REGULATION OF THE DROSOPHILA GLUE GENE *SGS-3*: SEQUENCES REQUIRED FOR PUFFING AND TRANSCRIPTION

Thesis by

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This thesis is dedicated to Ed Lewis, whose enthusiasm helped get me hooked, whose confidence in me helped give me confidence in myself, and whose support has helped ease the way many times.

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Abstract

The 68C intermolt puff of *Drosophila melanogaster* contains a cluster of three glue protein genes, *Sgs-3*, *Sgs-7*, and *Sgs-8*. Analyses of chromosomal rearrangments which break near the glue gene cluster show that a region of no more than 20 kilobase-pairs (kb) is required for expression of the genes and for formation of the 68C puff. This result is supported by P-element-mediated-transformation experiments in which defined segments of the 68C region are introduced back into the fly genome. Based on the criteria of correct tissue- and stage-specific expression, transcription of an RNA of appropriate size and abundance, and production of an sgs-3 protein, the correctly regulated expression of the *Sgs-3* gene requires less than 3.4 kb of total flanking sequences, approximately 2.3 kb 5' and 1.1 kb 3'. When upstream sequences are truncated at 130 base-pairs, low levels of *Sgs-3* expression are observed in some cases, with normal tissue- and stage-specific expression retained. Formation of a new intermolt puff at the site of insertion is observed for transformants in which the introduced DNA contains all three 68C glue genes, but not for those which contain only an introduced *Sgs-3* gene, even for cases in which the gene is abundantly expressed.

An attempt was made to recover lethal mutations in genes closely flanking the 68C glue protein genes by screening mutagenized chromosomes over large deficiencies which delete the 68AC region. Although a large number of lethal and semi-lethal mutations were recovered, including many which define new complementation groups, none maps close to the region of the 68C glue gene cluster.

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CHAPTER 1

Introduction: Development and Function of the Larval Salivary Glands in *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* is a holometabolous insect, meaning that it has distinct larval and adult stages, the transition from larva to adult occurring by complete metamorphosis during an immobile pupal stage. The major activity of the larva is feeding, and growth during this period is quite spectacular: in the course of four days (at 25°C) the larva increases in size over 100-fold. This rapid growth is accomplished primarily by an increase in cell size rather than in cell number. In many larval tissues DNA replication continues to occur, but the truncated mitotic cycle bypasses the segregation of replicated chromosomes, nuclear division, and cytokinesis. The resulting cells are highly polyploid. In most cases, the multiple copies of each chromosome remain in precise association along their entire length, resulting in a special type of polyploidy termed polyteny. In addition, homologous pairs of these huge chromosomes exhibit somatic pairing; thus, in polytene tissues there appears to be only half the normal complement of chromosomes (Bodenstein, 1950; Ransom, 1982).

The larval salivary glands are paired sac-like organs which are connected at their anterior ends by a common duct that opens into the pharynx. Determination of the larval salivary gland cells appears to occur early in embryogenesis (Hathaway and Selman, 1961) and by halfway through embryogenesis the cells have reached their maximum number. In a cluster near the duct of the gland are the imaginal cells of the adult salivary gland, which undergo further cell division during the larval stage. It is not known whether the salivary glands serve any function during the embryonic and early larval stages (Sonnenblick, 1950; Berendes and Ashburner, 1978).

During the larval stages the glands increase rapidly in size, relative to the size of the larva as well as absolutely. In the first instar larva they are usually confined to the first two thoracic segments; by the end of the third larval instar they extend into the second abdominal segment (Ross, 1939). This rapid growth is

accomplished solely by an increase in cell size and is accompanied by a dramatic increase in ploidy. The salivary gland cells are the most highly polytenized in the larva, up to 2048C in the posterior cells of the gland during the late third larval instar. Since there is a gradient in the degree of polytenization from the anterior to the posterior cells, the average salivary gland chromosome is present in about 1000 copies per cell in the late third instar larva (Lane et al., 1972; Berendes and Ashburner, 1978).

The function of the glands during the third larval instar is well established. They produce a mucoprotein "glue" which is expelled at the end of the instar and serves to anchor the newly forming puparium to the surface upon which it rests (Fraenkel and Brookes, 1953). The glue is composed of about 30% sugars and stains heavily with periodic acid-Schiff reagent. At about the middle of the third instar PAS-positive granules appear in the cytoplasm of the posterior gland cells. Toward the end of the third instar (in the laboratory, a few hours after the animals have left the food to climb up the sides of the bottle), the PAS-positive substance is secreted into the lumen of the glands, and shortly after, at puparium formation, is expelled (Lesher, 1952; Zhimulev and Kolesnikov, 1975; Berendes and Ashburner, 1978).

The glands continue to show a complex and changing pattern of protein synthesis and gene activity (inferred from puff activity) during the 12-hour period between puparium formation and pupation (Ashburner, 1967; Tissieres et al., 1974; Zhimulev et al., 1981; Sarmiento and Mitchell, 1982). However, their function during this period is again unknown. At this time highly vacuolated structures assumed to be lysosomes appear in the cytoplasm of the gland cells. Like many other larval tissues the glands are completely histolyzed during the prepupal and early pupal stages (Ross, 1939; Lane et al., 1972).

The polytene chromosomes of Dipteran larval salivary glands and other tissues have been the subject of intense study. The first observation of such polytene chromosomes was made by Balbiani (1881). It was more than 50 years before the utility of these huge and somatically paired chromosomes for genetic analysis was recognized (Painter, 1933, 1934). In the jubilant words of Theophilus Painter,

> . . . it was clear that we had within our grasp the material of which everyone had been dreaming. We found ourselves out of the woods and upon a plainly marked highway and . . . it was clear that the highway led to the lair of the gene (Painter, 1934).

The distinctive banding pattern exhibited by the chromosomes is very similar, although not identical, in all stages and tissues within a particular species (Beermann, 1950; Pavan and Breuer, 1952; Holden and Ashburner, 1978; see Ribbert, 1979, for a possible exception; for discussion see Zhimulev and Belyaeva, 1975a, and Richards, 1983). There are less subtle differences in the pattern of puffs observed. Puffs are regions of local chromatin decondensation producing small areas of the chromosome which have increased diameter and indistinct banding pattern. One of the most conspicuous features of polytene chromosomes, puffs have been shown to be sites of highly active transcription (Pelling, 1964; Zhimulev and Belyaeva, 1975b; Belyaeva and Zhimulev, 1976). The pattern of puffs visible is dependent upon the tissue in which and the stage at which they are observed (Ashburner and Berendes, 1978; Richards, 1980). A distinctive set of puffs, the heat shock puffs, appears under certain conditions of environmental stress (Ashburner and Berendes, 1978; Ashburner and Bonner, 1979; Petersen and Mitchell, 1983).

In the larval salivary glands the pattern of puffs on the polytene chromosomes can be analyzed from the third instar through the prepupal stage. During this time the number and location of puffs change rapidly. A system devised by Becker (1959) and extended by Ashburner (1967,1969; see also

Zhimulev, 1974) describes 21 different puff stages (PS) at which characteristic sets of puffs are observed. PS1 is observed in mid to late third instar larvae still in the food; animals starting to climb out of the food are in PS2, which is initiated by an increase in the insect hormone ecdysterone; secretion of the salivary gland glue into the lumen occurs over several puff stages after PS4; and puparium formation occurs at PS10/11.

A number of the salivary gland puffs are responsive to the insect steroid hormone, ecdysterone (which is discussed below). On the basis of observations on salivary glands *in vivo* and experiments in which salivary glands were cultured *in vitro*, Ashburner (1967; 1973) classified these puffs into four groups. The first group is the intermolt puffs, which are active before exposure to ecdysterone and are repressed by an increase in ecdysterone titer. The intermolt puffs are observed during PS1. The second group are the early puffs which appear rapidly, within minutes, after exposure to ecdysterone, respond quantitatively over a range of hormone concentrations, require constant exposure to ecdysterone to remain puffed, and which regress after about four hours. The early puffs first appear at PS2 and regress at PS7 or 8. There are two groups of late puffs which appear at PS5 or later in a delayed response to increased ecdysone titer. Both late groups fail to appear if RNA or protein synthesis is inhibited during the period of exposure to ecdysterone; they differ in that the earlier group requires a constant high level of ecdysterone to maintain the puff for the normal duration, and the later group does not.

Molecular and genetic experiments have established that the intermolt puffs are the sites of the genes which encode the polypeptides of the salivary gland glue (Korge, 1975, 1977; Akam et al., 1978; Muskavitch and Hogness, 1980; Velissariou and Ashburner, 1980, 1981; Crowley et al., 1983; Guild and Shore, 1984). These genes are abundantly transcribed in mid to late third instar larvae.

It has been suggested (Poel, 1972; Boyd and Ashburner, 1977) that the early puffs may encode substances which are required for the initiation of the process of glue protein secretion into the lumen of the gland. With the exception of the heat shock puffs, the functions of the other 100-200 puffs observed at various times are not known.

Many of the developmental events in the larval salivary glands are regulated by the insect steroid hormone ecdysterone. Insect development is regulated by three major classes of hormones: ecdysteroids, ecdysiotropins, and juvenile hormones (for reviews see Doane, 1973; Gilbert, 1980; Richards, 1981). Classical studies have shown that the process of molting, the transition from one stage to the next during which the cuticle is shed, requires a diffusible substance originating in the anterior of the animal which was termed molting hormone. The last step in the process of molting is ecdysis (shedding of the cuticle) and this substance was later named ecdysone. It is now known that there is a whole family of related substances which are called ecdysteroids. β-ecdysone (20-OH ecdysone) is believed to be the most active form in most insect tissues, whereas the primary function of α-ecdysone appears to be as the precursor for the β-form. As used here, "ecdysone" refers to either α-ecdysone or β-ecdysone. "Ecdysterone" refers only to β-ecdysone. In the larva, ecdysone is produced by the prothoracic gland cells of the ring gland, which is located behind the brain. The prothoracic gland is stimulated to produce ecdysone by a hormone released from the brain, ecdysiotropin or prothoracicotropic hormone. The action of a third type of hormone, juvenile hormone, produced by the corpus allatum, plays a role in determining the type of molt that occurs, larval to larval, larval to pupal, or pupal to adult. This classical model is a based on experiments using many different species of insect and is a useful but oversimplified generalization. Ecdysone affects many of the developmental processes in *Drosophila*

melanogaster, sometimes in opposite directions at different times during development (Hansson and Lambertsson, 1983). How one hormone can serve so many different functions is one the most intriguing aspects of ecdysone research. In some cases, for example during embryogenesis, different ecdysteroids may be involved (Kaplanis et al., 1973; Ohnishi et al., 1977). However, most larval and pupal functions appear to be controlled by 20-OH ecdysone (ecdysterone or β-ecdysone; Borst et al., 1974). Changes in the ecdysone titer in whole animals or hemolymph have been carefully assayed in third instar larvae and later stages (Hodgetts et al., 1977; Berreur et al., 1979; Handler, 1982). There is a small peak during mid third instar, followed by a dramatic rise at the end of the instar. This increase peaks slightly after puparium formation and then the hormone titer drops precipitously prior to pupation. There is a slight surge after pupation, then a steep climb to reach maximal levels during the mid pupal period. Low titers are found in adults.

Mutants which have low levels of ecdysone are unable to progress beyond the third larval instar (for example, *l(3)ecd-1*^{ts}, Garen et al., 1977, Berreur et al., 1984; *l(3)3*^{DTS}, Holden and Ashburner, 1978; *l(1)npr-1*, Kiss et al., 1978; *l(1)grg* and *l(1)su(f)*$^{mad-ts}$, Klose et al., 1980; and *l(1)su(f)*^{ts67g}, Hansson and Lambertson, 1983). Some of these mutants are rescued by an exogenous supply of ecdysterone; others are not. Many of the processes involved in puparium formation and pupation appear to be regulated by changes in ecdysone titer. These include production of salivary gland glues, secretion of the glue, tanning of the pupal and adult cuticle, and eversion of the imaginal discs (Boyd and Ashburner, 1977; Seligman et al., 1977; Kraminsky et al., 1980; Fristrom and Yund, 1980; Crowley and Meyerowitz, 1984). In adult females ecdysone appears to control the production of yolk proteins by the fat body (Jowett and Postlethwait, 1980; Bownes et al., 1983).

Much of the work on ecdysone and the ecdysone receptor in *D. melanogaster* has been done in cell culture (Maroy et al., 1980; Sage et al., 1982; Bonner, 1982; Berger and Morganelli, 1984). Theories on the hormone's action are based to some extent on models of vertebrate steroid hormone action. According to one currently accepted theory, the hormone enters the cell passively and interacts with a receptor protein, then the receptor-ecdysone complex is rapidly transported to the nucleus where it binds to specific DNA sequences, thereby regulating transcription. Pong's group has shown that ecdysone becomes localized to specific regions on the larval salivary gland polytene chromosomes; among those regions are sites of the intermolt puffs, which regress in the presence of ecdysone (Gronemeyer and Pongs, 1980; Dworniczak et al., 1983).

The molecular characterization of a number of gene systems which exhibit a response to ecdysone in *D. melanogaster* is currently in progress. These include larval serum proteins (Smith et al., 1981), dopadecarboxylase (Gilbert el al., 1984; Marsh et al., 1985), the ecdysone-inducible polypeptides found in cell culture (Savakis et al., 1984); the salivary gland glue proteins (Muskavitch and Hogness, 1982; Garfinkel et al., 1983; McGinnis et al., 1983; Guild and Shore, 1984; Bourouis and Richards, 1985), and the products of the early puffs (Moritz et al., 1984). These studies may uncover important clues relating to the ecdysone-mediated control of these genes. The work described in this thesis contributes to analysis of the glue genes located at the 68C intermolt puff, defining the sequences required in cis for the normal function of the *Sgs-3* glue protein gene and for the formation of an intermolt puff.

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CHAPTER 2

Regulation of the Drosophila Glue Gene *Sgs-3*: Sequences Required for Puffing and Transcription

Summary

The 68C intermolt puff of *Drosophila melanogaster* contains a cluster of three glue gene protein genes, *Sgs-3*, *Sgs-7*, and *Sgs-8*. By analysis of chromosomal rearrangements which break near the glue gene cluster, we have established that a region of no more than 20 kb is required for expression of the glue genes and for formation of the 68C puff. Using P-element-mediated transformation, we have introduced defined segments of the 68C region into the fly genome and assayed the expression of the *Sgs-3* gene. Based on the criteria of correct tissue- and stage-specific expression, transcription of an RNA of appropriate size and abundance, and production of an sgs-3 protein, the correctly regulated expression of the *Sgs-3* gene requires less than 3.4 kb of total flanking sequences, approximately 2.3 kb 5' and 1.1 kb 3'. Formation of a new intermolt puff at the site of insertion is not observed for all transformants which produce high levels of *Sgs-3* RNA. Only for transformants in which the introduced DNA from 68C also contains the *Sgs-7* and *Sgs-8* genes is a new intermolt puff observed at the chromosomal location of the insert.

Introduction

The fruit fly *Drosophila melanogaster* begins life as a fertilized egg, passes through three larval instars prior to pupation, then undergoes metamorphosis during a pupal stage, and emerges as a mature adult approximately nine days after hatching from the egg. During the second half of the last larval instar the cells of the highly specialized larval salivary glands begin to produce copious amounts of eight to nine small proteins. Toward the end of the third instar these polypeptides are secreted into the lumen of the gland, and at the time of puparium formation are expelled through the salivary gland duct as part of a mucoprotein glue that

serves to anchor the puparium to the surface upon which it rests (Fraenkel and Brookes, 1953; Korge, 1975, 1977a; Beckendorf and Kafatos, 1976).

The larval salivary glands are one of a number of larval tissues that are highly polyploid. By the end of the third larval instar each chromosome is present in over 1000 copies, all of which remain tightly associated and in register, forming huge polytene chromosomes. These giant chromosomes have a distinctive banding pattern which is virtually identical in all tissues containing polytene chromosomes. There are small regions along the chromosomes that are transiently wider in diameter and have a diffuse banding pattern. These regions are called puffs and have been shown to be sites of highly active transcription (Pelling, 1964; Zhimulev and Belyaeva, 1975; Belyaeva and Zhimulev, 1976). Some puffs appear and regress at precise developmental timepoints and others are responsive to environmental stimuli (reviewed in Ashburner and Berendes, 1978). A number of prominent puffs, called the intermolt puffs, are visible on the polytene chromosomes of the salivary glands during the period of glue protein production. Genetic and molecular experiments have shown that these puffs contain the structural genes for the polypeptides that constitute the salivary gland glue (Korge, 1975, 1977b; Akam et al., 1978; Muskavitch and Hogness, 1980; Velissariou and Ashburner, 1980, 1981; Crowley et al., 1983; Guild and Shore, 1984). On the basis of genetic experiments, the 68C puff on the left arm of the third chromosome has been identified as the site of the *Sgs-3* glue gene locus (Akam et al., 1978). Molecular cloning and analysis of DNA from the 68C region have revealed three closely linked genes which encode the glue polypeptides sgs-3, sgs-7, and sgs-8 (Meyerowitz and Hogness, 1982; Crowley et al., 1983). The DNA sequence and the boundaries of the transcription units have been determined for an approximately 7 kb region which includes the glue gene cluster (Garfinkel et al., 1983).

The regression of the intermolt puffs, which occurs several hours before puparium formation, coincides with an increase in the hemolymph titer of the insect steroid hormone ecdysterone (Becker, 1962). In experiments on salivary glands cultured *in vitro*, exogenously applied ecdysterone causes rapid regression of the intermolt puffs (Ashburner, 1973; Ashburner and Richards, 1976). Puff regression due to increased ecdysterone titer is still observed when cyclohexamide is present in the medium, indicating that the hormone is not acting indirectly by induction of a regulatory protein (Ashburner, 1974). Crowley and Meyerowitz (1984) have demonstrated that the effect of ecdysterone on the behavior of the 68C puff is correlated with the response of the 68C glue gene mRNAs to the hormone. *In vitro*, the levels of the 68C glue gene transcripts decline more rapidly when cultured in the presence of physiological concentrations of ecdysterone. This decline is due, at least in part, to a reduction in the rate at which newly synthesized 68C transcripts accumulate.

There is evidence, however, that ecdysterone is also required for the transcription of the glue protein genes. A small peak in ecdysterone titer has been observed at early-to-mid-third instar, at the time transcription of the glue genes is first detected (Berreur et al., 1979). Using animals with a temperature-sensitive mutation that fail to show the normal developmental increases in ecdysterone titer at restrictive temperatures, Hansson and Lambertson (1983) have observed that when shifted to restrictive temperature prior to third instar such animals fail to produce detectable levels of mRNA from the four glue genes tested (*Sgs-3*, *Sgs-4*, *Sgs-7*, and *Sgs-8*). If the animals are fed ecdysterone after being shifted to the restrictive temperature, transcription from the glue genes is observed. Glue gene transcription also occurs if the animals are not shifted to the higher temperature until a few hours into the third larval instar.

In the experiments presented here, we have begun to define the extent of the region surrounding the *Sgs-3* gene that is necessary for its regulated expression and for formation of an intermolt puff. First, by analysis of chromosomal rearrangements that break in or near 68C5-6, we establish that a region of approximately 20 kb is sufficient for expression of *Sgs-3* and probably *Sgs-7* and *Sgs-8* as well. This 20-kb region also contains all of the sequences required for puff formation. Using P-element-mediated transformation (Spradling and Rubin, 1982; Rubin and Spradling, 1982), we determine more precisely that 2.3 kb 5' of the *Sgs-3* gene and 1.1 kb 3' are sufficient for abundant expression of an appropriately regulated transcript. For transformed lines in which the introduced DNA includes all three genes of the 68C cluster, a new intermolt puff is observed at the site of insertion. If only *Sgs-3*, the largest of the 68C glue genes, is contained within the introduced DNA no new puff is observed, despite the fact that the introduced *Sgs-3* gene is abundantly expressed in an appropriate tissue- and stage-specific manner.

Results

Analysis of Chromosomal Rearrangements that Break in the Region of the 68C Glue Gene Cluster

Several known rearrangements break in or near 68C5-6; specifically, *In(3L)HR15* (Ashburner, 1972b) and *Df(3L)vin*^{3} (Akam et al., 1978) were found to have breakpoints close to the 68C glue gene cluster. The positions of these breakpoints on a restriction map of the region were determined by observing anomalies in genome blot patterns, using labeled cloned DNA probes corresponding to different segments of the wild-type 68C5-6 region. Figure 1 shows a restriction map of the region surrounding the 68C glue gene cluster and the recombinant clones used in all of the following experiments. DNA isolated from

adult flies was cut with restriction endonucleases, size-fractionated on an agarose gel, blotted to nitrocellulose, and hybridized with ^{32}P-labeled probes. When hybridized with λaDm1501-10, EcoRI-cut DNA from homozygous *In(3L)HR15* exhibits a change in the leftmost 4-kb EcoRI fragment shown in Figure 1; all other EcoRI restriction fragments are identical to those of wildtype. The location of the breakpoint was verified by *in situ* hybridization to salivary gland polytene chromosome spreads. A tritium-labeled recombinant clone, λcDm2021, containing a 15.6-kb insert from the left half of the region (see Figure 1), was hybridized to salivary gland squashes of homozygous *In(3L)HR15*. Signal was detected at both 68C and 64D, showing that λcDm2021 spans the proximal breakpoint of *In(3L)HR15*. For *Df(3L)vin*^{3}*/TM3*, also cut with EcoRI, new bands appear upon hybridization with λbDm2033. Which specific band is affected is obscured by the background of bands from the *TM3* chromosome. A recombinant library of genomic fragments was constructed from *Df(3L)vin*^{3}*/TM3* adult DNA and a clone spanning the deficiency breakpoint, λbDm2054, was isolated. *In situ* hybridization with λbDm2054 to wild-type salivary gland polytene chromosomes shows signal at 68C and 68E, verifying that this clone contains the deficiency breakpoint. Analysis of λbDm2054 by restriction enzyme digestion indicates that the breakpoint of *Df(3L)vin*^{3} is within a SalI fragment less than 2 kb from the 3' end of the *Sgs-3* gene (see Figure 1).

To determine whether the expression of the glue genes is affected by either of these rearrangements, total salivary gland RNA was separated by electrophoresis on an agarose-formaldehyde gel, blotted to nitrocellulose, and hybridized with labeled DNA sequences homologous with each of the different glue genes. In the homozygous *In(3L)HR15* stock the expression of the three glue genes is unaffected by the rearrangement. Since *Df(3L)vin*^{3} is viable only when heterozygous, the RNA transcribed from the 68C5-6 region of this chromosome

cannot be distinguished from that produced by the homologous chromosome unless size variants or null mutations can be used. There are no known null mutations for any of the 68C glue genes, nor are there known size variants for *Sgs-7* or *Sgs-8*. For *Sgs-3*, however, there are several naturally occurring size variants. $Df(3L)vin^3$ was induced on a chromosome with the Oregon-R size variant, which is larger than the variants produced by the Hikone-R strain. An RNA blot of $Df(3L)vin^3/Sgs\text{-}3^{HR}$ shows that both homologues are producing abundant amounts of *Sgs-3* RNA.

A strain containing a 9.2-kb transposable element 750 bp distal to the glue gene cluster was previously analyzed for glue gene expression (Meyerowitz and Hogness, 1982; see Figure 1). The transposon, designated "roo," is found at this site within the 68C5-6 region in the Oregon-R strain used in our laboratory, but not at this location is most other D. melanogaster strains (including other Oregon-R strains). The three 68C glue genes are expressed normally in this strain.

The Sequences Necessary for Puff Formation are Located within a 20-kb Region

In order to determine whether the disruption of the 68C region caused by these rearrangements affects the formation of the 68C intermolt puff, for each rearrangement orcein squashes of salivary gland polytene chromosomes at puff stage 1-2 (Ashburner and Berendes, 1978) were made. The size of the puff at 68C5-6 was measured, using 69A1-3 as a reference band (Table 1). A normal-sized puff appears at the proximal end of the *HR15* inversion (Figure 2A), demonstrating that the region beyond 14 kb to the left (distal) of the glue gene cluster is not required in cis for puff formation. Starting within 2 kb to the right of the glue gene cluster, many hundreds of kilobases of DNA are missing entirely from $Df(3L)vin^3$, yet this chromosome also produces a normal 68C puff (Figure 2B). Salivary chromosome squashes of OR16f, the roo-containing strain, also show a typical 68C intermolt puff.

The rearranged chromosomes were also analyzed at a later stage, puff stage 7-9. In wild-type animals the 68C puff regresses after puff stage 2. For all three chromosomes at puff stage 7-9 the relative diameter of the chromosome at 68C5-6 has decreased when compared to puff stage 1-2 (Table 1). The "ballet skirt" (Lefevre, 1976) at 68C1-4 is present during all puff stages and contributes to the diameter of the chromosome in the 68BC region. In the case of *In(3L)HR15*, 68C1-4 has been separated from 68C5-6 and the large diameter observed at puff stage 1-2 is due entirely to the intermolt puff.

P-Element-Mediated Transformation of the *Sgs-3* Gene

To define more precisely the extent of the region necessary for the regulated expression of the 68C glue genes, P-element-mediated transformation (Spradling and Rubin, 1982; Rubin and Spradlin, 1982) was used. Of the three glue genes at 68C, the *Sgs-3* gene is most amenable to this approach since naturally occurring size variants are available. $Sgs\text{-}3^{OR}$ and $Sgs\text{-}3^{HR}$, size variants present in the Oregon-R strain and Hikone-R strain, respectively, produce distinguishable species of both mRNA and protein. The Hikone-R strain contains two tandem *Sgs-3* genes (see Appendix), each coding for mRNAs smaller than that of the Oregon-R gene. Since neither the presence nor absence of a glue gene confers a selectable phenotype, it was necessary to co-transform with a selectable gene, alcohol dehydrogenase (*Adh*).

The first transformation plasmids were constructed by inserting a 6.0-kb BglII restriction fragment from the 68C5-6 region into the P-element-Adh^+ plasmid pAP5 (Goldberg et al., 1983); plasmids containing the 68C DNA in both orientations were isolated. The 6.0 kb of 68C DNA contains the complete $Sgs\text{-}3^{OR}$ gene plus 2.3 kb of 5' and 2.5 kb of 3' flanking DNA (Figure 3). In the plasmid designated pGA6.0a the Adh^+ gene is located 6.5 kb upstream of the *Sgs-3* gene and is transcribed in the same direction; in pGA6.0b the Adh^+ gene is 6.7 kb

downstream of the *Sgs-3* gene and is convergently transcribed. These transformation plasmids were each mixed with the intact P element plasmid pπ25.1 (Spradling and Rubin, 1982; O'Hare and Rubin, 1983) and injected into Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$ embryos prior to pole cell formation. Survivors of the injection procedure (G0) were backcrossed singly to Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$ and adult offspring (G1) of these matings tested for ethanol resistance. One copy of Adh^{+} confers resistance to ethanol, which is lethal to ADH-null flies. Resistant G1 flies were again crossed singly to Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$ and stocks maintained by selection every other generation. A summary of the transformation procedure is shown in Figure 4.

Progeny (G2) of the first resistant G1 animals to be recovered were dissected at late third larval instar and the salivary glands removed. The carcass of each larva was subjected to a histochemical spot test to detect ADH activity (Grell et al., 1968). Total RNA was prepared from the salivary glands, fractionated on an agarose-formaldehyde gel, blotted to nitrocellulose and hybridized with a ^{32}P-labeled probe homologous with *Sgs-3* (aDm2023, see Figure 1). The RNA from the salivary glands of a single animal was run in each lane; samples from sibling animals which were scored as positive or negative for ADH activity were run in adjacent lanes. All G2 animals that showed ADH activity also expressed the introduced $Sgs\text{-}3^{OR}$ gene. In those animals that were negative for ADH activity only the $Sgs\text{-}3^{HR}$ mRNAs were present.

Two ethanol-resistant G1 animals were obtained in the first experiments using pGA6.0a. These were progeny of a single G0 animal and proved, on the basis of genomic blots and chromosomal localization by *in situ* hybridization, to be the result of a single insertion event. Thus, only one independent transformant line, designated *TfGA6.0-1*, was established. DNA was isolated from an ethanol-selected stock of *TfGA6.0-1*, cut with restriction endonucleases, size-fractionated

on an agarose gel, blotted to nitrocellulose, and hybridized with ^{32}P-labeled aDm2023 (see Figure 1) or pπ25.1. From these genomic blots it was determined that the inserted DNA is intact and also that the strain contains complete P elements acquired during the transformation procedure. Using the ethanol-resistant phenotype, the location of the insertion of *TfGA6.0* was determined by conventional mapping procedures (see Experimental Procedures). The *Adh*$^+$ phenotype mapped to the third chromosome; the transformant line was subsequently designated *Tf(3)GA6.0-1*. The chromosomal location was verified by *in situ* hybridization to salivary gland polytene chromosomes using an ^{35}S-labeled probe, aDm2023, which contains the *Sgs-3* gene. One site of hybridization in addition to 68C is observed, at 66E1-3 (Figure 5A). More grains are observed at 68C for two reasons: there is twice the sequence homology due to the duplication of the 68C glue gene cluster in the Hikone-R strain, and the animal used for the *in situ* preparation shown was probably heterozygous for the introduced *Sgs-3* gene.

Subsequently, additional P-element transformation experiments produced one more transformed line using pGA6.0a (*Tf(3)GA6.0-2*) and two independent lines using pGA6.0b (*Tf(1)GA6.0-3* and *Tf(3)GA6.0-4*; see Table 2). In these experiments if a single G0 animal produced more than one *Adh*$^+$ offspring, they were assumed to have resulted from the same insertion event and only one line derived from a single G1 animal was maintained. The chromosomal location indicated in the transformant designations were determined by genetic mapping of the *Adh*$^+$ phenotype. With the exception of *Tf(3)GA6.0-2*, which is inviable when homozygous, the transformed lines are maintained as homozygous stocks. *Tf(1)GA6.0-3* has been mapped by *in situ* hybridization to the cytological chromosome location 10D-F and *Tf(3)GA6.0-4* has been mapped to 76F-77A.

2.3 kb of 5' Flanking DNA Allow Production of Abundant Levels of the *Sgs-3* Transcript in the Correct Tissue and Stage

To determine whether the inserted copy of *Sgs-3* in the *TfGA6.0* transformants exhibits normal tissue and stage specificity, total RNA was isolated from whole second instar larvae, and from the salivary glands and the remaining carcasses of early, mid, and late third instar larvae, white prepupae, and tan prepupae from *Tf(3)GA6.0-1*. An RNA blot was made and hybridized with a ^{32}P-labeled *Sgs-3* gene probe, aDm2023 (Figure 6). In mid and late third instar salivary glands, where the original $Sgs\text{-}3^{HR}$ genes are abundantly transcribed, the introduced $Sgs\text{-}3^{OR}$ genes are also abundantly transcribed. In other stages and tissues neither gene is expressed at detectable levels. Therefore, the sequences present in pGA6.0, 2.3 kb 5' of the *Sgs-3* gene and 2.5 kb 3', include all the sequences required in cis for the production of *Sgs-3* RNA in the correct tissue and at the correct developmental stage. Since the 68C insert in *Tf(3)GA6.0-1* contains only the 3' portion of the *Sgs-7* gene and none of *Sgs-8*, an *Sgs-3* gene which is separated from the other 68C glue genes is still expressed normally.

Although the introduced *Sgs-3* genes in the *TfGA6.0* lines produce abundant levels of RNA, these levels are slightly reduced from those of the wild-type $Sgs\text{-}3^{OR}$ gene. The amount of RNA produced from the introduced gene was determined relative to the amount of RNA produced from the resident $Sgs\text{-}3^{HR}$ genes. RNA isolated from the salivary glands of individual third instar larvae was fractionated on an agarose-formaldehyde gel, blotted to nitrocellulose, and hybridized with a ^{32}P-labeled probe homologous with *Sgs-3*. After obtaining an autoradiogram to determine the positions of the $Sgs\text{-}3^{OR}$ and $Sgs\text{-}3^{HR}$ RNA species, the corresponding positions on the nitrocellulose filter were cut out, submerged in scintillation fluid, and the amount of hybridized radioactivity contained on each fragment of the filter determined. Measurements from

homozygous *TfGA6.0* larvae (N=4) were compared to animals of the genotype *Sgs-3*^{OR}/*Sgs-3*^{HR} (N=3). Corrections were made for the amount of background and for the differences in gene size and copy number. In animals that are heterozygous for *Sgs-3*^{OR} and *Sgs-3*^{HR}, RNA from the Oregon-R *Sgs-3* variant accumulates to a higher level per gene than does RNA from the Hikone-R variant. The *Sgs-3*^{OR} RNA is present at 1.3 times the level of the *Sgs-3*^{HR} RNA, when corrected for gene size and number. For *Tf(3)GA6.0-1* animals the amount of RNA from each of the introduced *Sgs-3*^{OR} genes is slightly lower, 0.90±0.05 (mean ± standard error of mean) times that from each of the resident *Sgs-3*^{HR} genes. This is 70% of the level of *Sgs-3*^{OR} RNA accumulation observed in animals of the genotype *Sgs-3*^{OR}/*Sgs-3*^{HR}. In *Tf(1)GA6.0-3*, RNA from the introduced *Sgs-3* gene accumulates to a level of 0.95 ± 0.06 times that from the resident *Sgs-3*^{HR} *genes, which is 75% of the level observed for the Sgs-3*^{OR} gene in heterozygous control animals.

The *Sgs-3* gene product is an unusual protein: it is composed of 42% threonine (Garfinkel et al., 1983; Crowley et al., 1983). This characteristic provides a simple assay for the sgs-3 protein. To determine whether the RNA transcribed from the *Sgs-3*^{OR} genes in *Tf(3)GA6.0-1* is processed and translated normally to produce sgs-3 protein, salivary glands were dissected from animals in late third larval instar and cultured in a medium containing ^{3}H-threonine. Proteins were extracted from the labeled glands, alkylated, and separated by electrophoresis on an acid-urea gel. The ^{3}H-threonine-labeled sgs-3 proteins were detected by fluorography (Figure 7). Two different sgs-3 proteins, which comigrate with the sgs-3 Oregon-R variant and the sgs-3 Hikone-R variant, are produced in approximately equal amounts in *Tf(3)GA6.0-1* salivary glands. These results indicate that the RNA transcribed from the introduced *Sgs-3*^{OR} gene

undergoes normal processing, including excision of the intron, and is correctly translated.

An Inserted Copy of *Sgs-3* which Produces Abundant Amounts of RNA Does Not Form a New Puff

Orcein squashes of salivary gland chromosomes of homozygous *Tf(3)GA6.0-1* larvae were prepared to analyze the new site of the *Sgs-3* gene. The 66E region was compared to the 68C intermolt puff and to the 66E region in orcein squashes of the original stock, Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$ (Figure 5B-C). There are no consistent differences between the transformant stock and the original Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$ stock. The diameter of the 66E1-3 bands was measured in reference to control bands at 66C1-4 at puff stage 1-2, the stage at which the intermolt puffs are normally observed. For both the transformant stock and the recipient stock this ratio is 1.49 ± 0.08 (mean ± the standard error of the mean; N=5 for each stock). Despite the fact that the introduced *Sgs-3* gene is abundantly transcribed, there is no intermolt puff detectable at the site of the insertion. A qualitative analysis of orcein salivary gland squashes using the other two homozygous *TfGA6.0* lines produced the same result. No puff is observed at the sites of insertion during puff stage 1-2.

1.1 kb of 3' Flanking DNA are Sufficient for Regulated and Abundant Expression

A second transformation plasmid, pGA8.1, was constructed using the P-element-Adh^{+} plasmid pAP2 (Goldberg et al., 1983). The 3.75-kb XhoI fragment from pAP2 was removed and an 8.1-kb SalI partial restriction fragment from the 68C5-6 region was inserted. The 68C recombinant clone from which pGA8.1 was derived is from the Oregon-R 16f strain, which contains the transposable element roo. The 8.1-kb fragment starts within the roo element, includes all three glue genes, and extends 1.1 kb 3' of the *Sgs-3* gene (Figure 3). The glue gene cluster is

downstream of the *Adh*$^+$ gene, with *Sgs-3* and *Sgs-7* transcribed in the same direction as the *Adh*$^+$ gene. The *Adh*$^+$ gene is 7.3 kb from *Sgs-3* and 3.9 kb from *Sgs-8*. Following the transformation procedure described (Figure 4), two ethanol-resistant animals were recovered among the first G1. Again, these were the progeny of a single G0 animal and were shown to be the result of a single insertion event. A genomic blot showed the introduced DNA to be intact; the transformed line also contains complete P elements acquired during the P-element transformation procedure. Subsequently, seven more independent *TfGA8.1* lines were established (see Table 2).

For two of the *TfGA8.1* lines, RNA was isolated from whole second instar larvae and from the salivary glands and remaining carcasses of early, mid, and late third instar larvae, white prepupae and tan prepupae. RNA blots hybridized with ^{32}P-labeled aDm2023, a probe homologous with *Sgs-3*, revealed that the inserted copies of *Sgs-3* from *Tf(2)GA8.1-1* and *Tf(3)GA8.1-5* are abundantly expressed in the correct tissue and at the appropriate time, at precisely the same times the smaller RNAs from the resident *Sgs-3*^{HR} genes are observed. The relative quantity of RNA produced from the introduced *Sgs-3* gene in three of the *TfGA8.1* lines was determined following the procedure described above. In homozygous *Tf(2)GA8.1-1* animals *Sgs-3*^{OR} RNA accumulates to 1.15±0.21 times the level of RNA from the *Sgs-3*^{HR} genes, when corrected for gene size and number. This is 89% of the level of RNA accumulation observed from the wild-type *Sgs-3*^{OR} gene in the genotype *Sgs-3*^{OR}/*Sgs-3*^{HR}. In *Tf(3)GA8.1-3* the introduced *Sgs-3*^{OR} gene is expressed at 98% the level of an Oregon-R control gene in heterozygous animals. For *Tf(3)GA8.1-5* the introduced gene is expressed at 80% the level of the control gene. A gel of ^{3}H-threonine-labeled salivary gland proteins from *Tf(2)GA8.1-1* shows two sgs-3 protein species produced in approximately equal amounts, corresponding to the Oregon-R and Hikone-R

variants (Figure 7). Thus no more than 1.1 kb of 3' flanking DNA appear to be necessary for the correctly regulated and abundant expression of the *Sgs-3* gene and for correct processing of the *Sgs-3* mRNA.

An 8.1 kb Fragment from the 68C Region Produces a New Intermolt Puff

Several of the *TfGA8.1* lines were analyzed for the appearance of a new intermolt puff at the site of insertion (Figure 8). In the three lines analyzed, a large puff is observed during puff stage 1 and a slightly smaller puff at puff stage 2. By puff stage 4-7 all trace of the puffs has disappeared. Thus these new puffs appear and regress at precisely the same times as the intermolt puffs and reach their maximum size during the period of abundant glue gene expression.

Discussion

Definition of the Functional Region of the 68C Glue Protein Gene Cluster by Analysis of Chromosomal Rearrangments

Our analyses of chromosomal rearrangments which break in or near 68C5-6 establish that the 16- to 20-kb region defined by the breakpoints of *In(3L)HR15* and $Df(3L)vin^3$ contains all the sequences required in cis for the expression of *Sgs-3*, and probably of *Sgs-7* and *Sgs-8*. This region also contains all of the DNA sequences required in cis for formation of an intermolt puff. Analysis of a strain containing the transposble element roo at 68C5-6 indicates that the 7 kb between the site of the roo insertion and the $Df(3L)vin^3$ breakpoint may be sufficient for expression of the glue genes; if the sequences to the left of roo are required for expression of the glue genes, a mechanism able to span over 9 kb of irrelevant sequences must be invoked. The insertion of this 9.2 kb transposon into the middle of the 20-kb region defined above has little, if any, effect upon puff formation.

Less Than 3.4 kb of Flanking Sequences Are Required for Correctly Regulated Expression of the *Sgs-3* Gene

Using P-element-mediated transformation we have begun to further delimit the region required for the regulated expression of *Sgs-3*, the largest of the three glue protein genes located at 68C. Normally, expression of the 68C glue genes is accompanied by changes in chromatin structure of a large chromosomal region, resulting in the formation of a puff. However, we establish that a relatively small region of adjoining sequences is sufficient for appropriate expression of *Sgs-3*. In two different sets of transformed lines, one with 2270 base-pairs of 5' flanking sequences and the other with 1100 base-pairs of 3' flanking sequences, normal expression of the introduced *Sgs-3* gene is observed. Analysis of these transformants shows that less than 3.4 kb of flanking sequences are necessary for normal tissue- and stage-specific production of an *Sgs-3* RNA which accumulates to abundant levels, is of correct size, and which is translated to produce sgs-3 protein. An *Sgs-3* gene that is no longer linked to *Sgs-7* and *Sgs-8* still exhibits abundant and correctly regulated expression. These results corroborate those of Richards et al. (1983; Bourouis and Richards, 1985). Using the P-element-mediated system, they have recovered a number of transformants with 2750 base-pairs of 5' flanking sequences, all of which exhibit correctly regulated and abundant expression of the RNA from the introduced *Sgs-3* gene. Several of these transformants contain only 320 base-pairs of downstream sequences, further delimiting the extent of 3' sequences required for appropriate expression of the *Sgs-3* gene.

P-element-mediated transformation experiments with other developmentally controlled protein-coding genes from Drosophila have yielded similar results. For both the xanthine dehydogenase gene (the rosy locus; Spradling and Rubin, 1983) and the dopa decarboxylase gene (Scholnick et al., 1983) less than 4

kb of flanking sequences are required for correctly regulated expression. An insert of less than 12 kb supports correct developmental expression of the *Adh* gene, including appropriate promoter utilization (Goldberg et al., 1983). In all three cases approximately normal levels of expression are observed for the transformed lines.

Abundant Transcription is Neither Necessary nor Sufficient for Formation of a Puff

Puffs normally appear to be, as classically described (Beermann, 1952), morphological manifestations of local genome activity. A number of studies have shown that puffs are sites undergoing active transcription (Pelling, 1964; Zhimulev and Belyaeva, 1975; Belyaeva and Zhimulev, 1976). In experiments in which heat shock or ecdysterone-inducible puffs were observed, RNA synthesis at puff sites was detectable by autoradiography within minutes of induction (Berendes, 1968; Belyaeva and Zhimulev, 1976). On the basis of work done in *Chironomus tentans* using actinomycin D and other inhibitors of RNA synthesis (Beermann, 1965; Clever, 1967), it was proposed that the synthesis and accumulation of RNA is a prerequisite for, if not the cause of, puff formation. However, in experiments with *Drosophila hydei* (Berendes, 1968) and *Drosophila melanogaster* (Ashburner, 1972a) application of puff-inducing stimuli to larvae or cultured salivary glands in which RNA synthesis was suppressed did result in the appearance of puffs. The puffs that appear under these conditions are significantly smaller, however, about one-third the maximal observed size. These results do indicate that in Drosophila initiation of puffing does not require RNA synthesis. Since it has been observed in the case of heat shock puffs that newly synthesized RNA is retained within the puff region for up to 20-25 minutes (Berendes, 1968), it has been suggested that the puff structure facilitates the processing of the RNA during this time. On the basis of P-element-mediated transformation experiments using the promoter

region of the heat shock gene *hsp70*, Simon et al. (1985) propose that both a high rate of transcription and a long transcription unit are required to produce a large puff. Transformed heat shock fusion genes which produce long transcripts also form large puffs (Simon et al., 1985), whereas those which encode relatively short transcripts produce only small puffs (Bonner et al., 1984; Cohen and Meselson, 1984; Dudler and Travers, 1984).

The results of our transformation experiments demonstrate that abundant glue gene expression and puff formation can be uncoupled. We observe large puffs at the sites of insertion for the *TfGA8.1* transformants in which the introduced DNA contains all three genes from the 68C glue gene cluster. Deletion of the *Sgs-7* and *Sgs-8* genes and the DNA sequences between them in the *TfGA6.0* transformants allows abundant expression of the remaining *Sgs-3* gene, but abolishes the puffing behavior. For two different transformants Richards et al. (1983) also observed that there are no visible intermolt puffs formed at the new chromosomal sites of *Sgs-3* genes which are abundantly and appropriately expressed. This is somewhat surprising, in view of the fact that the other intermolt puffs all appear to contain only one glue protein gene. Despite the absence of a visible puff, transcripts from an introduced *Sgs-3* gene appear to be correctly processed, as indicated by the production of a normal sgs-3 protein.

The reverse case of this uncoupling phenomenon can also occur. Certain non-pupariating lethal mutations which map to 2B5 (*l(1)t435* and *l(1)npr-1*) appear to render the third instar larval salivary glands refractory to changes in ecdysterone titer (Belyaeva et al., 1980; Crowley et al., 1984). The intermolt puffs are formed and attain normal size, but fail to regress. Crowley et al. (1984) found that *l(1)npr-1* larvae produce very little or none of the 68C glue gene mRNAs. The 68C intermolt puff is present despite the fact that the 68C glue genes are not being transcribed at detectable levels.

For salivary glands cultured in the presence of ecdysterone, Crowley and Meyerowitz (1984) observed that regression at the 68C puff is slower than the reduction levels of transcription of the 68C glue genes. While this may simply be due to the mechanics of puffing and "unpuffing", for instance if unprocessed RNA remains for a time within the region of the puff, it is also possible that there are different mechanisms governing the two processes.

One explanation for the results of our transformation experiments is that the total level of transcription within the small region of the glue gene cluster is sufficiently high to produce a puff only when all three genes are present. None of the 68C glue genes produces a long transcript; the largest, *Sgs-3*, is less than 1.2 kb in length. However, if only the total level of transcription is critical, transcription of a longer RNA from the *Sgs-3* promoter may be sufficient to produce a puff. An alternate explanation is that a particular DNA sequence is required in cis for puff formation and that this sequence is present in the introduced 68C DNA in the *TfGA8.1* transformants but has been deleted from the *TfGA6.0* transformants. Excluding the roo sequences, this is a region of less than 2 kb. Although such a specific sequence would usually be closely linked to the transcribed genes and would normally function coordinately, its action may actually be independent. Thus, by mutation or physical separation, puff formation could be uncoupled from high levels of gene expression. These and other possibilities are currently being tested in our laboratory.

Experimental Procedures

Drosophila Strains and Culture

In(3L)HR15 is described in Ashburner (1972b) and was obtained from L. Craymer (California Institute of Technology, Pasadena, CA).

Df(3L)vin^{3} is described in Akam et al. (1978) and was obtained from the Mid-America Drosophila Stock Center at Bowling Green, OH.

OR16f is a third-chromosome-homozygous strain derived from Oregon-R (Meyerowitz and Hogness, 1982).

Hikone-R is a wild-type strain obtained from Stanford University, Stanford, CA.

Formosa is a wild-type strain obtained from the Umea Drosophila Stock Center, Umea, Sweden.

Adh^{fn23} *pr cn*; *b Adh*^{n2} *osp*^{76e} *cn*; and *CyO, Adh*^{nB} were obtained from L. Craymer. *Adh*^{fn23} is described in Benyajati et al. (1983); *Adh*^{n2} is described in O'Donnell et al. (1975); *CyO, Adh*^{nB} is a second chromosome balancer which carries a null allele of *Adh* (Gerace and Sofer, 1972); *osp*^{76e} is described in Woodruff and Ashburner (1979); other mutants are described in Lindsley and Grell (1968).

T(2;3)Ata; *TM3*; *TM6*; *Sb*; and *C(1)M3* were obtained from the Caltech Drosophila Stock Center, Pasadena, CA, and are described in Lindsley and Grell (1968).

Drosophila cultures were fed standard cornmeal-sucrose-agar food and were kept at 22°C, except as indicated in Transformation Procedures.

DNA Methods

Drosophila DNA used for genomic blots was prepared as described in Meyerowitz and Hogness (1982). High molecular weight Drosophila DNA used for genomic library construction was prepared as described in Meyerowitz and Martin (1984). Plasmid and bacteriophage DNA preparations followed Davis et al. (1980), with minor modifications.

Restriction endonuclease digestions were performed as described in Davis et al. (1980). The genomic library from *Df(3L)vin*^{3}*/TM3* was made as described in

Meyerowitz and Martin (1984). Plaque filters were prepared as in Davis et al. (1980). DNA was subjected to electrophoresis in agarose gels cast in Tris-borate-EDTA buffer (Peacock and Dingman, 1968); restriction fragments of bacteriophage lambda DNA served as size standards. DNA gels were denatured and neutralized as in Southern (1975), and transferred to nitrocellulose by the Southern procedure, using 20X SSPE (Davis et al., 1980). Drosophila genome blots contained 1-2 μg of DNA per lane.

^{3}H-, ^{35}S-, and ^{32}P-labeled DNA was prepared by nick translation, following the method of Rigby et al. (1977). Hybridizations were in 50% formamide, 5X SSPE, 100 μg/ml sonicated and boiled salmon testis DNA, 1X Denhardt's solution (0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin; Denhardt, 1966), 0.1% SDS, at 43°C. After hybridization filters were washed in 1X SSPE, 0.1% SDS at 43°C.

Cytological Techniques

In situ hybridizations were performed as described in Pardue and Gall (1975) with the following modifications: the RNase treatment was replaced with two successive 30 min heat treatments at 65°C in 2X SSPE; 10 mM DTT was included in the hybridization mixture when using ^{35}S-labeled probes; after hybridization, slides were washed in 50% formamide, 600 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5, four washes of 5 min each, prior to successive washes in 2X SSPE following Pardue and Gall.

For orcein squashes, salivary glands were dissected from a late third instar larva in 45% acetic acid and transferred to a drop of aceto-lactic orcein stain solution (2% (w/v) orcein in 1:1 lactic:acetic acid) on a siliconized coverslip. After approximately 1 min the glands were squashed between the coverslip and a glass slide. The stained chromsomes were photographed using a Zeiss photomicroscope and diameters of the chromosomes were measured from the photographs.

RNA Methods

Dissected larval salivary glands and carcasses to be used for RNA preparations were immediately transferred to extraction buffer (100 mM NaCl, 100 mM Tris-HCl pH 8.5, 20 mM EDTA, 1% (w/v) Sarkosyl) and frozen on dry ice. In some cases they were stored at -70°C for one to several days. RNA was prepared by two phenol-chloroform extractions followed by a chloroform extraction and ethanol precipitation. 100 µg of yeast tRNA was added as carrier prior to ethanol precipitation. The RNA was resuspended in 3X MOPS buffer (see below) and prepared for electrophoresis immediately.

Prior to electrophoresis RNA was treated at 55°C for 15 min in 50% formamide, 6% formaldehyde in 1X MOPS buffer (40 mM MPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA). The RNA was subjected to electrophoresis in 1.5% agarose gels cast with 1X MOPS buffer plus 6% formaldehyde; running buffer was 1X MOPS buffer. Following electrophoresis the gels were rinsed several times in water, treated for approximately 45 min with 50 mM NaOH, 100 mM NaCl, then neutralized in 100 mM Tris-HCl pH 7.5. Blotting procedures and hybridization conditions were as described in DNA Methods.

For relative quantitation, RNA isolated from the salivary glands of individual third instar larvae was fractionated on an agarose-formaldehyde gel, blotted to nitrocellulose and hybridized with a ^{32}P-labeled probe. After obtaining an autoradiogram to determine the positions of the RNA species, the corresponding positions on the nitrocellulose filter were cut out, submerged in Liquifluor scintillation fluid (NEN), and the number of counts per minute contained on each fragment of the filter determined. All pieces of the nitrocellulose filter were of the same size. The level of background in each lane was determined by cutting out a piece of equal size from a region above the

largest RNA species. Relative levels of expression are indicated as the mean ± the standard error of the mean.

Construction of Transformation Plasmids

pGA6.0a and pGA6.0b were constructed by deleting a small BglII fragment from pAP5 (Goldberg et al., 1983) and inserting the 6.0 kb BglII fragment from λbDm9010 (M. D. Garfinkel, unpublished experiments; see Figure 3). The vector ends were treated with alkaline phosphatase prior to the ligation reaction; the methods of Davis et al. (1980) were used. The total length of DNA between the P-element termini is 20.5 kb.

pGA8.1 was constructed by removing a 3.75-kb XhoI fragment from pAP2 (Goldberg et al., 1983) and inserting an 8.1-kb SalI partial digestion fragment from λbDm2002 (Meyerowitz and Hogness, 1982; see Figure 3). The total length of DNA between the P-element termini is 19 kb.

λbDm9010 and λbDm2002 were both obtained from the Oregon-R 16f strain.

Transformation Procedures and Selection of Transformants

The techniques of embryo microinjection followed Spradling and Rubin (1982). Injected embryos were of the genotype Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$. $Sgs\text{-}3^{HR}$ is the variant of the *Sgs-3* region found in the Hikone-R wild-type strain. The egg collection, dechorionation, and dessication steps were performed at 22°C; microinjection was performed at 18°C. The injected embryos remained at 18°C until hatching and were then transferred to 22°C. The DNA solution injected contained 300 μg/ml of the transformation plasmid and 50 μg/ml of the pπ25.1 P-element helper plasmid.

Injected animals (G0) that survived to adulthood were backcrossed singly to the recipient strain, Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$. After being aged 5 days, G1 adults

were subjected to an ethanol selection test, using 6-7% ethanol in water for 16-20 hr (Vigue and Sofer, 1976). G1 animals that survived the ethanol selection were backcrossed singly to Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$ and a separate line for each was maintained by ethanol selection every other generation.

For selection of Adh^{+} larvae for RNA preparations or *in situ* hybridizations, a histochemical spot test for ADH activity was used (Grell et al., 1968; Ursprung et al., 1970). A small portion of the carcass is sufficient to reveal ADH activity, leaving the salivary glands and the remainder of the carcass for use.

Genetic Analysis of Transformants

Adh^{+} G2 males were crossed to *b* Adh^{n2} osp^{76e} *cn*; *Sb/TM6* females. The progeny of this mating (the F1) were subjected to ethanol selection. Survival of only females in the F1 indicated that the site of insertion was on the X-chromosome. If males survived they were crossed to Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$ females and the progeny of this mating (the F2) subjected to ethanol selection. Survival of only *pr cn* F2 animals indicated that the site of insertion was on the second chromosome; survival of only Sb^{+} or $TM6^{+}$ F2 animals indicated that the insertion site was on the third chromosome. Initially, these F2 animals were used to construct homozygous stocks.

Occasionally exceptional results were observed in the course of these genetic manipulations. These problems arose with lines that were determined by genome blots to contain intact P elements. The anomalous results were shown to be caused by a low but significant amount of male recombination, presumably due to hybrid dysgenesis. Subsequently, mapping and construction of homozygous or balanced stocks was accomplished using a double balancer stock, the second chromosome balancer containing an *Adh* null allele: *T(2;3)Ata/CyO*, Adh^{nB}; *TM3*. X-linked transformant lines were crossed to *C(1)M3*; Adh^{fn23}*pr cn*; $Sgs\text{-}3^{HR}$ and maintained as attached-X stocks.

Protein Analysis

Analysis of the sgs-3 proteins, including labeling of the salivary glands in culture, isolation of a crude protein fraction, electrophoresis, and fluorography were performed as described in Crowley et al. (1983).

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Table 1. Measurements of Chromosomal Diameter at 68C5-6 for Rearranged Chromosomes

	Puff Stage 1-2	Puff Stage 7-9
In(3L)HR15	1.75 ± 0.06 (N=8)	0.99 ± 0.06 (N=8)
Df(3L)vin^3	1.89 ± 0.09 (N=7)	1.37 ± 0.08 (N=4)
Oregon-R 16f	1.77 ± 0.08 (N=6)	1.46 ± 0.07 (N=8)

The diameter of the chromosome at 68C5-6 was compared to that at the reference bands 69A1-3; expressed as the mean ± the standard error of the mean. The number of chromosomes measured is indicated in parentheses. In the case of *Df(3L)vin*^{3}, which is not viable when homozygous, only asynapsed chromosomes were analyzed. Oregon-R 16f contains a roo transposable element just distal to the 68C glue gene cluster.

Table 2. Characteristics of *Sgs-3* Transformant Lines

Transformant Line	*Viability of Homozygote*	*Cytological Location*	*Level of Expression*	*Produces New Puff*
Tf(3)GA6.0-1	+	66E1-3	70%	No
Tf(3)GA6.0-2	-	(ND)	(ND)	(ND)
Tf(1)GA6.0-3	+	10D-F	75%	No
Tf(3)GA6.0-4	+	76F-77A	(ND)	No
Tf(2)GA8.1-1	+	chromocenter	89%	(ND)
Tf(3)GA8.1-2	-	71E-F	(ND)	(ND)
Tf(3)GA8.1-3	+	97F-98A	98%	Yes
Tf(3)GA8.1-4	+	91D-F	(ND)	Yes
Tf(3)GA8.1-5	+	79B-C	80%	Yes
Tf(3)GA8.1-6	Highly dysgenic; not analyzed; original location on X chromosome			
Tf(3)GA8.1-7	+	3E-F	(ND)	(ND)
Tf(3)GA8.1-8	+	88C	(ND)	(ND)

ND = not determined

Figure 1. Cloned Segments Used in Analysis of Rearrangements Which Break in the 68C5-6 Region

A restriction map of the region surrounding the 68C glue gene cluster is diagrammed on the top line (Meyerowitz and Hogness, 1982). Restriction endonucleases are abbreviated as follows: B: BglII, H: HindIII, R: EcoRI, S: SalI, X: XhoI. The approximate coding regions and the directions of transcription for *Sgs-3*, *Sgs-7*, and *Sgs-8* are represented by arrows immediately below the restriction map (Garfinkel et al., 1983). The cloned segments which were used to analyze *Df(3L)vin*^{3} and *In(3L)HR15* are diagrammed at the bottom of the figure (λ clones are described in Meyerowitz and Hogness, 1982; aDm2023 is described in Garfinkel et al., 1983). Above the restriction map the positions of the proximal *In(3L)HR15* breakpoint, the 9.2-kb roo transposable element, and the *Df(3L)vin*^{3} breakpoint are indicated.

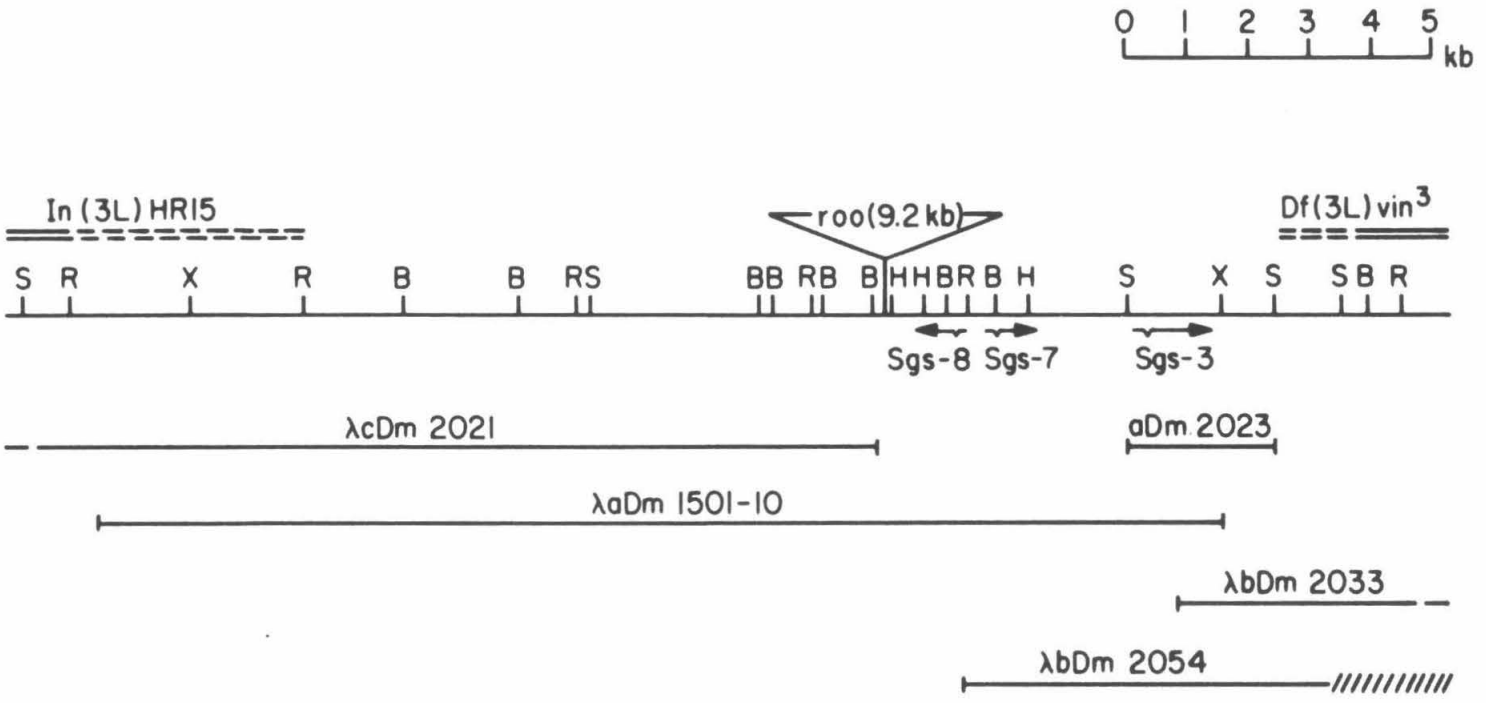
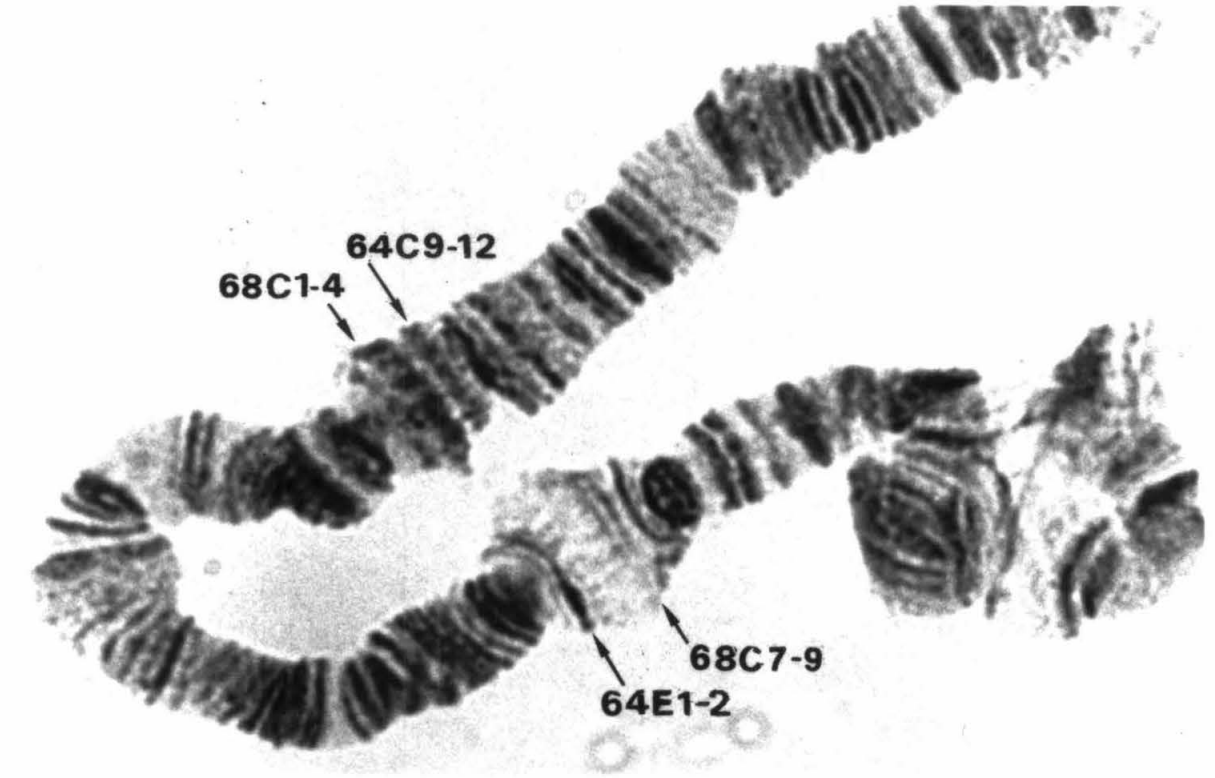

0 1 2 3 4 5
kb
In (3L) HR15
roo(9.2 kb)
Df(3L) vin3
S R X R B B RS BB RB B HH B R B H S X S S B R
Sgs-8 Sgs-7 Sgs-3
λcDm 2021
aDm 2023
λaDm 1501-10
λbDm 2033
λbDm 2054

Figure 2. 68C Intermolt Puff Observed in *In(3L)HR15* and *Df(3L)vin*^{3}

Orcein-stained preparations of polytene chromosomes from the salivary glands of late third instar larvae; puff stage 1-2. A) Homozygous *In(3L)HR15*. A large puff is present near the proximal breakpoint, where the 68C glue gene cluster is located. At the distal breakpoint is the "ballet skirt" (Lefevre, 1976), a region of expanded chromosomal diameter observed at 68C1-4 during all puff stages. B) *Df(3L)vin*^{3}*/TM3*. In the region shown the *Df(3L)vin*^{3} chromosome is asynapsed and appears half the diameter of the adjacent chromosome. A puff is present in the region of the deficiency breakpoint.


64C9-12
68C1-4
68C7-9
64E1-2

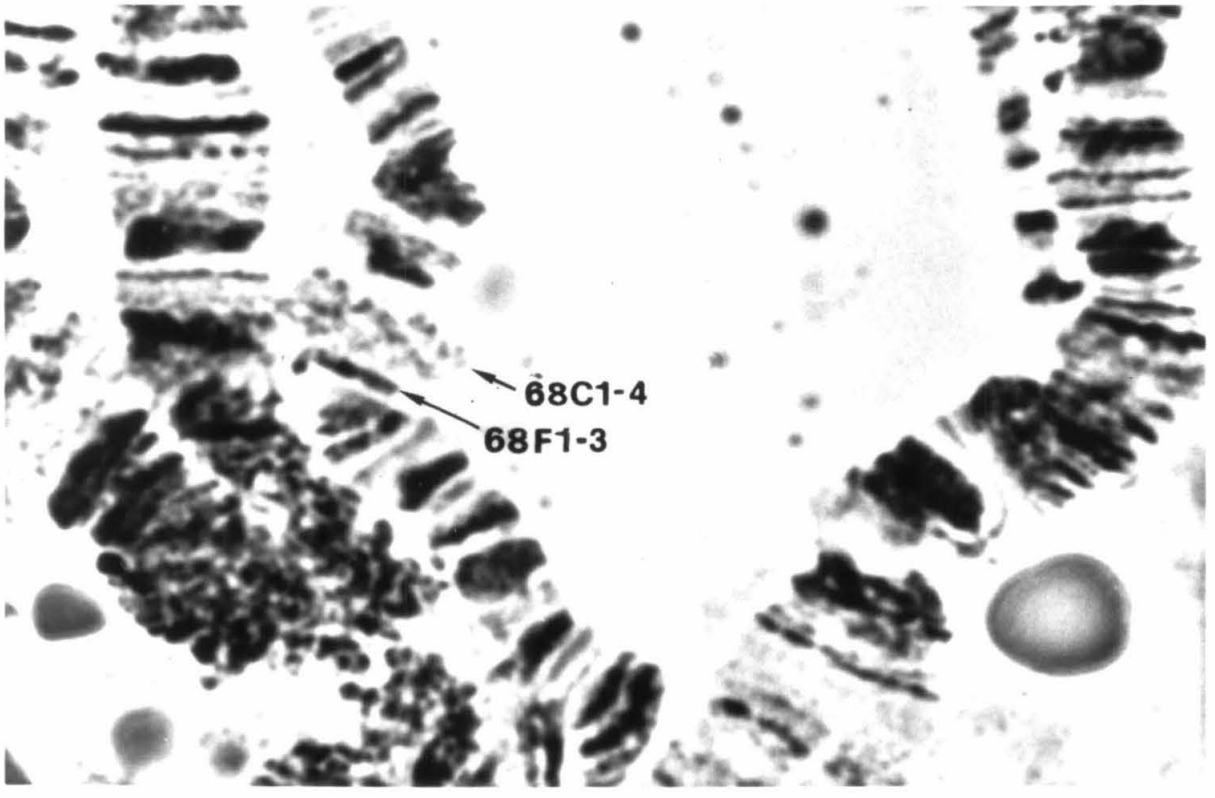

68C1-4
68F1-3

Figure 3. Restriction Maps of the Transformation Plasmids Used

Restriction endonucleases are abbreviated as follows: B: BamHI, Bg: BglII, H: HindIII, R: EcoRI, S: SalI, X: XhoI. The location and direction of transcription of *Sgs-3* and *Adh* genes are represented by arrows immediately below the restriction maps. Below the restriction maps are indicated the P-element-bounded fragments which are inserted into the genome and their length. pGA6.0b is identical to pGA6.0a except that the 6.0 kb 68C fragment is in the opposite orientation and it lacks the small insertion into the pBR322 region.

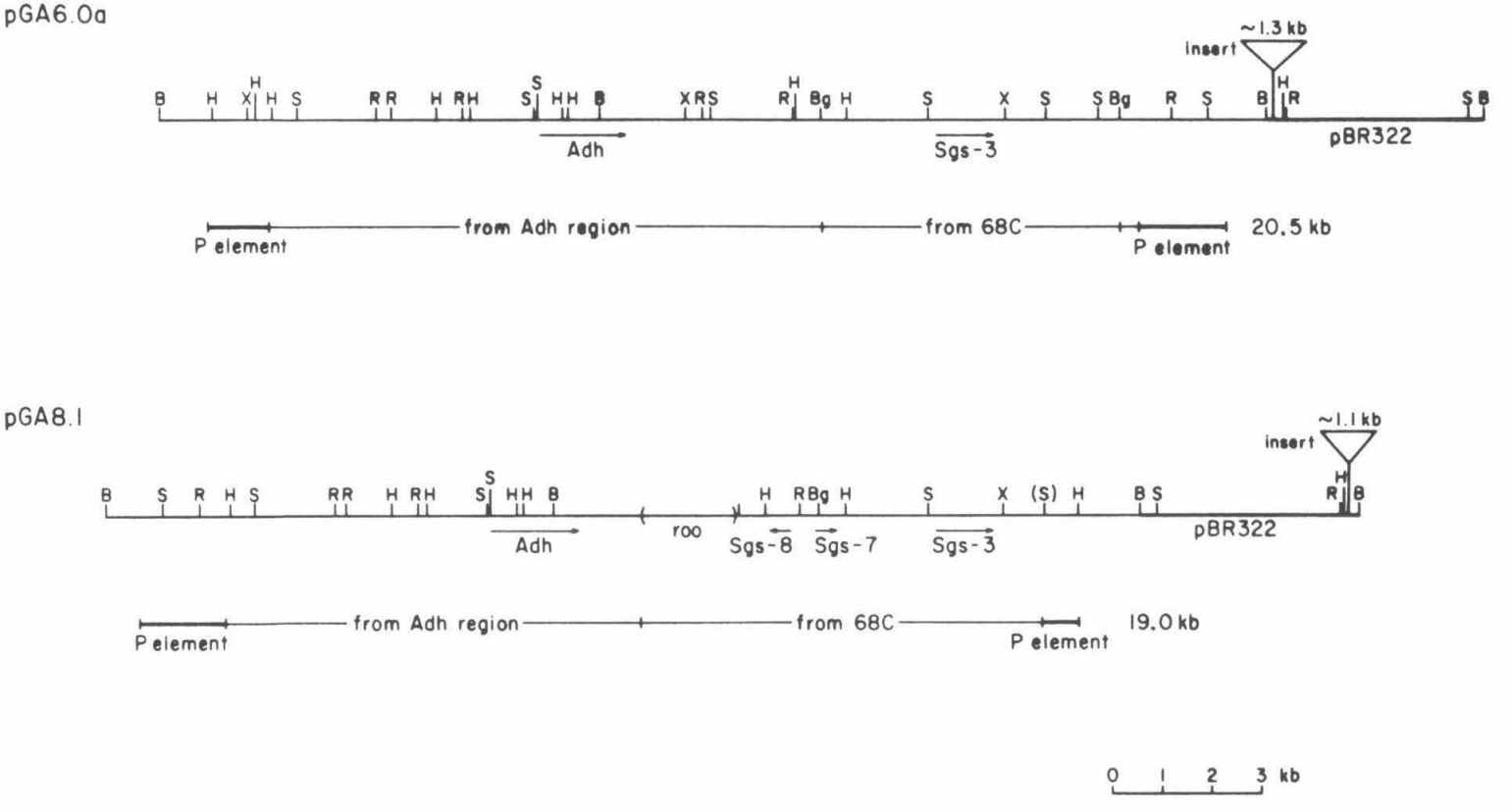

pGA6.0a
~1.3 kb
insert
B H X H H S R R H R H S S H H B X R S R H Bg H S X S S Bg R S B H R S B
Adh
Sgs-3
pBR322
P element
from Adh region
from 68C
P element
20.5 kb
pGA8.1
~1.1 kb
insert
B S R H S R R H R H S S H H B
roo
H R Bg H S X (S) H B S R B
Adh
Sgs-8
Sgs-7
Sgs-3
pBR322
P element
from Adh region
from 68C
P element
19.0 kb
0 1 2 3 kb

Figure 4. Isolation of *Sgs-3* Transformants

TRANSFORMATION PROTOCOL

1. Inject P-element plasmid which contains *Adh* gene and $Sgs\text{-}3^{OR}$ gene into Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$ embryos; co-inject intact P-element plasmid, pπ25.1.

2. Injected animals which survive to adulthood, designated G0, backcrossed singly to Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$.

3. G1 adults subjected to ethanol selection; survivors backcrossed singly to Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$.

4. G2 larvae tested for ADH activity by histochemical spot test; ADH^{+} animals analyzed for expression of $Sgs\text{-}3^{OR}$.

5. Stocks maintained by ethanol selection every other generation. Chromosomal location mapped using Adh^{+} phenotype. Homozygous or balanced stocks constructed using standard genetic procedures.

Figure 5. Cytological Analysis of *Tf(3)GA6.0-1*

A) *In situ* hybridization to determine chromosomal location; hybridization is observed at 68C, the site of the resident *Sgs-3* genes, and at the site of the insertion, 66E1-3. B) Orcein-stained preparation of the recipient strain, Adh^{fn23} *pr cn*; $Sgs\text{-}3^{HR}$, showing distal 3L at puff stage 1-2. C) Orcein-stained preparation showing distal 3L in *Tf(3)GA6.0-1* at puff stage 1-2. There is no visible change in the chromatin structure at 66E1-3.

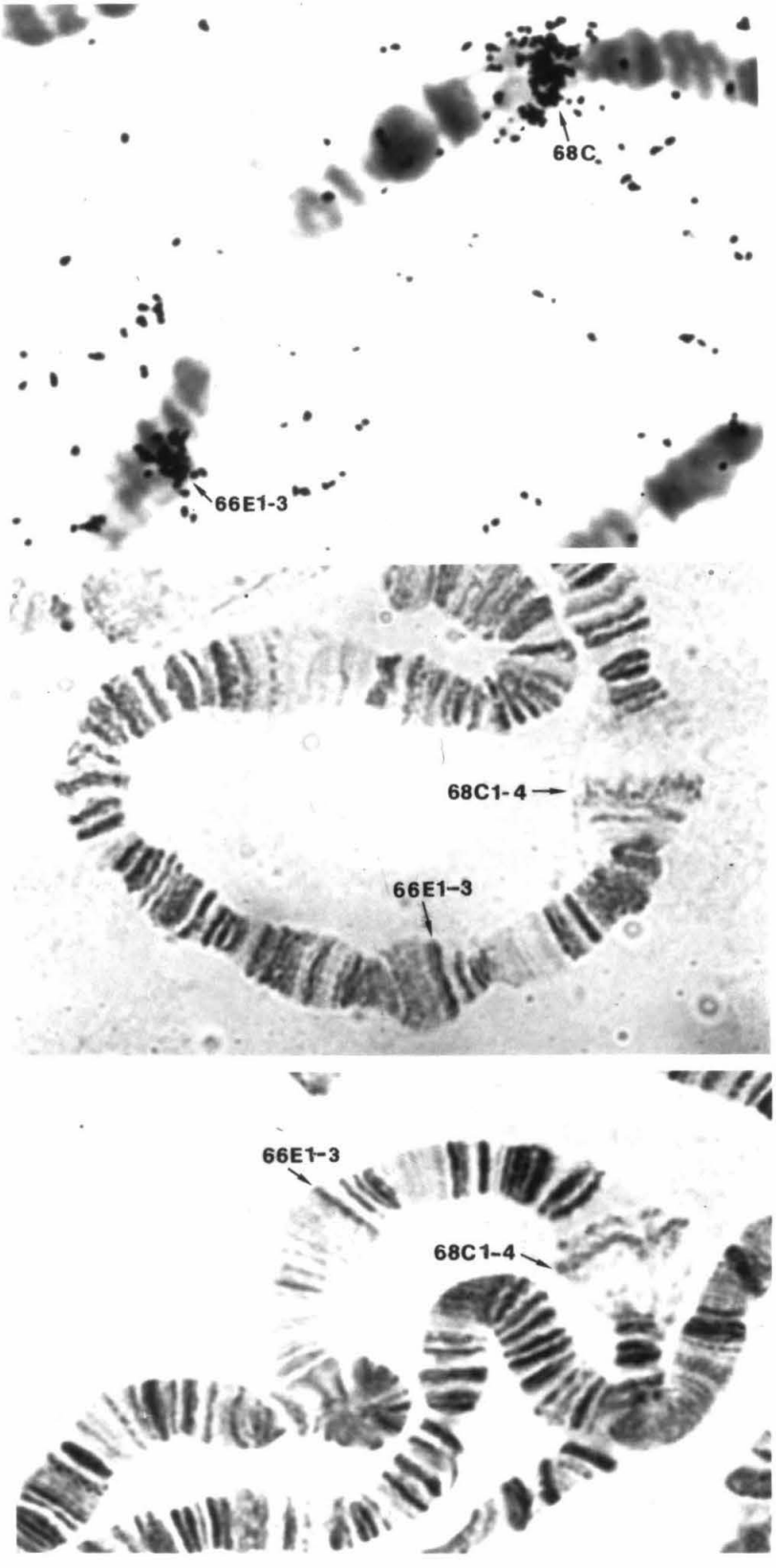

68C
66E1-3
68C1-4
66E1-3
66E1-3
68C1-4

Figure 6. RNA Blot of *Tf(3)GA6.0-1* Transformants at Different Developmental Stages

RNA isolated from the salivary glands and carcasses of homozygous *Tf(3)GA6.0-1* animals at different developmental stages was fractionated by size on a 1.5% agarose-formaldehyde gel. Except where indicated otherwise, RNA from two animals was used for each lane. The gel was base-treated, blotted to a nitrocellulose filter, and the filter was hybridized with ^{32}P-labeled probes.

Lane 1: salivary glands of third instar larvae of Oregon-R strain (standard).

Lane 2: salivary glands of third instar larvae of Hikone-R strain (standard).

Lane 3: salivary glands of mid third instar larvae.

Lane 4: salivary glands of late third instar larvae (pre-climbing).

Lane 5: salivary glands of late third instar larvae (early climbing).

Lane 6: salivary glands of late third instar larvae (late climbing).

Lane 7: salivary glands of white prepupae.

Lane 8: salivary glands of tan prepupae.

Lane 9: whole second instar larvae; ten animals.

Lane 10: whole early third instar larvae; six animals.

Lane 11: carcasses (with salivary glands removed) of mid third instar larvae; three animals.

Lane 12: carcasses (with salivary glands removed) of late third instar larvae (pre-climbing).

Lane 13: carcasses (with salivary glands removed) of late third instar larvae (early climbing).

Lane 14: carcasses (with salivary glands removed) of late third instar larvae (late climbing).

Lane 15: carcasses (with salivary glands removed) of white prepupae.

Lane 16: carcasses (with salivary glands removed) of tan prepupae.

A) The probe used was aDm2023, which is homologous with *Sgs-3* RNA. The *Sgs-3*^{OR} RNA is 1120 nt, prior to polyadenylation. In lanes 2,3,4 and 9 the Hikone-R band is an unresolved doublet. The Hikone-R strain contains a small, apparently tandem, duplication of the region of the 68C glue gene cluster. The two copies of the *Sgs-3* gene produce RNAs of slightly different sizes, approximately 950 nt and 1000 nt, which can be resolved on an acrylamide gel (see Appendix). B) The probe was removed from the filter in (A) with boiling 0.01 x SSPE and the filter was rehybridized with λbDm103, which contains the Drosophila rRNA coding region. This control experiment reveals that there is relatively less RNA in lanes 12 and 13 than in other lanes containing RNA from larval carcasses; the experiment was repeated for these stages and showed no detectable *Sgs-3* homologous RNA in the carcasses of late third instar larvae.

A
B
Sgs-3^{OR} —
Sgs-3^{HR} —
rRNA
1 standard: Oregon-R
2 standard: Hikone-R
3 S.G. of mid 3rd
4 S.G. of late 3rd
5 S.G. of late 3rd
6 S.G. of late 3rd
7 S.G. of white prepupae
8 S.G. of tan prepupae
9 whole 2nd
10 whole early 3rd
11 mid 3rd (-S.G.)
12 late 3rd (-S.G.)
13 late 3rd (-S.G.)
14 late 3rd (-S.G.)
15 white prepupae (-S.G.)
16 tan prepupae (-S.G.)

Figure 7. A Normal sgs-3 Protein is Produced from the Introduced *Sgs-3* Genes in *Tf(3)GA6.0-1* and *Tf(2)GA8.1-1*

For each genotype 10 pairs of salivary glands from late third instar larvae were cultured in medium containing ^{3}H-threonine (see Experimental Procedures). Proteins were extracted from the glands, alkylated and separated by electrophoresis in an acid-urea gel; the labeled proteins were detected by fluorography. The sgs-3 proteins, which are comprised of 42% threonine, label much more strongly than other proteins in the salivary glands. Lanes 1 and 2 were exposed for 48 hr; lanes 3 and 4 were exposed for 16 hr.

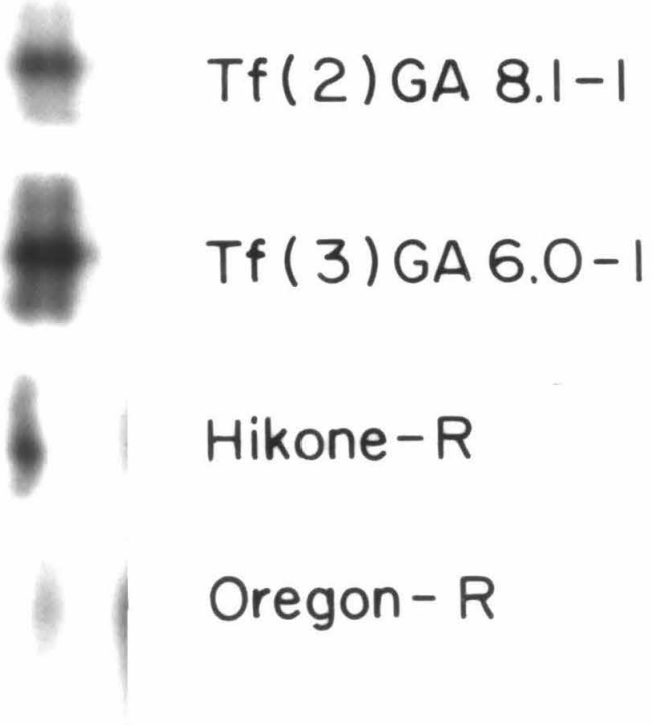
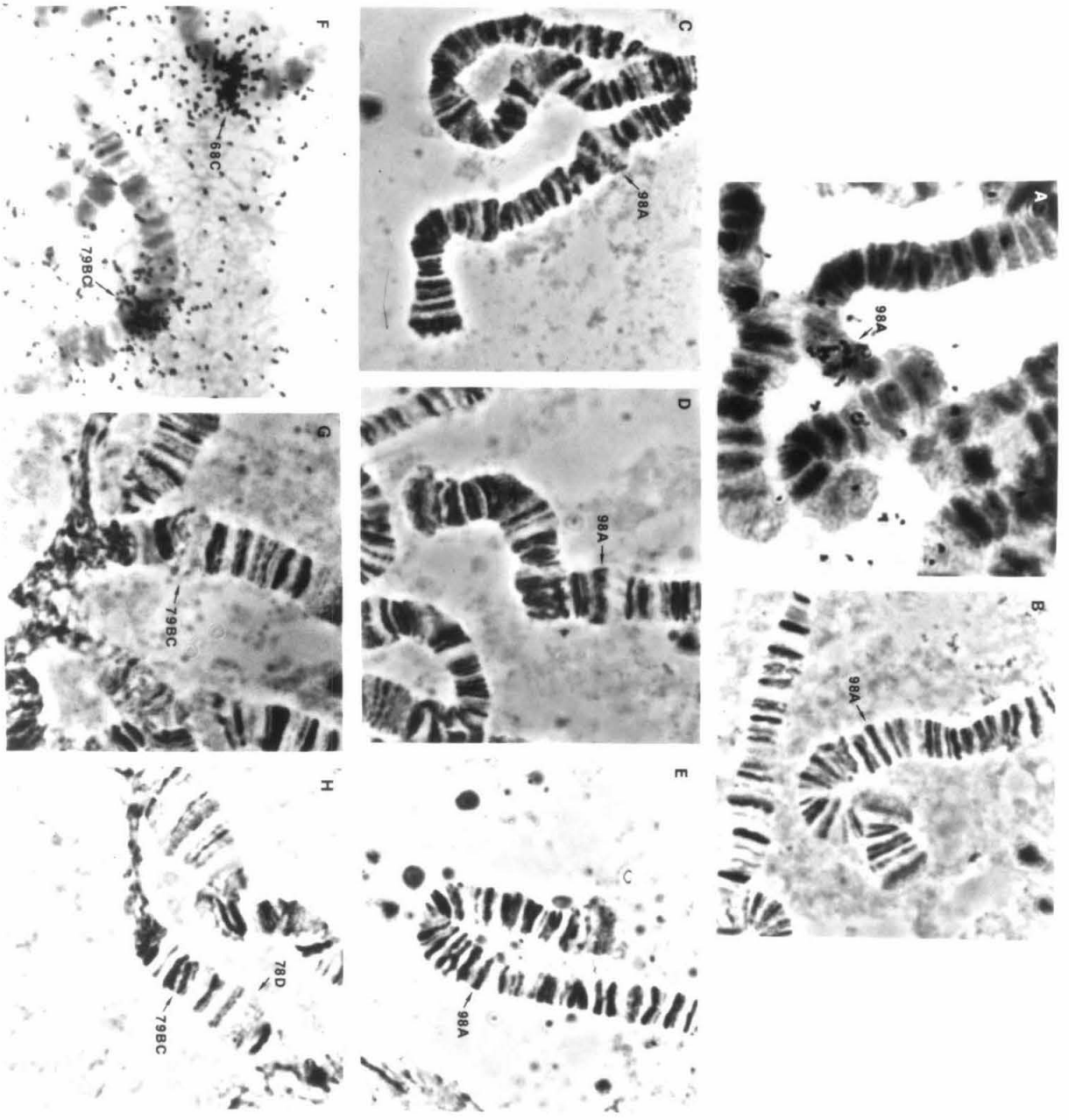

Tf(2)GA 8.1-1
Tf(3)GA 6.0-1
Hikone-R
Oregon-R

Figure 8. *TfGA8.1* Transformants Produce a New Intermolt Puff at the Site of Insertion

Tf(3)GA8.1-3 (A) *In situ* hybridization indicates that site of insertion is 97F-98A. (B) Orcein-stained preparation of salivary gland chromosomes of control animal at puff stage 1, showing no puff at 97F-98A. (C) Orcein squash from *Tf(3)GA8.1-3* animal at puff stage 1, showing large puff at 97F-98A. (D) Orcein squash of *Tf(3)GA8.1-3* at puff stage 2, showing smaller puff at 97F-98A. (E) *Tf(3)GA8.1-3* at puff stage 4-5, at which 97F-98A puff has completely regressed.

Tf(3)GA8.1-5 (F) *In situ* hybridization shows hybridization at 68C (site of the resident *Sgs-3* genes) and at 79BC. (G) Orcein-stained preparation of salivary gland chromosomes from *Tf(3)GA8.1-5* animal at puff stage 1-2, showing puff at 79BC. (H) Orcein squash of *Tf(3)GA8.1-5* at puff stage 6, at which time the 79BC puff has completely regressed. Visible at 78D is a puff that normally appears at this stage.

The probe used for the in situ hybridization experiments was ^{35}S-labeled aDm2023.


A
98A
B
98A
C
98A
D
98A
E
98A
F
68C
79BC
G
79BC
H
78D
79BC

CHAPTER 3

The *Sgs-3* Gene of Drosophila is Controlled by Two Separable Regulatory Regions

Summary

We have continued our analysis of the sequences required for the expression of the Drosophila glue gene *Sgs-3* utilizing the technique of P-element-mediated transformation. A DNA fragment which contains the entire *Sgs-3* gene, 130 base-pairs of 5' flanking sequences and 1100 base-pairs of 3' flanking sequences has been introduced into the fly genome using two different transformation vectors. In one case, low levels of appropriate tissue- and stage-specific expression are observed. In the second case, there is no detectable expression from the introduced *Sgs-3* genes. We conclude that sequences required in cis for normally regulated developmental expression are contained within the 130 base-pairs upstream of the gene or within the gene itself. Sequences necessary for abundant expression of the gene are located further upstream, and have been deleted from these transformed genes.

Introduction

The salivary gland secretion (Sgs) or glue genes of *Drosophila melanogaster* are a dispersed set of hormonally regulated genes which are expressed only in the larval salivary glands and only during a defined period in the third larval instar. *Sgs-3* is the largest in a cluster of three glue genes located at site 68C on the left arm of the third chromosome. By P-element-mediated transformation experiments it has been established that a total of 3.4 kilobase-pairs (kb) of flanking sequences, 2.3 kb 5' and 1.1 kb 3', are sufficient for normally regulated and abundant expression of this gene (Chapter 2). An *Sgs-3* gene that has been separated from the other two genes is the 68C glue gene cluster is expressed normally.

In the experiments discussed below, the sequences upstream of *Sgs-3* have been truncated at 130 base-pairs 5' of the gene, and this fragment of 68C DNA has

been used in P-element-mediated transformation experiments. Under some conditions tissue- and stage-specific expression is still observed, but the level of expression is greatly reduced.

Results

Two series of P-element-mediated transformation experiments (Spradling and Rubin, 1982; Rubin and Spradling, 1982) were conducted using a small fragment of DNA from the 68C region. Identical fragments were inserted into an *Adh*$^{+}$ (alcohol dehydrogenase) transformation vector and into a rosy^{+} (Xdh, xanthine dehydrogenase) transformation plasmid (Figure 1). The 68C DNA contains the *Sgs-3* gene with 130 base-pairs of 5' flanking sequences and 1100 base-pairs of 3' flanking sequences. The 3' end is identical to that of pGA8.1 (see Chapter 2), which has been observed to allow normal and abundant expression of *Sgs-3* in transformed animals.

Expression of the introduced genes is distinguised from that of the resident *Sgs-3* genes by the use of naturally occurring size variants. The introduced gene is the variant found in the Oregon-R strain, *Sgs-3*^{OR}. Two smaller size variants are used at the glue gene locus at 68C, *Sgs-3*^{HR} from the Hikone-R strain and *Sgs-3*^{For} from the Formosa strain.

The first transformation plasmid, pGA2.4, was constructed by replacing the 3.75-kb XhoI fragment of pAP2 (Goldberg et al., 1983) with a 2.4-kb SalI fragment containing *Sgs-3*^{OR} (Figure 1). This plasmid was co-injected with the P element plasmid pπ25.1 (Spradling and Rubin, 1982; O'Hare and Rubin, 1983) or pπ25.7wc (Karess and Rubin, 1984) into *Adh*^{fn23} *pr cn*; *Sgs-3*^{HR} embryos. Following the transformation procedure described (Chapter 2), six independent transformants were recovered from six different G0 animals. By genetically mapping the *Adh*$^{+}$ phenotype it was established that four of the inserts are on the second

chromosome, one is on the third chromosome, and one is on the X chromosome. In five of the lines the chromosome carrying the insertion is viable when homozygous; in the sixth line it is lethal. Genomic blots showed the inserted DNA to be intact in all tested cases and that one of the lines also contains an inserted P element.

The first salivary gland RNA blots of the *TfGA2.4* lines showed no detectable expression of the introduced *Sgs-3* genes, even in the homozygous lines. Since a low level of expression would be obscured by overlap of the signal from the $Sgs\text{-}3^{HR}$ RNAs, in three of the lines the third chromosome containing the $Sgs\text{-}3^{HR}$ variant was replaced by a third chromosome containing the Formosa size variant, $Sgs\text{-}3^{For}$, which codes for an mRNA smaller than those in the Hikone-R strain. In all three cases, when salivary gland RNA blots were performed with the *TfGA2.4*; $Sgs\text{-}3^{For}$ lines, RNA from the introduced $Sgs\text{-}3^{OR}$ gene is observed, but at levels reduced from normal. The amount of *Sgs-3* RNA from the introduced genes was determined relative to the amount of RNA produced by the $Sgs\text{-}3^{For}$ genes. Three to five measurements, representing different individual animals, were made for each of three lines. Corrections were made for the amount of background and for the larger size of the $Sgs\text{-}3^{OR}$ RNA. In two cases, the expression of the introduced genes is approximately 10-fold less than that of the Formosa genes. For *Tf(2)GA2.4-1* the RNA from the introduced genes accumulates to 11.1 ± 1.5% the level of the Formosa RNA, and for *Tf(2)GA2.4-4* it accumulates to 8.1 ± 1.4% of the level of the Formosa RNA. For these two lines homozygous transformed animals were used. In the case of *Tf(2)GA2.4-5*, even when corrected for the fact that heterozygous animals must be used, the level of expression of the introduced *Sgs-3* gene is much lower, 2.5 ± 0.7% the level of expression from the $Sgs\text{-}3^{For}$ genes.

For the transformed lines *Tf(2)GA2.4-1* and *Tf(2)GA2.4-4*, RNA was isolated from whole second instar and early third instar larvae, and from the salivary glands and remaining carcasses of mid to late third instar larvae and prepupae. An RNA blot hybridized with a labeled probe homologous with *Sgs-3* reveals that the introduced $Sgs\text{-}3^{OR}$ genes, despite their reduced level of expression, are appropriately expressed in the salivary glands during the latter half of the third larval instar (Figure 2). They are not expressed in any other tissue at this time, nor are they expressed in second instar or early third instar larvae. The introduced genes, but not the resident Formosa genes, are also expressed at very low levels in the salivary glands of tan prepupae, a stage at which *Sgs-3* is not normally expressed.

A second transformation plasmid, pGX2.4 (Figure 1), was constructed by inserting the same 2.4-kb SalI fragment containing the $Sgs\text{-}3^{OR}$ gene into the rosy transformation vector, Carnegie 20 (Rubin and Spradling, 1983). pGX2.4 was co-injected with pπ25.1 into $Sgs\text{-}3^{OR}$ ry^{42} embryos. G0 survivors were backcrossed to $Sgs\text{-}3^{OR}$ ry^{42} and transformants were identified in the G1 by their ry^{+} phenotype. Three independent transformed lines were established. On the basis of genetic crosses designed to detect the segregation of the ry^{+} gene, the insertions were mapped to the third chromosome in two of the lines and to the second chromosome in the third line.

In order to assay expression of the introduced $Sgs\text{-}3^{OR}$ gene, it was necessary to replace by genetic manipulations the resident $Sgs\text{-}3^{OR}$ gene at 68C with the $Sgs\text{-}3^{For}$ variant. This was done for two of the three lines. Salivary gland RNA blots from these two homozygous *TfGX2.4* lines show no detectable accumulation of RNA from the introduced $Sgs\text{-}3^{OR}$ genes (Figure 3). Only the smaller $Sgs\text{-}3^{For}$ species is observed.

Discussion

Using P-element-mediated transformation experiments we have analyzed the expression of an introduced *Sgs-3* gene which includes only 130 base-pairs of 68C DNA upstream of the RNA transcription initiation site. Expression is observed in some cases but not in others, and appears to be dependent upon the particular transformation vector used.

In the *TfGX2.4* lines, for which a rosy transformation vector was used, no detectable accumulation of RNA from the introduced *Sgs-3* genes is observed. The transformation plasmid used in these experiments is almost identical to one used by Bourouis and Richards (1985). They also observed no, or barely detectable, levels of RNA accumulation from their transformed *Sgs-3* genes. In the pGX2.4 transformation plasmid, the rosy gene is located 3' of the *Sgs-3* gene (Figure 1). Upstream of the *Sgs-3* gene are the P element sequences. In the transformed lines DNA sequences from the site of insertion are within 1 kb of the 5' end of the glue gene, so the upstream flanking sequences vary from one transformant line to the next.

When an identical segment of DNA from the 68C region is introduced via an *Adh* transformation vector, the results are very different. The introduced *Sgs-3* genes in the *TfGA2.4* lines are expressed; they are expressed only in larval salivary glands and at the appropriate time during development. However, the *Sgs-3* transcripts accumulate to only 10% or less of normal levels and the introduced genes are also inappropriately expressed at very low levels in the salivary glands of tan prepupae. In these transformed lines, DNA from the *Adh* region is located upstream of the introduced *Sgs-3* gene (Figure 1). In its tissue distribution and stage specificity, the expression observed is characteristic of a glue gene mRNA, not that of *Adh* (Goldberg et al., 1983). The exception to this is the low level of *Sgs-3* RNA found in the salivary glands of tan prepupae: *Adh* may

be expressed at low levels in the salivary glands during this stage (Ashburner, 1978). There does appear to be some element within the *Adh* DNA which affects the level of expression of the *Sgs-3* gene.

Based on the fact that the introduced *Sgs-3* genes in the *TfGA2.4* transformants exhibit a pattern of expression almost identical to that of normal *Sgs-3* genes, it appears that a regulatory region necessary in *cis* for appropriate tissue- and stage-specific expression is located very close to or within the *Sgs-3* gene. There may, however, be sequences required for repression of *Sgs-3* expression in tan prepupal which have been deleted. At the white prepupal stage, during which the ecdysterone titer is very high (Hodgetts et al., 1977; Berreur et al., 1979), the expression of the glue genes is not observed, both in normal animals and in these transformed animals. By the beginning of the tan prepupal stage the ecdyterone titer has started to drop sharply and briefly reaches low levels comparable to those observed during the intermolt stage. This similarity between the two stages may be involved in the inappropriate expression of *Sgs-3* observed in the *TfGA2.4* transformants.

An additional, separable, regulatory region is required for abundant levels of expression of *Sgs-3*. Since *TfGA6.0* transformants with 2270 base-pairs of 68C DNA 5' of *Sgs-3* do accumulate abundant levels of RNA from the transformed *Sgs-3* genes (Chapter 2), this second regulatory region appears to be located between 130 and 2270 base-pairs 5' of the gene. Other flanking DNA sequences may fortuitously substitute for this regulatory element, as in the case of the *TfGA2.4* transformants. Another case of this type of event has been observed by K. VijayRaghavan (unpublished results). Using a transformation plasmid similar to *TfGX2.4*, with the *Sgs-3* gene fused in frame with an *E. coli lacZ* gene (Bonner et al., 1984), he has obtained three independent transformed lines. Two of these lines have been analyzed for β-galactosidase activity in the salivary glands. One line

shows no activity; the other shows low levels of activity that is observed only in the salivary glands in third instar larvae and prepupae. As is the case for the *TfGX2.4* transformants, flanking DNA from the site of insertion is within 1 kb 5' of the introduced *Sgs-3-lacZ* gene. It might be expected that some *TfGX2.4* transformants would also show expression of the introduced *Sgs-3* gene, dependent upon the site of insertion. Additional *TfGX2.4* lines have been established and are currently being analyzed (K. VijayRaghavan, unpublished results).

The hypothesis that there are two separable regulatory regions that control the expression of *Sgs-3* may be an oversimplification. Bourouis and Richards (1985) find that transformants which contain 1.0 kb or 1.5 kb of 68C DNA upstream of the inserted *Sgs-3* are consistently expressed, but at significantly reduced levels. Transformants which contain 2.3 kb of 5' flanking sequences, that is, all of the sequences between *Sgs-3* and *Sgs-7*, exhibit slightly reduced levels of expression, between 70-75% of normal (Chapter 2).

Appropriately regulated but differing levels of expression are also observed in P-element-mediated transformation experiments in which varying amounts of the 5' flanking region of the heat shock gene *hsp70* were fused to an Adh^{+} gene (Dudler and Travers, 1984). Introduced fusion genes which contain 68 base-pairs of the *hsp70* 5' flanking region are appropriately expressed in response to heat shock, but at much reduced levels. Introduced genes with 97 base-pairs of upstream sequences are abundantly transcribed as well as correctly regulated. There are two copies of a heat shock promoter consensus sequence in the *hsp70* 5' region, one between -50 and -65 and the other between -70 and -85 (Pelham, 1982). It has been suggested that within this region are located two similar protein-binding regulatory elements (Topol et al., 1985). The presence of the closer element allows some expression, but both are required for high levels of expression.

Binding of a protein fraction to the distal site appears to be cooperative, requiring occupancy of the first site (Topol et al., 1985).

It is possible that the regulation of the *Sgs-3* gene is controlled in a similar way. However, there are no obvious regions of shared homology within the 5' flanking sequences of *Sgs-3* (Garfinkel et al., 1983). Another interpretation is that the two regulatory regions detected in these experiments serve two distinct functions, one controlling correct developmental expression and the other required for abundant levels of expression.

Experimental Procedures

Most of the experimental procedures used are described in Chapter 2.

The double balancer stock *T(2,3)Ata/CyO;TM3*, ry^{RK} was used to map the sites of insertion in the rosy$^+$ transformed lines. *TM3*, ry^{RK} was obtained from Barbara Wakimoto; the ry^{RK} allele was induced by EMS mutagenesis on a standard *TM3, Sb Ser* chromosome.

pGA2.4 was constructed by replacing the 3.75-kb XhoI fragment of pAP2 (Goldberg et al., 1983) with the SalI fragment from aDm2023 (Garfinkel et al., 1983). The vector ends were treated with alkaline phosphatase prior to the ligation reaction. The total length of the DNA within the P element termini is 13.3 kb.

pGX2.4 was constructed by M.D.Garfinkel by inserting the 2.4-kb SalI fragment from aDm2023 into the SalI site in the polylinker of Carnegie 20 (Rubin and Spradling, 1983). The total length of the DNA between the P element termini is 10.5 kb.

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Figure 1. Restriction Maps of the Transformation Plasmids pGA2.4 and pGX2.4

Restriction endonucleases are abbreviated as follows: B: BamHI, H: HindIII, R: EcoRI, S: SalI, X: XhoI. The approximate coding regions and directions of transcription for *Sgs-3*, *Adh*, and *Xdh* are represented by arrows immediately below the restriction maps. The length of the DNA within the P element termini and the derivation of the fragments composing each plasmid are also indicated.

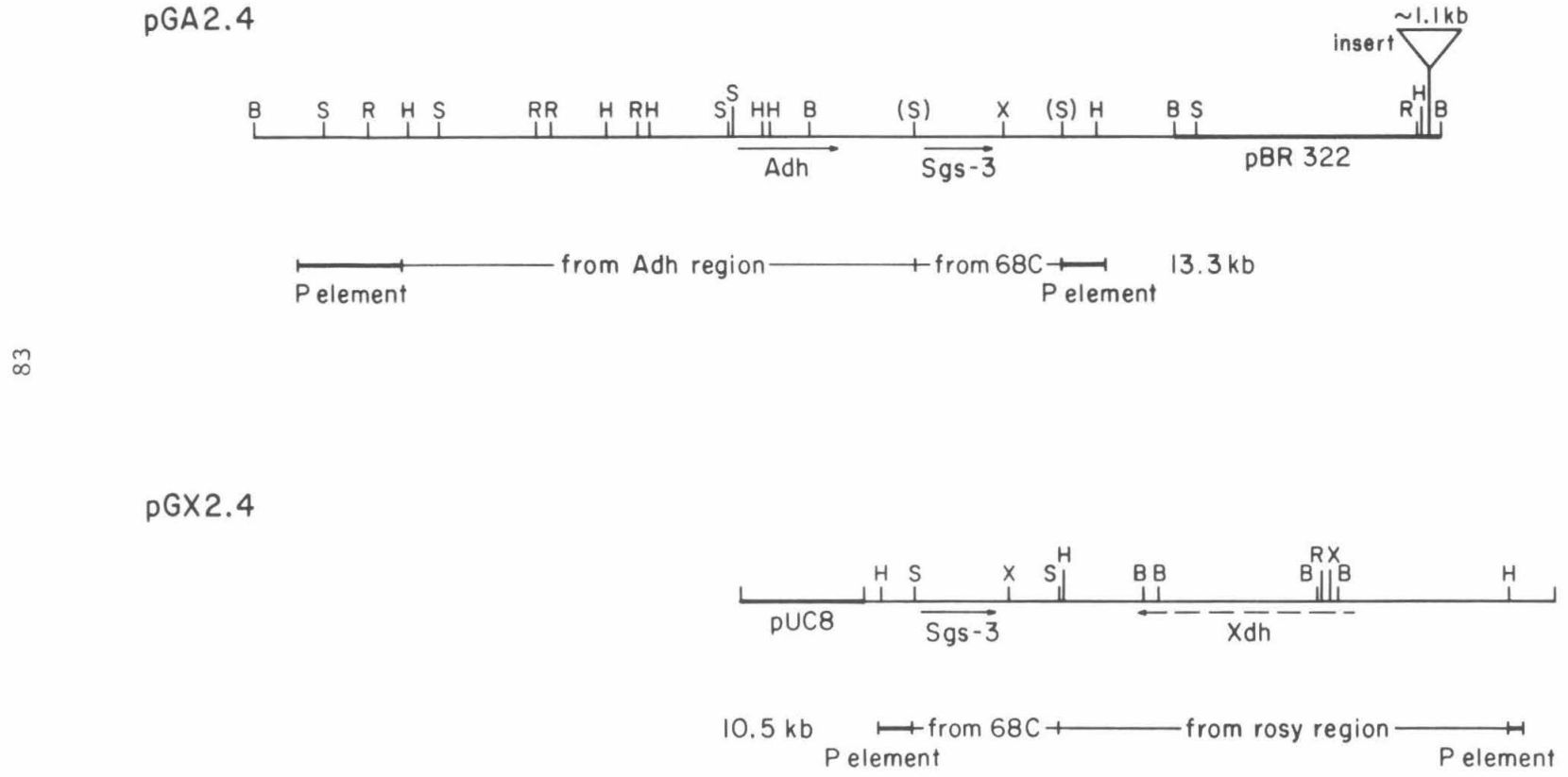

pGA2.4
~1.1kb
insert
B S R H S RR H RH S S HH B (S) X (S) H B S R H B
Adh
Sgs-3
pBR 322
P element
from Adh region
from 68C
P element
13.3 kb
pGX2.4
H S X S H B B R X B B H
pUC8
Sgs-3
Xdh
10.5 kb
P element
from 68C
from rosy region
P element
0 1 2 3 kb

Figure 2. RNA blot of *Tf(2)GA2.4-1* Transformants at Different Developmental Stages

RNA isolated from the salivary glands and carcasses of homozygous *Tf(2)GA2.4-1* animals at different stages was fractionated by size on a 1.5% agarose-formaldhyde gel. Except where indicated otherwise, the RNA from two animals was used for each lane. The gel was base-treated, blotted to a nitrocellulose filter, and the filter was hybridized with ^{32}P-labeled probes.

Lane 1: whole second instar larvae, ten animals.

Lane 2: whole early third instar larvae, six animals.

Lane 3: carcasses (with salivary glands removed) of mid third instar larvae, three animals.

Lane 4: carcasses (with salivary glands removed) of late third instar larvae (pre-climbing)

Lane 5: carcasses (with salivary glands removed) of late third instar larvae (early climbing).

Lane 6: carcasses (with salivary glands removed) of late third instar larvae (late climbing).

Lane 7: carcasses (with salivary glands removed) of white prepupae.

Lane 8: carcasses (with salivary glands removed) of tan prepupae.

Lane 9: salivary glands of third instar larvae of Oregon-R strain (standard).

Lane 10: salivary glands of third instar larvae of Formosa strain (standard).

Lane 11: salivary glands of mid third instar larvae; three animals.

Lane 12: salivary glands of late third instar larvae (pre-climbing).

Lane 13: salivary glands of late third instar larvae (early climbing).

Lane 14: salivary glands of late third instar larvae (late climbing).

Lane 15: salivary glands of white prepupae.

Lane 16: salivary glands of tan prepupae.

A) The probe used was aDm 2023, which is homologous with *Sgs-3* RNA. Prior to polyadenylation the $Sgs\text{-}3^{OR}$ RNA is 1120 nt and the $Sgs\text{-}3^{For}$ RNA is 820 nt. A longer exposure of lanes 9-16 is shown to the right. B) The probe was removed from the filter in (A) with boiling 0.01 x SSPE, 0.1% SDS and the filter was rehybridized with bDm103, which contains the Drosophila rRNA coding region. This control experiment reveals that there is very little RNA in lane 7; a repeat experiment for this data point was performed and showed no detectable *Sgs*-3-homologous RNA in the carcasses of white prepupae.

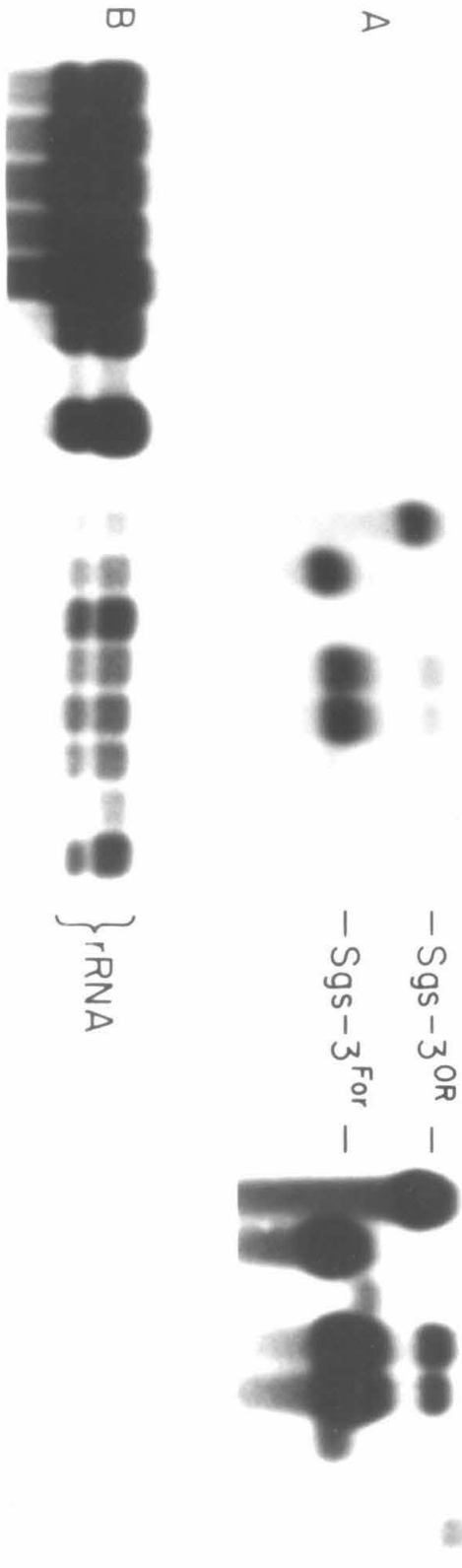

B
A
rRNA
— Sgs-3 OR —
— Sgs-3 For —
1 whole 2nd
2 whole early 3rd
3 mid 3rd (-S.G.)
4 late 3rd (-S.G.)
5 late 3rd (-S.G.)
6 late 3rd (-S.G.)
7 white prepupae (-S.G.)
8 tan prepupae (-S.G.)
9 standard: Oregon-R
10 standard: Formosa
11 S.G. of mid 3rd
12 S.G. of late 3rd
13 S.G. of late 3rd
14 S.G. of late 3rd
15 S.G. of white prepupae
16 S.G. of tan prepupae
9 standard: Oregon-R
10 standard: Formosa
11 S.G. of mid 3rd
12 S.G. of late 3rd
13 S.G. of late 3rd
14 S.G. of late 3rd
15 S.G. of white prepupae
16 S.G. of tan prepupae

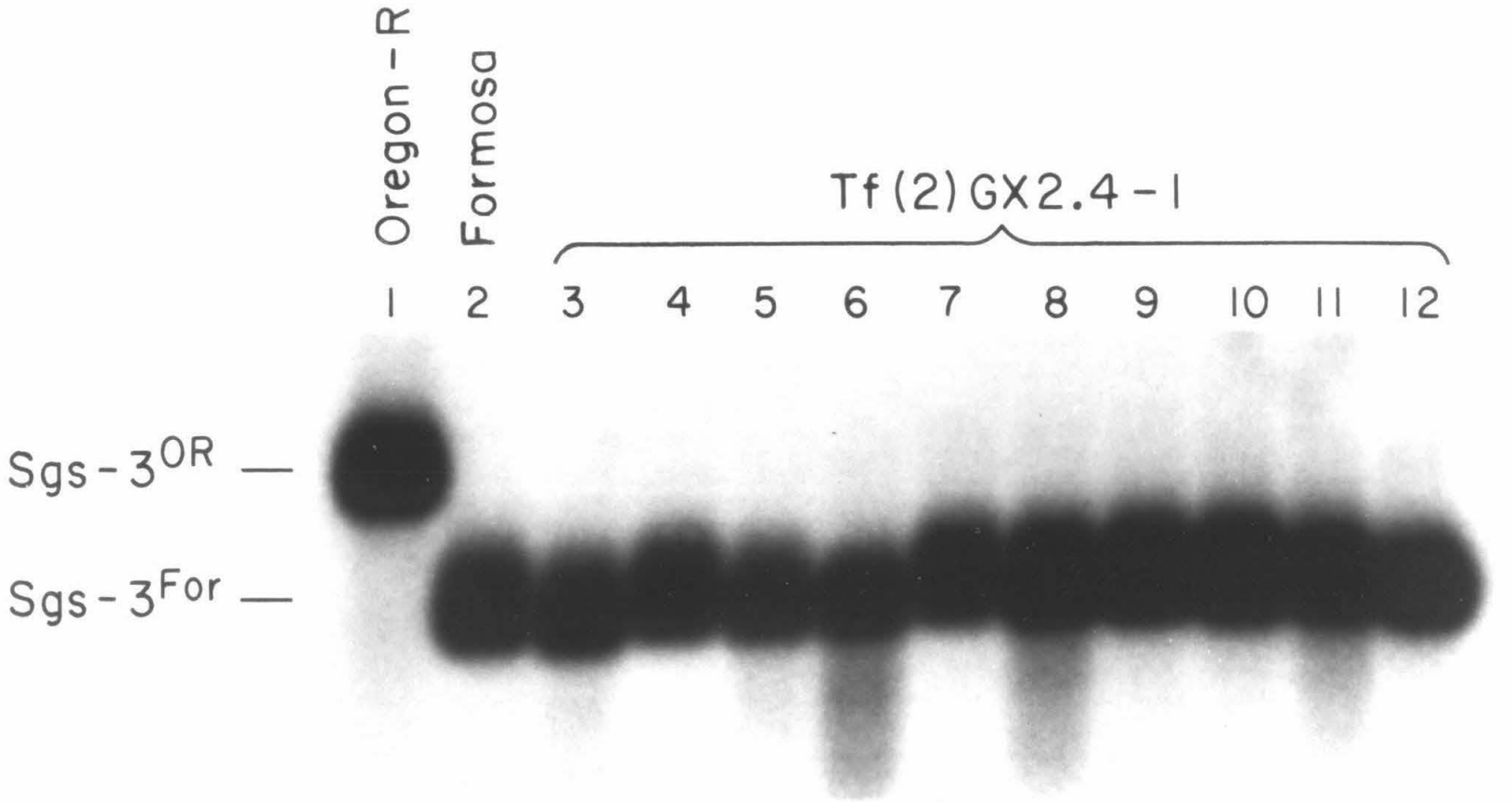
Figure 3. RNA Blot of Salivary Gland RNA from *Tf(2)GX2.4-1* Transformants

RNA isolated from the salivary glands of homozygous *Tf(2)GX2.4-1* late third instar larvae was fractionated by size on a 1.5% agarose-formaldehyde gel. RNA from the salivary glands of a single animal was used in each lane. The gel was base-treated, blotted to a nitrocellulose filter, and the filter was hybridized with a ^{32}P-labeled probe homologous with *Sgs-3*.

Lane 1: salivary glands of third instar larvae of Oregon-R strain (standard); prior to polyadenylation this RNA is 1120 nt in length.

Lane 2: salivary glands of third instar larvae of Formosa strain (standard); prior to polyadenylation this RNA is 820 nt in length.

Lanes 3-12: salivary glands of single Tf(2)GX2.4-1 third instar larvae.


Oregon-R
Formosa
Tf(2)GX2.4-1
1
2
3
4
5
6
7
8
9
10
11
12
Sgs-3OR
Sgs-3For

CHAPTER 4

Lethal mutations flanking the 68C glue gene cluster on chromosome 3 of *Drosophila melanogaster*

(accepted for publication in Genetics)

Summary

We have conducted a genetic analysis of the region flanking the 68C glue gene cluster in *Drosophila melanogaster* by isolating lethal and semi-lethal mutations uncovered by deficiencies which span this region. Three different mutagens were used: ethyl methanesulfonate (EMS), ethyl nitrosourea (ENU), and diepoxybutane (DEB). In the region from 68A3 to 68C11, 64 lethal, semi-lethal, and visible mutations were recovered. These include alleles of 13 new lethal complementation groups as well as new alleles of rotated, low xanthine dehydrogenase, lethal(3)517 and lethal(3)B76. Six new visible mutations from within this region were recovered on the basis of their reduced viability; all proved to be semi-viable alleles of lethal complementation groups. No significant differences were observed in the distributions of lethals recovered using the three different mutagens. Each lethal was mapped on the basis of complementation with overlapping deficiencies, mutations that mapped within the same interval were tested for complementation, and the relative order of the lethal groups within each interval was determined by recombination. The cytological distribution of genes within the 68A3-68C11 region is not uniform: the region from 68A2,3 to 68B1,3 (seven to ten polytene chromosome bands) contains at least 13 lethal complementation groups and the mutation low xanthine dehydrogenase; the adjoining region from 68B1,3 to 68C5,6 (six to nine bands) includes the 68C glue gene cluster, but no known lethal or visible complementation groups; and the interval from 68C5,6 to 68C10,11 (three to five bands) contains at least three lethal complementation groups and the visible mutation rotated. The developmental stage at which lethality is observed was determined for a representative allele from each lethal complementation group.

Introduction

The salivary gland secretion (sgs) or glue protein genes of *Drosophila melanogaster* constitute a dispersed set of hormonally regulated genes that are expressed in one specific tissue during one defined stage of development. They encode a group of at least eight polypeptides that are synthesized in the salivary glands during the second half of the third larval instar and are subsequently, near the end of the larval period, secreted into the lumen of the gland. At the time of puparium formation this secretion is expelled, serving as a glue that anchors the puparium to the surface upon which it rests (Fraenkel and Brookes, 1953; Korge, 1975, 1977a; Beckendorf and Kafatos, 1976).

A number of prominent puffs, called the intermolt puffs, are visible on the giant polytene chromosomes of the larval salivary glands during the period of glue protein production. The regression of these puffs, which occurs several hours before puparium formation, coincides with an increase in the hemolymph titer of the steroid hormone ecdysterone (Becker, 1962). Genetic and molecular experiments have shown that the intermolt puffs contain the structural genes for the polypeptides that constitute the salivary gland glue (Korge, 1977b; Akam *et al.*, 1978; Muskavitch and Hogness, 1980; Velissariou and Ashburner, 1980, 1981; Crowley, Bond and Meyerowitz, 1983; Guild and Shore, 1984). On the basis of genetic mapping experiments, the 68C puff on the left arm of the third chromosome has been identified as the site of the *Sgs-3* glue gene locus (Akam *et al.*, 1978). Molecular cloning and analysis of DNA from the puff region have revealed three closely linked genes, contained within a region of DNA less than 5 kilobase-pairs in length, which encode the glue polypeptides sgs-3, sgs-7, and sgs-8 (Meyerowitz and Hogness, 1982; Crowley, Bond and Meyerowitz, 1983, Garfinkel, Pruitt and Meyerowitz, 1983). The effect of ecdysterone on the behavior of the 68C puff has been shown to be correlated with the response of the 68C glue gene

mRNAs to the hormone (Crowley and Meyerowitz, 1984). By *in situ* hybridization (Kress, Meyerowitz and Davidson, 1985) and analysis of chromosomal rearrangements (M. A. Crosby and E. M. Meyerowitz, unpublished results), a more precise cytological localization of the glue gene cluster has been made to 68C5,6.

In an effort to facilitate genetic analysis of the 68C glue genes, we have conducted a series of mutagenesis experiments designed to recover lethal mutations within a large region which includes the glue gene cluster. The results of this study are presented here. Although we were successful in obtaining a large number of lethal and semi-lethal mutations, many of which define previously unknown lethal complementation groups, none of the mutations recovered are in genes closely flanking the glue gene cluster.

Materials and Methods

Drosophila culture conditions and stocks: Unless indicated otherwise, Drosophila cultures were raised at 22° on standard cornmeal-sucrose-yeast-agar medium (Lewis 1960; application of live yeast was omitted). Allopurinol-supplemented medium was prepared by adding a solution of allopurinol (4-hydroxy-pyrazolo-pyrimidine, HPP; Sigma) to Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply). Initial *lxd* complementation tests were performed using 240 µg/ml allopurinol. At this concentration only adults that eclosed during the first two to three days could be reliably scored; among animals that eclosed later, some that were heterozygous for *lxd* showed a red-brown eye phenotype. Critical complementation tests were repeated using 150 µg/ml allopurinol (V. Finnerty, personal communication).

The Oregon-R, Canton-S, and Hikone-R wild-type strains were originally obtained from the Department of Biochemistry, Stanford University Medical Center, Stanford, California. The Oregon-R strain was used for all mutagenesis

experiments. In order to eliminate any pre-existing lethal or sterile mutations, prior to the mutagenesis experiments most of the third chromosome was made isogenic by isolating a single third chromosome over a *TM3* balancer chromosome and then creating a homozygous stock. This stock was designated Ore-R-16f.

The chromosomal deficiencies used are listed in Table 1. The *Df(3L)vin* series of deficiencies is described in Akam *et al.* (1978) and was obtained from the Mid-America Stock Center, Bowling Green, Ohio. The *Df(3L)lxd* deficiencies were induced by D. R. Schott, M. C. Baldwin and V. Finnerty (unpublished experiments) and were obtained from Bruce Baker, University of California, San Diego, California. The mutations *l(3)B76*, *l(3)v4-2*, and *vin* are described in Akam *et al.* (1978) and were obtained from David Roberts, University of Oxford, Oxford, England. The lethal mutation *l(3)517* was obtained from Victoria Finnerty, Emory University, Atlanta, Georgia. *Tb* is described in Craymer (1980) and *TM6B* is described in Craymer (1984a); both were obtained from Loring Craymer, California Institute of Technology, Pasadena, California. Other mutations and balancer chromosomes used are described in Lindsley and Grell (1968) and were obtained from the Caltech Drosophila Stock Center, Pasadena, California.

Isolation of lethal and semi-lethal mutations in the 68A3 to 68C11 region: Ore-R-16f males less than two days old were fed a chemical mutagen (see below) and mated *en masse* to *Ser* Dr^{Mio} / *In(3R)C, l(3)a Sb* virgin females. Cultures were transferred twice, usually after three days and after six days; after nine days the parents were discarded. The scheme used to recover lethals in the 68A3-69A1 region is diagrammed in Figure 1 (see also Craymer, 1984b). All mutagenesis experiments were performed at 22°.

Originally, F_1 males of the genotype $+^*$ / *Ser* Dr^{Mio} were crossed to *Df(3L)vin*^{5}, Dr^{Mio} / *TM3, Sb Ser* females. It was expected that the *Ser* Dr^{Mio}/*TM3, Sb Ser* class would die and that the absence of Drop animals in the F_2

would indicate that the mutagenized chromosome contained a lethal mutation uncovered by *Df(3L)vin*^{5}. However, the *Ser Dr*^{Mio} chromosome proved not to be lethal over other *Ser* chromosomes. The lethal associated with *Ser* is apparently separable from it and was lost during the construction of the *Ser Dr*^{Mio} chromosome. The F_2 animals from the original experiment were screened by checking for the absence of a Stubble^{+} Drop class. Since a simpler screen that could be performed by looking through the sides of the culture vials was desirable, F_1 males of the genotype $+^{*}$ / *Ser Dr*^{Mio} were subsequently crossed to *Df(3L)vin*^{66}*+In(3LR)TM3, Sb Ser / Pr Dr* females as indicated in Figure 1.

All three mutagens were administered by adding to a 1% sucrose solution *and* allowing the flies to feed for 24 hours, following the method described by Lewis and Bacher (1968). Each mutagen was tested in small-scale experiments in which mutagenized males were crossed to attached-X females and the relative survival of males and females in the F_1 was observed. The highest concentration of ethyl methanesulfonate (EMS; Sigma) tested, 42 mM, did not significantly reduce male fertility and resulted in a mutation rate equivalent to 0.84 lethal hits per chromosome arm. This is in good agreement with results obtained by other investigators (for example, Nicklas and Cline, 1983). Since a high rate of mutation was desired, 42 mM EMS was used for the mutagenesis experiment. Ethyl nitrosourea (ENU; Sigma) was first made up as stock solution of 10 mg/ml in 10 mM acetic acid and 1 ml aliquots were frozen at -80^{o} for up to several months prior to use. In the attached-X tests, a range of 0.2 mg/ml to 0.4 mg/ml ENU was used (McCarron and Chovnick, 1981). The mutation rate was found to be independent of concentration within this range and was calculated to be equivalent to 0.62 lethal hits per chromosome arm. Both 0.2 mg/ml and 0.4 mg/ml ENU were used in the mutagenesis experiment. After these experiments were completed it was discovered that the mutation rate using ENU could be increased

by increasing the duration of the feeding period (E. B. Lewis, personal communication). Diepoxybutane (1,3-butadiene diepoxide; DEB; Aldrich) was tested in the attached-X assay at concentrations of 5 mM, 50 mM, and 500 mM. Use of 500 mM DEB resulted in death of the parental males; 50 mM DEB resulted in complete sterility of the mutagenized males. Mutagenesis using 5 mM DEB produced X-linked lethal mutations at a rate equivalent to 0.46 hits per chromosome arm. The first DEB mutagenesis experiments were performed using the mutagen at a concentration of 5 mM; later, a higher frequency of lethal mutations was obtained using 10 mM DEB.

The mutations recovered were assigned to one of eight subregions by determining whether they were uncovered by deficiencies with breakpoints in the region. The series of *Df(3L)vin* deficiencies listed in Table 1 was used. Only mutations which were uncovered by *Df(3L)vin*^{5} but were not uncovered by *Df(3L)vin*^{6}, mapping to the region from 68A3 to 68C11, were characterized further.

Assignment of mutations to complementation groups: Complementation crosses were raised in half-pint milk bottles to avoid crowding. Crosses were done between mutations balanced over *TM3, Sb Ser* and the absence of any Stubble^{+} flies in more than 100 progeny was used as the criterion for failure to complement. Of the 61 lethal or semi-lethal mutations recovered in the 68A3-68C11 interval, 58 fell into one subregion, that uncovered by *Df(3L)vin*^{5} or *Df(3L)vin*^{66}, but not by *Df(3L)vin*^{4}. The first 11 mutations that mapped to this subregion were tested for complementation in all possible pair-wise combinations. As complementation groups were established a single allele from each group was used in tests with subsequent mutations. Originally, three different alleles from the complex *l(3)C28* locus, representing the complementation groups designated *l(3)C28*^{a}, *l(3)C28*^{b}, and *l(3)C28*^{c}, were used in tests of new mutations. For later

complementation tests only one allele, representing *l(3)C28*^{a}, was used. After all new mutations had been assigned to a complementation group, alleles of each group were tested in all pair-wise combinations. After the approximate order and lethal stage of the genes in this region were determined (see below) it was discovered that two pairs of adjacent loci exhibited lethality at the same stage. All combinations of alleles between each pair of loci were tested and all proved to complement all the alleles of the other locus.

The three remaining mutations mapped to the subregion uncovered by *Df(3L)vin*^{7}, but not by *Df(3L)vin*^{6}. This region also contains the visible mutation rotated (*rt*). The new mutations were tested for complementation in all pair-wise combinations and each was tested for complementation with *rt*.

Complementation tests were not performed with the mutations which mapped to subregions uncovered by *Df(3L)vin*^{6}.

Cytology: Temporary larval salivary gland squashes were made by dissecting the glands in 45% acetic acid and staining in 2% orcein in equal parts lactic acid and glacial acetic acid. Cytological breakpoints were assigned using the maps of Bridges (1941) as reproduced in Lindsley and Grell (1968) and of Lefevre (1976).

Mapping crosses: An allele from each complementation group was crossed to *ru h th st cu sr e*^{s} *ca* and recombinant lethal chromosomes marked with *ru h* or *th st* (*cu sr e*^{s} *ca*) were recovered. These were used in the following mapping cross:

ru h l(3)x / l(3)y th st ♀♀ X *Df(3L)vin*^{5}, *ru h th st cp / Ser Dr*^{Mio} ♂♂.

In the F_1 all classes survive over the *Ser Dr*^{Mio} chromosome. Only the recombinant class resulting from a crossover between the two lethal mutations, producing *l(3)x*$^{+}$ *l(3)y*$^{+}$, will survive over *Df(3L)vin*^{5}, *ru h th st cp*. The mutation *Dr*^{Mio} causes an extreme reduction in the size of the eye; one Drop^{+} animal can

be easily scored amidst hundreds of Drop animals. Flanking markers can be scored immediately, indicating the order of the two lethal genes. In most cases, only *h* and *th* could be reliably scored. The expressivity of *ru* was quite variable. Due to the fact that some of the stocks used to detect and balance the lethal mutations contained the sex-linked mutation vermilion (*v*), recombinant males were often *v*, which cannot be distinguished from *st*. Reciprocal mapping crosses were made for each pair tested. For very close loci only the order of the genes was determined. Three or more recombinants recovered with at least one from each reciprocal class was considered to establish the order of a given pair. If at least ten recombinants were recovered between a pair of genes, the map distance was determined. Map distances were calculated as follows: (number of $Drop^+$) x 200 / (number of Drop). The standard deviation for each map distance was determined. Mapping crosses were performed at either 22^o or 25^o.

Determination of lethal stage: An allele from each complementation group was used in one of the following crosses:

l(3)x / Tb	♂♂	X	*Df(3L)lxd*^{8} / *TM6B, Tb*	♀♀
l(3)x / Tb	♂♂	X	*Df(3L)lxd*^{9} / *TM6B, Tb*	♀♀
l(3)x / Tb	♂♂	X	*Df(3L)vin*^{4} / *TM6B, Tb*	♀♀ .

The cultures were examined periodically to determine if $Tubby^+$ animals were present at each stage. If no $Tubby^+$ animals were observed for a particular stage, $Tubby^+$ animals from the preceding stage were removed to vial cultures and observed. By this procedure the hemizygous phenotypes of mutations which cause lethality at the second instar larval period or later were determined.

The *Tb* phenotype is very difficult to score in embryos and first instar larvae. For mutations in which $Tubby^+$ second instar larvae were not observed in the crosses described above, the following procedure was used to observe the

homozygous phenotype of embryos. For each mutation the outcrossed stocks used for recombination mapping experiments (see above) were crossed to two different wild-type stocks, Canton-S and Hikone-R. Crosses were performed with these heterozygous animals: *l(3)x th st* / + (C-S) X *ru h l(3)x* / + (H-R). After mating in vials for one to two days the animals were transferred to apple-agar plates (Nusslein-Volhard, Wieschaus and Kluding 1984) and allowed to lay eggs for two- to four-hour intervals. After 48 hours the plates were examined for dead embryos and the proportion of fertile eggs which resulted in dead embryos was determined.

For mutations for which the lethal stage appeared to be in the first larval instar the procedure above was repeated with the following changes: the lethal stock was crossed to one wild-type stock, and an appropriate deficiency (see above) was crossed to another wild-type stock. The offspring of these crosses were mated and allowed to lay eggs on apple-agar plates. After three days the plates were examined for the presence of first instar larvae, dead or alive. Live first instar larvae were transferred to vials for observation. Again, the hemizygous phenotypes were observed in these cases.

The crosses described above are designed to detect the latest stage to which animals homozygous or hemizygous for a given mutation are able to survive. Thus a mutation which causes lethality at more than one stage would not be accurately characterized.

Results

Using the mutagenesis scheme diagrammed in Figure 1, a total of 108 hemizygous lethal or semi-lethal mutations were recovered in the region from 68A3 to 69A1. The distribution of the mutations recovered, based upon their complementation behavior with overlapping deficiencies, is shown in Figure 2. The distribution is not uniform: the number of mutations recovered correlates

neither with the number of bands within a given interval nor with the physical length of the interval along the polytene chromosome. The majority of mutations map to a region of seven to ten bands, from 68A2,3 to 68B1,3. Within an interval which includes eight to twelve bands, from 68B1,3 to 68C8,10, no new lethal or semi-lethal mutations were recovered. One previously isolated semi-lethal mutation is known to exist within this region, however. Since the 68C glue gene cluster is located at 68C5,6, we were unsuccessful in obtaining mutations in loci closely flanking the glue protein genes.

In mutagenesis experiments with EMS, 2227 chromosomes were screened (1320 over *Df(3L)vin*^{5} and 907 over *Df(3L)vin*^{66}). Of the 64 lethal or semi-lethal EMS-induced mutations recovered, 30 are located within the 68A3 to 68C11 interval. From 2770 ENU-treated chromosomes screened (1223 over *Df(3L)vin*^{5} and 1547 over *Df(3L)vin*^{66}), 34 lethal or semi-lethal mutations were recovered and 23 of these are within the 68A3 to 68C11 interval. In DEB mutagenesis experiments 2950 chromosomes were screened, primarily over *Df(3L)vin*^{66} (2756; 194 were screened over *Df(3L)vin*^{5}). Ten lethal DEB-induced mutations were recovered, with eight of these mapping to the 68A3 to 68C11 region.

Failure to recover any lethals within the relatively large interval from 68B1,3 to 68C8,10 prompted us to determine whether animals heterozygous for deficiencies which overlap in this region (see Table 1) would survive. Animals of the genotype *Df(3L)lxd*^{6} / *Df(3L)vin*^{4} survive to adulthood and exhibit an upright or spread-wing phenotype with variable penetrance; the females show reduced fertility. Animals of the genotype *Df(3L)lxd*^{2} / *Df(3L)vin*^{4} also survive to adulthood and have the same wing phenotype; they also have a rough eye phenotype and the females are completely sterile. Thus a region of six to nine bands, from 68B1,3 to 68C5,7, is devoid of any genes required for the survival of the fly under laboratory conditions.

Complementation tests with the 58 mutations which mapped to the 68A2,3 to 68B1,3 region indicate that there are at least 13 lethal complementation groups within this interval (see Table 2), one of which exhibits intragenic complementation (discussed below). Two lethal mutations, *l(3)517* and *l(3)B76*, had been previously mapped to the 68A region; new alleles of both were recovered. This region is subdivided by two deficiencies that have breakpoints within it, *Df(3L)lxd*^{8} and *Df(3L)lxd*^{9} (Table 1). The biochemical mutation low xanthine dehydrogenase (*lxd*) and one of the lethal complementation groups described above, *l(3)C117*, fall within the interval uncovered by both *Df(3L)lxd*^{8} and *Df(3L)lxd*^{9}. The four alleles of *l(3)C117* were tested for complementation with *lxd*. All were found to complement the *lxd* red-brown eye phenotype that is visible when the flies are cultured on a medium supplemented with the xanthine dehydrogenase inhibitor, allopurinol (Keller and Glassman, 1965). Thus, there are at least 14 complementation groups within this region that spans seven to ten polytene chromosome bands.

The three mutations recovered within the 68C8,10 to 68C10,11 interval define two different complementation groups, one of which is semi-lethal (see Table 2). All three mutations complement the visible mutation rotated (*rt*).

The number of alleles from each complementation group recovered with EMS, ENU, and DEB is indicated in Table 2. On the basis of χ^2 tests applied to each complementation group, there is no significant difference in the distribution of lethals recovered with the three different mutagens. Excluding mutations within the complex complementation group *l(3)C28*, which are subject to interpretation, no mutation was observed to span more than one complementation group.

In addition to the lethal and semi-lethal mutations discussed above, three visible mutations from within the 68A3 to 68C11 region were recovered during

these experiments. An EMS-induced allele of *rt* was discovered by observation and an ENU-induced *rt* allele was recovered because it arose simultaneously with a lethal within the 68F region. A third mutant, recovered in an EMS mutagenesis experiment, was investigated because it was observed to have a prolonged time of development to adulthood. When homozygous or hemizygous, this mutant exhibits a red-brown eye color phenotype (on regular medium). By deficiency mapping it was determined that the mutation is located within the same interval as *l(3)C117* and *lxd*. Tested both on regular food and on medium supplemented with allopurinol, the new mutation complements all alleles of *l(3)C117*. On regular medium it also complements *lxd*; however, on medium with allopurinol it fails to complement *lxd*. Thus, it appears to be a new, visible, allele of *lxd*.

Alleles of the lethal complementation group designated *l(3)C28* exhibit complex complementation interactions. These are diagrammed in Figure 3. Two of the complementing alleles ($l(3)C28^{b1}$ and $l(3)C28^{c1}$) have been separated by recombination and are approximately 0.07 (+0.04) cM apart.

Figure 4 shows a map of the genes within the 68A3 to 68C11 region, based both on deficiency mapping and recombinational mapping. Attempts to map *l(3)B76* by recombination were abandoned since both alleles are semi-lethal and surviving adults have a phenotype of variable penetrance, overlapping wildtype. The only genes known to be located within a region spanning approximately 1.0 cM, between *l(3)C28* and *l(3)v4-2*, are the 68C glue genes. Immediately distal to this region there are at least 14 genes within approximately 0.7 cM. Proximal to the 68C glue genes there are at least four complementation groups within a region of approximately 0.4 cM.

In the course of the recombinational mapping experiments, exceptional recombinants were obtained in a number of cases. The most commonly observed exceptional event (ten cases) was the appearance of a wild-type recombinant in

crosses in which all other recombinants were of the *h th* phenotype. This was observed for many different pairs of genes. The reciprocal event was never observed. In the second type of event, a recombinant which retained one of the parental combinations of flanking markers was observed. This occurred in three cases, all between the same two alleles: $l(3)C493^{a}$ and $l(3)C605^{b}$. In all three cases the recombinant animal displayed the flanking markers of the $l(3)C605^{b}$ parent.

Discussion

This study adds the region from 68A3 to 68C11 to the expanding list of small chromosomal regions for which the distribution of lethal and semi-lethal mutations has been examined (see, for example, Judd, Shen and Kaufman, 1972; Woodruff and Ashburner, 1979; Hilliker *et al.*, 1980; Wright *et al.*, 1981; Nicklas and Cline, 1983; Roberts *et al.*, 1985). As has been the case in many such studies (Barrett, 1980), significant differences in gene mutability produced results which fail to fit a Poisson distribution, thus no attempt has been made to estimate the number of undetected lethal loci within the region. The distribution of lethal complementation groups within the 68A3-68C11 interval is striking in its lack of uniformity. There are at least 13 lethal complementation groups within the 68A2,3 to 68B1,3 interval, a region which contains seven to ten bands (or chromomeres) in larval salivary gland polytene chromosomes. The adjacent interval from 68B1,3 to 68C5,6, which spans six to nine polytene chromosome bands, contains no known vital genes. In fact, this region is completely expendable for survival of the fly under laboratory conditions. Animals heterozygous for deficiencies which overlap within this region survive to adulthood; they do exhibit some phenotypic abnormalities and the females are sterile or reduced in fertility. The interval from 68C5,6 to 68C10,11, which spans

three to five bands, contains at least three lethal complementation groups. These results contribute to the growing evidence that there can be a wide variation in the number of lethal complementation units per polytene chromosome band (Lefevre 1981; Zhimulev *et al.* 1981; WRIGHT *et al.* 1981; Nicklas and Cline 1983). While the function of the chromomeres of polytene chromosomes is still unknown, our observations are consistent with the interpretation of Zhimulev and Belyaeva (1975) and Skaer (1977) that interbands and puffs are the transcriptionally active regions of the chromosome. Bands are condensed, quiescent regions of the DNA (as opposed to control regions for the adjoining interbands, see Crick 1971). The banding pattern observed in different stages and tissues is relatively constant due to the large number of genetic functions necessary for the basal metabolism of the cell. According to this model, either a band or an interband may contain more than one transcription unit.

The region from 68A3,4 to 68B4,C1 has also been studied by Campbell, Hilliker and Phillips (1985) who have recovered 28 EMS-induced and 73 PM-hybrid-dysgenesis-induced mutations uncovered by $Df(3L)lxd^{9}$. By complementation analysis they establish that there are at least ten lethal complementation groups in this region. We find 11 lethal complementation groups within the same interval. Which genes defined by Campbell, Hilliker and Phillips (1985) correspond to those described in this work has not been established.

We have been unsuccessful in our attempt to find mutations in genes which closely flank the glue protein genes at 68C5,6. The glue genes *Sgs-3*, *Sgs-7*, and *Sgs-8* are the only known genes within a region of approximately 1.0 cM, between *l(3)C28* and *l(3)v4-2*. Since animals that are completely deficient for most of this region do exhibit visible abnormalities and are female-sterile, it is reasonable to assume that several nonessential loci are located within the interval. Similar, relatively barren, chromosomal regions appear to exist around the Notch (Lefevre,

1981) and *Sxl* (Nicklas and Cline, 1983) loci and in the 34C5 region (Wright *et al.*, 1981). Of course, these regions may simply contain "cryptic" genes, lesions in which do not confer lethal or visible phenotypes. The glue protein genes fall into this category.

The distributions of lethals obtained for the three different mutagens used, EMS, ENU, and DEB, are not significantly different (see Table 2). Only among genes for which a very small number of alleles were recovered are there cases of complementation groups detected by just one of the three mutagens. None of the mutations recovered in these experiments uncovers more than one complementation group (see discussion of *l(3)C28*, below). In the cases of EMS and ENU, this result agrees with findings of Lim and Snyder (1974) and others (Liu and Lim, 1975; Hilliker *et al.*, 1980; Wright *et al.*, 1981; Roberts *et al.* 1985) that monofunctional alkylating agents rarely produce deletions in euchromatic regions of the Drosophila genome. There is evidence that EMS does produce a high frequency of chromosomal rearrangements in heterochromatic regions (Bishop and Lee, 1969, 1973). In the case of DEB, Shukla and Auerback (1980) have postulated that this bifunctional mutagen produces a very high frequency of small deletions, of sufficient size to uncover two or more adjacent loci. We obtained only eight DEB-induced mutations in the 68A3-68C11 region; none of these uncovers more than one known lethal complementation group.

One of the lethal complementation groups defined in this study, *l(3)C28*, exhibits intragenic complementation (Figure 3). We classify this cluster of mutations as a single complementation group for the following reasons: first, there exist two alleles which fail to complement all other alleles; second, the two complementing alleles for which the stage of lethality has been determined show the same lethal phenotype; and third, although the complementation pattern could also be explained by assuming that a number of the mutations are small deletions

uncovering two or more adjacent genes, the occurrence of five such small deficiencies in this one region and none elsewhere is very unlikely. Many of the complex loci in Drosophila have complementing alleles, including dumpy (Carlson, 1959; Grace, 1980), Notch (Welshons and von Halle, 1962), and bithorax (Lewis, 1963, 1967, 1978). Molecular clones from the Notch and bithorax regions have been isolated (Artavanis-Tsakonas, Muskavitch and Yedvobnick, 1983; Kidd, Lockett and Young, 1983; Bender *et al.*, 1983; Artavanis-Tsakonas *et al.*, 1984) and the bases for the complex complementation interactions observed in these systems may soon be elucidated. The complementation pattern for the *l(3)C28* locus is most similar to that observed in the case of the rudimentary locus (Carlson, 1971; Jarry, 1979). DNA sequences corresponding to the rudimentary gene have also been cloned (Segraves *et al.* 1983, 1984). The rudimentary locus encodes a multifunctional polypeptide which catalyzes the first three steps in *de novo* pyrimidine biosynthesis (Rawls and Fristrom, 1975). The multifunctional character of the gene product explains many of the unusual complementation interactions observed.

In the course of the recombinational mapping experiments a number of exceptional recombinants were observed. These fall into two classes. In the less frequent class (three cases) the recombinant displayed one of the parental combinations of flanking markers. These can be explained by the occurrence of a double crossover or by gene conversion (Ballatyne and Chovnick, 1971). A spontaneous reversion of one of the lethals could also produce this type of result. The predominant class (ten cases) of exceptional events was the occurrence of a wild-type recombinant in crosses otherwise producing *h th* recombinants. This was observed for many different pairs of genes. The reciprocal case was never observed. Although this type of event has been observed in other studies (Carlson, 1971), that it should be the most common exceptional occurrence is surprising. It

requires three crossovers within a region of 16.7 cM or a gene conversion accompanied by a reciprocal recombination outside the region subject to the gene conversion event. (A gene conversion coupled with reciprocal recombination within the same region produces a chromosome containing one of the two lethals and would not be detected in these experiments.) An explanation invoking multiple crossovers or gene conversion is probably incorrect or only partially correct, considering the relatively high frequency with which this event occurred and the fact that the reciprocal event was never observed.

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Table 1

Description of deficiencies used in this study

Deficiency	Breakpoints	Reference
Df(3L)vin^3	68C5,6; 68E3,4	AKAM *et al.* 1978
Df(3L)vin^4	68B1,3; 68F3,6	"
Df(3L)vin^5	68A2,3; 69A1,3	"
Df(3L)vin^6	68C8,11; 69A4,5	"
Df(3L)vin^7	68C8,11; 69B4,5	"
Df(3L)vin^{66}	68A2,3; 68D3	"
Df(3L)lxd^2	68A2,3; 68C5,7	D. R. SCHOTT, M. C. BALDWIN & V. FINNERTY, unpublished experiments
Df(3L)lxd^6	67E1,2; 68C1,2	"
Df(3L)lxd^8	68A2,3; 68A5,6	"
Df(3L)lxd^9	68A3,4; 68B4,C1	"

The breakpoints of *Df(3L)vin*^{3} and *Df(3L)vin*^{4} are those observed in this laboratory and differ from the breakpoints designated in AKAM *et al.* Cytological analyses of the *Df(3L)lxd* deficiencies were performed by BRUCE BAKER (personal communication) and in this laboratory.

Table 2

Alleles of the lethal genes within the 68A3 to 68C11 region

Gene	Alleles	Mutagen	Lethal Stage	Comments
l(3)C288				New nomenclature: *l(3)68Aa*
	a*	EMS	Early third instar	
	b, c, d	EMS		
	e	ENU	Semi-lethal	Adults small with thin bristles.
	f, g	ENU		
	h, i	DEB		
l(3)C493				New nomenclature: *l(3)68Ab*
	a*	DEB	First instar	
	b, c	EMS		
	d	EMS	Semi-lethal	Adults have rough eye phenotype.
l(3)C117				New nomenclature: *l(3)68Ac*
	a*	EMS	Second instar	
	b, c	ENU		
	d	EMS		
l(3)517				New nomenclature: *l(3)68Ad*
	l	?		Previously isolated allele.
	b*	EMS	Third instar	
	c	ENU		
	d	EMS	Semi-lethal	Adults have dusky wings; males sterile

Table 2 (continued)

l(3)C178				New nomenclature: *l(3)68Ae*
	a*	EMS	Early pupal	
	b	ENU		
	c	ENU	Semi-lethal	Adults have small wings, malformed legs; variable penetrance.
	d, e	DEB		
l(3)C206				New nomenclature: *l(3)68Af*
	a*	ENU	Early pupal	
	b	ENU		
l(3)C404				New nomenclature: *l(3)68Ag*
	a*	ENU	Second instar	
	b, c, d	ENU		
	e	EMS	Semi-lethal	Adults have small, thin bristles.
l(3)C11				New nomenclature: *l(3)68Ah*
	a*	EMS	Embryonic	
	b	ENU		
l(3)C46				New nomenclature: *l(3)68Ai*
	a*	EMS	Late first/early second instar	
	b, c	EMS		
	d	EMS	Semi-lethal	Adults have upright or outspread wings; penetrance

Table 2 (continued)

				variable; females sterile, males reduced in fertility.
	e, f, g	ENU		
	h	ENU	Semi-lethal (hemizygous)	Phenotype as for *l(3)C46*^{d}; viability of homozygotes fair to good.
	i	DEB		
l(3)C141				New nomenclature: *l(3)68Aj*
	a*	EMS	Early pupal	
	b	EMS		
	c	DEB		
l(3)C557				New nomenclature: *l(3)68Ak*
	a*	ENU	Early pupal	
l(3)C28				New nomenclature: *l(3)68Al* Complex complementation interactions.
	a1, a2	EMS		
	b1*	EMS	Late embryonic/early first instar	
	b2	EMS	Temperature sensitive	
	c1*	EMS	Late embryonic/early first instar	
	c2	EMS		
	c3, c4	ENU		

Table 2 (continued)

	d1	EMS		
	e1	ENU		
l(3)B76				New nomenclature: *l(3)68Am*
	1	?	Late pupal/adult (semi-lethal)	Previously isolated allele; phenotype as for *l(3)B76*^{b}.
	b	EMS	Late pupal/adult (semi-lethal)	Adults have malformed legs and, less often, stunted wings; variable penetrance.
l(3)v4-2				New nomenclature: *l(3)68Ca*
	1*	EMS	Late pupal/adult (semi-lethal); temperature sensitive	Previously isolated allele.
l(3)C605				New nomenclature: *l(3)68Cb*
	a*	EMS	Late third instar	
	b*	DEB		
l(3)C70				New nomenclature: *l(3)68Cc*
	a*	EMS	Late pupal/adult (semi-lethal)	Adults have dusky wings, thin bristles.

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*alleles used for recombinational mapping

EMS=ethyl methane sulfonate

ENU=ethyl nitrosourea

DEB=diepoxybutane

New nomenclature is that proposed by DAN LINDSLEY and will be used in the revised edition of Genetic Variations of *Drosophila melanogaster*.

FIGURE LEGENDS

Figure 1. Mutagenesis scheme used to recover recessive lethal and semi-lethal mutations in the region from 68A3 to 69A1. All crosses were performed at 22°.

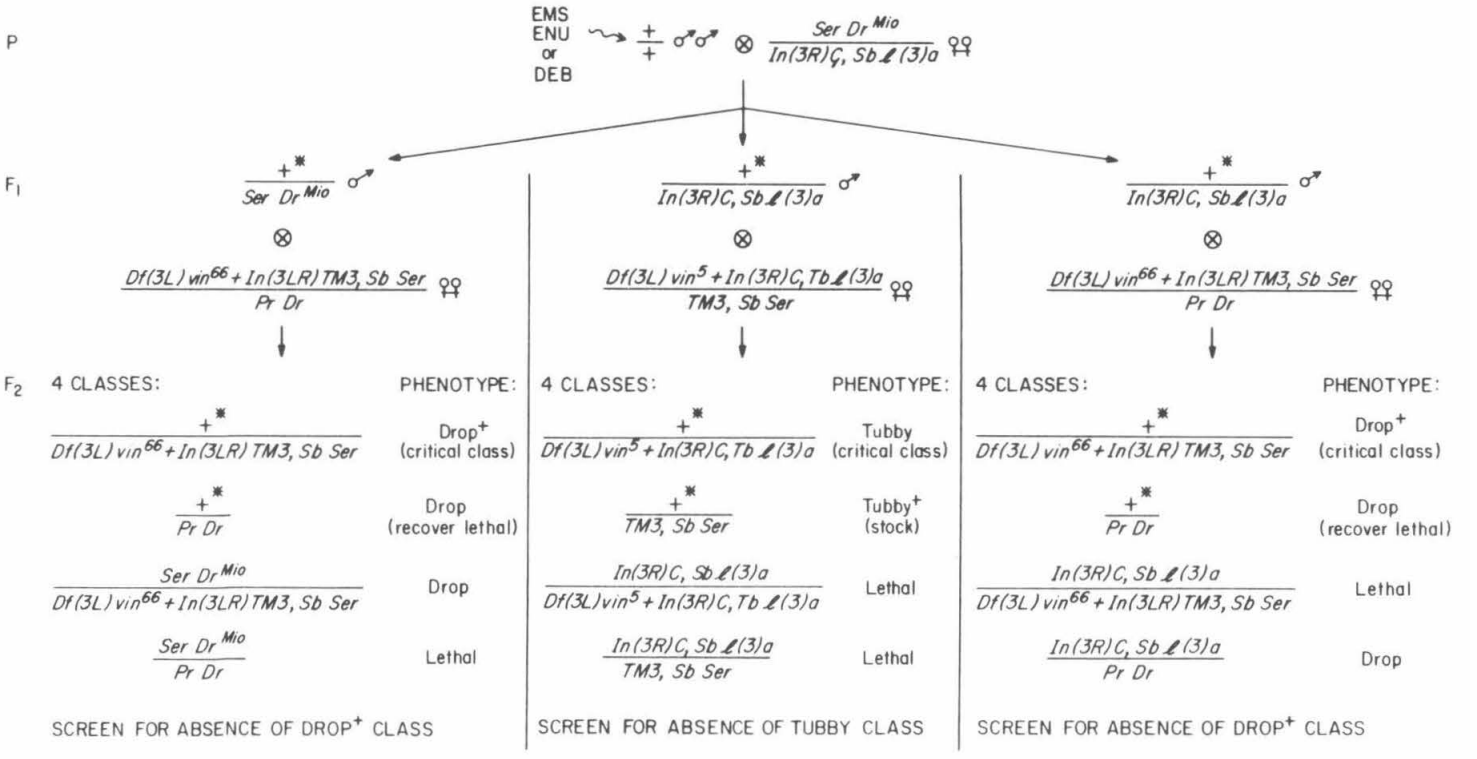
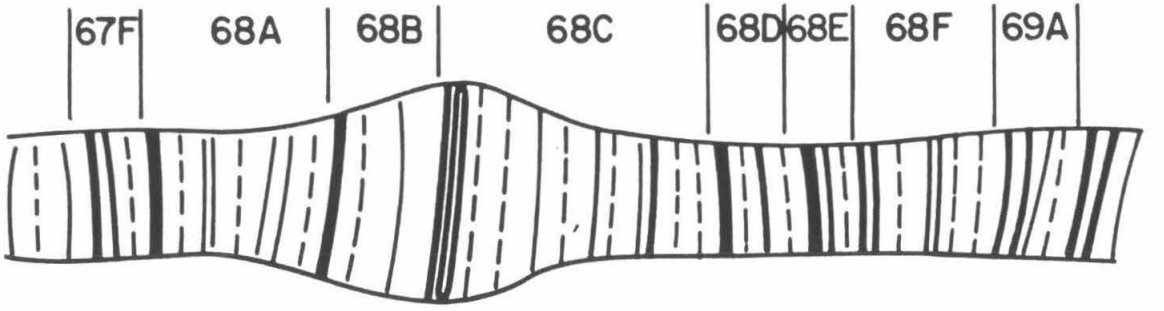
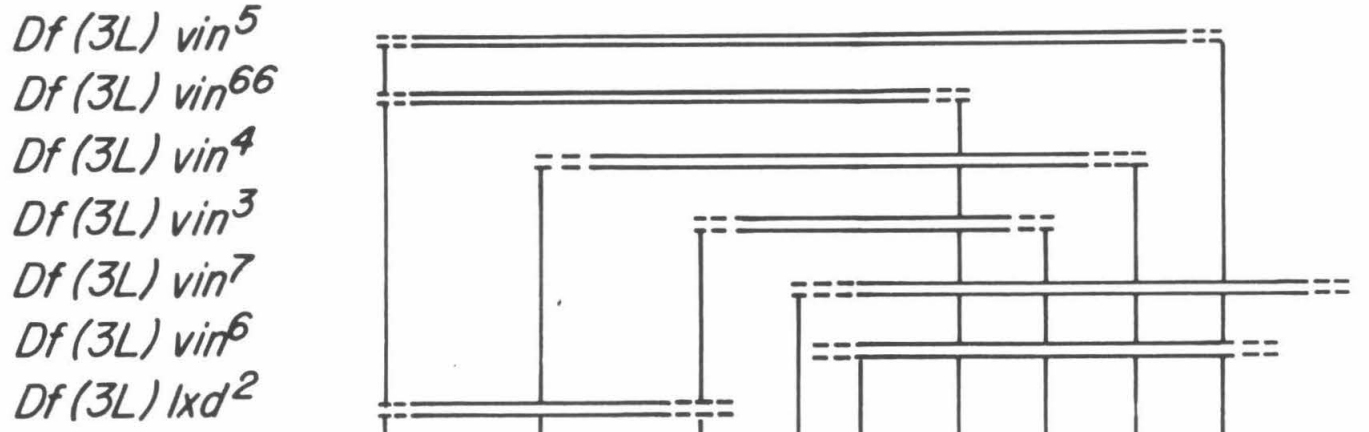

P
EMS
ENU
or
DEB
+/+ ♂♂ ⊗ Ser Dr Mio / In(3R)Ç, Sb ℓ(3)a ♀♀
F1
+* / Ser Dr Mio ♂
⊗
Df(3L) vin66 + In(3LR) TM3, Sb Ser / Pr Dr ♀♀
+* / In(3R)C, Sb ℓ(3)a ♂
⊗
Df(3L) vin5 + In(3R)C, Tb ℓ(3)a / TM3, Sb Ser ♀♀
+* / In(3R)C, Sb ℓ(3)a ♂
⊗
Df(3L) vin66 + In(3LR) TM3, Sb Ser / Pr Dr ♀♀
F2
4 CLASSES:
PHENOTYPE:
+* / Df(3L) vin66 + In(3LR) TM3, Sb Ser
Drop+ (critical class)
+* / Pr Dr
Drop (recover lethal)
Ser Dr Mio / Df(3L) vin66 + In(3LR) TM3, Sb Ser
Drop
Ser Dr Mio / Pr Dr
Lethal
SCREEN FOR ABSENCE OF DROP+ CLASS
4 CLASSES:
PHENOTYPE:
+* / Df(3L) vin5 + In(3R)C, Tb ℓ(3)a
Tubby (critical class)
+* / TM3, Sb Ser
Tubby+ (stock)
In(3R)C, Sb ℓ(3)a / Df(3L) vin5 + In(3R)C, Tb ℓ(3)a
Lethal
In(3R)C, Sb ℓ(3)a / TM3, Sb Ser
Lethal
SCREEN FOR ABSENCE OF TUBBY CLASS
4 CLASSES:
PHENOTYPE:
+* / Df(3L) vin66 + In(3LR) TM3, Sb Ser
Drop+ (critical class)
+* / Pr Dr
Drop (recover lethal)
In(3R)C, Sb ℓ(3)a / Df(3L) vin66 + In(3LR) TM3, Sb Ser
Lethal
In(3R)C, Sb ℓ(3)a / Pr Dr
Drop
SCREEN FOR ABSENCE OF DROP+ CLASS

Figure 2. Distribution of lethal and semi-lethal mutations recovered in the 68A3 to 69A1 region. At the top of the figure is a representation of the region from 67F to 69A on the left arm of the third chromosome (after BRIDGES 1941, as reproduced in LINDSLEY and GRELL 1968). Immediately below are diagrammed the extents of the deficiences that were used for mapping the newly induced mutations. The number of mutations recovered in each interval is indicated at the bottom of the figure.





Bands in interval	7-10	6-9	2-4	1-3	6-9	6-8	3-7	4-8
Number of mutants recovered:								
With *Df(3L) vin*^{5}	27	0	0	0	6	4	11	17
With *Df(3L) vin*^{66}	31	0	0	3	9	–	–	–

Figure 3. Complementation data for the complex locus *l(3)C28*. Survival of the *trans* heterozygote is indicated by "+"; "-" indicates no surviving heterozygotes. The number of alleles in each complementation sub-group is indicated in parentheses. Complementation crosses were performed at 22°, except in the case of the temperature-sensitive allele, $l(3)C28^{b2}$, for which they were performed at 25°.

	l(3)C28^{a}	*l(3)C28*^{b}	*l(3)C28*^{c}	*l(3)C28*^{d}	*l(3)C28*^{e}
l(3)C28^{a} (2)	−	−	−	−	−
l(3)C28^{b} (2)		−	+	−	−
l(3)C28^{c} (4)			−	+	−
l(3)C28^{d} (1)				−	+
l(3)C28^{e} (1)					−

HYPOTHETICAL
COMPLEMENTATION UNITS

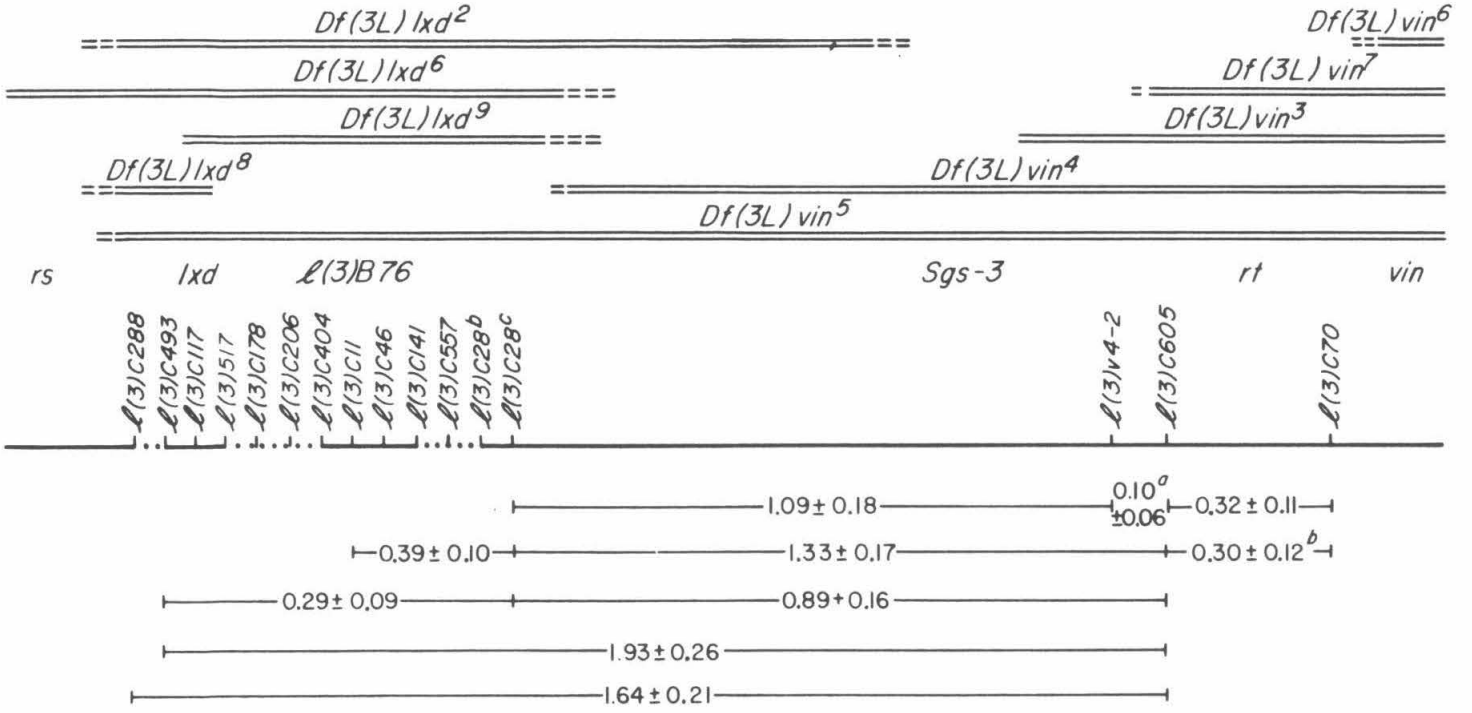
I II III

l(3)C28^{a}

l(3)C28^{b} *l(3)C28*^{c}

l(3)C28^{d} *l(3)C28*^{e}

Figure 4. Map of the 68A3 to 68C11 region based on recombinational and deficiency mapping. The deficiencies used are diagrammed at the top of the figure. Immediately below are indicated the loci mapped by deficiency mapping only (written horizontally). Genes mapped by recombinational analysis (written vertically) are listed along the central line. For pairs of genes separated by a dotted line, fewer than three recombinants were recovered; they are listed in probable order. Indicated at the bottom of the figure are the recombinational map distances obtained. For each pair of genes for which a map distance was determined, at least ten recombinants were obtained (except in the case marked *a*) and at least 5000 animals were scored (except in the case marked *b*).


Df(3L) lxd2
Df(3L) lxd6
Df(3L) lxd9
Df(3L) lxd8
Df(3L) vin6
Df(3L) vin7
Df(3L) vin3
Df(3L) vin4
Df(3L) vin5
rs
lxd
ℓ(3)B76
Sgs-3
rt
vin
ℓ(3)C288
ℓ(3)C493
ℓ(3)C117
ℓ(3)517
ℓ(3)C178
ℓ(3)C206
ℓ(3)C404
ℓ(3)C11
ℓ(3)C46
ℓ(3)C141
ℓ(3)C557
ℓ(3)C28b
ℓ(3)C28c
ℓ(3)v4-2
ℓ(3)C605
ℓ(3)C70
1.09 ± 0.18
0.10a ±0.06
0.32 ± 0.11
0.39 ± 0.10
1.33 ± 0.17
0.30 ± 0.12b
0.29 ± 0.09
0.89 + 0.16
1.93 ± 0.26
1.64 ± 0.21

APPENDIX

The Hikone-R Strain Carries a Small Duplication which Includes the 68C Glue Gene Cluster

On the basis of whole genome DNA blots it was determined that the Hikone-R wild-type strain carries a duplication of the region surrounding the 68C glue gene cluster. It is probable that this duplication is tandem, based on the following: (1) A consistent restriction map based on the results of the whole genome DNA blots (Figure 1); (2) Both *Sgs-3* genes segregate with the third chromosome in genetic crosses; (3) Only one location is observed in *in situ* hybridizations to salivary gland polytene chromosomes; (4) The 68C puff in this strain is larger than in other wild type strains. The two copies of *Sgs-3* in Hikone-R produce RNAs of slightly different size, which can be resolved on acrylamide gels.

Figure 1 shows a restriction map of the 68C region of the Oregon-R strain used in our laboratory in comparison to the probable restriction map of this region in Hikone-R. This map is based upon data from whole genome DNA blots and should be considered tentative. If it is correct, then the duplication in Hikone-R is tandem and approximately 12 kb in length. In Figures 2 and 3 are shown some of the genome blots upon which the restriction map is based.

Puff measurements of 68C in puff stage 1 animals from Hikone-R lines show the puff to be larger in this strain. The diameter of 68C5,6 is approximately 2.4 times the diameter of control bands at 69A1,3 (n=4), as compared to other wildtypes in which it is twice the diameter, or less (see Chapter 2).

In the course of analyzing *in situ* hybridizations for many transformants which were recovered in an Hikone-R-derived stock, only one additional site of hybridization, at 68C, was ever observed for the resident genes.

The two *Sgs-3* genes in Hikone-R are slightly different in size. This was suspected on the basis of whole genome blots; an example is in Figure 3A. It was confirmed by RNA blots of total RNA from the salivary glands of late third instar larvae (Figure 2C). Using the sizes of the Oregon-R *Sgs-3* variant (1120 nt prior

to polyadenylation) and the Formosa variant (820 nt prior to polyadenylation) as standards, the two *Sgs-3* RNA species in Hikone-R are approximately 950 nt and 1000 nt in length.

Figure 1. Restriction maps of the 68C region in Oregon-R and Hikone-R wild-type strains.

Restriction endonucleases are abbreviated as follows: H: HindIII, R: EcoRI, S: SalI. The map of Oregon-R 16f is from Meyerowitz, E. M. and Hogness, D. S. (1982) Cell *28*, 165-176. Below the maps are indicated the extents of the genomic subclones used for the whole genome Southern analysis. The regions which encode the *Sgs-3* gene are indicated by arrows; for the Hikone-R strain these are postulated on the basis of the Oregon-R data. There are small inverted repeats which are present about six times in the genome that are found at the HindIII sites which bound aDm2026; these are indicated by a slight increase in the thickness of the line of the restriction map.

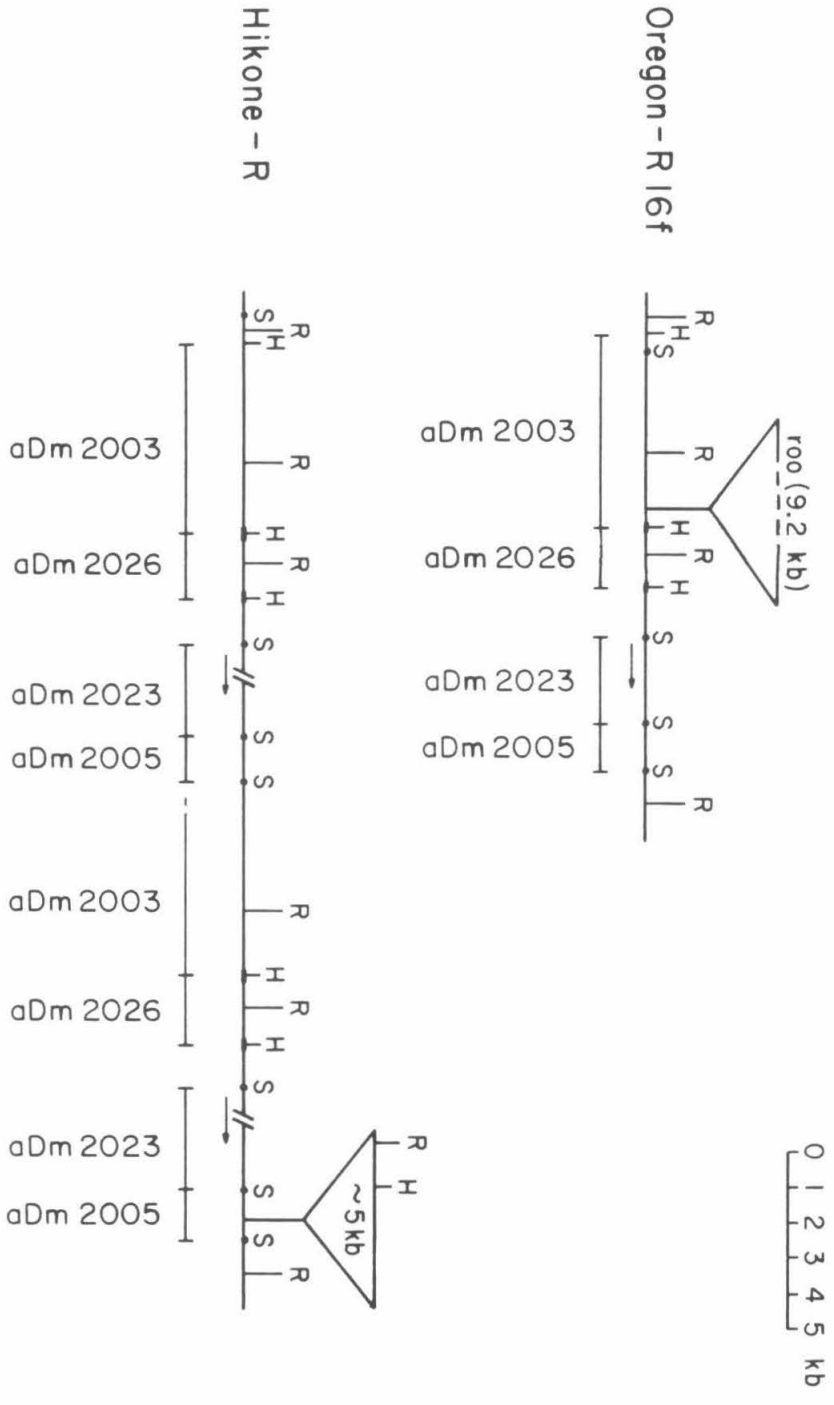

Hikone-R
Oregon-R 16f
aDm 2003
aDm 2026
aDm 2023
aDm 2005
aDm 2003
aDm 2026
aDm 2023
aDm 2005
roo (9.2 kb)
~5kb
R
H
S
0 1 2 3 4 5 kb

Figure 2A. Whole genome DNA blot, DNA cut with EcoRI and probed with ^{32}P-aDm2026.

In all other wild-type strains, two fragments 7.2 kb and 2.8 kb are observed (the Oregon-R strain is one without the roo insertion). The Hikone-R strain retains the 2.8 kb fragment (probably a doublet), but the 7.2 kb fragment is replaced by a fragment approximately 9.6 kb and one approximately 5.8 kb. Faint fragments are from inverted repeats at other locations in the genome.

Figure 2B. Whole genome DNA blot, DNA cut with HindIII and probed with ^{32}P-aDm2003.

The two Oregon-R strains show different size fragments, 9.0 kb and 5.6 kb, due to the presence or absence (respectively) of the roo insertion. In addition to the 5.6 kb fragment observed in Oregon-R strains lacking the roo insertion, Hikone-R contains a 10.1 kb fragment.

Figure 2C. RNA blot of total RNA from salivary glands of Hikone-R late third instar larvae demonstrates that the Hikone-R strain produces two species of *Sgs-3*, mRNA.

RNA samples were prepared as for a formaldehyde gel (see Chapter 2) and run on a 5% acrylamide gel cast in MOPS buffer (without formaldehyde). The RNA was transferred by electroblot to a nylon filter (NEN) and the filter was probed with ^{32}P-aDm2023. The Formosa size variant of *Sgs-3* is 820 nt prior to polyadenylation and the Oregon-R variant is 1120 nt prior to polyadenylation.

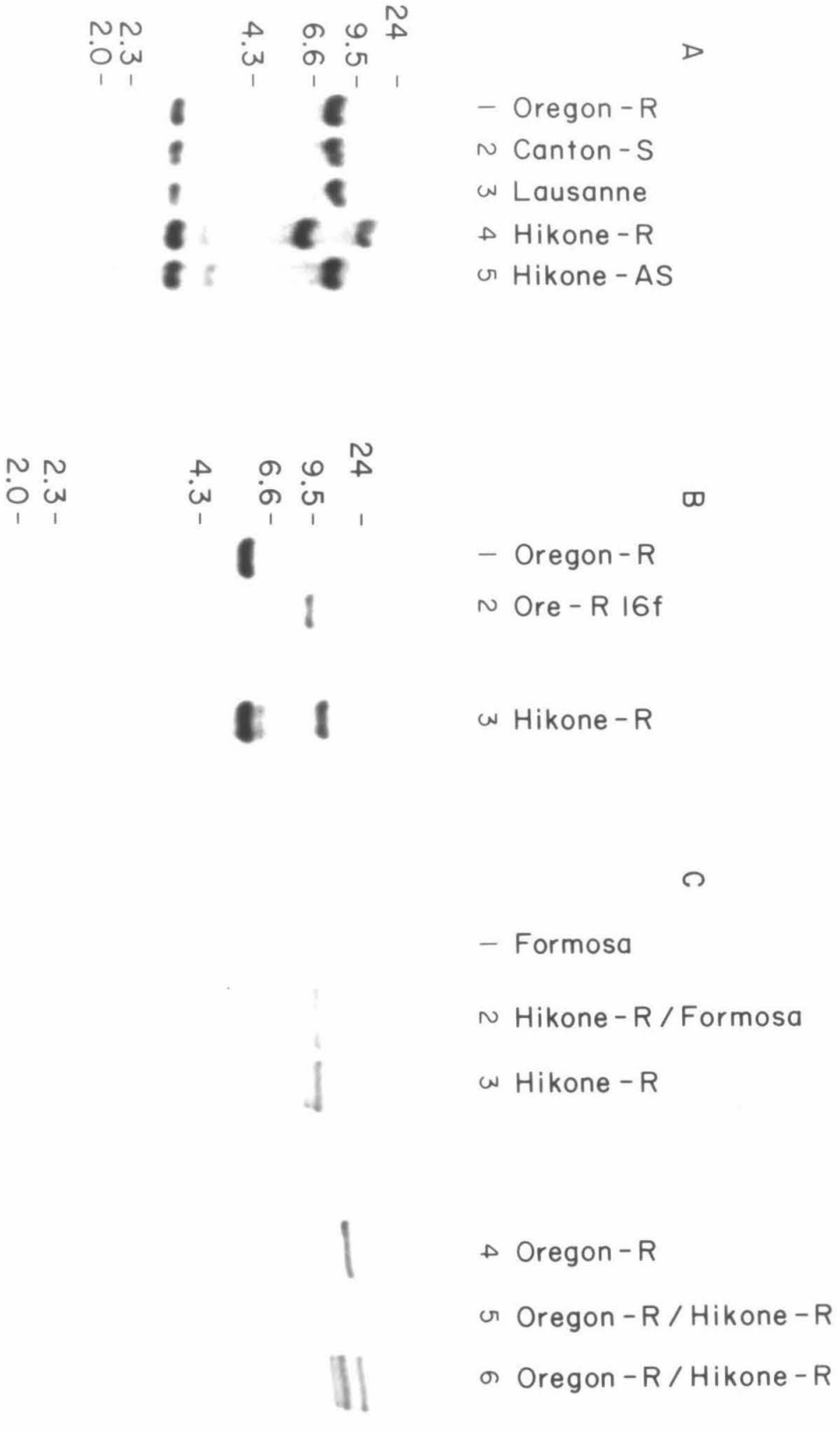

A
24 -
9.5 -
6.6 -
4.3 -
2.3 -
2.0 -
1 Oregon - R
2 Canton - S
3 Lausanne
4 Hikone - R
5 Hikone - AS
B
24 -
9.5 -
6.6 -
4.3 -
2.3 -
2.0 -
1 Oregon - R
2 Ore - R 16f
3 Hikone - R
C
1 Formosa
2 Hikone - R / Formosa
3 Hikone - R
4 Oregon - R
5 Oregon - R / Hikone - R
6 Oregon - R / Hikone - R

Figure 3A. Whole genome blot, DNA cut with SalI and probed with ^{32}P-aDm2023.

The Oregon-R strains show the expected fragment of 2.4kb; the Hikone-R strain shows a doublet at approximately 2.3 kb. The faint bands are due to partial digestion of the DNA.

Figure 3B. Whole genome blot, DNA cut with SalI and probed with ^{32}P-aDm2005.

In addition to the expected fragment of approximately 1.4 kb, the Hikone-R strain shows a fragment of 6.6 kb.

Figure 3C. Whole genome blot, DNA cut with EcoRI and probed with ^{32}P-aDm2005.

The Oregon-R strain shows a fragment of 6.6kb. With a probe 1.4 kb in length, fragments of 5.5 kb, 6.2 kb and 9.6 kb are detected in Hikone-R. The interpretation most consistent with these and other results (see Figure 3B) is that there is an insertion of approximately 5 kb at the proximal end of the duplicated region.

A
1 Oregon - R
2 Oregon - R 16f
3 Hikone - R
4 Hikone - R
24
9.5
6.6
4.3
2.3
2.0
B
1 Oregon - R
2 Oregon - R 16f
3 Hikone - R
4 Hikone - R
24
9.5
6.6
4.3
2.3
2.0
C
1 Oregon - R 16f
2 Hikone - R
3 Hikone - R