular-weight, cysteine-rich proteins. The predicted 75-amino-acid protein from RNA II would be expected to have methionine residues at positions 1, 13, and 56,

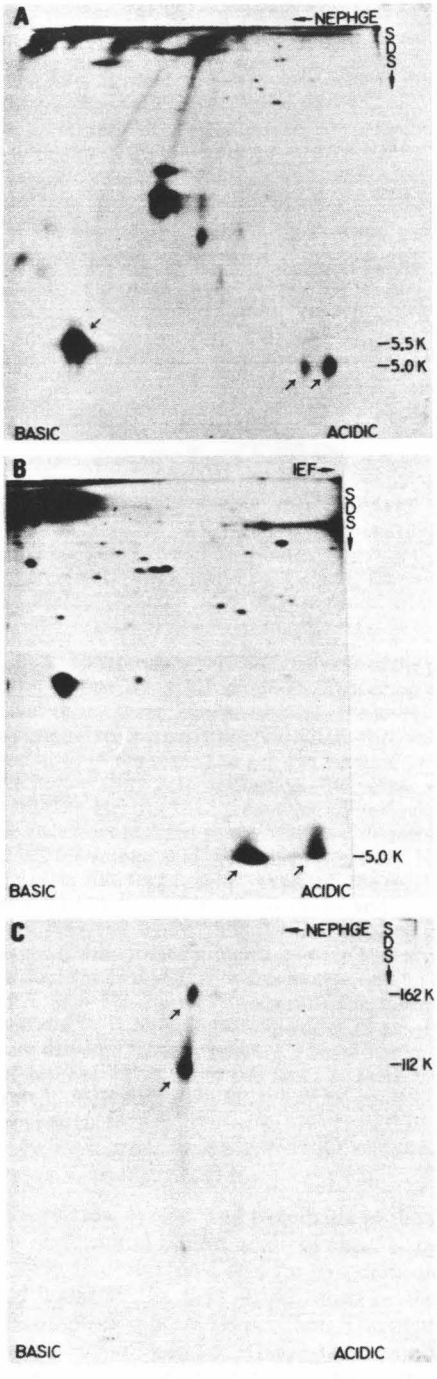

FIG. 2. Two-dimensional gel electrophoresis of salivary gland proteins. (A) Putative products of RNAs II and III. Thirty salivary gland lobes were labeled with [^{35}S]cysteine (see text), and one-third of the labeled protein was used. Proteins were denatured in 8 M urea–5% 2-mercaptoethanol–1% NP-40 before electrophoresis. NEPHGE was from right to left for 2,000 Vh. The SDS gel was 15% polyacrylamide. Fluorographic exposure was for 11 h. The putative 68C proteins are indicated by arrowheads. (B) Eight larvae were injected with [^{35}S]cysteine, their salivary gland lobes were dissected, and one-half of the protein from the gland lobes was loaded (see text). The proteins were denatured in 8 M urea–1% 2-mercaptoethanol–2% NP-40 before electrophoresis. IEF was from left to right for 7,100 Vh. The SDS gel was 15% polyacrylamide. Exposure was for 18 days. The two proteins indicated by arrowheads correspond to the two 5K spots in A. In the presence of urea and NP-40 these spots focus at a more acidic position in IEF gels than in nondenaturing gels, where they migrate as a single species (see text). (C) Putative protein IV products. Thirty salivary gland lobes were labeled with [^{3}H]threonine, and one-fourth of the isolated protein was loaded (see text). Proteins were denatured in 8 M urea–5% 2-mercaptoethanol–1% NP-40 before electrophoresis. NEPHGE was from right to left for 1,000 Vh. The SDS gel was 10% polyacrylamide. Exposure was for 42 h.

whereas the predicted 74-amino-acid protein III should have methionines only at positions 1 and 73. A treatment of the proteins with cyanogen bromide, which breaks peptide bonds on the carboxyl side of methionine residues (11), should therefore only detectably affect protein II or its processed derivatives; such treatment would only shorten protein III or its derivatives by a maximum of two amino acids. The three low-molecular-weight proteins were labeled in cultured salivary glands with [^{35}S]cysteine, and each protein was purified by preparative NEPHGE–SDS-PAGE as described above. Each purified protein was then treated with cyanogen bromide, and the reaction products were analyzed by one-dimensional SDS-PAGE (Fig. 3). Both of the 5K proteins were cleaved identically by CNBr, to yield at least one peptide which was smaller than the intact peptide, but large enough to be retarded on the 20% polyacrylamide gel. The 5.5K basic protein was not visibly affected by CNBr treatment. Thus, the 5K proteins could both be products of the group II RNA and probably not of the group III RNA; the 5.5K protein could be coded by RNA III, but probably not by RNA II.

To resolve this issue conclusively, the proteins were labeled with various radioactive amino acids in cultured salivary glands, purified by preparative two-dimensional gel electrophoresis, and subjected to sequential removal of amino-terminal amino acids by successive Edman degradations. The step of Edman degradation that released free labeled amino acid indicated the position of each labeled amino acid in the protein sequence. With an automated protein microsequenator (13), it was possible to determine whether any amino acid was present or absent in at least the first 20 amino acid positions of each protein. This determination was made

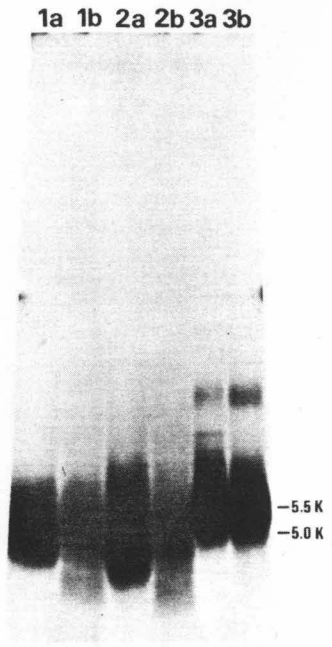

FIG. 3. Cyanogen bromide cleavage of the putative RNA II and RNA III products. Dissected salivary gland lobes were incubated with [^{35}S]cysteine and separated by preparative NEPHGE–SDS-PAGE as described in the text. The gel was vacuum dried, and autoradiography was performed. Individual proteins were then electroeluted from excised and rehydrated gel slices, lyophilized to dryness, and dissolved in 25 μl of 70% formic acid. Five microliters of CNBr (300 mg/ml) in 70% formic acid was added, the reaction was incubated at 4°C for 16 h, and CNBr and formic acid were removed by lyophilization. Untreated proteins (a) and CNBr-treated proteins (b) were heated at 100°C for 3 min in 50 mM Tris-hydrochloride (pH 6.8)–0.6 to 0.8% SDS–5% 2-mercaptoethanol–10% glycerol and then run on a 20% polyacrylamide-SDS gel. Proteins were detected by fluorography. Lanes: 1, more acidic 5K protein, 19,500 cpm per lane; 2, more basic 5K protein, 26,900 cpm per lane; 3, 5.5K protein, 38,800 cpm per lane.

for cysteine, lysine, and isoleucine in the first 30 amino-terminal amino acids of the 5K putative protein II derivatives and for cysteine, proline, and lysine in the first 40 positions of the 5.5K presumptive protein III product. The results are shown in Fig. 4 and 5. The sequences obtained showed both 5K peptides to have the same amino acid sequence as far as it was determined and demonstrated the likelihood that both are translation products of the 68C RNA II. Furthermore, this amino-terminal sequence explains why the molecular weight of the in vivo RNA II product is lower than the molecular weight predicted from the nucleotide sequence of the RNA and the molecular weight of the peptide obtained by in vitro translation of the RNA II: the amino-terminal 23 amino acids of the predicted protein II are absent in the salivary gland protein derived from this RNA. Nineteen of the 23 missing amino acids are hydrophobic in character (7); thus the RNA II-derived salivary gland protein

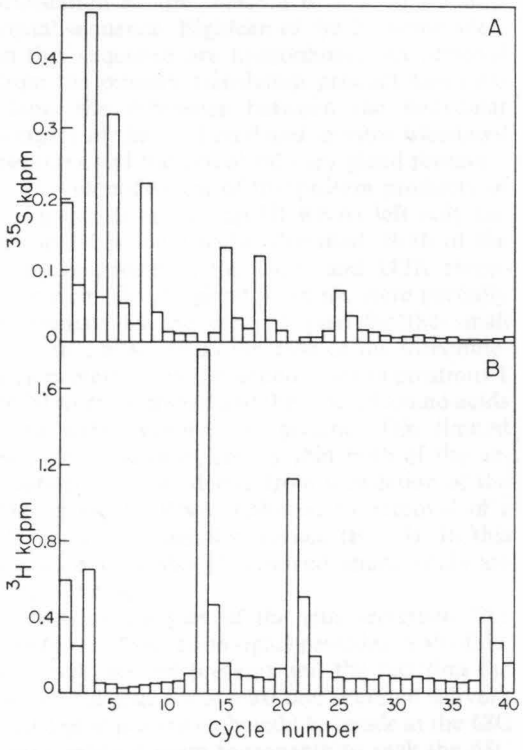

FIG. 4. Determination of the amino-terminal amino acid sequence of the putative group III RNA product. Thirty-two salivary gland lobes were labeled in medium containing [^{35}S]cysteine, and proteins were extracted as described in the text. [^{3}H]lysine-labeled protein from 80 lobes was prepared in the same manner. One-sixteenth of the ^{35}S-labeled protein was mixed with one-half of the ^{3}H-labeled protein, and the 5.5K cysteine-rich putative group III RNA product was purified from this mixture by preparative NEPHGE–SDS-PAGE (see text). The purified protein, containing 43,300 ^{3}H cpm and 16,300 ^{35}S cpm, was reduced and alkylated and then introduced into an automated spinning cup Edman microsequenator. The radioactivity of the resulting fractions was determined by scintillation spectrometry as described in the text. A Beckman LS 9000 liquid scintillation counter was used to quantitate the counts per minute in a low-energy window (^{3}H plus ^{35}S) and in a high-energy window (^{35}S). The counts per minute were then converted to disintegrations per minute by correcting for quenching, scintillation counter efficiency, and ^{35}S disintegrations per minute in the low-energy window by using *n*-dioctyl-[^{35}S]sulfide as a standard. (A) ^{35}S released by the first 40 cycles of Edman degradation. (B) ^{3}H released.

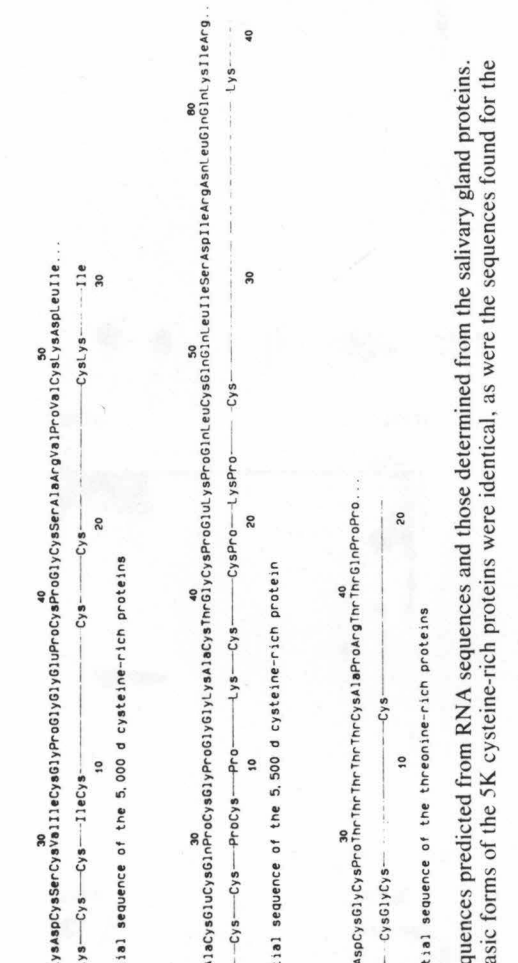

FIG. 5. Comparison of amino-terminal amino acid sequences predicted from RNA sequences and those determined from the salivary gland proteins. The sequences determined for both the acidic and the basic forms of the 5K cysteine-rich proteins were identical, as were the sequences found for the 162K and 112K threonine-rich proteins (see text).

appears to have been processed after translation to remove a hydrophobic, amino-terminal signal sequence of the type found to be post-translationally removed from many secreted proteins (6). The partial amino acid sequence of the basic 5.5K salivary gland protein shows that it is almost certainly the product of the group III 68C RNA, and that it also has been processed after translation by the removal of a 23-amino-acid signal sequence. Eighteen of the 23 amino acids in this sequence are hydrophobic; its removal from the primary translation product again explains the difference between the molecular weights of the predicted and in vitro translated peptides and the actual salivary gland protein.

The identification of the protein products of the group II and group III RNAs left only the group IV product to be identified. Both of the putative products, the 162K and 112K threonine-rich salivary gland proteins, were partially sequenced by the method used for the small proteins, although in the case of the threonine-rich proteins only the amino acids in positions 1 to 20 were examined and the labeled amino acids used were cysteine and glycine. This limited sequence indicates clearly that both of the sequenced proteins derive from translation of the 68C group IV RNA, followed by removal of a 23-amino-acid signal sequence (Fig. 5). In this instance 15 of the 23 removed amino acids are hydrophobic.

Proteins are part of the glue secretion. The existence of secretion signal peptides in all of the 68C puff-derived proteins and the fact that the structural gene for at least one secreted salivary gland glue protein is thought to reside at the 68C locus made it seem reasonable to seek the 68C proteins in the secreted larval glue. To do this, mid-third-instar larvae were either fed or injected with radioactively labeled amino acids, and after puparium formation the secreted, radiolabeled glue was collected from the larval substrate and subjected to two-dimensional PAGE. When [^{35}S]cysteine was used as the radiolabel, the secreted glue showed labeled proteins that comigrated with the 5K RNA II products and with the basic 5.5K RNA III product (Fig. 6A and B). When [^{3}H]threonine was used, only one labeled glue protein was seen on gels; this comigrated with the 162K threonine-rich product of the group IV RNA (Fig. 6C). Thus, each of the three RNAs transcribed from the 68C puff codes for a component of the secreted salivary gland glue.

The next question is whether these glue components have been previously recognized and named. From the similarity of our SDS-PAGE gels and those of Beckendorf and Kafatos (5), it seems possible that the group II and III RNA products are included in their glue protein frac-

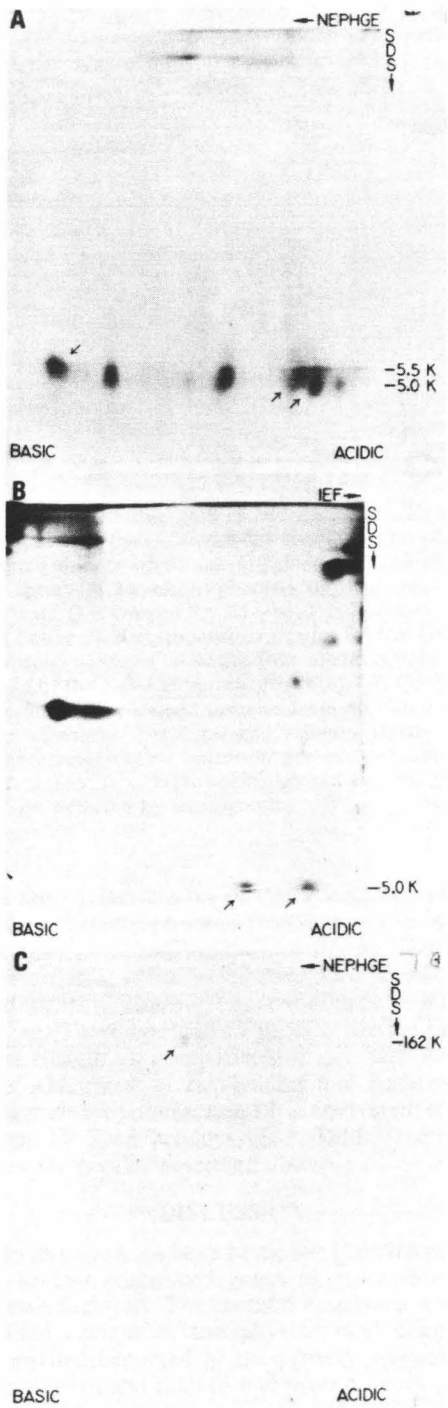

FIG. 6. Two-dimensional gel electrophoresis of secreted glue proteins. Glue proteins were labeled in vivo, purified after secretion as described in the text, and separated by two-dimensional gel electrophoresis. The resulting gel was analyzed by fluorography. (A) [^{35}S]cysteine-labeled proteins after migration in an NEPHGE first dimension and an SDS-15% polyacrylamide second dimension. Proteins were denatured in 8 M urea–5% 2-mercaptoethanol–1.5% NP-40 before electrophoresis (electrophoresis parameters as in Fig. 2A). The proteins marked by arrows are as follows (from left to right): the 5.5K 68C protein, the basic 5K 68C protein, and the acidic 5K 68C protein. The three additional, labeled proteins are part of the glue. These three comigrate with the known 68C products in the SDS-PAGE dimension, implying that their molecular weights are in the range of 5K to 5.5K. They are present in [^{35}S]cysteine-labeled whole salivary gland proteins as well as in secreted glue and are far less abundant than the three identified 68C proteins in whole salivary glands. Their relation to the 68C proteins is unknown. (B) [^{35}S]cysteine-labeled proteins in an IEF–SDS-PAGE gel. Proteins were denatured in 8 M urea–1% 2-mercaptoethanol–2% NP-40 before electrophoresis (electrophoresis parameters as in Fig. 2B). (C) [^{3}H]threonine-labeled proteins after NEPHGE–SDS-PAGE. Proteins were denatured in 8 M urea–0.3% SDS–5% 2-mercaptoethanol–4% NP-40 before electrophoresis (electrophoresis parameters as in Fig. 2C).

tion P6, although their measurement of molecular weight is quite different from ours. Korge's (16) analysis of salivary glues does not include these proteins. Since these proteins are newly identified as separate species, we named the salivary gland secretions translated from the 68C group II RNA sgs-8 and the 68C gene II *Sgs-8*; we named the secretion protein derived from the group III RNA sgs-7, corresponding with the structural gene *Sgs-7*. Since the group II RNA gives two different proteins on some types of gel, we subdivide sgs-8 into sgs-8a, the more acidic of the two 5K proteins, and sgs-8b, the more basic, as measured in urea- and NP-40-containing IEF gels. This leaves the group IV protein product. Since genetic information indicates that the glue protein sgs-3 is coded by the 68C puff, it seemed likely that the gene IV product was the previously named sgs-3. Korge (17) showed that the Formosa wild-type strain of *D. melanogaster* produces an sgs-3 protein (3d) which, after alkylation, migrates more rapidly on acid-urea gels than the alkylated sgs-3 protein (3a) of the Oregon R wild-type strain. To show that the threonine-rich RNA IV product is sgs-3, we compared the electrophoretic migration of Coomassie blue-stained sgs-3 and [^{3}H]threonine-labeled protein IV on acid-urea gels after alkylation. Figure 7A shows the migration of Coomassie blue stained, alkylated sgs-3 from the Oregon-R and Formosa wild-types; Fig. 7B is an autoradiograph showing that the threonine-rich alkylated Oregon-R protein IV migrates identically in an acid-urea gel with sgs-3. Figure 7C demonstrates that Oregon-R protein IV and the homologous threonine-rich protein extracted from Formosa wild-type salivary glands show

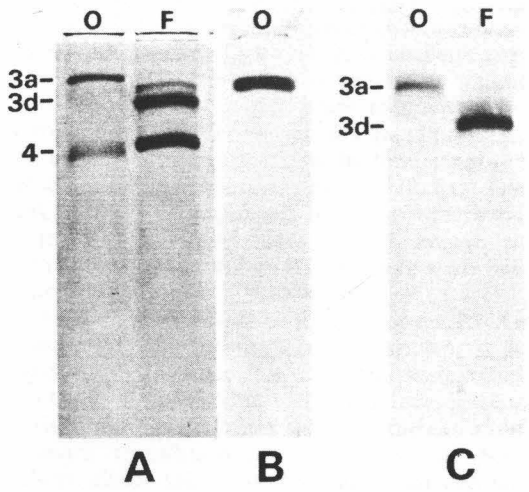

FIG. 7. Acid-urea gels of reduced and alkylated proteins. Proteins isolated from dissected glue plugs, secreted glue, or whole salivary glands were alkylated and separated by electrophoresis on acid-urea gels (see text). O is Oregon R wild type; F is Formosa wild type (see text). Electrophoresis was for 6.5 h at 100 V. The proteins 3a and 3d derive from alleles *Sgs-3a* and *Sgs-3d* of the *Sgs-3* structural gene (16). (A) Coomassie brilliant blue-stained proteins from dissected glue plugs of ethanol-fixed, bloated salivary glands. (B) [^{3}H]threonine-labeled secretion protein detected by fluorography. (C) [^{3}H]threonine-labeled salivary gland proteins detected by fluorography.

the same relative rates of migration, after alkylation and electrophoresis in acid-urea gels, as do the sgs-3 proteins from the two strains. Thus, by two criteria, identical electrophoretic mobility and identical strain-dependent change in mobility, sgs-3 and the RNA IV product are the same. It is clear from comparison of our SDS-PAGE gels with those of Beckendorf and Kafatos (5) that their protein fraction P1 is equivalent to the group IV RNA product sgs-3. Table 1 summarizes the results described above.

DISCUSSION

In this work we have found the proteins coded by several sequenced genes of previously unknown function. The method employed was to predict certain of the physical and chemical properties expected of the protein products of these genes and then to find proteins with these properties in the tissue in which the genes were known to be active. This is a method which could be applied to many genes of known structure and tissue of expression, but unknown product, although with current techniques the successful application of this method is probably restricted to genes which transcribe abundant mRNAs. In the instance described here, this method allowed assignment of three secreted salivary gland glue proteins to the gene cluster found at 68C on the salivary gland polytene chromosomes. One of these glue protein genes, *Sgs-3*, had already been determined to reside at the 68C locus by recombination mapping of electrophoretic variants (16) and by gene dosage studies (1). The two newly identified 68C structural genes are *Sgs-7* and *Sgs-8*, which code for the glue proteins here designated sgs-7 and sgs-8, respectively. The addition of these new proteins brings to four the number of glue polypeptides whose structural genes have been cloned and located at specific polytene chromosome loci: the three at 68C and sgs-4, whose structural gene *Sgs-4* is at location 3C on the X chromosome (15, 22). In addition to these cloned loci, there are two additional genes whose genetic dosage correlates exactly with the amount of particular glue proteins: the gene *Sgs-1*, located at 25B on the second chromosome, correlates

TABLE 1. Secreted glue polypeptides coded at 68C[a]

Polypeptide	Apparent mol wt	pI	Coded by RNA	Mol wt from protein sequence
sgs-3	162K	10.8	IV	29,782
sgs-7	5K	10.3	III	5,481
sgs-8a	5K	7.0	II	5,592
sgs-8b	5K	7.0	II	5,592

[a] Apparent molecular weights were calculated by comparing the mobilities of Oregon R salivary gland proteins on SDS-polyacrylamide gels with the mobilities of the following proteins: *E. coli* RNA polymerase I (β and β′ subunits, 160K and 155K), phosphorylase A (94K), bovine serum albumin (66K), egg albumin (45K), carboxypeptidase A (35K), trypsinogen (24K), β-lactoglobulin (18.4K), myoglobin (17.2K), and lysozyme (14.3K). The cyanogen bromide cleavage products of myoglobin (8,270, 6,420, 2,550 [28]) were used as standards for the low-molecular-weight range. Isoelectric points were calculated by comparing the mobilities of salivary gland proteins with the mobilities of marker proteins of known isoelectric point on nondenaturing IEF gels, as described in the text. The isoelectric points of the marker proteins were as follows: C phycocyanin, 4.65, 4.85; azurin, 5.65; ovotransferrin, 6.05; porcine myoglobin met, 6.45; equine myoglobin met, 7.3; sperm whale myoglobin met, 8.3; and cytochrome *c*, 10.6. Proteins sgs-8a and sgs-8b migrate as one on nondenaturing IEF gels, but separate into an acidic and a basic component in IEF gels containing urea and NP-40 (see text). The molecular weights derived from the protein sequence were calculated from a conceptual translation of the known RNA sequences (Garfinkel et al., submitted for publication), taking into account the fact that the amino-terminal 23 amino acids predicted from these sequences are absent in the mature salivary gland proteins.

with production of glue protein sgs-1 (30), and *Sgs-6*, at 71C-F on the third chromosome, correlates with protein sgs-6 (31). It is virtually certain that these genes are glue protein structural genes. All six of the glue genes mapped so far correspond to prominent salivary gland polytene chromosome puffs present at the time when the glue proteins are being synthesized (3, 31). The 68C puff is distinguished from the others by being the only puff locus that is known to contain the structural genes for more than one glue protein.

Since the nucleotide sequences of the RNAs coding for each of the 68C are known, it is possible to calculate the molecular weight expected of these products. Table 1 includes the results of this calculation, based on the expected primary translation products from the 68C RNAs minus the 23-amino-acid signal peptide known to be removed from each protein before its secretion. The correspondence of measured and expected molecular weight is good for sgs-7 and sgs-8, but not for sgs-3. The reason for this is almost certainly glycosylation. sgs-3 is known to be an extensively glycosylated protein (5, 16), and such proteins migrate more slowly than would be expected from their molecular weight in SDS-polyacrylamide gels (27). Glycosylation also provides a plausible explanation for the two forms of sgs-3 seen in salivary glands after 20 min of [^{3}H]threonine labeling of the isolated tissue. Since only the form that migrates in gels with an apparent molecular weight of 162K is found in secreted glue, it may be that the form migrating at 112K is a less glycosylated precursor of the mature protein, and that labeling of the proteins of isolated salivary glands followed by an incubation in unlabeled amino acids might show conversion of the apparent 112K form to the secreted form of sgs-3. One possible function of sgs-3 glycosylation may be to make the protein soluble; the in vitro translation product of the sgs-3 mRNA (RNA IV) would not enter SDS-polyacrylamide gels, whereas the glycosylated sgs-3 protein does. Chemical deglycosylation of at least one soluble mammalian glycoprotein has been shown to render it insoluble in a wide variety of aqueous buffers (9).

That the molecular weights determined for sgs-7 and sgs-8 by SDS-PAGE were close to those predicted from the gene sequences implies that these glue polypeptides are not extensively glycosylated. This is consistent with the finding of Beckendorf and Kafatos (5) that their glue fraction P6, which probably includes sgs-7 and sgs-8, was not stained by the periodic acid-Schiff reaction, which stains most carbohydrates. It is possible to make a rough estimation of the isoelectric point expected of a polypeptide from its amino acid sequence (32). If this is done for sgs-7 the expected pI is in the approximate range 7.5 to 8.5; sgs-8 would be expected to have a pI in the approximate range 7.2 to 8.2. That the measured pI of sgs-7 is 10.3 may indicate that some sort of prosthetic group is attached to it or that some other modification has occurred. The two different isoelectric forms of sgs-8 that are seen after migration in some gel types also imply that some sort of post-translational modification in addition to removal of the signal peptide may be suffered by at least one form of this protein.

One question raised by the demonstration that the 68C puff codes for three glue proteins derives from the investigation of Beckendorf and Kafatos (5) of the developmental times of synthesis of the salivary gland secretion polypeptides. Their observation was that their fraction P1, or sgs-3, and fraction P6, which probably includes sgs-7 and sgs-8, had very different developmental profiles in third-instar larvae. Observations on the RNAs now known to code for these polypeptides (19; Crowley, Kendall, and Meyerowitz, unpublished observations) indicate that their developmental profiles may be identical. More detailed observations of the developmental regulation of both RNA and protein will be necessary to find whether this RNA-protein difference is a consequence of differential control of translation of the 68C mRNAs.

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CHAPTER 3

Steroid Regulation of RNAs Transcribed from the *Drosophila* 68C Polytene Chromosome Puff

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Steroid Regulation of RNAs Transcribed from the *Drosophila* 68C Polytene Chromosome Puff

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The 68C region of the *Drosophila melanogaster* salivary gland polytene chromosomes harbors the structural genes for the three salivary gland glue proteins sgs-3, sgs-7, and sgs-8. This region is puffed during the third larval instar, the stage when glue proteins are being produced in the salivary glands. The puff regresses near the end of the third instar as a result of an increased titer of the steroid hormone ecdysterone in the larval hemolymph. The experiments reported here were designed to determine whether the ecdysterone effect on puffing at 68C is correlated with hormone effects on expression of the three puff RNAs. In the first series of experiments, it is shown that there is a more rapid disappearance of 68C RNA transcripts from salivary glands cultured in the presence of ecdysterone than from glands cultured in its absence. The second set of experiments, in which 68C transcripts were pulse-labeled in salivary glands cultured in the presence or absence of hormone, demonstrates that one effect of ecdysterone is to cause a sharp reduction in the rate at which newly synthesized 68C transcripts accumulate. The final experiments follow the time required for ecdysterone to produce this effect, and show that it occurs in salivary glands exposed to the hormone for as little as 15 min. In all of the experiments, the RNA products of the *Sgs-3*, *Sgs-7*, and *Sgs-8* genes acted coordinately.

INTRODUCTION

Puffs are visible regions of highly active transcription on dipteran polytene chromosomes (Pelling, 1964). The 68C puff of *Drosophila melanogaster*, on the left arm of the third chromosome in the larval salivary glands, is the site of transcription of three different messenger RNAs. They are transcribed from the three genes *Sgs-3*, *Sgs-7*, and *Sgs-8*, and are translated to produce the polypeptides sgs-3, sgs-7, and sgs-8. Each of these proteins is synthesized in salivary gland cells during the third larval instar; at the end of this stage they are secreted from the cells to the lumen of each salivary gland lobe, then expelled from the lumen through the salivary duct as part of the salivary glue (Crowley *et al.*, 1983). The glue causes the newly formed puparium to adhere to the surface upon which it rests for the duration of the prepupal and pupal periods (Fraenkel and Brookes, 1953).

The 68C puff is visible from mid-third larval instar, when specific locations on the salivary gland polytene chromosomes can first be reliably identified, to several hours before puparium formation, at which time the puff regresses. This regression is caused by an increase in the hemolymph titer of the steroid hormone ecdysterone. This is evidenced by the correlation of puff regression and hormone titer increase *in vivo*, and by the ability of exogenously applied ecdysterone to cause normal puff regression in salivary glands that have been cultured *in vitro* in the absence of the hormone (Ashburner, 1973; Ashburner and Richards, 1976). That the effect of the hormone is due to a direct steroid action on the 68C puff (presumably mediated by a specific steroid receptor protein—Maroy *et al.*, 1978), is shown by two pieces of evidence: in cultured salivary glands the puff regresses under the influence of ecdysterone even when cycloheximide is present in the culture medium at a concentration sufficient to inhibit general protein synthesis (Ashburner, 1974), and indirect immunofluorescence experiments show that the steroid is localized at the puff site at the time of puff regression (Gronemeyer and Pongs, 1980).

The DNA sequence organization of the 68C puff is known. The three genes contained within the puff are clustered in a region of about 4500 base pairs of contiguous chromosomal DNA. Their order in the chromosome is telomere, *Sgs-8*, *Sgs-7*, *Sgs-3*, centromere; they are found in the region of the bands 68C 3-5. *Sgs-8* is transcribed from the centromeric to the telomeric end, the other two genes are transcribed in the opposite direction. The region between the 5′ ends of the divergently transcribed pair *Sgs-8* and *Sgs-7* is less than 500 base pairs; from the 3′ end of the *Sgs-7* transcription unit to the 5′ start of the adjacent *Sgs-3* gene is less than 2000 base pairs. Not including the 3′ poly(A) tracts, which are present in each of the RNAs, the sgs-8 mRNA is approximately 360 bases, the sgs-7 mRNA about 320 bases, and the sgs-3 mRNA approximately 1120 bases in length. Each of the messenger RNAs arises from a precursor RNA by removal of a single intervening se-

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quence near the 5′ end of the primary transcript. The intervening sequences are 69, 66, and 73 bases for the *Sgs-8*, *Sgs-7*, and *Sgs-3* transcripts, respectively (Garfinkel *et al.*, 1983). The 68C RNA transcripts are found only in salivary glands, and not in other tissues, and are found there only in the third larval instar stage of development (Meyerowitz and Hogness, 1982).

The 68C puff is thus a chromosomal region with known DNA sequence, known transcriptional abilities and known protein products. It is also a region known to change its puff activity as a result of direct interaction with a steroid hormone. The experiments presented here are designed to find if the ecdysterone regulation of the 68C puff is paralleled by ecdysterone regulation of the synthesis or accumulation of puff RNA, and to determine the time course and nature of any steroid effects on 68C puff RNA metabolism.

MATERIALS AND METHODS

Materials. Aminophenylthioether paper was prepared according to the procedure of Seed (1982). Polaroid Coaterless Land Pack Film 667 or Type 55 Land Film (when negatives were required) was used for photography of stained gels. Kodak XAR-5 film was used for autoradiography. Photographs and autoradiographs were scanned with a Joyce, Loebl microdensitometer. Kodak Plus-X pan (PX 135-36) film and a Zeiss photomicroscope were used for photography of salivary gland squash preparations. Edcysterone (β-ecdysone, 20-OH ecdysone) was obtained from Sigma Chemical Co., dissolved at a concentration of ~10 mg/ml in 100% ethanol and stored at −20°C. The exact concentration of the ecdysterone stock solution was determined by measuring its absorbance at 242 nm assuming a value of e = 12,400 (Hoffmeister *et al.*, 1965; Hocks and Wiechert, 1966). Yeast tRNA was from Miles Laboratories Inc. Deoxycytidine 5′-[α^{32}P]triphosphate (~3000 Ci/mole) and [^{3}H]guanosine were obtained from Amersham Corp. Proteinase K was purchased from EM Reagents. [^{3}H]Adenosine, [^{3}H]cytidine, [^{3}H]uridine, Aquasol-2 (liquid scintillation counting cocktail), and Liquifluor (PPO-POPOP toluene concentrate) were obtained from New England Nuclear Corp. Toluene (scintillar grade) was from Mallinckrodt, Inc. Flies of the OR16f strain which has a homozygous third chromosome were used for all experiments (Meyerowitz and Hogness, 1982).

Growth conditions for developing animals. In order to obtain first or second instar larvae, eggs were collected in small petri dishes on medium consisting of 2% agar and 5% sucrose onto which live yeast had been sprinkled. For third instar larvae and prepupae, milk bottles containing standard cornmeal-agar food were used. All eggs and developing animals were kept at 22°C. The times after egg laying at which the animals were collected were as follows: first instar larvae, 25 to 39 hr; second instar larvae, 59 to 68 hr; third instar larvae (for analysis of developmental regulation), 72 t0 120 hr; third instar larvae (for experiments with ecdysterone), 80 to 100 hr; prepupae, 120 to 124 hr.

Ecdysterone treatment and pulse-labeling of salivary glands in vitro. For all experiments involving ecdysterone, salivary glands were dissected from larvae which were near the middle of the third instar (about 80 to 100 hr after egg laying). At this point in development the concentration of the hormone in the animals is still relatively low (Berreur *et al.*, 1979; Maroy *et al.*, 1980). The culture medium used for *in vitro* incubation of salivary glands contained: 10 m*M* morpholinopropanesulfonic acid (pH 7), 80 m*M* NaCl, 10 m*M* KCl, 1 m*M* $CaCl_2$, and 0.1 m*M* $MgCl_2$ (Mitchell *et al.*, 1978); glands were cultured at 21-24°C. The medium was oxygenated before use, and mock experiments with cultured glands followed by polytene chromosome spreads showed that heat shock puffs were not induced by the culture conditions. For ecdysterone treatments, 6 to 20 salivary gland lobes were incubated in 10- to 20-μl drops of culture medium containing the appropriate concentration of hormone. For pulse-labeling, 20 lobes were incubated in 10-μl drops of culture medium containing the appropriate concentration of ecdysterone plus 20 μCi of [^{3}H]nucleosides per μl. The specific activities (curies per millimole) of the nucleosides were as follows: [^{3}H]adenosine, 52; [^{3}H]cytidine, 27; [^{3}H]guanosine, 21; and [^{3}H]uridine, 40.

Isolation of RNA from whole animals. Larvae or prepupae were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The tissue was solubilized in extraction buffer [20 m*M* NaCl, 20 m*M* Tris-hydrochloride (pH 7.5), 1% sodium dodecyl sulfate (SDS), 40 m*M* EDTA] and extracted three times with equal volumes of phenol and chloroform-isoamyl alcohol (100:1). After the last phenol-chloroform extraction, the aqueous phase was transferred to another tube and the organic phase was reextracted with an equal volume of extraction buffer which was then combined with the other aqueous solution. The pooled aqueous solution was then extracted with chloroform and finally with ether. Nucleic acid was precipitated by adding 2.5 volumes of 100% ethanol and incubating the solution at −20°C for 1 hr or longer.

Isolation of unlabeled RNA from dissected salivary glands. Two to 10 salivary gland lobes were transferred to a tube containing 20 μl of extraction buffer plus 10 μg of yeast tRNA. The tube and its contents were then frozen in liquid nitrogen. Twenty microliters of phenol plus 20 μl of chloroform were added and the tube was vortexed vigorously as the aqueous solution thawed. Extractions were performed as described above, after which

yeast tRNA (10 μg) and sodium acetate (final concentration = 0.2 *M*) were added. Nucleic acid was precipitated by adding 2.5 volumes of 100% ethanol and freezing the tube and its contents in dry ice.

Isolation of [^{3}H]RNA from dissected salivary glands by phenol extraction. Sixty salivary gland lobes were transferred to a tube containing 40 μl of extraction buffer and then frozen in liquid nitrogen. Forty microliters of phenol plus 40 μl of chloroform were added and the tube was vortexed vigorously as the aqueous solution thawed. Extractions were performed as described above, after which sodium acetate was added (final concentration = 0.2 *M*) and nucleic acid precipitated by adding 2.5 volumes of 100% ethanol and freezing the tube and its contents in dry ice for 20 min. The solution was then allowed to thaw at 2°C and the nucleic acid was pelleted by spinning the tube at 15,000 rpm, 15 min, 2°C in an Eppendorf microcentrifuge. The supernatant was removed and the nucleic acid resuspended in 20 μl of TE [10 m*M* Tris-hydrochloride (pH 7.5), 1 m*M* EDTA]. The resuspended RNA was heated at 95 to 100°C for 1 min, then rapidly cooled to 0°C. Five microliters of 0.03% (w/v) bromcresol purple was added and the sample was passed through a 1 ml column of Bio-Gel P-60 (Bio-Rad Laboratories) in TE to remove unincorporated [^{3}H]-nucleosides. RNA was precipitated from the excluded volume and pelleted as described above. After removing the supernatant the RNA was resuspended in 57 μl of water. One microliter was removed for scintillation counting to determine the number of cpm in the sample and 6 μl was removed for ultraviolet spectrophotometry to determine the mass of nucleic acid in the sample.

Isolation of [^{3}H]RNA from dissected salivary glands by CsCl centrifugation. Twenty gland lobes were transferred to a tube containing 72 μl of lysis buffer [7 *M* urea, 15 m*M* sodium acetate (pH 5.5), 1% SDS, 10 m*M* EDTA, 15 m*M* EGTA, 20 μg/ml polyvinyl sulfate] plus 20 μg yeast tRNA and 35 μg proteinase K. The lysate was incubated at 68°C for 30 min, then 0.95 ml of 5.7 *M* CsCl—10 m*M* sodium acetate (pH 6)—0.2% diethylpyrocarbonate (density = 1.7 g/ml; this solution was passed through a 0.45-μm Millipore filter before use) was added. The tube was spun at 15,000 rpm, 2 min, 22°C in an Eppendorf microcentrifuge and the aqueous solution containing nucleic acid was removed from underneath the floating layer of cellular debris and protein. The clarified solution was then transferred to a thick-walled polyallomer tube and spun in a Beckman SW50.1 rotor at 33,000 rpm, 22 hr, 20°C which pelleted the RNA to the bottom of the tube while leaving protein and DNA in the supernatant (Glisin *et al.*, 1974). After centrifugation, the supernatant was removed and the walls of the tube wiped with Kimwipes to remove any remaining protein. The pelleted RNA was resuspended in 50 or 100 μl of H_2O and transferred to a microcentrifuge tube. Sodium acetate was added to a final concentration of 0.2 *M* and RNA was precipitated by adding 2.5 volumes of 100% ethanol and freezing the tube and its contents in dry ice for 20 min. The solution was then allowed to thaw at 2°C and the RNA was pelleted by spinning the tube at 15,000 rpm, 15 min, 2°C in an Eppendorf microcentrifuge. After removing the supernatant the RNA was resuspended in 79 μl of water and 1 μl was removed for scintillation counting to determine the number of cpm in the sample.

Gel electrophoresis, blotting, and hybridization of RNA. RNA samples were treated with glyoxal, fractionated by electrophoresis in agarose gels, and transferred to activated paper as described by Alwine *et al.* (1980) except that diazophenylthioether paper (freshly converted from aminophenylthioether paper; Seed, 1982) was used in place of diazobenzyloxymethyl paper. The blots were then pretreated with hybridization buffer plus 1% (w/v) glycine and subsequently hybridized to nick translated ^{32}P-labeled DNA probes (Rigby *et al.*, 1977) as described by Alwine *et al.* (1980). After washing, blots were autoradiographed with Kodak XAR-5 film and DuPont Cronex Lightning Plus intensifying screens at −60°C. When signals on the autoradiograph were to be quantitated by microdensitometer scanning, no intensifying screen was used.

Preparation of aminophenylthioether DNA filters. Plasmid DNAs were purified by CsCl equilibrium centrifugation as described by Meyerowitz *et al.* (1980), sheared by heating at 95 to 100°C for 10 min in 0.3 *N* NaOH (0.35 to 1.1 mg of DNA per ml), neutralized, and ethanol precipitated. The fragmented DNA was then dissolved at 3.3 mg/ml in 13 m*M* citric acid, 17 m*M* Na_2HPO_4 (pH 4), heated at 80°C for 1 min, then rapidly cooled to 0°C. Four volumes of dimethyl sulfoxide were added and the DNA transferred to disks of aminophenylthioether paper (1.1-cm diameter) that had been freshly converted to diazophenylthioether paper (Alwine *et al.*, 1980). A 15-μl aliquot of the DNA solution (10 μg DNA) was spotted on each filter disk and the filters were incubated at 22°C for 12 hr. The filters were then washed three times in water for 2 min at 22°C, once in 0.4 *N* NaOH at 42°C for 15 min, twice in water for 2 min at 22°C and stored in 20 m*M* PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8), 0.02% SDS, 1 m*M* EDTA at 4°C.

Preparation of nitrocellulose DNA filters. Plasmid DNAs were purified by CsCl equilibrium centrifugation as described by Meyerowitz *et al.* (1980), sheared by heating at 95 to 100°C for 10 min in 0.3 *N* NaOH (0.1 mg of DNA per ml), neutralized by adding an equal volume of 2 *M* ammonium acetate, and cooled to 0°C. A platform was constructed which consisted of a stack

of paper towels (5 to 8 cm high), overlaid with 3 sheets of Whatman 3MM paper (prewetted in water) which in turn were overlaid with a nitrocellulose filter (same dimensions as the 3MM paper; also prewetted in water). Nitrocellulose filter squares (4 × 4 mm) were wetted in H_2O, then placed on the large nitrocellulose filter on top of the platform. Aliquots of the DNA solution were slowly pipetted onto the small square filters using a siliconized 100 μl capillary pipet (0.1 ml per filter, i.e., 5 μg DNA per filter). Just before and just after transferring the DNA, 2 drops of 1 *M* ammonium acetate (0°C) were placed on the filter and allowed to blot through. After DNA had been transferred to each of the filters, the platform was flooded with 1 *M* ammonium acetate (0°C) and this solution allowed to blot through. The filters were then washed three times with 4× SSC [0.6 *M* NaCl, 0.06 *M* sodium citrate (pH 7.1)] and baked under vacuum at 80°C for 2 to 11 hr. The DNA filters were stored at −20°C.

Hybridization of [^{3}H]RNA to aminophenylthioether filter-bound DNA. Pulse-labeled RNA from 18 salivary gland lobes (~10^5 cpm) was dissolved in 17 μl H_2O, heated at 95 to 100°C for 1 min then rapidly cooled to 0°C. Aliquots from concentrated stock solutions were then added to the RNA sample resulting in a 75 μl hybridization solution with the following final concentrations: 60% formamide (deionized), 0.4 *M* NaCl, 20 m*M* PIPES (pH 6.8), 0.2% SDS, 1 m*M* EDTA. The hybridization solution was then incubated with an aminophenylthioether DNA-filter in a flat-bottom, siliconized glass vial at 42°C for 17 hr with gentle agitation. At the end of this period, the unhybridized RNA was removed and the filter washed three times in 5 ml of 20 m*M* PIPES (pH 6.8), 0.2% SDS, 1 m*M* EDTA at 60°C with vigorous agitation. The specifically hybridized RNA was then eluted by incubating the filter with 0.3 ml of 99% formamide (deionized) at 68°C with vigorous agitation for 30 min. The formamide was removed and the elution repeated. The eluants were pooled and transferred to a scintillation vial. Ten milliliters of Aquasol-2 was added to each vial and cpm determined with a Beckman LS-250 scintillation counter. Each sample was counted for 100 min. When aliquots of a salivary gland [^{3}H]RNA preparation corresponding to 14, 28, or 55 lobes were hybridized to identical DNA filters (10 μg DNA per filter), the cpm hybridized specifically to the RNA-coding DNA was directly proportional to the total input cpm. This indicates that the DNA is in excess even when [^{3}H]RNA from 55 lobes is used in one of these hybridizations. For all experiments using aminophenylthioether DNA filters, RNA from 18 lobes was used for each hybridization. When equivalent aliquots of a [^{3}H]RNA preparation were hybridized to identical DNA filters (10 μg DNA per filter) for 11, 22, 31, or 40 hr, the filter from the 40 hr hybridization had as many cpm as the filter from the 11 hr hybridization indicating that hybridizations under these conditions are complete after 11 hr. All hybridizations using aminophenylthioether DNA filters were 17 hr long.

Hybridization of [^{3}H]RNA to nitrocellulose filter-bound DNA. The prehybridization solution for the DNA filters contained 0.6 *M* NaCl, 0.12 *M* Tris-hydrochloride (pH 8), 0.1% SDS, 4 m*M* ETDA, 5× Denhardt's solution [1× is 0.02% (w/v) each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone] and 1 mg/ml yeast RNA. Nitrocellulose filters (4 × 4mm) bearing 5 μg of plasmid DNA were placed in the bottom of a 5-ml plastic test tube and 0.1 ml of the prehybridization solution was then pipetted into the tube. Each tube contained up to three filters bearing recombinant plasmid DNA (vector plus RNA-coding DNA) and one filter bearing nonrecombinant plasmid DNA (vector only). The aqueous solution containing the filters was then overlaid with mineral oil to prevent evaporation, and the tube incubated at 68°C with gentle agitation for at least 2 hr. Pulse-labeled RNA from 20 salivary gland lobes (~10^5 cpm) was dissolved in 78 μl of water, heated at 95 to 100°C for 2 min, then rapidly cooled to 0°C. Small aliquots from concentrated stock solution were then added to the RNA sample resulting in a 0.1 ml hybridization solution with the following final concentrations: 0.6 *M* NaCl, 0.12 *M* Tris-hydrochloride (pH 8), 0.1% SDS, 4 m*M* EDTA, and 1× Denhardt's solution [0.02% (w/v) each of bovine serum albumin, Ficoll, and polyvinylpyrrolodine]. The prehybridization solution was removed from the filters and replaced with 0.1 ml of [^{3}H]RNA in hybridization solution. Hybridizations were for 22 hr at 68°C with gentle agitation. At the end of this period, the filters were washed in 5 ml of high salt wash solution [0.6 *M* NaCl, 0.12 *M* Tris-hydrochloride (pH 8), 0.1% SDS, 4 m*M* EDTA] at 68°C with vigorous agitation for 1 hr. The high salt wash was repeated, followed by three 1 hr washes in low salt wash solution [0.15 *M* NaCl, 0.03 *M* Tris-hydrochloride (pH 8), 0.1% SDS, 1 m*M* EDTA] at 68°C. The filters were then dried at 42°C for 10 to 20 min and transferred to scintillation vials. Ten milliliters of toluene:Liquifluor (15:1) was added to each vial and cpm determined with a Beckman LS-250 scintillation counter. Each sample was counted three times for 50 or 100 min (150 or 300 min total per sample), and the results of the three determinations were averaged. To determine the cpm specifically hybridized to the RNA-coding DNA, the cpm obtained for the vector-only filter were subtracted from the cpm obtained for the filters bearing vector plus RNA-coding DNA. Vector-only filters produced ~20 cpm as did filters bearing no DNA indicating that there was no hybridization of ^{3}H-labeled salivary gland RNA to vector DNA sequences.

When aliquots of a salivary gland [^{3}H]RNA preparation corresponding to 14, 29, or 48 lobes were hybridized to identical DNA filters (5 μg DNA per filter), the cpm hybridized specifically to the RNA-coding DNA was directly proportional to the total input cpm. This indicates that the DNA is in excess even when [^{3}H]RNA from 48 lobes is used in one of these hybridizations. For all experiments using nitrocellulose DNA filters, RNA from 20 lobes was used for each hybridization. When equivalent aliquots of a [^{3}H]RNA preparation were hybridized to identical DNA filters for 11, 22, or 33 hr, the 22-hr filter had as many cpm as the 33-hr filter indicating that hybridizations under these conditions are complete by 22 hr. All hybridizations using nitrocellulose DNA filters were 22 hr long. Aminophenylthioether filters and nitrocellulose filters gave similar results, but less background hybridization was encountered in control experiments using nitrocellulose filters. Unless otherwise noted, DNA hybridizations were performed using nitrocellulose filters.

RESULTS

68C RNA Levels Are Affected by Ecdysterone

The first step in analyzing the effects of ecdysterone on expression of the 68C puff RNAs was to determine the developmental time of appearance and disappearance of each of the RNAs *in vivo*. Equal amounts of total RNA extracted from animals at various stages of development were fractionated by gel electrophoresis, transferred to diazophenylthioether paper (DPT paper—Seed, 1982), and then hybridized with ^{32}P-labeled DNA probes specific for each of the 68C-encoded glue protein RNAs. The results, shown in Fig. 1, demonstrate that none of the RNAs are detectable in first or second instar larvae, that all of them become abundant RNA components in third instar larvae, and that all three RNAs are again undetectable immediately after the third larval instar stage, in white prepupae. In a general way, therefore, the levels of all of the 68C RNAs parallel the activity of the steroid-regulated puff.

The rapid regression of the 68C puff in cultured salivary glands has been shown to be caused by ecdysterone in concentrations above 10^{-7} *M*, with the maximal rate of puff regression occurring in the presence of 10^{-6} *M* or higher concentrations of the hormone. The puff also regresses in the absence of ecdysterone in cultured salivary glands, though at a rate much lower than that seen in the presence of a sufficient concentration of the hormone (Ashburner, 1973). Salivary glands dissected from mid-third instar larvae were cultured either in

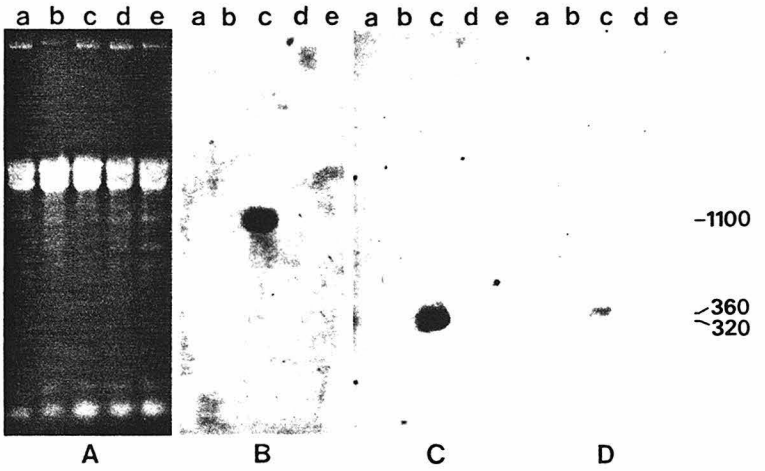

FIG. 1. Developmental regulation of 68C RNAs. Total RNA from animals at various stages of development (Materials and Methods) was denatured, glyoxal-treated, fractionated by electrophoresis (15 μg per lane) in a 1.8% agarose gel, and then transferred to diazophenylthioether (DPT paper; Seed, 1982). Lanes: RNA from first instar larvae (a), RNA from second instar larvae (b), RNA from third instar larvae (c), RNA from white (early) prepupae (d), and RNA from tan (late) prepupae (e). (A) Ethidium bromide stained RNA in gel before transfer. (B) Autoradiograph of DPT paper after transfer of RNA and hybridization to the ^{32}P-labeled insert of clone aDm2023 (a 2.4-kilobase fragment originally derived from λ bDm2002) which contains the entire coding region for the sgs-3 mRNA. Exposure was for 23 hr. (C) Autoradiograph of the same blot after elimination of the previously hybridized DNA fragment by boiling in 0.01× SSPE and subsequent hybridization to the ^{32}P-labeled cDNA insert from clone adm127C8 which is homologous with the sgs-7 mRNA. Exposure was for 18 hr. (D) Same blot after hybridization to the ^{32}P-labeled cDNA insert from clone adm109F4 which is homologous with the sgs-8 mRNA. Exposure was for 65 hr. The RNA lengths are shown on the right in nucleotides. The DNA clones used for probes and determination of the RNA lengths have been described by Meyerowitz and Hogness (1982).

the absence of added steroids, or in the presence of 10^{-5} *M* ecdysterone, for various lengths of time. RNA was then extracted from these glands and the relative quantities of each of the 68C RNAs determined by microdensitometry of autoradiographs of RNA gel blots that had been probed with ^{32}P-labeled DNA sequences homologous with each of the RNAs. The RNA quantity determined for each gel lane was normalized for the total amount of RNA loaded in each lane, as determined by measurement of the ethidium bromide fluoresence of the ribosomal RNA in each lane (Jordan *et al.*, 1976; Lis *et al.*, 1978). Figure 2 shows the ethidium-stained RNA gel, and autoradiographs of the gel blots after hybridization to labeled RNA-specific DNA probes. Figure 3 shows the quantitative results: the mass of each of the mRNA species (corrected for the difference in ribosomal RNA mass in each preparation) is plotted as a function of time of salivary gland culture with or without ecdysterone. After 4 hr of culture, the hormone caused a reduction of about twofold in the level of all three 68C RNAs. After about 11 hr, none of the RNAs were detected in the hormone-treated glands, although they were present in the untreated controls. Thus, the effect of ecdysterone on 68C puff RNA levels is similar to its effect on puff diameter: the hormone causes an increased rate of puff regression, and of 68C RNA disappearance, in cultured salivary glands.

The 68C RNAs were not detected in salivary glands cultured for 11 hr in the presence of 10^{-5} *M* ecdysterone, but were detected in glands incubated for the same period of time without hormone. To find the minimum concentration of the steroid necessary for this effect, salivary glands from mid-third instar larvae were cultured for 10 to 12 hr in the presence of various ecdysterone concentrations, then assayed for the presence or absence of sgs-3 mRNA. Figure 4 shows the result: that ecdysterone at a concentration of 10^{-9} *M* or greater is effective in eliminating sgs-3 RNA from glands, as measured by this assay.

Ecdysterone Reduces Accumulation of Newly Synthesized RNA

The abundance of any RNA is determined by the rates of its transcription, processing, nuclear degradation, and cytoplasmic degradation (Harpold *et al.*, 1981; Salditt-Georgieff *et al.*, 1981). To find which of these rates might be affected by the hormone in the ecdysterone-mediated

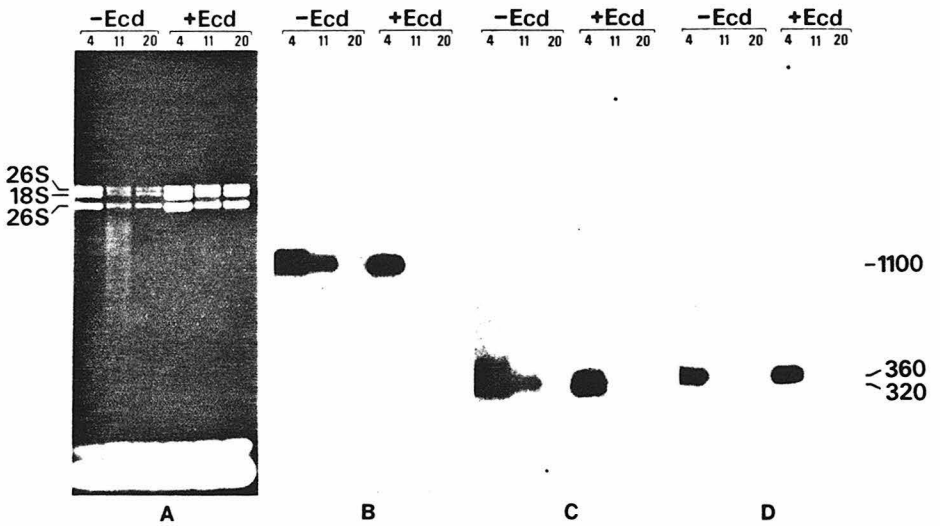

FIG. 2. Steady-state levels of 68C RNAs during culture of salivary glands with or without ecdysterone. Salivary glands were dissected from mid-third-instar larvae and transferred to drops (10 gland lobes per 10-μl drop) of culture medium (Materials and Method) containing no ecdysterone (−Ecd) or 10^{-5} *M* ecdysterone (+Ecd). After incubation for ~4, ~11, or ~20 hr at 21 to 24°C, RNA was extracted and analyzed as described for Fig. 1. Each lane contained RNA from 10 salivary gland lobes except −Ecd, 20 hr which contained RNA from 8 lobes. (A) Ethidium bromide stained RNA in gel before transfer. Positions of 18 S rRNA and the two longest processed fragments of the 26 S rRNA are indicated on the left (Jordan *et al.*, 1976; Lis *et al.*, 1978). (B) Autoradiograph of DPT paper after transfer of RNA and hybridization to ^{32}P-labeled aDm2023 DNA which is homologous with the sgs-3 mRNA. Exposure was for 24 hr. (C) Autoradiograph of same blot after elimination of the previously hybridized DNA fragment and subsequent hybridization to adm127C8 DNA, a probe for the sgs-7 mRNA. Exposure was for 23 hr. (D) Same blot after hybridization to adm109F4 DNA, a probe for the sgs-8 mRNA. Exposure was for 17 days. The RNA lengths are shown on the right in nucleotides.

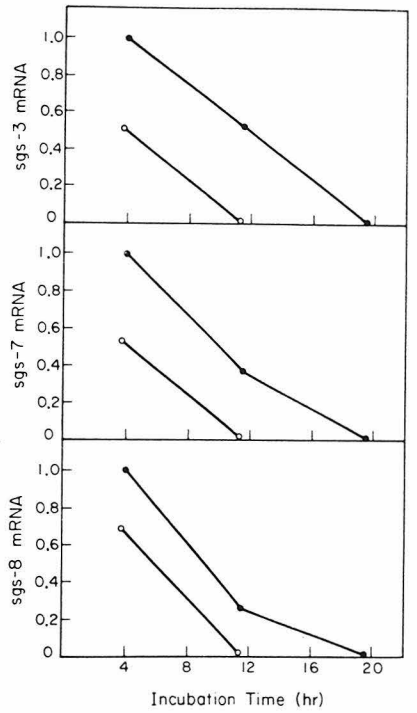

FIG. 3. Relative mass of glue protein mRNAs on gel in Fig. 2. The negative from the Polaroid photograph shown in Fig. 2A and autoradiographs of the filter, similar to those shown in Figs. 2B-D, were scanned with a Joyce, Loebl microdensitometer (shorter exposures were used for scanning). All signals were in the linear response range of the film. Ordinate: the amount of each glue protein mRNA in each RNA preparation, corrected for different total RNA amounts in each preparation by normalization to 18 S and 26 S rRNA levels. Abscissa: incubation time in hours. (●), No ecdysterone; (○) 10^{-5} *M* ecdysterone.

reduction in 68C RNA abundance, salivary glands were dissected from mid-third instar larvae and cultured in various ecdysterone concentrations for times long enough to achieve maximal hormone effect (see below); then RNAs in the process of being synthesized were labeled by addition of [^{3}H]adenosine to the culture medium for a 15-min pulse-labeling period. Salivary gland RNA was then extracted and purified by sedimentation through a CsCl gradient, and the purified RNA hybridized to completion to an excess of filter-bound cloned DNA homologous with each of the 68C RNAs. The amount of labeled nucleoside incorporated into newly synthesized sgs-3, sgs-7, or sgs-8 RNA during the 15-min pulse labeling period was then measured. The results, shown in Fig. 5, demonstrate that pretreatment of cultured glands with 10^{-5} *M* ecdysterone causes a 10-fold reduction in the amount of label incorporated into the sgs-3 RNA being synthesized during the labeling period, and a greater than twofold drop in incorporation into the sgs-7 and sgs-8 RNAs. Similar results are obtained with [^{3}H]uridine as the labeled nucleoside, indicating that the effect of the hormone is not restricted to RNA polyadenylation. Increasing the hormone concentration to 10^{-3} *M* does not produce a greater effect (data not shown); decreasing the hormone concentration to 10^{-9} *M* or less eliminates the effect.

These results could indicate that ecdysterone reduces the rate of accumulation of newly synthesized 68C RNAs, or that the hormone prevents the specific activity of the RNA precursor pool from reaching the levels that it would in the absence of the hormone. That the first possibility is correct is shown by two facts: first, ecdysterone has essentially no effect on incorporation of label into total salivary gland RNA in pulse-labeling experiments (Table 1). Thus, the data shown in Fig. 5 are nearly identical when plotted either as absolute amount of label incorporated into 68C RNA, as in parts A, B, and C of the figure, or as a ratio of 68C RNA incorporation to incorporation of label into total salivary gland RNA, as in parts, D, E, and F. If the effect of ecdysterone were on the specific activity of the RNA precursor pool, and not on rates of accumulation of specific RNAs, the ratio of label accumulated in the 68C RNAs to label incorporated in total salivary gland RNA

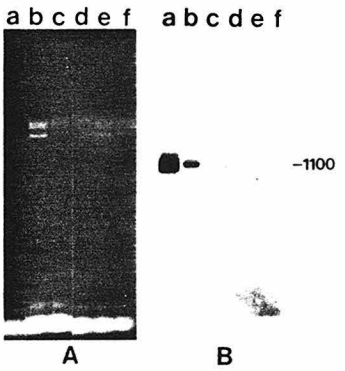

FIG. 4. Steady-state level of sgs-3 mRNA after culture of salivary glands with various concentrations of ecdysterone. Salivary glands were dissected from mid-third-instar larvae and cultured as described for Fig. 2. (10 gland lobes per 10-μl drop of culture medium). Incubations were for 10 to 12 hr with the following ecdysterone concentrations: 0 (a); 10^{-11} *M* (b); 10^{-9} *M* (c); 10^{-7} *M* (d); 10^{-6} *M* (e); 10^{-5} *M* (f). RNA was then extracted (Materials and Methods) and analyzed as described for Fig. 1. Each lane contains RNA from 10 salivary gland lobes. (A) Ethidium bromide stained RNA in gel before transfer. (B) Autoradiograph of DPT paper after transfer of RNA and hybridization to a ^{32}P-labeled DNA fragment homologous with the sgs-3 mRNA (aDm2023). Exposure was for 29 hr. The length of the RNA is indicated on the right in nucleotides. Microdensitometric scans of lanes c, d, e, and f showed no signal above background.

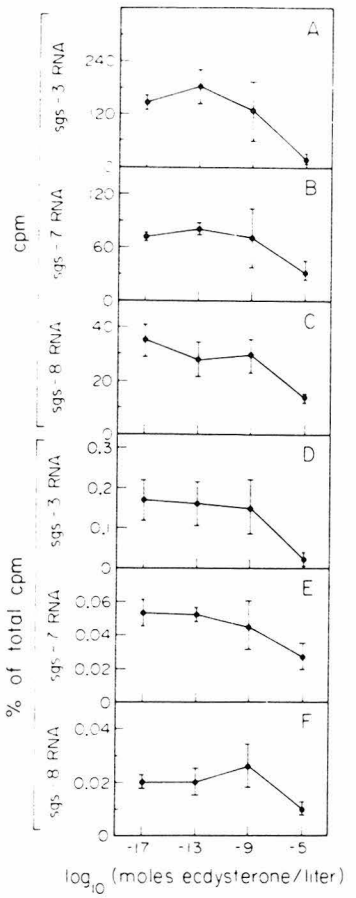

FIG. 5. Pulse-labeling of 68C RNAs in salivary glands cultured with various concentrations of ecdysterone. Salivary glands from mid-third-instar larvae were cultured as described for Fig. 2 (20 gland lobes per 10- or 20-μl drop) in the indicated concentrations of ecdysterone for 65 to 86 min (A and D), 17 to 20 min (B and E), or 18 to 71 min (C and F) prior to labeling. Labeling incubations were 14 to 16 min long in 10 μl drops of culture medium containing the appropriate concentration of ecdysterone plus 20 μCi of [^{3}H]adenosine per μl. The ^{3}H-labeled RNA was then purified by lysing the salivary gland cells, treating the lysate with Proteinase K, and pelleting the RNA through a CsCl gradient (Materials and Methods). The purified, labeled RNA was then hybridized to an excess of filter-bound DNA. The number of ^{3}H cpm hybridized specifically to the DNA of interest was then quantitated by scintillation counting. Each point on the graphs represents the mean of at least three determinations except the points for 10^{-17} *M* ecdysterone in panels A, B, D, and E which represent the mean of two determinations. The bars indicate the standard error for each set of data. (A) ^{3}H cpm hybridized to DNA homologous with the sgs-3 RNA (aDm2023). (B) ^{3}H cpm hybridized to DNA homologous with sgs-7 RNA (adm127C8). (C) ^{3}H cpm hybridized to DNA homologous with the sgs-8 RNA (adm109F4). (D) Percentage of input cpm hybridized to DNA homologous with the sgs-3 RNA. (E) Percentage of input cpm hybridized to DNA homologous with the sgs-7 RNA. (F) Percentage of input cpm hybridized to DNA homologous with the sgs-8 RNA.

TABLE 1
INCORPORATION OF [^{3}H]ADENOSINE INTO RNA IN 20 SALIVARY GLAND LOBES[a]

Experiment	Ecdysterone concentration (moles/liter)			
	10^{-17}	10^{-13}	10^{-9}	10^{-5}
1	1.2	1.2	1.3	1.0
2	1.7	1.4	1.0	1.1
3	2.2	1.8	2.0	2.4
4	1.5	1.2	1.0	1.2
5	1.1	1.3	1.8	1.0
6	1.7	1.8	1.4	1.3
Mean	1.6	1.5	1.4	1.3

[a] Salivary gland incubations, RNA labeling and RNA purification were performed as described for Fig. 5. Each number in the table represents 10^{-5} times the number of ^{3}H cpm incorporated into RNA in 20 salivary gland lobes.

in the course of the experiment would be unaffected by the hormone. In addition, control experiments were performed in which incorporation of labeled precursors into both the sgs-3 RNA and a mitochondrial transcript was measured after purifying both RNAs from batches of salivary glands cultured in the presence or absence of ecdysterone. The ratio of accumulated label in sgs-3 RNA to that in the mitochondrial transcript after a 30-min pulse labeling with [^{3}H]adenosine was 4.7 in the absence of added hormone, and 1 after salivary glands were cultured in the presence of 10^{-5} *M* ecdysterone (Table 2). If RNA precursor specific activity in the cytoplasm and nucleus of the salivary gland cells were the parameter changed by ecdysterone, this ratio should be unaffected by hormone.

Thus, ecdysterone reduces the rate of accumulation of newly synthesized RNA from the 68C puff. To confirm this conclusion, and to study this hormone effect further, another series of experiments was conducted. Salivary glands from mid-third instar larvae were cultured in the presence of 10^{-5} *M* ecdysterone, or in the absence of added steroid, for 30 min (the effect of the hormone is complete within 30 min—see below), then pulse-labeled for times varying from 5 to 20 min with a mixture of [^{3}H]cytidine, [^{3}H]guanosine, and [^{3}H]uridine. The amount of label incorporated into each of the 68C RNA species was determined by hybrid selection and scintillation counting, as in the previous experiments. The results are summarized in Fig. 6. Reduced synthesis of the sgs-3 and sgs-7 RNAs resulting from hormone treatment could be detected with a labeling period of only 5 or 10 min. The overall rate of label accumulation in sgs-8 RNA was so low that significant incorporation was not achieved before 15 min. For the other two RNAs, however, it is clear that if the hormone effect is on the half-life of puff RNA after its transcription rather than

TABLE 2
INCORPORATION OF [^{3}H]ADENOSINE INTO THE sgs-3 RNA AND A MITOCHONDRIAL TRANSCRIPT[a]

Experiment	No Ecdysterone		10^{-5} *M* Ecdysterone	
	sgs-3	Mitochondrial	sgs-3	Mitochondrial
1	—	—	6	7
2	—	—	9	25
3	231	51	—	—
4	44	9	14	7
Mean	140	30	10	10
Ratio $\left(\frac{\text{sgs-3 cpm}}{\text{mitochondrial cpm}}\right)$	4.7		1.0	

[a] Sixty salivary gland lobes were incubated in 20-μl drops of culture medium containing the indicated concentrations of ecdysterone for 2 hr prior to labeling. Labeling incubations were 30 min long in 20-μl drops of culture medium containing the appropriate concentration of hormone plus 20 μCi of [^{3}H]adenosine per μl. The ^{3}H-labeled RNA was then purified by phenol extraction (Materials and Methods). After removing aliquots for RNA concentration determination, each RNA sample was divided into three equal fractions (each fraction contained the RNA from 18 salivary gland lobes). These fractions were then incubated with aminophenylthioether paper disks containing an excess of aDm2023 DNA (homologous with the sgs-3 RNA), adm125G9 DNA (homologous with a mitochondrial transcript—Villeneuve and Meyerowitz, unpublished), or pBR322 DNA (the vector which is included in both the aDm2023 DNA and adm125G9 plasmids). After washing away unbound RNA, the RNA specifically hybridized to the DNA on the filters was eluted and quantitated by scintillation counting (Materials and Methods). The cpm eluted from the pBR322 filter (background) were subtracted from the cpm eluted from the aDm2023 and adm125G9 filters. The numbers in this table are the number of cpm above background for each hybridization.

on the rate of transcription, the hormone must be reducing the half-life of the newly synthesized RNA to the order of 5 min or less. It is again shown that the steroid has an effect on a very early step in RNA production, at least for the sgs-3 and sgs-7 RNAs.

The Hormone Effect Is Very Rapid

To find the minimum time of hormone treatment required to cause full reduction in the accumulation rate of 68C RNAs in cultured salivary glands, glands from mid-third larval instar were incubated in 10^{-5} *M* ecdysterone for different amounts of time, then pulse-labeled with either [^{3}H]adenosine or [^{3}H]uridine for 15 min. In each experiment all of the salivary glands were in culture for the same amount of time, with only the length of the hormone treatment being varied between cultures. The labeled RNA was purified and specific RNA species selected by hybridization to filter-bound DNA as before. The results are shown in Fig. 7. The effect of ecdysterone on all three 68C RNA species was complete by 30 min, and almost complete at the earliest times checked, from 14 to 17 min. This is true of incorporation of either adenosine or uridine. In addition to showing that the hormone acts almost immediately, these experiments again confirm that the effect of the hormone is to repress accumulation of newly synthesized 68C RNAs, and again show that the mass of RNA accumulated in the presence or absence of ecdysterone in a 15-min period is greatest for *Sgs-3* transcripts, followed by *Sgs-7*, then *Sgs-8* RNAs.

The effect of the steroid on the glue protein RNAs appears to be much more rapid than the effect of the hormone on the size of the 68C puff. The rate of regression of the puff in the presence of ecdysterone is charted in Fig. 7A, and is also discussed in Ashburner *et al.* (1973) and Bonner and Pardue (1977), where it is shown that hormone-induced puff regression takes over 2 hr to be complete.

DISCUSSION

The experiments reported in this paper allow three major conclusions: that ecdysterone causes the three glue RNAs transcribed from the 68C puff to disappear

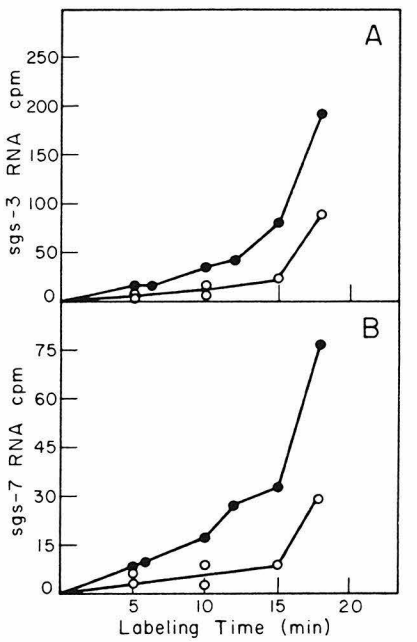

FIG. 6. Effect of ecdysterone on accumulation of 68C RNAs in cultured salivary glands. Salivary glands from mid-third-instar larvae were cultured as described for Fig. 2 (20 gland lobes in 10 μl) with or without ecdysterone for 30 min prior to labeling. The glands were then transferred to medium containing equimolar amounts of [^{3}H]cytidine, [^{3}H]guanosine, and [^{3}H]uridine, as well as ecdysterone if the salivary glands were hormone treated before labeling. After labeling, RNA was purified and hybridized to filter-bound DNA as described for Fig. 5. (●) no hormone treatment; (○) incubation with 10^{-5} *M* ecdysterone. Each point represents RNA from 20 salivary gland lobes. (A) ^{3}H cpm hybridized to DNA homologous with the sgs-3 RNA (aDm2023). (B) ^{3}H cpm hybridized to DNA homologous with the sgs-7 RNA (adm127C8).

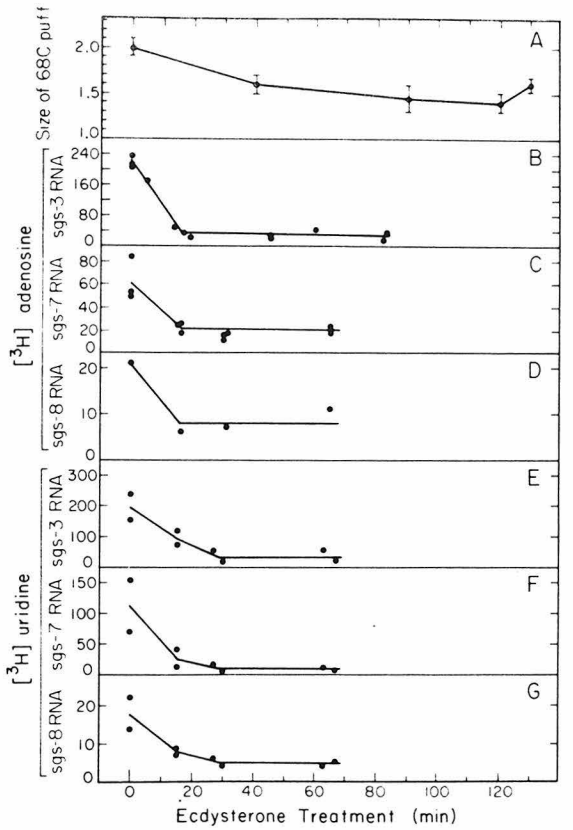

FIG. 7. Size of 68C puff and pulse-labeling of 68C RNAs in salivary glands after various times of preincubation with ecdysterone. Salivary glands from mid-third-instar larvae were cultured as described for Fig. 2 [6 or 7 gland lobes per 10-μl drop (A), 20 gland lobes per 10- or 20-μl drop (B–G)] with 10^{-5} *M* ecdysterone for various amounts of time or without hormone ($T = 0$). All glands were in culture for the same amount of time, only the length of the ecdysterone treatment (shown on the abscissa) varied between cultures in each experiment. (A) To determine the relative size of the 68C puff, the lobes were fixed and stained for 2 to 4 min in a 20-μl drop of 1:1 lactic acid:acetic acid containing 2% orcein, then squashed between a siliconized coverslip and a glass slide. The stained polytene chromosomes were photographed using a Zeiss photomicroscope. The maximum diameter of the 68C puff region and the diameter of the 69A1-3 bands (not involved in puffing activity in third instar larvae) were measured from the photographs. The numbers on the ordinate represent the ratio of the diameter of the 68C puff to the diameter of the 69A1-3 bands. Each point represents the mean of more than one determination: 0 min, four determinations; 40 min, six determinations; 90 min, two determinations; 120 min, five determinations; 130 min, three determinations. The bars represent the standard error for each set of data. (B–D) Salivary gland RNA was pulse-labeled with [^{3}H]adenosine, purified, and hybridized to filter-bound DNA as described for Fig. 5. (E–G) Same as (B–D) except that [^{3}H]uridine was used as the label. (B and E) ^{3}H cpm hybridized to DNA homologous with the sgs-3 RNA (aDm2023). (C and F) ^{3}H cpm hybridized to DNA homologous with the sgs-7 RNA (adm127C8). (D and G) ^{3}H cpm hybridized to DNA homologous with the sgs-8 RNA (adm109F4).

from cultured salivary glands more rapidly than they would in the absence of the hormone; that one of the predominant effects caused by ecdysterone on 68C puff RNAs is a sharp reduction in the rate at which they accumulate; and that this steroid effect occurs very rapidly after the hormone is administered to salivary glands. The concentration of ecdysterone necessary to produce effects on the 68C RNAs is similar to that which causes regression of the 68C puff in cultured salivary glands, although the effect of the hormone on the RNAs is complete in a shorter time than is required for complete puff regression.

Exactly what process in RNA production is the one affected by ecdysterone in these experiments is not known. Among the possibilities are an effect on the initiation of transcription or on the rate of elongation during transcription, or an effect on stability of the 68C RNAs after they have been synthesized. Reduction in stability might result from either of two processes. The first is that the initial RNA transcript is extremely unstable and is processed to a relatively stable RNA, with the fraction of the primary transcript that is processed depending on ecdysterone concentration. The second is that the hormone affects the half-life of all of the newly transcribed RNA, reducing it to a time short compared to the maximal 15 min from the start of RNA synthesis in which the ecdysterone regulatory step occurs. The most likely level of hormone effect is at initiation of transcription, the process regulated by steroid hormones in murine mammary tumor viruses (Ucker *et al.*, 1983). In the case of the murine virus, though, the steroid effect is to stimulate transcriptional initiation; in the 68C puff case the hormone effect is in the opposite direction. The possibility of an effect on frequency of RNA processing must also be considered, since the only recognizable sequences in or near each of the three 68C genes that are held in common are those surrounding the single intervening sequence of each RNA (Garfinkel *et al.*, 1983). There may also be processes other than those that influence accumulation of newly synthesized 68C RNA that are ecdysterone regulated: the *in vivo* disappearance of all three 68C RNAs at the time of puparium formation, and the *in vitro* disappearance of the RNAs in cultured salivary glands, may result from more than one hormone effect. The rapidity with which ecdysterone causes a drop in 68C RNA accumulation rates indicates, however, that this is the most direct, primary effect of the hormone on the puff RNAs. The time required for the complete ecdysterone effect on the 68C RNAs is similar to that required for glucocorticoid induction of murine mammary tumor virus transcription in MTV-infected rat hepatoma tissue culture cells (Ucker *et al.*, 1981). Since induction of this viral transcription probably results from binding of activated glucocorticoid receptor protein to the viral DNA (Payvar *et al.*, 1981,

1982; Pfahl, 1982; Chandler *et al.*, 1983), it is possible that the 68C RNA effects of ecdysterone are also direct, and involve binding of ecdysterone and ecdysterone receptor protein to the puff DNA sequence.

The more rapid response of puff RNA synthesis than puff diameter to hormone indicates that the rate of RNA accumulation at a puff site is not the only determinant of puff size in polytene chromosomes. Newly synthesized RNA has been shown to remain at the puff site of its synthesis for over 30 min in the salivary gland polytene chromosomes of *Drosophila hydei* (Berendes, 1968); it is possible that puff diameter is proportional to the amount of RNA at the puff site, which is a function of both rate of accumulation and rate of departure of the RNA.

Throughout all of the experiments reported here, a strict coordination of expression of the three 68C glue genes has been observed: *in vivo* they first appear at the same developmental time and in the same tissue, and they disappear together as well. *In vitro*, all of the genes respond to ecdysterone in the same way, with the same time course, and to similar extents. The rate of accumulation of the three RNAs is different in all of the pulse-labeling experiments, however. *Sgs-3* synthesizes several-fold more RNA than *Sgs-7*, in a ratio approximately proportional to the sizes of the *Sgs-3* and *Sgs-7* transcripts. *Sgs-8*, with a transcript slightly larger than that of the *Sgs-7* gene, expresses considerably less RNA than *Sgs-7*. That the levels to which these RNAs accumulate in salivary glands *in vivo* are approximately proportional to the rate at which they accumulate in pulse-label experiments on cultured salivary glands can be seen in Fig. 1, where probes of similar specific activity were used to locate each of the RNAs on the same RNA gel blot filter. The autoradiographic intensity of the sgs-8 signal is clearly less than that of the sgs-3 or sgs-7 RNAs, even after an autoradiographic exposure about three times as long as that used for the sgs-3 and sgs-7 filters. Figure 2 shows a similar variation in amounts of the 68C transcripts *in vitro*.

The coordination of the 68C gene transcripts in tissue and in developmental time, and the response of the level of these transcripts to ecdysterone leave a number of general questions to be answered. Among them are: do the three 68C RNAs respond identically to ecdysterone through an interaction of the hormone and hormone receptor with a single sequence that affects transcription of all three RNAs, or would each gene function autonomously if separated from the others? If ecdysterone does act by interacting with specific DNA sequences, what are they? Both questions are in the process of being answered by taking various fragments of the cloned 68C glue puff and reintegrating them into the fly genome, then assaying the expression of the reintroduced sequences in third instar larvae. Normal time and tissue distribution of *Sgs-3* expression from reintroduced fragments of the 68C puff have already been observed (M. Crosby and E. Meyerowitz, work in progress).

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CHAPTER 4

A *Trans*-Acting Regulatory Product Necessary for Expression of the *Drosophila melanogaster* 68C Glue Gene Cluster

(Draft for submission to *Cell*)

A Trans-Acting Regulatory Product Necessary for Expression of the Drosophila melanogaster 68C Glue Gene Cluster

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Summary

When salivary glands of Drosophila melanogaster larvae homozygous or hemizygous for the lethal(1)t435 mutation, which is located at cytological position 2B5 on the X chromosome, are cultured in the presence of ecdysterone, the 68C puff does not regress. Such a treatment does cause the puff to contract in wild-type glands. Here we show that another allele of this mutation, lethal(1)npr-1 (l(1)npr-1), also inhibits the steroid-induced regression of the puff at 68C in cultured salivary glands. In addition, RNA blot hybridizations and ^{3}H-uridine pulse-labeling experiments demonstrate that the l(1)npr-1 mutation prevents expression of the 68C glue protein genes, Sgs-3, Sgs-7 and Sgs-8. Since a deletion of the 2B5 band also prevents expression of the 68C glue RNAs, the effect of the mutation is to remove a wild-type product required in trans for expression of the 68C genes. The l(1)npr-1 mutation was also crossed into a Drosophila stock that contains a copy of the Sgs-3 gene introduced by DNA-mediated transformation, with only 2.3 kb of upstream sequence and 2.5 kb of downstream sequence. This gene expresses the glue RNA normally in the presence of a wild-type X chromosome; in a l(1)npr-1 background the introduced gene is not expressed. Thus, the wild-type product of the 2B5 locus interacts, either directly or indirectly, with the DNA of the 68C glue gene cluster to allow its expression. Transcription autoradiogram experiments were also performed, and show that no detectable transcription is occurring at the 68C puff in l(1)npr-1 mutants. Therefore, a final conclusion is that the 68C puff is not caused by high levels of RNA transcription and accumulation.

Introduction

In Drosophila melanogaster the 68C region of the third chromosome contains three genes, Sgs-3, Sgs-7 and Sgs-8, which are transcribed in third larval instar salivary glands to produce messenger RNAs for salivary glue polypeptides. These three polypeptides, and several others, are synthesized in salivary gland cells throughout the third instar, are secreted into the lumen of the gland at the end of this stage, and then are expelled through the salivary duct (Crowley et al., 1983). This mucoprotein secretion then hardens and allows the newly formed puparium to adhere to a solid support (Fraenkel and Brookes, 1953).

About midway through the third larval instar salivary gland chromosomes achieve a degree of polyteny that allows them to be easily observed with a light microscope. This is the earliest time at which chromosomal puffs can be distinguished. The 68C region is puffed at this time and remains so until just before the end of the third instar, when the concentration of the steroid hormone ecdysterone in the larval hemolymph increases dramatically. This causes regression of the puff and a sharp reduction in expression of the Sgs-3, Sgs-7 and Sgs-8 genes. The evidence for this is that the 68C puff contracts and expression of the glue genes ceases when ecdysterone concentration increases during normal development in vivo; and that increasing the hormone concentration in cultured salivary glands duplicates the effects seen in vivo (Ashburner, 1967; Ashburner, 1973; Ashburner et al., 1973; Crowley and Meyerowitz, 1984).

Larvae which are homozygous or hemizygous for the lethal(1)t435 mutation (l(1)t435, Belyaeva et al., 1980) never pupariate. They spend up to a week as third instar larvae, then die without molting. In salivary glands of these larvae a puff is visible at 68C on the third chromosome during the latter part of the third instar, as in wild-type larvae. However, when the mutant glands are cultured in the

presence of ecdysterone the puff does not regress as it does in wild-type glands. Also, puffs at other loci which are induced by ecdysterone in wild-type glands are not fully induced by the hormone in this mutant. l(1)t435 has been cytologically mapped to the 2B5 band of the X chromosome. Apparently a product of the 2B5 locus is necessary for normal hormonal control of puffing in salivary glands (Belyaeva et al., 1981). The mutation known as lethal(1)npr-1 (l(1)npr-1, Kiss et al., 1976; Kiss et al., 1978) is an allele of l(1)t435. Like the l(1)t435 larvae, males hemizygous and females homozygous for l(1)npr-1 are non-pupariating. They do not progress beyond the third instar larval stage of development, remaining as third instar larvae for about one week, then dying (Fristrom et al., 1981).

Examination of polytene chromosomes stained with metachromatic dyes and autoradiographs of polytene chromosomes pulse-labeled with ^{3}H-uridine has led to a widely accepted belief that puffs are sites of rapid RNA synthesis (Pelling, 1964; Berendes, 1968; Ellgaard and Clever, 1971; Tissieres et al., 1974; Zhimulev and Belyaeva, 1975; Belyaeva and Zhimulev, 1976; Bonner and Pardue, 1977; Mitchell et al., 1978). This hypothesis seems to be confirmed by experiments which show that ecdysterone causes a reduction in glue RNA synthesis as well as puff size at the 68C locus in wild-type salivary glands (Crowley and Meyerowitz, 1984; see above). A mutant strain in which the 68C puff does not regress in response to the hormone provides an opportunity to further examine the relationship between puffing and transcription. In this paper we first show that the 68C region is puffed in the l(1)npr-1 salivary glands and that the puff fails to respond to ecdysterone, as was the case in l(1)t435 glands. We then demonstrate that transcription of the 68C glue genes, Sgs-3, Sgs-7 and Sgs-8, is drastically reduced in l(1)npr-1 compared to wild-type and, in addition, that there are no other RNAs being produced at the 68C puff in the mutant glands. Thus, the 68C puff can exist without serving as a site of high-level RNA

synthesis. We further show that 6 kb or less of 68C DNA is all that is necessary for the repression of Sgs-3 by the l(1)npr-1 mutation, and thus that the normal product of the 2B5 locus, or some product induced by this locus, interacts with the DNA of the 68C glue gene cluster to allow expression of the Sgs-3 gene.

Results

Wild-type Activity of the 2B5 Locus is Necessary for Steroid-induced Regression of the 68C Puff

To analyze the effect of the l(1)npr-1 mutation on the 68C region, salivary glands hemizygous for l(1)npr-1, or glands from control larvae hemizygous for the balancer chromosome Binsn (Lindsley and Grell, 1968), and with the same autosomal background as that of the l(1)npr-1 larvae were cultured either in 10^{-5} molar ecdysterone, or in the absence of hormone, for 2 hours, after which the chromosomes were stained and spread for light microscopic examination. The diameters of the 68C and 69A1-3 regions of the third chromosome were measured and the ratio of 68C diameter to 69A1-3 diameter calculated, as a quantitation of the extent of puffing at 68C (Ashburner, 1973; Crowley and Meyerowitz, 1984). The 69A1-3 bands are used as a reference because they are not involved in puffing activity in third instar salivary glands. The results are shown in Table 1. Ecdysterone did not affect the size of the 68C puff in the l(1)npr-1 salivary glands, although it did reduce 68C diameter by 30% in the control glands. Thus the l(1)npr-1 mutation inhibits the hormone-mediated regression of the 68C puff in cultured salivary glands. Belyaeva and coworkers (1981) obtained essentially the same result with the allelic l(1)t435 mutation.

The 68C Glue RNAs are Neither Accumulated nor Transcribed in l(1)npr-1 Salivary Glands

Since the 68C puff response was found to be abnormal in the l(1)npr-1 glands, we began an examination of glue gene expression to determine if that was also affected by the mutation. RNA was extracted from salivary glands of hemizygous l(1)npr-1, hemizygous Binsn or wild-type (OR16f) larvae, fractionated by electrophoresis, transferred to a nitrocellulose filter and the RNA filter subsequently probed with ^{32}P-labeled DNA fragments homologous with each of the 68C RNAs. Figure 1 depicts the results: none of the 68C glue RNAs is detectable in the l(1)npr-1 glands but all three are quite abundant in the Binsn and wild-type control salivary glands (summarized in Table 2). By comparison of autoradiographs of filters exposed to film for various amounts of time after each hybridization we determined that the abundances of the 68C RNAs in the l(1)npr-1 glands are at least 180-fold reduced compared to Binsn or wild-type.

Since the hemizygous l(1)npr-1 larvae have a prolonged third instar, it was possible that 68C glue gene expression was simply delayed in these animals. To determine if this was the case we analyzed RNA from salivary glands of hemizygous l(1)npr-1 larvae which were 4 to 11 days old, a range that covers the entire third instar for these animals, in RNA gel blots. None of the 68C glue RNAs were detectable at any point in the third instar (data not shown). Thus, the 68C RNAs never accumulate to a significant level in hemizygous l(1)npr-1 larvae.

To determine which process in glue RNA metabolism is affected by the l(1)npr-1 mutation the rates of accumulation of newly synthesized RNAs were measured in salivary glands of hemizygous l(1)npr-1, and control Binsn salivary glands. This was done by pulse-labeling the RNA being synthesized in the glands with ^{3}H-uridine, purifying the RNA, hybridizing it to an excess of filter-bound

DNA homologous with each of the 68C glue RNAs and quantitating the amount of hybridized RNA by scintillation spectrometry. The results of these experiments are summarized in Table 3. In the l(1)npr-1 glands the amount of label incorporated into newly synthesized Sgs-3, Sgs-7 and Sgs-8 RNAs is at least 360-, 42- and 8-fold reduced, respectively, compared to the controls. Overall incorporation of ^{3}H-uridine into salivary gland RNA is unaffected by the mutation. Thus the rates of synthesis of all three 68C glue RNAs are drastically and specifically reduced in the l(1)npr-1 salivary glands.

The Effects of l(1)npr-1 at 68C are Due to Absence of a Normal Product

Among the possible ways in which a mutation leads to a specific phenotype is the simple elimination of the wild type activity of the affected locus. The classical test for this is comparison of the phenotype of organisms bearing the mutation with that of organisms with a deletion for the locus in question (Muller, 1932). A deletion for the 2B5 band, which contains the l(1)npr-1 locus, was constructed starting with males that have a Dp(1;Y)Sz280, y^2 Y chromosome, which carries the 1A to 2C1-2 region of the X chromosome with an internal deletion from 2B3-4 to 2B7-8 (Belyaeva et al., 1982). These were crossed to females carrying Df(1)S39, cho^2, an X chromosome deleted for the region from 1E1-2 through 2B5 (Belyaeva et al., 1980). Male third instar larval progeny of this cross of the constitution Df(1)S39, cho^2/Dp(1;Y)Sz280, y^2 totally lack 2B5, and are recognizable by the chocolate (cho) phenotype (Belyaeva et al., 1981). Such larvae were collected, their salivary glands dissected and RNA extracted from them. This RNA was subjected to gel electrophoresis in parallel with RNA from salivary glands of sibling larvae that were not deficient for 2B5. The gel was blotted to nitrocellulose, and the filter hybridized with ^{32}P-labeled λcDm2007, a lambda

clone containing sequences that hybridize to all three of the 68C glue RNAs (Meyerowitz and Hogness, 1982). All of the RNAs were present in the control lane; none were detectable in the lane with RNA from the 2B5-deficient larvae. This shows that the 68C RNA phenotype of the l(1)npr-1 mutation is identical to that of a complete deficiency for the 2B5 locus, and thus that the l(1)npr-1 phenotype is due to absence of the wild-type product of the locus. That a deficiency for 2B5 has the same phenotype as the l(1)npr-1 mutation also confirms that the absence of the 68C RNAs in the l(1)npr-1 strain is due to the l(1)npr-1 mutation, and not to any other mutation that might be carried in the strain. Further evidence that this is the case is the absence of the 68C RNAs in strains bearing other alleles of l(1)npr-1. Males hemizygous for l(1)d.norm.-1^{a} (Stewart et al., 1972), or females homozygous for l(1)t435, both of which are alleles of l(1)npr-1 induced at different times, and in the case of l(1)t435 in a different genetic background than l(1)npr-1, also lack the 68C glue RNAs. This was determined in RNA gel blot experiments identical to those described for l(1)npr-1 and for the 2B5 deficiency.

l(1)npr-1 Repression of Sgs-3 Requires Only 6 kb of 68C DNA

The l(1)npr-1 mutation is at position 2B on the X chromosome but prevents transcription of genes at 68C on the third chromosome. This implies that a product of the 2B5 locus or some product induced by the action of the wild-type 2B5 locus interacts with the DNA or chromatin at the unlinked 68C puff. The next experiments performed were directed toward determining which DNA sequences in the 68C region were interacting with the product of the 2B5 locus, or with a product induced by this locus. We started with a Drosophila strain that contains a normal 68C glue puff locus derived from the Hikone-R wild type

strain. This strain produces an Sgs-3 mRNA approximately 1000 nucleotides in length, rather than the 1120 base Sgs-3 mRNA characteristic of the Oregon-R wild type strain from which the locus was originally cloned (Meyerowitz and Hogness, 1982). The strain also contains an additional copy of the Sgs-3 gene of the Oregon-R type, on a 6 kb DNA fragment inserted into the 66E region of the third chromosome by P factor-mediated transformation. This insertion is designated Tf(3)GA6.0-1. The 6 kb piece has 2.3 kb of 5' upstream sequence, the entire Sgs-3 gene including the intron, and 2.5 kb of 3' downstream sequence. The Tf(3)GA6.0-1, Sgs-3^{HR} strain, with both the Oregon-R Sgs-3 allele (Sgs-3^{OR}) and the Hikone-R Sgs-3 allele (Sgs-3^{HR}), produces both the Oregon-R and Hikone-R Sgs-3 RNA transcripts in normal quantity, tissue and developmental stage, as assayed in RNA gel blot experiments (Crosby, 1983; Crosby and Meyerowitz, in preparation). Female flies carrying a l(1)npr-1 X chromosome balanced over the Binsn chromosome were crossed with males of the Tf(3)GA6.0-1, Sgs-3^{HR} strain, producing male larvae hemizygous for l(1)npr-1 and heterozygous for the third chromosome of the transformed strain. These larvae were dissected and their salivary glands removed. RNA was extracted from the glands and from control glands taken from Binsn siblings of the experimental larvae, and both samples assayed for the the presence of the Sgs-3 RNA by electrophoresis followed by blot hybridization. RNA samples from hemizygous l(1)npr-1 or Binsn male larvae from the original mutant stock were tested for Sgs-3 expression at the same time. The results are shown in Figure 2. In the control Binsn; Tf(3)GA6.0-1, Sgs-3^{HR} both the 1000 base and the 1120 base Sgs-3 RNAs are present. Neither were detected in the l(1)npr-1; Tf(3)GA6.0-1, Sgs-3^{HR} salivary glands. The other RNA samples gave the expected results. That the introduced 6 kb Sgs-3^{OR} gene fails to express in the absence of a wild-type l(1)npr-1 locus shows that the 68C DNA sequences necessary for interaction with this locus are contained within the 6 kb fragment,

and are thus no more than a few thousand base pairs from the Sgs-3 gene, and within the 68C glue gene cluster.

The Puff at 68C is not Caused by RNA Synthesis

A puff is present at 68C in l(1)npr-1 salivary glands, although the three glue genes located there are not transcribed. Since puffs are generally associated with high levels of RNA synthesis, it seemed possible that the 68C puff in l(1)npr-1 larvae was a result of transcription of some genes in the puff other than the glue genes. To determine if the puff at 68C is caused by synthesis of RNAs other than the glue RNAs, transcription in salivary chromosomes of hemizygous l(1)npr-1 and Binsn larvae was analyzed using transcription autoradiography. Salivary glands were incubated in medium containing ^{3}H-uridine for 10 min, then stained with orcein and squashed between a glass slide and a coverslip. The unincorporated uridine was washed away, the slide dipped in a photographic emulsion, exposed for three days, then developed. Silver grains over the chromosomes on the developed slides indicate RNA synthesis (Pelling, 1964; Zhimulev and Belyaeva, 1975). Figure 3 shows the results. No more than one grain was found at 68C in the l(1)npr-1 chromosome spreads, while 11 to 12 grains were detected on this puff in chromosome spreads of the control Binsn larvae. The grain counts are summarized in Table 4. Thus the rate of RNA synthesis at 68C in l(1)npr-1 glands is at least 11-fold reduced compared to Binsn glands. We have shown that, in the absence of added ecdysterone, the puff at 68C is the same size in the mutant and control glands (see Table 1). Therefore the size of the 68C puff is not related to the amount of RNA being made there. Also, there are no RNAs being synthesized at 68C in the l(1)npr-1 glands at the rate at which the glue RNAs are made at this locus in Binsn control or wild-type glands.

Discussion

Our analysis of the l(1)npr-1 locus has demonstrated it to be a trans-acting regulator of the 68C glue genes. In addition, we have shown that the nature of the l(1)npr-1 mutation is that it prevents production of the normal product of the locus, and further, that the normal function of the wild-type product of the locus involves either direct or indirect interaction with DNA sequences within the 68C glue gene cluster. Finally, our results show that puffing at 68C can occur in the absence of any measurable RNA synthesis in the entire 68C region.

The l(1)npr-1 mutation maps to position 2B5 on the X chromosome but prevents expression of the glue protein genes at 68C on the third chromosome. We have demonstrated that the effect of the mutation is on transcription of the glue genes, and that a product of the 2B5 region is involved in activating transcription at 68C. The simplest explanation is that a protein coded by an RNA transcribed from the 2B5 region interacts with the DNA within or surrounding the 68C glue genes and thereby allows RNA polymerase II to transcribe the genes. It is also possible that the normal 2B5 product only induces another locus, or a set of loci whose products are the ones that directly interact with the 68C DNA. There are several known examples in eukaryotic systems of genes which require interaction with a trans-acting regulatory protein in order to be transcribed. Transcription of mammary tumor virus (MTV) DNA in MTV-infected rat cells is induced by binding of the glucocorticoid receptor protein to sequences within or near the transcribed region. In the presence of glucocorticoid hormones the receptor binds to one site upstream of the transcription initiation site and four sites between 4 and 8 kb downstream of the initiation site. This appears to be a prerequisite for transcription of the viral DNA (Ucker et al., 1981; Payvar et al., 1983). The progesterone receptor is thought to induce transcription of the

ovalbumin and conalbumin genes in chicken oviduct. In the presence of the steroid hormone progesterone the receptor binds to sequences just 5' of these genes and allows them to be transcribed (Mulvihill et al., 1982; Compton et al., 1983). In Neurospora crassa the qa-2, qa-3 and qa-4 genes, which code for enzymes involved in the degradation of quinic acid, are regulated by the protein product of the qa-1 gene. When the concentration of quinic acid is high the qa-1 protein induces transcription of the other qa genes presumably by binding to DNA sequences within or near them (Case and Giles, 1975; Patel et al., 1981; Geever et al., 1983). The SV40 early transcription unit is regulated in a similar fashion. The Sp1 protein must bind to a series of tandem repeats upstream of the early promoter in order for RNA polymerase II to begin transcribing there (Dynan and Tjian, 1983). When a plasmid containing the adenovirus E2 gene is transfected into mouse L cells the gene is not expressed efficiently unless a plasmid containing the adenovirus E1A gene or the pseudorabies virus genome is cotransfected with it. This implies that the protein produced by the E1A gene and a protein coded on the pseudorabies virus genome can interact with the E2 gene and activate its transcription (Imperiale et al., 1983). The results of our experiments indicate that the Drosophila 68C glue genes are further examples of eukaryotic genes whose transcription is regulated by a protein coded by another gene. In the 68C case there are several clustered and coordinately regulated genes that all respond to the same trans-acting signal.

The normal expression of Sgs-3 transcription as regulated by the l(1)npr-1 locus involves at most 6 kb of 68C DNA. This was revealed by analyzing the expression of an Sgs-3 gene in a 6 kb fragment which was introduced into the fly genome by P factor-mediated transformation. The 6 kb region extends 2.3 kb on the 5' side of Sgs-3 and 2.5 kb on the 3' side. This result implies that the l(1)npr-1 mutation affects a regulatory element which normally interacts with the DNA

within this 6 kb region to allow for Sgs-3 transcription. We have not yet performed a similar analysis on the Sgs-7 and Sgs-8 genes.

The 68C region is puffed in l(1)npr-1 salivary glands just as in wild-type glands. However, our results show that the 68C glue RNAs are not synthesized in the mutant glands so they can not be responsible for puff formation. Further, our transcription autoradiograms showed no sign of RNA synthesis at 68C in the l(1)npr-1 glands. Apparently the 68C puff is not created by RNA synthesis. This is not consistent with the widely accepted belief that puffs on polytene chromosomes result from a high rate of transcription of the puff DNA. Pulse labeling of newly synthesized salivary gland RNA with ^{3}H-uridine followed by chromosome spreads and autoradiography has always shown a high degree of correlation between puffing and RNA synthesis. This is true of puffs induced by heat shock as well as puffs which arise during normal development (Pelling, 1964; Berendes, 1968; Zhimulev and Belyaeva, 1975; Belyaeva and Zhimulev, 1976). We have found that a puff of normal size can exist on a polytene chromosome in the absence of detectable RNA synthesis at that site. It thus seems that chromosomal puffs arise due to an alteration in chromatin structure which does not result from high levels of transcription, but which may allow for a high rate of transcription of the puff DNA. This interpretation is consistent with the results of Berendes (1968), who found that heat shock and ecdysterone treatment can both induce puffs at normal chromosomal positions in Drosophila hydei, in the presence of levels of Actinomycin-D sufficient to inhibit RNA accumulation at the puff sites. The puffs observed in these experiments never approached a normal size, however.

The l(1)npr-1 mutation inhibits ecdysterone-induced regression of the 68C puff and does not allow transcription of the 68C glue genes while having no effect on ecdysterone levels (Fristrom et al., 1981). Hansson and Lambertsson (1983)

have shown that accumulation of the Sgs-3, Sgs-7 and Sgs-8 glue RNAs does not occur in larvae that are genetically ecdysterone deficient, and that accumulation of these RNAs can be induced in ecdysterone deficient larvae by application of exogenous hormone. Thus, induction of 68C glue gene expression and regression of the 68C puff (Ashburner, 1973) both require ecdysterone, and both are inhibited by the l(1)npr-1 mutation. It is thus possible that the mutation prevents production of normal levels of functional ecdysterone receptor (Maroy et al., 1978; Yund et al., 1978) or of some component of this receptor required in third instar larvae. This speculation is consistent with the overall phenotype of the l(1)npr-1 mutation, which is evident in tissues other than salivary glands, and which undoubtedly involves failure of normal expression of many genes other than those of the 68C puff (Stewart et al., 1972; Fristrom et al., 1981; Mathers and Meyerowitz, work in progress).

Experimental Procedures

Materials

Ecdysterone (B-ecdysone, 20-OH ecdysone) was purchased from Sigma Chemical Co., dissolved at a concentration of approximately 10 mg/ml in 100% ethanol and stored at -20°C. The exact concentration of this stock solution was determined by diluting a small aliquot into methanol (Baker analyzed, HPLC grade) and measuring its absorbance at 242 nm, assuming a value of e = 12,400 (Hoffmeister et al., 1965; Hocks and Wiechert, 1966). Deoxycytidine 5'-[α-^{32}P]triphosphate (α-^{32}P dCTP, 1800-5400 Ci/mmole) was obtained from ICN Pharmaceuticals Inc. ^{3}H-uridine (38.4 Ci/mmol), Aquasol-2 (liquid scintillation counting cocktail) and Liquifluor (PPO-POPOP toluene concentrate) were from New England Nuclear.

Toluene (scintillar grade) was from Mallinckrodt Inc. Proteinase K was purchased from EM Reagents. Yeast tRNA was obtained from Miles Laboratories Inc. The following items were purchased from Kodak: XAR-5 film for autoradiography of RNA gel blot filters, NTB-2 nuclear track emulsion, D-19 developer and rapid fixer for autoradiography of pulse-labeled chromosomes, Plus-X pan film for photography of stained chromosomes and chromosome autoradiographs. A Zeiss photomicroscope was used for photography of chromosome spreads.

Drosophila

Strain OR16f, which has a homozygous third chromosome, was used as wild-type (Meyerowitz and Hogness, 1982). In the l(1)npr-1 mutant strain (provided by J. W. Fristrom, Department of Genetics, University of California, Berkeley) the X chromosome containing the l(1)npr-1 mutation was marked with yellow, white and maroonlike and balanced over Binsn. Male larvae hemizygous for l(1)npr-1 were identified by their golden brown mouth parts. Male Binsn larvae, which have black mouth parts, were used for controls (Fristrom et al., 1981). Strains with l(1)d.norm.-1^a, Dp(1;Y)Sz280 and Df(1)S39,cho^2 were provided by Dr. Istvan Kiss, Institute of Genetics, Hungarian Academy of Sciences, Szeged; the l(1)t435 strain (y l(1)t435/FM6/Dp(1;Y)67g,y^2) was a gift of Dr. Igor Zhimulev of the Institute of Cytology and Genetics of the USSR Academy of Sciences, Novosibirsk. Eggs were collected on standard cornmeal-agar food in half-pint milk bottles for 1 day at 22°C. Animals were allowed to develop at the same temperature. Mid-third instar larvae (i.e., 6- to 7-day-old animals which were still in the food) were used for all experiments. At this point in development the concentration of ecdysterone in the larval hemolymph is still relatively low (Berreur et al., 1979; Maroy et al., 1980).

Salivary Gland Cultures

Three to 44 salivary gland lobes were cultured in a 10 µl drop of culture medium (10 mM morpholinopropane sulfonic acid pH 7, 80 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$) on a piece of parafilm at 22°C (Mitchell et al., 1978). The buffer was oxygenated before use and analysis of salivary chromosomes after in vitro incubations has shown that heat shock puffs are not induced by the culture conditions. Ecdysterone when present was at 10^{-5} M. For pulse-labeling ^{3}H-uridine was included at 20 mCi/ml (if RNA was to be purified for hybridization) or 50 µCi/ml (for transcription autoradiograms).

Purification of Unlabeled RNA from Salivary Glands

Ten salivary gland lobes were transferred to a microcentrifuge tube containing 20 µl of extraction buffer (20 mM NaCl, 20 mM Tris-hydrochloride, pH 7.8, 40 mM ethylenediamine tetraacetic acid [EDTA], 1% sodium dodecyl sulfate [SDS]) plus 20 µg of yeast tRNA and immediately frozen in liquid nitrogen. Twenty µl of phenol plus 20 µl of chlorform-isoamyl alcohol (100:1) were added and the tube vortexed as the extraction buffer and glands thawed. The tube was then spun briefly in a microcentrifuge to separate the two phases and the organic phase was removed and discarded. The aqueous solution was extracted two more times with phenol/chloroform and then transferred to a clean tube. The organic solution from the last extraction was reextracted with 10 µl of extraction buffer which was then pooled with the previously extracted aqueous solution. The lysate was then extracted with 20 µl of chloroform-alcohol, transferred to a new tube and extracted with 20 to 40 µl of ethyl ether. Any residual ether was removed by directing a stream of air over the solution. One-fifteenth volume of 3 M sodium

acetate pH 5.5 and 2.5 volumes of 100% ethanol were added at 0°C. The solution was mixed by vortexing, transferred to a -20°C freezer for 15 min, then placed in dry ice for 20 min or longer. The tube was held at 2°C until the contents were completely thawed and then spun in an Eppendorf microcentrifuge at 15,000 rpm, 15 min, 2°C. The supernatant was removed, residual liquid removed by brief lyophilization and the pellet resuspended in 5.6 µl of water.

RNA Blots

RNA samples were treated with formaldehyde, fractionated by electrophoresis through agarose gels containing formaldehyde (Lehrach et al., 1977) and transferred to nitrocellulose, all as described by Maniatis et al. (1982) with the following modifications: 1X gel-running buffer was 20 mM morpholinopropane sulfonic acid pH 7, 5 mM sodium acetate, 1 mM EDTA, the gel contained 1.5% (w/v) agarose, the total reaction volume was 25 µl, after electrophoresis the gels were washed for 25 min in five changes of water then equilibrated in 20X SSPE (3.6 M NaCl, 0.2 M $NaPO_4$ pH 6, 0.02 M EDTA) before transfer to nitrocellulose (i.e., the alkaline hydrolysis and neutralization steps were omitted). Transfers were done in 20X SSPE. After transfer the filters were washed briefly in 2X SSPE, then placed on Whatman 3 MM filter paper and immediately transferred to a vacuum oven and baked for 2 hr or longer at 80°C.

RNA filters were pretreated, hybridized to ^{32}P-DNA probes, washed and autoradiographed as described by Alwine et al. (1980) with the following modifications: glycine was not included in the hybridization buffer for the pretreatment, the concentration of salmon sperm DNA was 100 µg/ml, after hybridization the filters were washed briefly in 50 ml of 1X SSPE, 0.1% SDS at room temperature then for 2 hr in two changes (225 ml each) of the same solution

at 42°C. The probes used were nick translated ^{32}P-DNA prepared as described by Rigby et al. (1977).

Purification and Hybridization of ^{3}H-RNA

Purification of pulse-labeled RNA from cultured salivary glands by CsCl centrifugation, hybridization of the RNA to an excess of nitrocellulose filter-bound DNA, and quantitation of the hybridized RNA were performed as described by Crowley and Meyerowitz (1984).

Chromosome Spreads for Puff Measurements

Three salivary gland lobes were transferred to a 20 µl drop of aceto-lactic orcein stain solution (2% [w/v] orcein in 1:1 lactic:acetic acid) on a siliconized coverslip. One minute later the glands were squashed between the coverslip and a glass slide. The stained chromosomes were photographed using a Zeiss photomicroscope and the diameter of the 68C and 69A1-3 regions were measured from the photographs. Puff size is expressed as a ratio of the diameter of the chromosome at 68C to the diameter at 69A1-3.

Transcription Autoradiograms

A pair of salivary gland lobes was pulse-labeled for 10 min in a 5 µl drop of culture medium containing 50 µCi/ml ^{3}H-uridine, washed briefly in 5 µl of culture medium with no radioactivity, then transferred to a 20 µl drop of aceto-lactic orcein stain on a siliconized coverslip. Two minutes later the glands were squashed between the coverslip and a glass slide and the stained chromosomes were photographed.

The slide was frozen on dry ice, the coverslip pried off with a razor blade and the slide immediately immersed in 100% ethanol. The slide was washed in two more changes of 100% ethanol, air-dried, washed in 5% trichloroacetic acid for 15 min at 0°C to remove unincorporated uridine, then dehydrated in 70% ethanol for 10 min, followed by 100% ethanol for 5 min. After air-drying the slide was dipped in Kodak NTB-2 nuclear track emulsion at 45°C under a red safelight then kept in a light-tight box at 4°C until ready to be developed. After an 85 hr exposure the slide was developed for 2.5 min in Kodak D-19 developer (under a red safelight), washed for 20 sec in water, fixed for 5 min in Kodak rapid fixer, washed in five changes of water, air-dried and mounted with a coverslip using Permount (Fisher Scientific Co.).

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Table 1. Relative Diameter of the 68C Puff in Cultured Salivary Glands

	l(1)npr-1	Binsn
- Ecdysterone	1.9 ± 0.05	2.0 ± 0.07
+ Ecdysterone	2.0 ± 0.07	1.3 ± 0.03

Salivary glands from mid-third instar larvae were cultured in the absence of ecdysterone (- Ecdysterone) or in the presence of 10^{-5} M ecdysterone (+ Ecdysterone) for 2 hr at 22°C prior to staining and squashing (see Experimental Procedures). Each number is the mean of at least five measurements (ratio of 68C diameter to 69A1-3 diameter). Standard error is indicated.

Table 2. Abundance of Specific RNAs in Third Instar Salivary Glands

RNA	Abundance of Specific Transcript	
	l(1)npr-1	Binsn
Sgs-3	-	++
Sgs-7	-	++
Sgs-8	-	++
18S + 26S rRNA	+++	+++

+++ = Very abundant

++ = Abundant

- = Undetectable

Abundances were determined by autoradiography of RNA gel-blot filters as described in Experimental Procedures.

Table 3. Incorporation of ^{3}H-uridine into Salivary Gland RNAs

	l(1)npr-1				Binsn			
Experiment	Total	Sgs-3	Sgs-7	Sgs-8	Total	Sgs-3	Sgs-7	Sgs-8
1	1.2×10^{5}	1	1	6	9.4×10^{4}	360	72	65
2	5.9×10^{4}	0	1	5	3.3×10^{4}	197	42	38

The number of cpm incorporated into total salivary gland RNA or each of the 68C RNAs during a 15 min pulse with ^{3}H-uridine was determined as described in Experimental Procedures. The numbers shown are the cpm above background. Background was 20 cpm.

Table 4. Number of Grains Over the 68C Puff in Transcription Autoradiograms

	l(1)npr-1	Binsn
Experiment 1	1	12
Experiment 2	1	12
Experiment 3	1	11
Mean	1	12

Salivary glands were incubated in ^{3}H-uridine for 10 min, stained, squashed and autoradiographed as described in Experimental Procedures. After an 85 hr exposure the autoradiographic emulsion was developed and silver grains counted. By counting grains over a region of the slide with no chromosomes we determined that the background level of grains over a region the area of the 68C puff in l(1)npr-1 larvae is 0.24.

Figure Legends

Figure 1. 68C Glue RNAs in Third Instar Salivary Glands.

RNA from salivary glands of male larvae hemizygous for l(1)npr-1 (npr), male larvae hemizygous for the Binsn X chromosome, and male wild-type Oregon R 16f (OR) larvae was fractionated by electrophoresis in a formaldehyde agarose gel, then transferred to a nitrocellulose filter as described in Experimental Procedures. Ten gland lobes were used for each RNA preparation. The filter was hybridized with the ^{32}P-labeled insert of clone aDm2023 (a 2.4 kb fragment originally derived from λbDm2002, homologous with the Sgs-3 transcript), the cDNA insert of adm127C8 (homologous with the Sgs-7 transcript) or the cDNA insert of adm109F4 (homologous with the Sgs-8 transcript). These clones are described in detail by Meyerowitz and Hogness (1982) and by Garfinkel, Pruitt and Meyerowitz (1983). To remove one probe before hybridizing the next, the filter was given two 5 min washes in 0.01X SSPE at 100°C. Exposure times were as follows: 30 min for the Sgs-3 probe, 30 min for the Sgs-7 probe and 34 min for the Sgs-8 probe. The signals in the Binsn and OR lanes were detectable after 15-17 min exposures, while no signals were detected in the npr lane even after much longer exposures (i.e., 62 hr for Sgs-3, 118 hr for Sgs-7 and 52 hr for Sgs-8). Hybridization of the filter with a labeled probe homologous with ribosomal RNA showed that each lane has approximately the same amount of total RNA. The RNA lengths are shown on the right in nucleotides.

Figure 2. Expression of Endogenous and Reintroduced Sgs-3 Genes in Third Instar Salivary Glands.

RNA was isolated from salivary glands of male larvae of the genetic constitution l(1)npr-1/Y; Tf(3)GA6.0-1, Sgs-3^{HR}/Sgs-3^{OR} and Binsn/Y; Tf(3)GA6.0-1, Sgs-3^{HR}/Sgs-3^{OR}. These males contain, in addition to the indicated Sgs-3 alleles at 68C, an Sgs-3^{OR} allele introduced to region 66E of the third chromososme by P factor-mediated transformation. This insertion is designated Tf(3)GA6.0-1. The introduced gene is on a 6 kb fragment, surrounded by non-68C sequences. For comparison RNA was extracted from males of the original l(1)npr-1/Binsn stock as well; these larvae are of genotype l(1)npr-1/Y or Binsn/Y, with the Sgs-3 allele characteristic of this stock on the third chromosome. This Sgs-3 allele produces an RNA of size equal to that of the Sgs-3^{OR} allele. These RNA samples were fractionated by electrophoresis through a formaldehyde-agarose gel, then transferred to a nitrocellulose filter. Ten salivary gland lobes were used for each RNA preparation. The filter was probed with the ^{32}P-labeled Drosophila insert of clone aDm2023, which contains the Sgs-3^{OR} gene, then exposed to film for 3 hr. The RNA samples from the Tf(3)GA6.0-1-containing larvae (Transformed Stock) are in the first two lanes, with no Sgs-3 RNA detectable from either the endogenous or the introduced genes when in a l(1)npr-1 background, but with both Sgs-3^{OR} and Sgs-3^{HR} RNAs produced when the same third chromosomes are in a background with a wild-type l(1)npr-1 locus. The third and fourth lanes show the parallel results from the original l(1)npr-1/Binsn stock. The RNA sizes are indicated in nucleotides.

Figure 3. RNA Synthesis at 68C in Mutant and Control Salivary Glands.

Salivary glands from mid-third instar male larvae carrying the l(1)npr-1 X chromosome (A,B and C) or the control Binsn X chromosome (D,E and F) were pulse-labeled with ^{3}H uridine for 10 min, stained, spread and exposed to an autoradiographic emulsion as described in Experimental Procedures. Exposure time was 85 hr. The arrow in each frame indicates the 68C puff.

Figure 1.

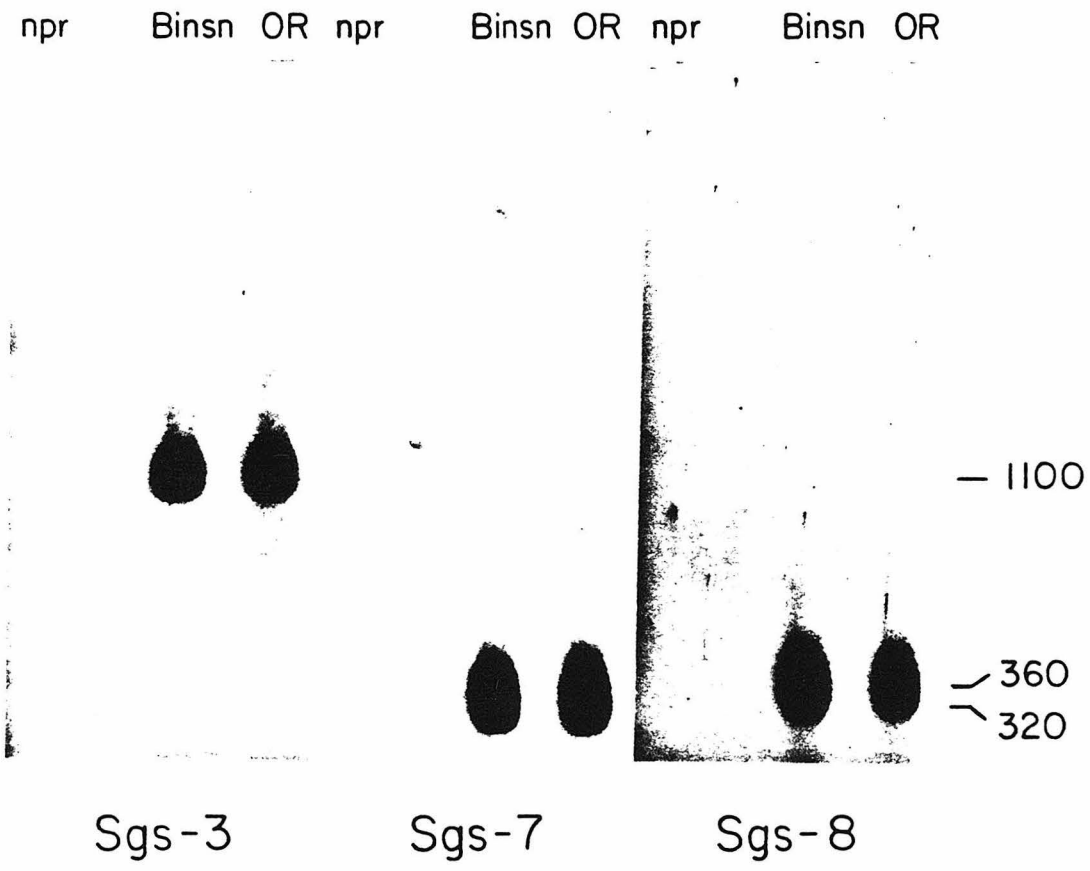

npr
Binsn OR
npr
Binsn OR
npr
Binsn OR
1100
360
320
Sgs-3
Sgs-7
Sgs-8

Figure 2.

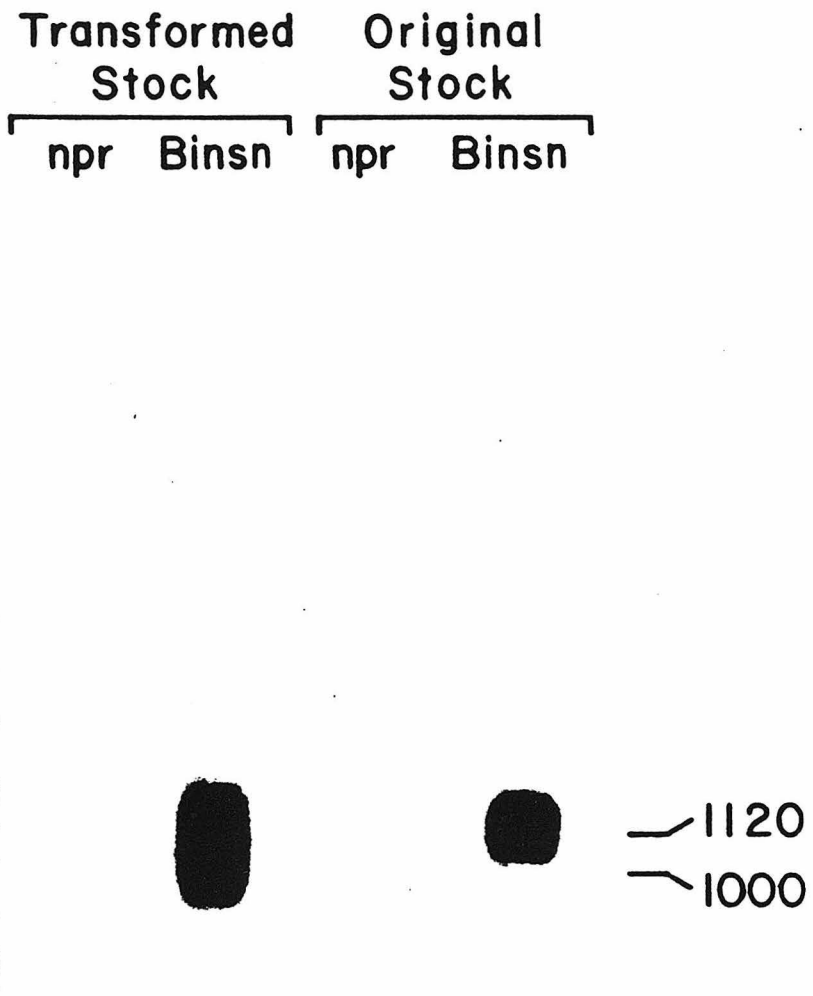
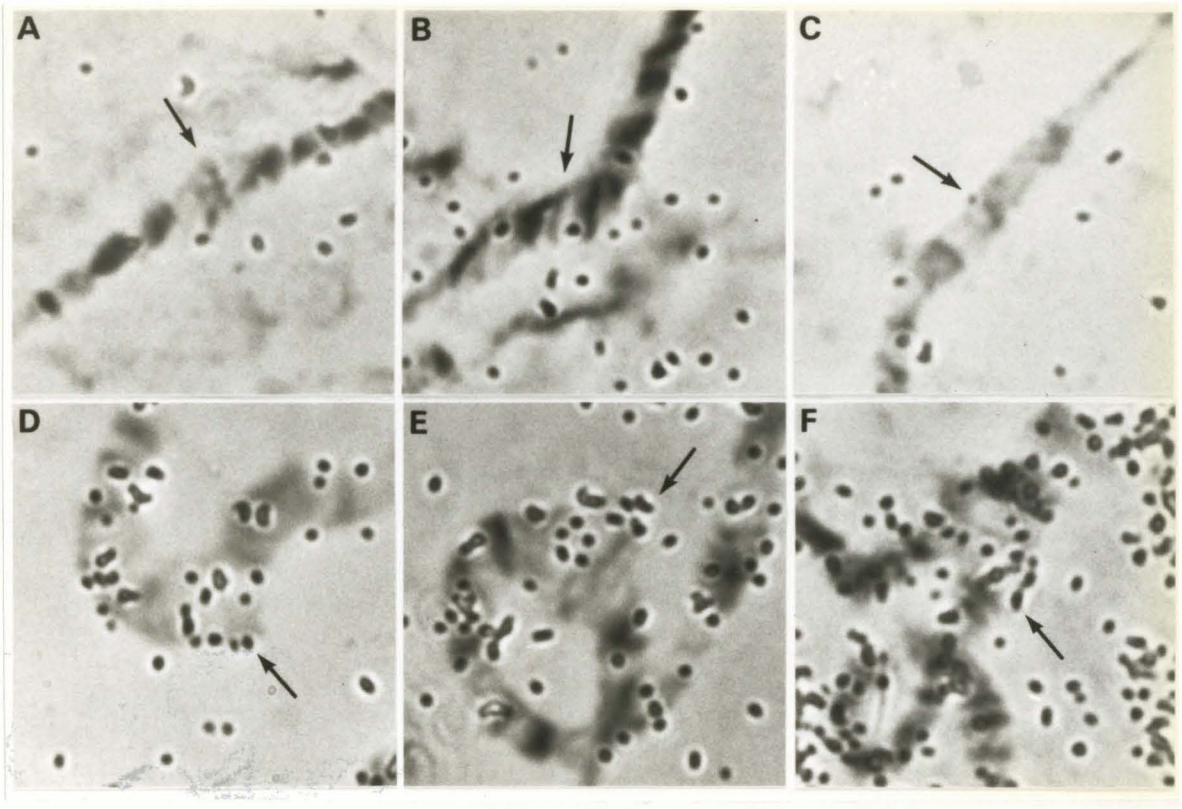

Transformed Stock
Original Stock
npr
Binsn
npr
Binsn
1120
1000

Figure 3.



CHAPTER 5

Conclusion

We have shown that the three abundant RNAs transcribed from the *Drosophila melanogaster* 68C puff are messengers for salivary glue proteins. These mRNAs are regulated by the steroid hormone ecdysterone and a transcription factor which is coded by or induced by a gene at position 2B on the X chromosome. Also, we have demonstrated that the puff at position 68C on the left arm of the third chromosome is not created by synthesis of glue RNAs or by synthesis of any other RNAs.

The proteins coded by the 68C RNAs along with several other proteins coded at other loci comprise the salivary gland glue. Some of these polypeptides, including the 68C protein sgs-3, are heavily glycosylated. It is likely that the carbohydrate which is attached to these proteins gives the secretion its stickiness (Fraenkel and Brookes, 1953; Beckendorf and Kafatos, 1976; see Chapter 2). The synthesis of the 68C glue proteins is correlated with the presence of the corresponding messenger RNAs indicating that expression of the 68C glue genes is controlled by regulation of mRNA accumulation and not by regulation of translation of the messengers (Beckendorf and Kafatos, 1976; see Chapters 2 and 3).

The molting hormone ecdysterone plays a key role in regulation of the 68C glue RNAs. The concentration of this steroid in the salivary glands increases slightly at the beginning of the third larval instar then drops off to the basal level until the end of the third instar at which time the concentration increases again. The second rise in hormone titer is much larger than the first increase (Berreur *et al.*, 1979; Maróy *et al.*, 1980). Hansson and Lambertsson (1983) have shown that the first rise in ecdysterone titer is at least partially responsible for induction of 68C glue RNA accumulation. We have demonstrated that the second increase in hormone concentration causes a sharp reduction in the rates of transcription of the *Sgs-3*, *Sgs-7* and *Sgs-8* genes.

At least one factor other than ecdysterone is necessary for transcription of the 68C glue genes. A gene located at position 2B on the X chromosome produces

or induces production of a *trans*-acting regulatory element which is involved in activating transcription at 68C. A mutation in this gene l(1)npr-1 prevents expression of *Sgs*-3, *Sgs*-7 and *Sgs*-8, and also prevents the hormone mediated regression of the 68C puff. Since both of these processes involve ecdysterone it is possible that the hormone receptor is coded at 2B and that the mutation prevents production of a functional receptor. Activation of *Sgs*-3 transcription by this *trans*-acting element requires at most 6 kb of 68C DNA. Contrary to the conclusions of others we find that the puff at 68C is not created by a high rate of transcription of the puff DNA, but that it can exist in the absence of detectable transcription in the 68C vicinity.

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