Developmental Regulation and Chromosomal Decondensation of the 68C Glue Gene Cluster in *Drosophila melanogaster*

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Dedicated to my parents, Dorothy Daw Mathers and William Clayton Mathers, for without their efforts, as with chemicals, life itself would be impossible. The love, support, encouragement, humor and sterling example of my family has been a great inspiration.

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Peter Hiram Mathers

Abstract of Dissertation:

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The larval salivary gland secretion genes (*Sgs-3, Sgs-7* and *Sgs-8*) at chromosome position 68C in *Drosophila melanogaster*, are developmentally regulated and coordinately expressed. Each gene codes for a component of the mucoprotein glue which is synthesized in the third instar salivary glands. Expression of these three genes is associated with the 68C intermolt puff present in the polytene chromosomes of the salivary gland secretory cells. Expression occurs only in the salivary glands of third instar larvae, and requires the steroid hormone ecdysterone. We show that synthesis of the 68C glue gene RNAs is prevented in larvae carrying *trans*-acting mutations within the *Broad-Complex* (*BR-C*), while a puff persists at 68C in these mutant larvae. We use the $l(1)su(f)^{ts67g}$ mutation (which has reduced ecdysterone levels and also prevents 68C glue gene expression) and a mutation in the *BR-C* ($npr1^3$) to study the *cis*-acting elements responsible for interaction with *trans*-acting factors by analyzing the protein:DNA contacts which occur upstream of the *Sgs-3* glue gene during active synthesis, using in vivo DMS-footprinting. The proximal promoter can direct tissue- and stage-specific expression and is shown to possess three protein-binding domains. Comparison of contacts at these three domains between expressing and non-expressing tissues (including salivary glands from $(1)su(f)^{ts67g}$ and $npr1^3$ mutant larvae) identifies a single binding domain responsible for controlling developmental expression of *Sgs-3*.

We also analyze the *cis*-acting sequences required for the chromosomal puffing associated with 68C glue gene expression. Examination of various fragments of 68C DNA reintroduced into the *Drosophila* chromosomes by P-element transformation identifies a region of 152 basepairs between *Sgs-7* and *Sgs-8* which is necessary, but not sufficient, to

promote puffing. Only when this region is accompanied by an adjacent promoter element is there a puff. The insertion sites containing these reintroduced fragments fail to puff in mutant larvae; therefore, formation of the 68C intermolt puff requires the products of the *su(f)* and *BR-C* loci, and the puff at 68C in mutant larvae is not the same puff as that associated with expression of *Sgs-3*, *Sgs-7* and *Sgs-8*.

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Chapter 1:

General Introduction

The larval salivary glands of Dipterans consist of a pair of sac-like lobes, first described in gadfly larvae by Swammerdam (1738). These lobes contain a small number of giant secretory cells (approximately 130 per lobe in *Drosophila melanogaster*) and still fewer non-secretory neck cells, all surrounding a lumen which connects to a common duct joining the pair of lobes. The chromosomes of the secretory cells, initially described by Balbiani (1881), are polytene, that is, comprised of many aligned chromatids. The polyploidy of these cells is the result of an early disruption in the mitotic process, such that nuclear division and cytokinesis are abandoned without an interruption in chromosomal replication. While the purpose of this disruption is unclear, the effect is profound on the cells, causing enormous increases in both nuclear and cellular volume. This chromosome replication can continue for several cycles, resulting in the average salivary gland secretory cell containing around 1000 genomic copies in *Drosophila melanogaster*. The arrangement of these chromatids is in register, so that they appear as a bundled cord, with precise alignment along their length. The effect of this alignment is compounded by the phenomenon of somatic pairing, so that a view of the nucleus gives the impression of a haploid constitution. The value of these giant chromosomes for cytogenetic analysis is well established (Bridges 1935).

Drosophila melanogaster salivary gland cells are determined to differentiate to appropriate cell types during early embryogenesis (Hathaway and Selman 1961). Under normal growth conditions (25°C), the embryonic period is approximately 24 hours, followed by three larval instars. Between each of these instars, the larva molts, shedding its cuticle. Each instar is identified by an increase in overall size and in the size of individual organs. The salivary glands also exhibit a drastic increase in size, but this is accomplished by increasing cell size, rather than cell number, which is set early in development. The increase in cell size is a result of the continued DNA synthesis mentioned above, in the absence of cell division (polyploidy). As *Drosophila* are holometabolous insects, this larval development is followed by metamorphosis to complete

the transformation to adult form. During the third instar, larvae stop their continual feeding and begin to climb out of the food. After several hours of wandering, they cease movement, evert their spiracles, harden their cuticle and begin the process of metamorphosis. This stage is called puparium formation. After several hours, true pupal development begins, denoted by head eversion. After several days, adults eclose from their pupal casing and quickly provide a subsequent generation.

Formation of these distinct developmental stages requires a mechanism for their creation and maintenance. Two hormones are thought to play an important role in regulating *Drosophila* development, the steroid ecdysterone (also known as β-ecdysone and 20-OH-ecdysone) and the isoprenoid juvenile hormone. Classic theories on insect development suggest that the concentrations of these two hormones in the larval hemolymph, both absolute and relative to each other, signifies whether a molt will be embryonic to larval, larval to larval or larval to pupal. How well this theory applies to the development in *Drosophila* is not fully settled, but it appears to be an oversimplification (see Richards and Ashburner 1984 for a good review of the insect hormones). It is known, however, that ecdysterone is an important factor in the regulation of gene expression. Gene regulation by steroid hormones is now well understood in mammalian systems (Yamamoto 1985; Evans 1988), and ecdysterone is thought to act in a similar fashion through a hormone-receptor-DNA-binding complex (Bonner 1982; Richards and Ashburner 1984). Indeed, ecdysterone-responsive elements have been identified (Mestril et al. 1986; Riddihough and Pelham 1987) as important in the hormone-inducible expression of the developmentally regulated heat shock genes in *Drosophila melanogaster*. In addition, isolation and cloning of an ecdysterone-binding protein with DNA-binding capabilities has been reported (D. Hogness, personal communication). The isolation and characterization of numerous ecdysterone-inducible genes has been reported and a specific set of these genes, the salivary gland secretion (or *Sgs*) genes, will be discussed later. The importance of ecdysterone in the process of developmentally regulated chromosome

puffing has also been shown (Becker 1959; Clever and Karlson 1960; Ashburner 1973; Dworniczak et al. 1983).

During the early part of this century, Dipterans became known as excellent organisms for genetic research, and because of this, the salivary gland chromosomes began to take on greater importance. The cytogenetic chromosome maps of Painter (1933, 1934) and Bridges (1935), using *Drosophila melanogaster*, depict these polytene chromosomes, with their distribution of banded and interband regions. This distinctive banding pattern is present throughout development in all tissues with polytene chromosomes (Beermann 1952; Pavan and Beurer 1952; Holden and Ashburner 1978), with only minor deviations. While a majority of the chromosomal architecture is invariant, selected regions of decondensation are observed which act to greatly alter both the localized banding patterns and chromosomal structure, often forming a puff. Poulson and Metz (1938) were the first to recognize the changing pattern of "puffs" and "bulbs" using *Sciara* salivary gland chromosomes. The proposal that these puffs reflect changes in gene activity was clearly presented by Beermann (1956), following his and other's work on the consistency of banding patterns between several tissues. Analysis of ^{3}H-uridine incorporation patterns helps to verify the transcriptional activity within puffed regions (Pelling 1959, 1964; Zhimulev and Belyaeva 1975; Belyaeva and Zhimulev 1976). Several good reviews have been written on polytene chromosome puffing (for example, Ashburner and Berendes 1978; Korge 1987).

The pattern of chromosomal puffing in the larval salivary glands is a reflection of the genes active at a particular time in development. A system has been conceived which recognizes as distinct stages in development the distribution of puffs throughout the chromosomes (Becker 1959; Ashburner 1967, 1969, 1972b). This system covers developmental stages from mid-third instar, when the polytenization is complete enough to make visualization possible and characterization accurate, to the late prepupae, when the larval salivary glands undergo histolysis. The individual puff stages (PS1 through PS21)

are characterized by the presence or absence of a large number of puffs, but few of these puffs have defined functions.

The ability to induce puffing with external stimuli has led to a better understanding of the function of puffed loci, and of the factors involved in the puffing process. Ritossa (1962), Ashburner (1970) and Tissières et al. (1972) found that treatment of salivary glands or of whole larvae with heat and other forms of environmental stress induces decondensation of a specific set of stress-induced loci, the heat shock puffs. Clever and Karlson (1960) and Ashburner (1972a, 1973) showed that specific sets of puffs could also be induced by the hormone ecdysterone. By analyzing the pattern of ecdysterone induction in salivary glands cultured under various conditions, Ashburner et al. (1973) devised a model to account for hormonal modulation of gene activity. Briefly, this model recognizes four classes of ecdysterone-responsive puffs: intermolt (present at PS1), early, early late and late. The intermolt puffs are found to regress in vivo and in vitro upon an increase in ecdysterone concentration. This same increase in hormone induces formation of the early puffs, in a manner independent of protein synthesis. Induction of the later puffing stages is susceptible to a disruption in protein synthesis, and their activation is thought to be regulated by the activity of the early puffs.

The function of some of the intermolt puffs has been elucidated from genetic studies and protein analysis of the salivary gland secretion. During the latter half of the third larval instar, the salivary glands are actively producing mucoproteins, which are stored in secretion granules until released into the lumen a few hours prior to puparium formation (Lane et al. 1972). Korge (1977b) found that these mucoproteins make up greater than 30% of the protein mass of the salivary gland at the end of the secretion phase. The secretion of these proteins into the lumen is in response to increases in the ecdysterone titer (Poels 1972). Once collected in the lumen, the mucoproteins are expelled through the common duct, which empties into the pharynx, and deposited onto the outer cuticle of the larvae just prior to puparium formation. These proteins had been shown earlier to serve as

the "glue" that helps the pupa to stick to a solid surface through the process of metamorphosis (Fraenkel and Brookes 1953).

Correlation was made between the production of these glue proteins and the activity of the intermolt puffs. Using gel electrophoresis, Korge (1975) identified four distinct fractions present within the secreted glue, and was able to partially characterize the chromosomal location for two of these fractions: *Sgs-4* and *Sgs-3*. While Beckendorf and Kafatos (1976) were able to identify two additional secretion proteins, the actual number is at least seven (Crowley et al. 1983). Korge (1977a) and Akam et al. (1978) verified the locations of the *Sgs-4* and *Sgs-3* genes using cytogenetic analysis, and found them correlated with an appearance of the intermolt puffs present at 3C and 68C. Using similar techniques, *Sgs-1* and *Sgs-6* have been mapped to puffs at positions 25B (Velissariou and Ashburner 1980) and 71C (Velissariou and Ashburner 1981), respectively. Three other glue protein genes were localized after characterization of their coding sequences and complementary DNA clones. In this way, *Sgs-5* was found to come from expression at the 90BC puff (Guild and Shore 1984), and the *Sgs-7* and *Sgs-8* glue protein genes were found associated with the *Sgs-3* gene at position 68C (Crowley et al. 1983; Meyerowitz and Hogness 1982). Another glue protein gene has been mapped to 68C, in addition to *Sgs-3, Sgs-7* and *Sgs-8*, but has not been named in the *Sgs* nomenclature as yet (Gautam 1983).

The cloning and characterization of five of these salivary gland glue genes has been reported (*Sgs-4*- Muskavitch and Hogness 1980; *Sgs-3, Sgs-7* and *Sgs-8*- Meyerowitz and Hogness 1982, Crowley et al. 1983; *Sgs-5*- Guild and Shore 1984). DNA sequencing of these genes and their flanking sequences has revealed features common among them and between these genes and other eukaryotic genes (Muskavitch and Hogness 1982; Garfinkel et al. 1983; Shore and Guild 1986). In particular, the three glue genes at position 68C appear to be evolutionarily related, with similarities in amino acid composition, intron

position and conserved upstream sequences (Garfinkel et al. 1983; also see Chapter 3). This may help to explain the coordinate regulation of expression of these three genes.

Studies on the *cis*-acting sequences regulating glue gene expression have benefited extensively from the introduction of methods for germline transformation of modified DNA fragments (Rubin and Spradling 1982; Spradling and Rubin 1982). Another technique which has proved valuable is the somatic expression assay (Martin et al. 1986), which involves analysis of expression from non-integrated DNA fragments after injection into pre-cellular blastoderm embryos. This technique is similar to the transient assay system used commonly in cultured cells. Application of these two techniques to the problem of glue gene expression and regulation has revealed much information regarding necessary sequences (see reviews by Meyerowitz et al. 1987; Martin et al. 1989; also see Giangrande et al. 1989; Roark et al., in press; Todo et al., submitted). In addition to these experiments utilizing injected DNA fragments, several sequence comparisons have found regions of altered DNA sequence upstream of the glue genes in strains showing a disrupted expression pattern (Muskavitch and Hogness 1982; McGinnis et al. 1983; Hofmann and Korge 1987; Shore and Guild 1987). In a similar fashion, Martin et al. (1988) sequenced the upstream region of *Sgs-3* homologs in species related to *Drosophila melanogaster*, in an effort to correlate conserved regions with functional regulatory domains. While the specific distribution of upstream regulatory elements appears to differ for each glue gene, there are certain conserved elements within the group (for example, see Todo et al., submitted), and the generalized conclusions from these studies suggest a set of genes which show regulatory domains similar to enhancers and promoters characterized for other eukaryotic systems (see Maniatis et al. 1987, Levine and Manley 1989).

The sequences upstream of the each of the three 68C glue genes have been shown to govern temporally and spatially specific expression in a concurrent manner. As few as 130 basepairs upstream of the *Sgs-3* gene (the proximal promoter element) are sufficient to direct transcriptional activity solely in the salivary glands during third instar (Vijay

Raghavan et al. 1986), but are insufficient to allow full level expression. Sequences at 600 basepairs upstream (the distal promoter element) are required for increased expression levels, and appear similar to the proximal element in their ability to direct stage- and tissue-specific expression of adjacent genes (Roark et al., in press). Sequences further upstream act as weak enhancers, boosting expression of *Sgs-3* by 3-fold (Giangrande et al. 1987), and may also have effects on the expression of the *Sgs-7* and *Sgs-8* glue genes (A. Hofmann, M. Garfinkel and E. Meyerowitz, in preparation). The proximal and distal promoter elements show functional redundancy, and may represent a duplication of regulatory domains. This is not an uncommon design for the promoter and enhancer elements in other eukaryotic genes (see Maniatis et al. 1987 for several examples). This level of complexity for promoter organization is similar to that found in the yolk protein genes of *Drosophila melanogaster* (Garabedian et al. 1986), but appears to be relatively simple in comparison to the regulatory hierarchies involved in the regulation of *Drosophila* early development (Gehring 1987; Levine and Manley 1989).

The *trans*-acting factors responsible for mediating the developmental regulation of the glue genes have received much less attention than their *cis*-acting counterparts. It appears that ecdysterone is required earlier in development for the initiation of glue RNA accumulation (Hansson and Lambertsson 1983), possibly at the second to third instar molt (see Richards 1981 for a review on developmental changes in ecdysteroid titers). Five individual loci have been identified in which mutations result in drastically reduced ecdysterone levels in the larval hemolymph (*l(3)ecd-1*, Garen et al. 1977, Berreur et al. 1984; *l(2)gl*, Korochkina and Nazarova 1977; $L(3)3^{DTS}$, Holden and Ashburner 1978; *l(1)grg*, Klose et al. 1980; and *l(1)su(f)*, Klose et al. 1980, Hansson and Lambertsson 1983). Several temperature-sensitive alleles of the *suppressor of forked* (*su(f)*) locus show this reduction and fail to pupariate if raised to non-permissive temperature at the proper time in development. Hansson and Lambertsson (1983) showed that $l(1)su(f)^{ts67g}$ larvae failed to express *Sgs-3, Sgs-4, Sgs-7* or *Sgs-8* RNAs, when raised to the restrictive temperature

of 30°C at 60 hours post-oviposition. In addition, they found that this temperature-dependent defect and a disruption in normal patterns of salivary gland protein synthesis could be overcome by feeding the *l(1)su(f)^{ts67g}* larvae exogenous ecdysterone (Hansson and Lambertsson 1983, 1984). Therefore, ecdysterone is necessary for activation of glue gene expression, but it is not known whether there is direct involvement in this process by the hormone or perhaps an indirect stimulation through activation of a regulatory cascade.

We also know that an increase in ecdysterone titers towards the end of the third instar plays an important role in the cessation of RNA synthesis (Crowley and Meyerowitz 1984) and the regression of the 68C intermolt puff (Ashburner 1973). The relatively short time required for repression of RNA synthesis by ecdysterone (15 minutes, Crowley and Meyerowitz 1984) could be used to argue for a direct involvement by the hormone in this repression. Indeed, ecdysterone has been found associated with the 68C puff through the use of photocross-linking and indirect immunofluorescence (Dworniczak et al. 1983). The timing of the reported association correlates with the active stages of glue gene expression, but not with repressed stages. Several models have been proposed for steroid-induced repression of gene expression (Levine and Manley 1989). The mechanism by which ecdysterone functions to both activate and repress glue gene expression is still a mystery.

Another factor had previously been reported which has affects on larval development and, in particular, on the ecdysterone-controlled regulation of the 68C puff. Kiss et al. (1978) and Fristrom et al. (1981) reported the isolation of mutations in the 2B5 region of the X chromosome (now referred to as the *Broad-Complex* or *BR-C*; Lindsley and Zimm 1986), which cause defects in the process of metamorphosis, and result in extended larval development followed by lethality. This failure to proceed in development to the pupal stage has led to the designation of *non-pupariating* or *npr* for the most severely affected alleles of the *BR-C* (Kiss et al. 1978, 1988). The genetic complexity of the *BR-C* has been examined (Belyaeva et al. 1980), as has the molecular complexity (Chao and Guild 1986). Significant to considerations of glue gene regulation, Belyaeva et al. (1981) found that

larvae carrying a non-pupariating allele of the *BR-C* fail to show the normal regression of the 68C puff when cultured in physiological concentrations of ecdysterone. This phenomenon has led to the experiments which constitute this thesis.

The work presented in this thesis was performed in an effort to better understand the process of developmental gene regulation as it pertains to the tissue- and stage-specific expression of the 68C glue genes. Chapter 2 presents results on the effects of mutation in the *BR-C* (*npr* alleles) on the RNA accumulation of the glue genes *Sgs-3, Sgs-4, Sgs-5, Sgs-7* and *Sgs-8*. The findings in Chapter 2 led to work on two different aspects of gene regulation: the *trans*-acting factors involved in glue gene regulation and their interactions with DNA sequences responsible for regulatory control (Chapter 3); and the structural changes of the chromosome associated with gene expression (puffing), the *cis*-acting factors which control puffing, and the relationship between chromosomal puffing and the act of transcription (Chapter 4). The appendices present additional information on interaction between *cis*-acting control sequences and the *trans*-acting product(s) of the *BR-C*.

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Chapter 2:

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

Explanation of contribution:

The inclusion of this paper as a chapter in this thesis serves to demonstrate the significant contributions made by me towards its completion. The work described in the following paper was performed either jointly with Thomas Crowley or solely by me, once Tom had left. In addition, contributions were provided in the preparation of the original manuscript and significant additions made upon review. The work was therefore a true collaboration.

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

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Summary

The mutation *l(1)npr-1* is located at cytological location 2B5 on the X chromosome in Drosophila melanogaster. We have found that this mutation causes absence of the normal product of the 2B5 locus and that it has the following phenotypes: the 68C glue puff on the third chromosome does not regress when mutant salivary glands are cultured in the presence of ecdysterone; the three 68C glue protein mRNAs are not synthesized; and a transformed Drosophila strain carrying both a normal resident 68C *Sgs-3* gene and an introduced functional *Sgs-3* gene with only a few kb of flanking sequences expresses neither *Sgs-3* RNA if the *l(1)npr-1* mutation is crossed into the stock. Thus the normal product of the *l(1)npr-1* gene is required for regression of the 68C puff, and the *l(1)npr-1* gene product allows expression of the *Sgs-3* gene by interacting, either directly or indirectly, with DNA sequences near this glue protein gene.

Introduction

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

At 25°C third instar begins approximately 72 hr after egg deposition, and lasts about two days. About midway through the third larval instar salivary gland chromosomes achieve a degree of polyteny that allows them to be easily observed with a light microscope. This is the earliest time at which chromosomal puffs can be clearly distinguished. The 68C region is puffed at this time and remains so until just before the end of the third instar, when the concentration of the steroid hormone ecdysterone in the larval hemolymph increases dramatically. This causes regression of the puff and a sharp reduction in expression of the *Sgs-3, Sgs-7,* and *Sgs-8* genes. The evidence for this is that the 68C puff contracts and expression of the glue genes ceases when ecdysterone concentration increases during normal development in vivo, and increasing the hormone concentration in cultured salivary glands duplicates these effects (Ashburner, 1967; Ashburner, 1973; Ashburner et al., 1973; Crowley and Meyerowitz, 1984).

Larvae that are homozygous or hemizygous for the *lethal(1)t435* mutation (*l(1)t435,* Belyaeva et al., 1980) never pupariate. They spend up to a week as third instar larvae, but die at this stage. In salivary glands of these larvae a puff is visible at 68C on the third chromosome during the latter part of the third instar, as in wild-type larvae. However, when the mutant glands are cultured in the presence of ecdysterone the puff does not regress as it does in wild-type glands. Also, puffs at other loci that are induced by ecdysterone in wild-type glands are not fully induced by the hormone in this mutant. *l(1)t435* has been cytologically mapped to the 2B5 band of the X chromosome. Apparently a product of the wild-type 2B5 locus is necessary for normal hormonal control of puffing in salivary glands (Belyaeva et al., 1981). The mutation known as *lethal(1)npr-1 (l(1)npr-1* (Kiss et al., 1976, 1978) is an allele of *l(1)t435.* Like the *l(1)t435* larvae, males hemizygous and females homozygous for *l(1)npr-1* are nonpupariating. They do not progress beyond the third instar larval stage of development, remaining as third instar larvae for about one week, then dying, despite having normal levels of ecdysterone (Fristrom et al., 1981).

A mutant strain in which the 68C puff does not regress in response to the hormone provides an opportunity to examine the relationship between puffing, transcription, and hormonal response. In this paper we first show that the 68C region is puffed in the *l(1)npr-1* salivary glands and that the puff fails to respond to ecdysterone, as is the case in *l(1)t435* glands. We then demonstrate that accumulation of RNA transcribed from the 68C glue genes, *Sgs-3, Sgs-7,* and *Sgs-8,* is reduced to below a detectable level in *l(1)npr-1* larvae. We further show that 6 kb or less of 68C DNA is all that is necessary for the repression of *Sgs-3* by the *l(1)npr-1* mutation, and thus that the normal product of 2B5 locus, or some product induced by this locus, interacts with the DNA of the 68C glue gene cluster to allow expression of the *Sgs-3* gene.

Results

Wild-Type Activity of the 2B5 Locus Is Necessary for Steroid-Induced Regression of the 68C Puff

To analyze the effect of the *l(1)npr-1* mutation on the 68C region, salivary glands hemizygous for *l(1)npr-1,* or glands from control larvae hemizygous for the balancer chromosome *Binsn* (Lindsley and Grell, 1968) and with the same autosomal background as that of the *l(1)npr-1* larvae, were cultured either in 10^{-5} molar ecdysterone or in the absence of hormone for 3 hr, after which the chromosomes were stained and spread for light microscopic examination. The diameters of the 68C puff (measured just proximal to 68C4) and the 69A1-3 region of the third chromosome were measured and the ratio of 68C puff diameter to the 69A1-3 diameter calculated as a quantitation of the extent of puffing at 68C (Ashburner, 1973; Crowley and Meyerowitz,

1984). The 69A1-3 bands are used as a reference because they are not involved in puffing activity in third instar salivary glands. The results are shown in Table 1 and Figure 1. Ecdysterone does not reduce the relative size of the 68C puff in the *l(1)npr-1* salivary glands, although it does cause puff regression in the control glands. Thus the *l(1)npr-1* mutation inhibits the hormone-mediated regression of the 68C puff in cultured salivary glands. Belyaeva and coworkers (1981) obtained essentially the same result with the allelic *l(1)t435* mutation.

The 68C Glue RNAs Never Accumulate in *l(1)npr-1* Salivary Glands

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

Since the hemizygous *l(1)npr-1* larvae have a prolonged third instar, it was possible that 68C glue gene expression was simply delayed in these animals. To determine if this was the case we analyzed RNA from salivary glands of hemizygous *l(1)npr-1* larvae which were 4, 5, 6, 7, 8, 9, 10, or 11 days old, a range that covers the end of the second and the entire third instar of these animals. None of the 68C glue RNAs was detectable at any point in this range (data not shown). Thus the 68C RNAs never accumulate to a significant level in hemizygous *l(1)npr-1* salivary glands. The 68C RNAs also fail to accumulate in cultured salivary glands dissected from male larvae hemizygous for *l(1)npr-1*. This is the case if the glands are cultured in the presence of 10^{-5} M ecdysterone for 3 hr or cultured in the absence of added hormone.

The Effects of *l(1)npr-1* Are on Transcription or Very Early Processing of the 68C RNAs

To determine which processes in glue RNA metabolism might be affected by the *l(1)npr-1* mutation, the rates of accumulation of newly synthesized RNAs were measured in salivary glands in hemizygous *l(1)npr-1* and control *Binsn* salivary glands. This was done by pulse-labeling the RNA being synthesized in the glands with ^{3}H-uridine for 15 min, purifying the RNA, hybridizing it to an excess of filter-bound DNA homologous with each of the 68C glue RNAs, and measuring the amount of hybridized RNA by scintillation spectrometry. The results of these experiments are summarized in Table 2. In the *l(1)npr-1* glands the amount of label incorporated into newly synthesized *Sgs-3, Sgs-*

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

	l(1)npr-1	*Binsn*
− Ecdysterone	1.69 ± 0.04	2.04 ± 0.05
+ Ecdysterone	1.87 ± 0.07	1.46 ± 0.05

Salivary glands from mid-third instar larvae were cultured in the absence of ecdysterone (− Ecdysterone) or in the presence of 10^{-5} M ecdysterone (+ Ecdysterone) for 3 hr at 22°C prior to staining and squashing (see Experimental Procedures). Each number is the mean of 24 measurements (six nuclei from each of four animals). Numbers represent the ratio of 68C puff diameter (measured just proximal to 68C3-4) to the diameter of the unpuffed 69A1-3 region. Standard error is indicated. Student's t-test analysis yields values of $p > 0.05$ for the *l(1)npr-1* classes and $p < 0.001$ for the *Binsn* classes, indicating that ecdysterone has no significant effect on the puff in the *l(1)npr-1* background, but that the hormonal effect in wild type is highly significant.

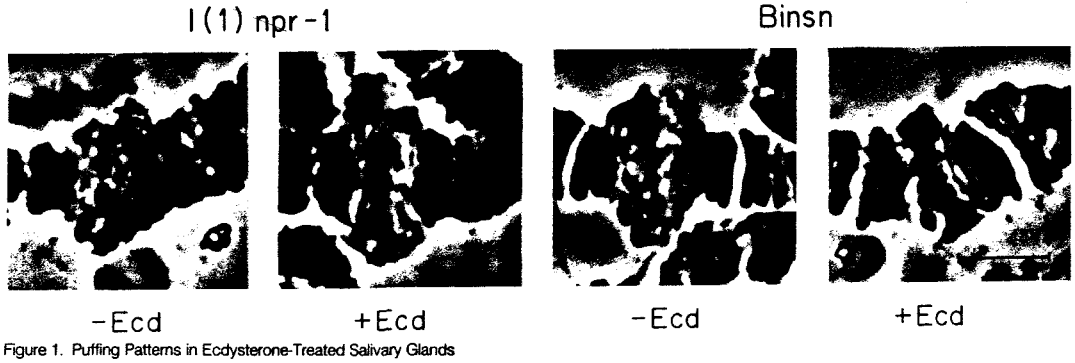

Figure 1. Puffing Patterns in Ecdysterone-Treated Salivary Glands

Salivary glands from male third instar larvae carrying either the *l(1)npr-1* X chromosome or the *Binsn* X chromosome were cultured with (+Ecd) or without (−Ecd) 10^{-5} M ecdysterone for 3 hr. Glands were then stained, spread, and photographed as described in Experimental Procedures. Squashes presented are representative samples from 24 nuclei photographed and measured in each class. Chromosomes are shown distally to proximally from left to right. The 68C1-4 region, usually represented by the distal half of the 68C puff, is unaffected by ecdysterone; its diameter remains relatively expanded throughout third instar. The bar represents 5 μm.

7, and *Sgs-8* RNAs is reduced at least 360-, 42-, and 8-fold, respectively, compared to the controls. Incorporation of ^{3}H-uridine into total salivary gland RNA is unaffected by the mutation. Thus the net rates of synthesis of all three 68C glue RNAs are drastically and specifically reduced in the *l(1)npr-1* mutant salivary glands, and the mutation either prevents new synthesis or causes immediate degradation of the 68C RNAs.

This experiment also rules out the possibility that the effect of the *l(1)npr-1* mutation is to cause the positions of initiation or termination of the 68C glue RNAs to be random. Were this the case they might be missed in RNA blot experiments, but would give normal or even greater than normal signal in a hybrid selection experiment such as this.

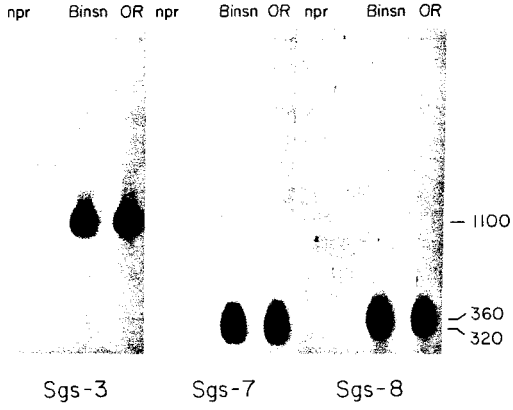

Figure 2. 68C Glue RNAs in Third Instar Salivary Glands

RNA from salivary glands of male larvae hemizygous for *l(1)npr-1* (npr), male larvae hemizygous for the *Binsn* X chromosome, and male wild-type Oregon R 16f (OR) larvae was fractionated by electrophoresis in a formaldehyde agarose gel, then transferred to a nitrocellulose filter as described in Experimental Procedures. Ten gland lobes were used for each RNA preparation. The filter was hybridized with the ^{32}P-labeled insert of clone aDm2023 (a 2.4 kb fragment originally derived from λbDm2002, homologous with the *Sgs-3* transcript), the cDNA insert of adm127C8 (homologous with the *Sgs-7* transcript) or the cDNA insert of adm109F4 (homologous with the *Sgs-8* transcript). These clones are described in detail by Meyerowitz and Hogness (1982) and by Garfinkel et al. (1983). To remove one probe before hybridizing the next, the filter was given two 5 min washes in 0.01× SSPE at 100°C. Exposure times were as follows: 6 hr for the *Sgs-3* probe, 5 hr for the *Sgs-7* probe, and 5 hr for the *Sgs-8* probe. The signals in the Binsn and OR lanes were detectable after 15–17 min exposures, while no signals were detected in the npr lane even after much longer exposures (i.e., 62 hr for *Sgs-3*, 118 hr for *Sgs-7*, and 52 hr for *Sgs-8*). Hybridization of the filter with a labeled probe homologous with ribosomal RNA showed that each lane has approximately the same amount of total RNA. The RNA lengths are shown on the right in nucleotides.

The Effects of *l(1)npr-1* at 68C Are Due to Absence of a Normal 2B5 Gene Product

Among the possible ways in which a mutation leads to a specific phenotype is the simple elimination of the wild-type activity of the affected locus. The classical test for this is comparison of the phenotype of organisms bearing the mutation with that of organisms with a deletion for the locus in question (Muller, 1932). A deletion for the 2B5 band, which contains the *l(1)npr-1* locus, was constructed starting with males that have a *Dp(1;Y)Sz280,y*^{2} Y chromosome, which carries the 1A to 2C1-2 region of the X chromosome with an internal deletion from 2B3-4 to 2B7-8 (Belyaeva et al., 1982). These were crossed to females carrying *Df(1)S39,cho*^{2}, an X chromosome deleted for the region from 1E1-2 through 2B5 (Belyaeva et al., 1980). Male third instar larval progeny of this cross of the constitution *Df(1)S39,cho*^{2}*/Dp(1;Y)Sz280,y*^{2} totally lack 2B5, and are recognizable by the chocolate (*cho*) phenotype (Belyaeva et al., 1981). Such larvae were collected, their salivary glands dissected, and RNA extracted from them. This RNA was subjected to gel electrophoresis in parallel with RNA from salivary glands of sibling larvae that were not deficient for 2B5. The gel was blotted to nitrocellulose, and the filter hybridized with ^{32}P-labeled λcDm2007, a lambda clone containing sequences that hybridize to all three of the 68C glue RNAs (Meyerowitz and Hogness, 1982). All of the RNAs were present in the control lane; none were detectable in the lane with RNA from the 2B5-deficient larvae. This shows that the 68C RNA phenotype of the *l(1)npr-1* mutation is identical to that of a complete deficiency for the 2B5 locus, and thus that the *l(1)npr-1* phenotype is due to the absence of the wild-type product of the locus. That a deficiency for 2B5 has the same phenotype as the *l(1)npr-1* mutation also confirms that the absence of the 68C RNAs in the *l(1)npr-1* strain is due to the *l(1)npr-1* mutation, and not to any other mutation that might be carried in the strain. Further evidence that this is the case is the absence of the 68C RNAs in strains bearing other alleles of *l(1)npr-1*. Males hemizygous for *l(1)d.norm.-1*^{a} (Stewart et al., 1972) or females homozygous for *l(1)t435*, both of which are alleles of *l(1)npr-1* induced at different times, and in the case of *l(1)t435* in a different genetic background than *l(1)npr-1*, also lack the 68C glue RNAs. This was determined in RNA gel blot experiments

Table 2. Incorporation of ^{3}H-Uridine into Salivary Gland RNAs

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

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

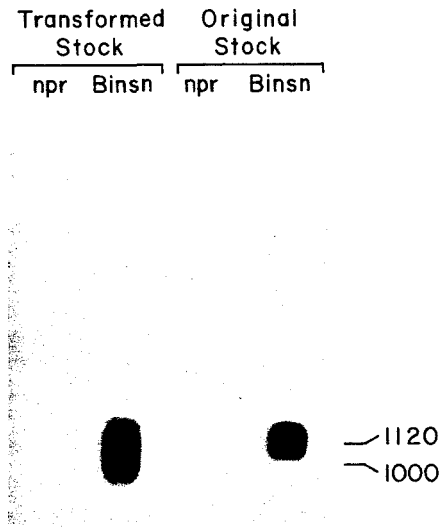

Figure 3. Expression of Endogenous and Reintroduced *Sgs-3* Genes in Third Instar Salivary Glands

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

identical to those described for *l(1)npr-1* and the 2B5 deficiency.

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

The *l(1)npr-1* mutation is at position 2B on the X chromosome but prevents expression of genes at 68C on the third chromosome. This implies that a product of the 2B5 locus or some product induced by the action of the wild-type 2B5 locus interacts with the DNA or chromatin at the unlinked 68C puff. The next experiments performed were directed toward determining which DNA sequences in the 68C region were interacting with the product of the 2B5 locus, or with a product induced by this locus. We started with a Drosophila strain that contains a normal 68C glue puff locus derived from the Hikone-R wild-type strain. This strain produces *Sgs-3* mRNA approximately 1000 nucleotides in length, rather than the 1120 base *Sgs-3* mRNA characteristic of the Oregon-R wild-type strain from which the locus was originally cloned (Meyerowitz and Hogness, 1982). The strain also contains an additional copy of the *Sgs-3* gene of the Oregon-R type, on a 6 kb DNA fragment inserted into the 66E region of the third chromosome by P-factor-mediated transformation. This insertion is designated *Tf(3)GA6.0-1*. The 6 kb piece has 2.3 kb of 5′ upstream sequence, the entire *Sgs-3* gene, and 2.5 kb of 3′ downstream sequence. The *Tf(3)GA6.0-1, Sgs-3^{HR}* strain, with both the Oregon-R *Sgs-3* allele (*Sgs-3^{OR}*) and the Hikone-R *Sgs-3* allele (*Sgs-3^{HR}*), produces both the Oregon-R and Hikone-R *Sgs-3* RNA transcripts in normal quantity, tissue and developmental stage, as assayed in RNA gel blot experiments (Crosby, 1983; Crosby and Meyerowitz, unpublished data). Female flies carrying a *l(1)npr-1* X chromosome balanced over the *Binsn* chromosome were crossed with males of the *Tf(3)GA6.0-1, Sgs-3^{HR}* strain, producing male larvae hemizygous for *l(1)npr-1* and heterozygous for the third chromosome of the transformed strain. These larvae were dissected and their salivary glands removed. RNA was extracted from the glands and from control glands taken from *Binsn* siblings of the experimental larvae, and both samples assayed for the presence of the *Sgs-3* RNA by electrophoresis followed by blot hybridization. RNA samples from hemizygous *l(1)npr-1* or *Binsn* male larvae from the original mutant stock were tested for *Sgs-3* expression at the same time. The results are shown in Figure 3. In the control *Binsn; Tf(3)GA6.0-1, Sgs-3^{HR}* both the 1000 base and the 1120 base *Sgs-3* RNAs are present. Neither were detected in the *l(1)npr-1; Tf(3)GA6.0-1, Sgs-3^{HR}* salivary glands. The other RNA samples gave the expected results. That the introduced 6 kb *Sgs-3^{OR}* gene fails to express in the absence of a wild-type *l(1)npr-1* locus shows that at least some of the 68C DNA sequences necessary for interaction with this locus are contained within the 6 kb fragment, and are thus no more than a few thousand base pairs from the *Sgs-3* gene.

Synthesis of Glue RNAs from 3C and 90BC Is Reduced in *l(1)npr-1*

To understand what effects the *l(1)npr-1* mutation has on other genes expressed in larval salivary glands, two other cloned genes were used as ^{32}P-labeled probes on a filter that carried a developmental profile of RNA from *l(1)npr-1* and *Binsn* larvae that had been cultured at 22°C (see Figure 4). The *Sgs-4* glue gene at position 3C on the X chromosome has been cloned and characterized (Muskavitch and Hogness, 1980). An *Sgs-4* clone, aDm1523, which contains the D Hind III fragment of the *Sgs-4* region (Muskavitch and Hogness, 1980), was modified by removing the Eco RI fragment that extends from the 5′ terminus of the *Sgs-4* gene upstream into the pBR322 vector. This leaves only *Sgs-4* sequences without flanking DNA in the new clone. This DNA was ^{32}P-labeled and hybridized to

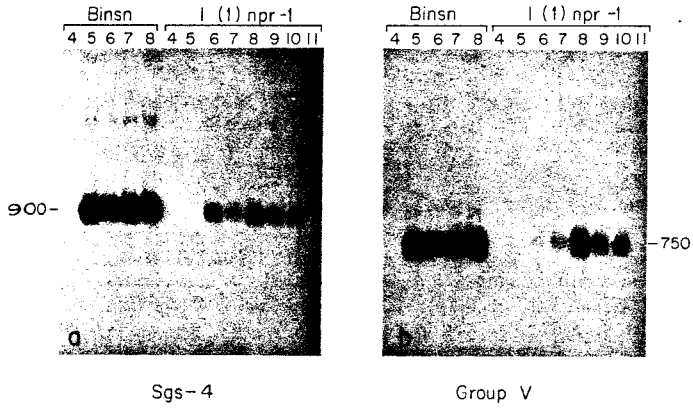

Figure 4. Developmental Expression of Glue RNAs from 3C and 90BC

RNA was extracted from salivary glands of male *Binsn* larvae from each of days 4, 5, 6, 7, and 8, and from male *l(1)npr-1* larvae of days 4, 5, 6, 7, 8, 9, 10, and 11. This RNA was fractionated in a formaldehyde-agarose gel and blotted to a nitrocellulose filter as described (see Experimental Procedures). Each lane contains RNA from salivary glands of five animals. (a) The filter was probed with DNA homologous with the *Sgs-4* glue gene. (b) After removal of the previous probe, this filter was hybridized with ^{32}P-labeled aDm1687 DNA, which carries sequences homologous with the Group V salivary gland RNA (Guild, 1984). Exposure time for each filter was 9.5 hr. Probes were removed with two 5 min washes in 0.01× SSPE at 100°C. RNA content in each lane was measured by hybridization with a probe homologous to ribosomal RNA and was found to be approximately equal in each lane. The lengths of each RNA are shown in nucleotides.

the nitrocellulose filter as described in Experimental Procedures. The results are presented in Figure 4a. They indicate that *Sgs-4* is transcribed and accumulated in a *l(1)npr-1* background, but that either the transcription rate or RNA stability is reduced, resulting in a level of *Sgs-4* mRNA 7% of that found in *Binsn* control salivary glands (measured by scanning densitometry of a series of autoradiographs, data not shown). Because the development of *l(1)npr-1* seems delayed, measurements were taken from the day of maximum accumulation (day 8). *Binsn* RNA from day 8 was used as the control. RNA abundance in different lanes was normalized by measurement of ribosomal RNA levels in each lane using a ^{32}P-labeled ribosomal DNA clone as a probe (cDm103, Glover and Hogness, 1977).

After removal of the aDm1523-derived DNA, the filter was probed with cloned DNA from the 90BC region of chromosome 3R. This clone, aDm1687, contains the Group V gene, which codes for an abundant salivary gland RNA that is probably a glue protein mRNA (Guild, 1984). Upon hybridization, washing, and exposure of the RNA filter, the results showed a reduction of Group V RNA in *l(1)npr-1* salivary glands as compared to *Binsn* controls (see Figure 4b). Scans of the autoradiographs showed the level of the Group V RNA in *l(1)npr-1* glands to be 27% of that found in control salivary glands. Again, day 8 was found to be the day of highest Group V RNA levels in *l(1)npr-1* larvae, and *Binsn* day 8 RNA was used as a control.

Analysis of these two transcripts in larvae carrying *l(1)t435, l(1)d.norm.-1*^{a}, or a deficiency for the 2B5 region again showed reductions in accumulation, although quantitative analysis was not attempted because of different developmental rates. Because all of the mutants carry X chromosomes that differ from their controls, we had to rule out the possibility that these *Sgs-4* genes are less active than those of the control stock. This was done by analyzing RNA from larvae of the genetic constitution *l(1)npr-1/ Dp(1:Y)67g,y*^{2}. These larvae, which carry the Sgs-4 gene of the *l(1)npr-1* X chromosome and a wild-type *l(1)npr-1* locus in the duplication, were found to produce wild-type levels of *Sgs-4* RNA from the *l(1)npr-1* X chromosome (data not shown).

Discussion

Our analysis of the *l(1)npr-1* locus has demonstrated it to be a *trans*-acting regulator of the 68C glue genes. In addition, we have shown that the nature of the *l(1)npr-1* mutation is such that it prevents production of the normal product of the locus, and further, that the normal function of the wild-type product of the locus involves either direct or indirect interaction with DNA sequences within the 68C glue gene cluster. This interaction is necessary for either the transcription or early stability of the 68C transcripts. The simplest model for the action of the 2B5 locus is that a protein coded by an RNA transcribed from the 2B5 region interacts with the DNA within or surrounding the 68C glue genes and thereby allows RNA polymerase II to transcribe the genes. It is also possible that the normal 2B5 product only induces another locus, or a set of loci whose products are the ones that directly interact with the 68C DNA. There are several known examples in eucaryotic systems of genes that require interaction with a *trans*-acting regulatory protein in order to be transcribed. Transcription of mammary tumor virus (MTV) DNA in MTV-infected rat cells is induced by binding of the glucocorticoid receptor protein to sequences within or near the transcribed region. In the presence of glucocorticoid hormones the receptor binds to one site upstream of the transcription initiation site and to four sites between 4 and 8 kb downstream of the initiation site. This appears to be a prerequisite for transcription of the viral DNA (Ucker et al., 1981; Payvar et al., 1983). The progesterone receptor is thought to induce transcription of the ovalbumin and conalbumin genes in chicken oviduct. In the presence of the steroid

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

The normal expression of *Sgs-3* transcription as regulated by the *l(1)npr-1* locus involves at most 6 kb of 68C DNA. This was revealed by analyzing the expression of an *Sgs-3* gene in a 6 kb fragment that was introduced into the fly genome by P-factor-mediated transformation. The 6 kb region extends 2.3 kb on the 5′ side of *Sgs-3* and 2.5 kb on the 3′ side. This result implies that the *l(1)npr-1* mutation affects a regulatory element which normally interacts with the DNA within this 6 kb region to allow for *Sgs-3* transcription. We have not yet performed a similar analysis on the *Sgs-7* and *Sgs-8* genes.

The *l(1)npr-1* mutation inhibits ecdysterone-induced regression of the 68C puff and does not allow expression of the 68C glue genes. The mutation, however, is thought to have no effect on ecdysterone levels (Fristrom et al., 1981). Hansson and Lambertsson (1983) have shown that accumulation of the *Sgs-3, Sgs-4, Sgs-7,* and *Sgs-8* glue RNAs does not occur in larvae that are genetically ecdysterone deficient, and that accumulation of these RNAs can be induced in ecdysterone-deficient larvae by application of exogenous hormone. Thus induction of 68C glue gene expression and regression of the 68C puff (Ashburner, 1973) both require ecdysterone, and both are inhibited by the *l(1)npr-1* mutation. It is possible that the mutation prevents production of normal levels of functional ecdysterone receptor (Maroy et al., 1978; Yund et al., 1978) or of some component of this receptor required in third instar larvae. This speculation is consistent with the overall phenotype of the *l(1)npr-1* mutation, which is evident in tissues other than salivary glands, and which undoubtedly involves failure of normal expression of many genes other than those of the 68C puff (Stewart et al., 1972; Fristrom et al., 1981). That some puffs respond normally to ecdysterone in a *l(1)t435* background (Belyaeva et al., 1981), and that the *Sgs-4* and 90BC glue RNAs are produced (though at a reduced level) in the mutant strains suggests that the *l(1)npr-1* gene product is not a component required for all cellular responses to ecdysterone.

A final aspect of the *l(1)npr-1* mutation deserves comment: the presence of a normal-appearing 68C puff in the mutant strains in the absence of detectable synthesis or accumulation of any of the three 68C glue RNAs. Polytene chromosome puffs are associated with chromosomal regions of active transcription (Pelling, 1964; Berendes, 1968; Ellgaard and Clever, 1971; Tissieres et al., 1974; McKenzie et al., 1975; Zhimulev and Belyaeva, 1975; Belyaeva and Zhimulev, 1976; Bonner and Pardue, 1977; Mitchell et al., 1978). In wild-type larvae the only abundant salivary gland RNAs from the region of DNA that give rise to the 68C puff are the three 68C glue messengers (Meyerowitz and Hogness, 1982; Crosby and Meyerowitz, unpublished data). It thus seems possible that the 68C puff in *l(1)npr-1* larvae may not be correlated with transcription in the region. Equally likely are a number of alternative possibilities. Among them: that the *l(1)npr-1* mutant strains produce an abundant salivary gland RNA at 68C different from those produced at 68C in wild-type larvae, but still giving rise to a puff at that location; that the transcription of the 68C glue RNAs is normal in *l(1)npr-1* salivary glands but that RNA degradation is so rapid that new RNA synthesis cannot be detected even in a 15 min pulse-labeling experiment; or that RNA transcription at 68C in the mutant strains has started and then stopped, with nascent transcripts and transcriptional proteins still present to give a normal puff appearance. Experiments to differentiate between some of these possibilities are in progress. Whether the results will require a reconsideration of the relation of puffing and transcription remains to be seen.

Experimental Procedures

Materials

Ecdysterone (β-ecdysone, 20-OH ecdysone) was purchased from Sigma Chemical Co., dissolved at a concentration of approximately 10 mg/ml in 100% ethanol and stored at −20°C. The exact concentration of this stock solution was determined by diluting a small aliquot into methanol (Baker analyzed, HPLC grade) and measuring its absorbance at 242 nm, assuming a value of $\epsilon = 12{,}400$ (Hoffmeister et al., 1965; Hocks and Wiechert, 1966). Deoxycytidine 5′-α-^{32}P-triphosphate (α-^{32}P dCTP, 1800–5400 Ci/mmole) was obtained from ICN Pharmaceuticals Inc. and Amersham Corporation. ^{3}H-uridine (38.4 Ci/mmole), Aquasol-2 (liquid scintillation counting cocktail), and Liquifluor (PPO–POPOP toluene concentrate) were from New England Nuclear. Toluene (scintillar grade) was from Mallinckrodt Inc. Proteinase K was purchased from EM Reagents. Yeast tRNA was obtained from Miles Laboratories Inc. The following items were purchased from Kodak: XAR-5 film for autoradiography of RNA gel blot filters, Technical Pan Film 2415 for photography of stained chromosomes, and HC-110 developer and rapid fixer for film processing. A Zeiss photomicroscope was used for photography of chromosome spreads. A Gilford Multi Media Densitometer and a Hewlett-Packard 3390A Reporting Integrator were used for densitometric scans of autoradiographs.

Drosophila

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

Salivary Gland Cultures

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

Purification of Unlabeled RNA from Salivary Glands

Ten salivary gland lobes were transferred to a microcentrifuge tube containing 20 μl of extraction buffer (20 mM NaCl, 20 mM Tris-hydrochloride [pH 7.8], 40 mM ethylenediamine tetraacetic acid [EDTA], 1% sodium dodecyl sulfate [SDS]) plus 20 μg of yeast tRNA and immediately frozen in liquid nitrogen. Twenty microliters of phenol plus 20 μl of chloroform-isoamyl alcohol (100:1) were added and the tube vortexed as the extraction buffer and glands thawed. The tube was then spun briefly in a microcentrifuge to separate the two phases and the organic phase was removed and discarded. The aqueous solution was extracted two more times with phenol/chloroform and then transferred to a clean tube. The organic solution from the last extraction was reextracted with 10 μl of extraction buffer which was then pooled with the previously extracted aqueous solution. The lysate was then extracted with 20 μl of chloroform-alcohol, transferred to a new tube, and extracted with 20 to 40 μl of ethyl ether. Any residual ether was removed by directing a stream of air over the solution. One-fifteenth volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes of 100% ethanol were added at 0°C. The solution was mixed by vortexing, transferred to a −20°C freezer for 15 min, then placed in dry ice for 20 min or longer. The tube was held at 2°C until the contents were completely thawed and then spun in an Eppendorf microcentrifuge at 15,000 rpm, 15 min, 2°C. The supernatant was removed, residual liquid removed by brief lyophilization and the pellet resuspended in 5.6 μl of water.

RNA Blots

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

RNA filters were pretreated, hybridized to ^{32}P-DNA probes, washed, and autoradiographed as described by Alwine et al. (1980) with the following modifications: glycine was not included in the hybridization buffer for the pretreatment, the concentration of salmon sperm DNA was 100 μg/ml, after hybridization the filters were washed briefly in 50 ml of 1X SSPE, 0.1% SDS at room temperature then for 2 hr in two changes (225 ml each) of the same solution at 42°C. The probes used were nick-translated ^{32}P-DNA prepared as described by Rigby et al. (1977). The Group V RNA clone, aDm1687, was a gift from Dr. Greg Guild (Department of Biology, University of Pennsylvania, Philadelphia, PA).

Purification and Hybridization of ^{3}H-RNA

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

Chromosome Spreads for Puff Measurements

Pairs of salivary gland lobes were transferred to 20 μl drops of aceto-lactic orcein stain solution (2% [w/v] orcein in 1:1 lactic:acetic acid) on a siliconized coverslip. One minute later the glands were squashed between the coverslip and a glass slide. The stained chromosomes were photographed using a Zeiss photomicroscope and the diameter of the 68C puff and the 69A1-3 region were measured from projections of the negatives. Puff size is expressed as a ratio of the diameter of the chromosome at the region of maximal 68C puff diameter, which is just proximal to 68C3-4, to the diameter of the unpuffed region at 69A1-3.

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Chapter 3:

Protein:DNA Interactions at the *Sgs-3* Glue Gene in *Drosophila* and Their Correlation with Developmental Expression

Protein:DNA Interactions at the *Sgs-3* Glue Gene in *Drosophila* and Their Correlation with Developmental Expression

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Abstract

The 68C glue genes of *Drosophila melanogaster* are coordinately expressed in a spatially and temporally limited manner. The proximal promoter element of *Sgs-3* is sufficient to properly regulate this expression (Vijay Raghavan et al. 1986). We have analyzed protein:DNA interactions in this region using in vivo dimethylsulfate (DMS) footprinting. The region contains three distinct protein binding domains. By footprinting various tissues and stages, we have identified a binding activity which correlates with the tissue and stage specificity of the *Sgs-3* proximal promoter element. Analysis of protein:DNA contacts in non-expressing *trans*-acting mutants shows a lack of binding in this region.

Introduction

The glue proteins of *Drosophila* are produced in the salivary glands of the third instar larva and serve to secure the pupal case to a solid surface during metamorphosis (Fraenkel and Brookes 1953). The 68C gene cluster contains three glue protein-coding genes, *Sgs-3*, *Sgs-7*, and *Sgs-8* (Crowley et al. 1983). These genes are regulated by the steroid hormone ecdysterone, which is required for both activation and cessation of RNA synthesis (Hansson and Lambertsson 1983; Crowley and Meyerowitz 1984). The activation of these glue genes is disrupted by two *trans*-acting mutations. Two non-pupariating lethal mutations, $npr1^3$ (also known as *l(1)npr-1*; Crowley et al. 1984) and $l(1)su(f)^{ts67g}$ (Hansson and Lambertsson 1983) cause failure to accumulate 68C glue RNAs to any detectable level, despite the appearance of normal chromosomal decondensation in the region. The $l(1)su(f)^{ts67g}$ mutation affects ecdysterone levels in the larval hemolymph and its effects can be overcome by administration of the steroid (Hansson and Lambertsson 1983, 1984). On the other hand, the $npr1^3$ mutation acts cell-autonomously (Vijay Raghavan et al. 1988) and its effects are not rescued by exogenous hormone (Belyaeva et al. 1981).

Analysis of *cis*-acting regulatory elements in the 68C region has concentrated primarily on those regulating the largest of the three genes, *Sgs-3*. P-element transformation studies have demonstrated important regulatory domains in the proximal element immediately upstream of the transcription start site (Vijay Raghavan et al. 1986; Martin et al. 1989; Giangrande et al. 1989; Todo et al., accompanying report), in a distal element approximately 600 basepairs (bp) from the start site (Bourouis and Richards 1985; Meyerowitz et al. 1986; Roark et al., in press) and in multiple remote regions from 1.5 to 2.5 kilobases (kb) upstream (Giangrande et al. 1987). In addition, changes in chromatin structure identified by DNase I-hypersensitive sites have been associated with regions at -750, -600, -470 and -75 bp relative to *Sgs-3* in third instar salivary glands (Ramain et al.

1986), with the site at -600 shown to be functionally important for abundant levels of expression (Ramain et al. 1988; Roark et al., in press). The site at -75 bp is probably associated with the proximal promoter element which has been shown to be capable of conferring tissue- and stage-specific expression to both the *Sgs-3* promoter (Vijay Raghavan et al. 1986) and a heterologous promoter (Martin et al. 1989; Roark et al., in press). These latter two studies have defined the functional sequences specific to the *Sgs-3* proximal element as -98 to -56 bp upstream of *Sgs-3*, while the TATA domain falls between -40 and -34 bp upstream.

Precise studies on protein:DNA interactions at *Sgs-3* have been limited in the past by technical difficulties. The DNase I hypersensitivity studies of Ramain et al. (1986,1988) provide a resolution of ± 30 bp and require as many as 200 hand-dissected glands per lane. Attempts at in vitro footprinting using isolated protein extracts (Gilbert et al. 1976) or in vivo footprinting using conventional methods (Church and Gilbert 1984; Jackson and Felsenfeld 1985) would require extremely large amounts of tissue (see for example Giangrande et al. 1989). Recently, Mueller and Wold (1989) have developed a novel method for in vivo footprinting, utilizing the polymerase chain reaction (PCR) to exponentially amplify genomic sequences, while still maintaining the specificity of a sequence ladder. This technique allows in vivo footprinting at single base resolution from as few as 10^5 genomes, the equivalent of approximately one salivary gland pair. Most importantly, in vivo footprinting allows analysis of protein binding under physiological conditions, providing information on actual protein:DNA interactions as opposed to the potential for binding seen under in vitro conditions.

Utilizing this new footprinting procedure, we have analyzed protein:DNA interactions in the proximal promoter element of *Sgs-3*. Comparisons were made between dimethylsulfate (DMS) sequence ladders from wild-type salivary glands collected during a stage of active glue transcript synthesis, and from naked DNA. These studies define three protein binding domains within the proximal element. The farthest upstream site in the

proximal element shows strong homology to the ecdysterone responsive element of *hsp23* (Mestril et al. 1986). The farthest downstream site is the TATA domain. Footprinting of salivary glands at various stages of development and in various tissues shows that binding to these homologies is invariant, whereas binding to the middle site occurs only in the proper tissue and stage. In addition, analysis of binding in the background of two *trans*-acting mutants, $npr1^3$ and $l(1)su(f)^{ts67g}$, shows patterns similar to non-expressing tissues and stages of development. Since the normal product(s) of the *BR-C* locus (site of the $npr1^3$ mutation) has been shown to regulate expression within the proximal element (Mathers 1989), we suggest that the lesion in the $npr1^3$ mutation may affect, directly or indirectly, the ability of a transcription factor to bind to the tissue- and stage-specific domain in the proximal promoter.

Results

DMS-footprinting in salivary glands

We have taken advantage of the properties of dimethylsulfate (DMS) for our in vivo footprinting studies in salivary glands. DMS passes through cell membranes, allowing minimal disruption of the glands prior to DNA methylation. Using standard Maxam and Gilbert (1980) conditions, limited DMS methylation followed by piperidine cleavage produces a series of DNA fragments specifically cleaved at G residues. Binding of protein to DNA can result in reductions and enhancements in the reactivity of DMS to specific residues, depending on the protein:DNA contact points and the boundaries of these interactions (Gilbert et al. 1976). Thus, by comparing the relative abundance of specific fragments in a ladder representing individual G residues in the sequence, a pattern of protein:DNA contacts can be visualized.

The ligation-mediated in vivo footprinting method of Mueller and Wold (1989) was utilized as a means of studying the protein contact points in the *Sgs-3* proximal promoter element, using minor modifications (Figure 1A and see Materials and Methods). Briefly, DMS-treated salivary gland DNA is isolated and cleaved with piperidine. After denaturation, a region of the *Sgs-3* promoter region is specifically replicated using a gene-specific oligonucleotide as primer. A linker (Figure 1B) is ligated to the G-specific end of this double-stranded region. Using oligonucleotides representing this linker region and a nested gene-specific region, exponential amplification by polymerase chain reaction (PCR) (Saiki et al. 1988) of randomly cleaved fragments is achieved and restricted to the *Sgs-3* promoter region. The final round of PCR involves the addition of more nested gene-specific primer, now end-labeled with ^{32}P. The resulting products are run over a standard denaturing gel and the resulting sequence ladder visualized by autoradiography.

Using the DNA from two salivary glands dissected from larvae at the mid-third instar stage of development, a comparison was made between in vivo methylation by DMS and in vitro methylation (Figure 2). Differences in DMS reactivity could be seen in three regions over the sequences analyzed (summarized in Figures 3 and 4). The first of these regions falls approximately in the sequences from -91 to -79 bp upstream of the RNA start site and is named Site 1. Binding domains are defined by the extent of contiguous G residues protected from DMS in vivo. Because this procedure visualizes only G residue contacts, protein interactions are probably extending beyond the limits stated here. Binding to Site 1 is also characterized by enhancements of DMS methylation at -98, -97, -96 and -93. These enhancements may help to define the left boundary of the binding site.

A second site of protein:DNA interaction lies between nucleotides -67 and -53, in a region of relatively high A-T content. Sequences in Site 2 have also been shown to be important for proper expression from the *Sgs-3* promoter (Martin et al. 1989; Todo et al., accompanying report). Protections are visualized at only three bases in this region, with strong protections at -66 and -58. The moderate reduction of -67 fragments may represent the left end of the binding site, as enhancements are seen at -73, -71 and -69. The enhancements in this region could also be used to define the rightmost limit for Site 1 binding. These enhancements may represent a pocket of hydrophobicity between proteins bound at Site 1 and Site 2. Alternatively, the enhancement might represent an altered helical structure caused by protein binding. The relatively weak enhancements at -52 and -50 may serve to limit the right boundary of the Site 2 domain. No known strong homologies to other protein-binding domains occur in this region, but a weak dyad symmetry is observed from -70 to -60 on one strand and -59 to -49 on the other strand (see Figure 4).

The third site of alteration between in vivo methylated DNA and naked DNA is defined by protections at -32 and -23 (Figure 2). These nucleotides are directly adjacent to the TATA homology, a region known to be important in many genes transcribed by RNA

polymerase II. The TATA domain has been shown to be important for in vivo *Sgs-3* expression (Giangrande et al. 1989). Binding to the TATA domain is also characterized by enhancements to reactivity at -36 and -33. We see no interactions outside of these four nucleotides, differing from the findings of Giangrande et al. (1989), which utilized tissue extracts for DNase I footprinting.

In an attempt to distinguish between strong and weak interactions, autoradiographic exposures of the sequencing gels (without the use of intensifying screens) were scanned by densitometry. Once shown to be within the linear range of the film, scans of gel tracks were integrated and the values for in vivo and naked DNA samples compared (see Materials and Methods). The results are presented in Figure 3 and summarized in Figure 4. The strongest reproducible protections (>30% reduction) occur at nucleotides -91, -86, -66 and -58. The strongest enhancements (>1.7-fold increase) occur at nucleotides -97 and -69. The relatively weak interactions seen overall might be explained in a number of ways. The salivary gland samples used here contain small portions of fat body as well as non-secretory neck cells within the gland which could act to dilute a strong binding signal by contributing fragments normally protected in expressing cells. In addition, we have observed that the onset of glue gene expression within a salivary gland occurs in a posterior to anterior gradient (P.M., unpublished), with the possibility that not all cells are actively synthesizing *Sgs-3* at a given time. Finally, binding to these sites could be relatively weak.

Analysis of protein:DNA interactions in non-expressing salivary glands and other tissues

Comparison of intact and purified salivary gland DNA defines three binding domains in the proximal promoter element. The ability to determine the protein-binding profile in the region using a relatively small amount of tissue allows us to analyze the proximal element under various conditions, including where the gene should not be active. The genes of the 68C glue cluster are expressed primarily, if not exclusively, in the salivary glands of the

mid-third instar larvae (Meyerowitz and Hogness 1982). Ramain et al. (1986) have previously shown that the DNase I-hypersensitive site present in mid-third instar salivary glands at -75 (±30 bp) is reduced in glands from white prepupae, several hours past the cessation of glue transcription. They also found that the hypersensitivity pattern in non-expressing cultured cell lines was the same as that found in white prepupae. This suggests that the DMS footprinting pattern should be altered in some fashion, but that binding should not be eliminated in salivary glands from stages other than mid-third instar and in other larval tissues.

Using the PCR footprinting technique, we have analyzed the protein:DNA interactions in salivary glands from early third instar, before the onset of glue transcription, and from late third instar, when the lumen of the salivary gland is bloated with the glue protein secretions and glue transcription has ceased. When compared with the sequence patterns of DNA methylated in vitro and in vivo, we find interactions in two regions of the promoter (Figure 5 and summarized in Figures 6 and 7). Binding, as defined by the pattern of protections and enhancements seen under expressing conditions, is seen in Site 1 and in the TATA domain. While the general pattern of protections and enhancements does not change, some interactions seen in mid-third instar glands are not observed here (compare Figures 3 and 6). Binding is not seen at Site 2. Interestingly, enhancement of position -73 is still seen despite the vacancy at Site 2. This suggests the Site 1 domain may extend as far as -74 or -75 and thus gives credence to the possibility that the putative right half-site of the inverted repeat is occupied despite its lack of functional necessity and weak protection from DMS reactivity. As seen in Figure 5, the strength of the interactions in these glands is weaker than those observed for mid-third instar glands. This weaker contact and slightly altered pattern suggest two possibilities: the binding to these sites is transient (possibly the result of a failure to form a stable complex) or alternatively, the factors binding to these sites at these stages are not identical to the factors involved in active transcription. The results can not distinguish between these possibilities, nor are they mutually exclusive.

Analysis of DMS footprint patterns in other, quiescent, tissues yields results similar to those seen in non-expressing stages of salivary glands. In vivo methylation of dissected fat body from mid-third instar larvae shows a protein:DNA contact pattern of weak binding in Site 1 and the TATA domain (Figure 5 and summarized in Figures 6 and 7). Footprinting analysis of carcass (the larvae devoid of salivary glands) and dissected gut tissue from mid-third instar larvae shows a similar contact pattern (data not shown). This pattern mimics that seen in the non-expressing salivary gland stages, suggesting that factors capable of binding to these two regions exist throughout third instar (and possibly other stages as well) and that their presence is not spatially limited to the salivary gland. These results coincide with the findings of Ramain et al. (1986), possibly explaining the reduction in hypersensitivity seen in this region and the finding that hypersensitivity is not completely eliminated. Significantly, our results point to the sequence within Site 2 as the region important for specifying both tissue- and stage-limited expression. The failure to see binding at Site 2 under any conditions except active glue synthesis suggests that a factor capable of binding in this region is synthesized or activated exclusively in salivary glands at a stage coincident with glue gene transcription.

Footprinting patterns in larvae carrying trans*-acting mutations affecting 68C glue expression*

The normal developmental expression of the 68C glue genes can be disrupted by introducing mutations from either of two X-linked loci. Certain mutations at the 2B5 locus eliminate the accumulation of RNA from all three 68C glue genes and greatly reduce the expression of two other cloned glue genes, *Sgs-4* and *Sgs-5* (Crowley et al. 1984). Mutations in the region also have profound effects on larval metamorphosis (Kiss et al. 1978), imaginal disc development (Fristrom et al. 1981; Kiss et al. 1988) and the ecdysterone-induced puffing patterns in salivary glands (Belyaeva et al. 1981). The 2B5

region shows both genetic complexity (Belyaeva et al. 1980) and molecular complexity (Chao and Guild 1986) and has been named the *Broad-Complex* (*BR-C*; Lindsley and Zimm 1986).

For our studies on glue gene expression, we have primarily used the $npr1^3$ mutation (also called *l(1)npr-1*), which acts in many ways similarly to a complete deficiency for the *BR-C* and is in the non-pupariating class of alleles, referred to as *l(1)2Bad* (Kiss et al. 1988). By studying expression of P-element transformation constructs from the 68C region in the $npr1^3$ mutant background, we have shown that as little as 130 bp of *Sgs-3* upstream sequence is required for proper regulation by the *BR-C* product(s) (Crowley et al. 1984; Vijay Raghavan et al. 1986). Mathers (1989) has gone on to show that the effect of the *BR-C* product(s) on *Sgs-3* expression is the result of sequences between -130 and +1, presumably within the proximal promoter element. These findings suggest that the product(s) of the *BR-C* is interacting, directly or indirectly, with sequences in the proximal promoter to activate transcription.

We set out to determine if salivary glands in $npr1^3$ mutant larvae display any altered profile of protein:DNA interactions when compared to wild-type binding patterns. Salivary glands from hemizygous $npr1^3$ larvae and from larvae cytologically deficient for the 2B5 region (genotype *Df(1)S39/Dp(1;Y)Sz280*; Belyaeva et al. 1981) were treated with DMS in vivo and the recovered DNA analyzed by PCR footprinting. The protein:DNA contact patterns are similar to those seen in non-expressing tissues and stages of salivary gland development (Figure 5). Weak binding is again observed over Site 1 and the TATA domain, but no apparent contact occurs in the region of Site 2. Whether Site 2 is occupied in wild-type glands by the *BR-C* product(s) or by factors downstream in a regulatory cascade can not be determined by these results and awaits the further characterization of the *BR-C* region.

In addition to the $npr1^3$ mutation, 68C glue gene expression is also blocked in larvae carrying the X-linked temperature-sensitive mutation, $l(1)su(f)^{ts67g}$, when raised at

non-permissive temperature (Hansson and Lambertsson 1983). Certain mutations at the *su(f)* locus have been proposed to result in lethality of mitotically active cells (see Wilson 1980) and result in a non-pupariating phenotype when reared at non-permissive temperature (Dudick et al. 1974). A specific effect of *l(1)su(f)* mutations is the strong reduction in titers of the steroid hormone, ecdysterone (Klose et al. 1980). When ecdysterone is exogeneously administered, $l(1)su(f)^{ts67g}$ larvae have been shown to initiate glue transcription and to proceed to the formation of pseudopupae (Hansson and Lambertsson 1983, 1984), thus displaying an ecdysterone rescue phenotype (unlike mutations in the *BR-C*). These studies have been cited to propose that glue gene activation requires the presence of ecdysterone in early third instar larvae. Therefore, we have analyzed the protein:DNA interactions in salivary glands from larvae carrying the $l(1)su(f)^{ts67g}$ mutation in order to discern which, if any, protein-binding domains are affected by the reduction of ecdysterone.

Larvae hemi- and homozygous for the $l(1)su(f)^{ts67g}$ mutation were collected over a 2 hr interval and reared at 25°C until 60 hr after oviposition. At this stage, larval cultures were shifted to the non-permissive temperature of 30°C and allowed to develop for 40 hr. Control larvae were maintained at 25°C. In vivo DMS treatment of the glands and subsequent amplification of the DNA produces footprint ladders as shown in Figure 5 and summarized in Figures 6 and 7. Protein:DNA contacts resemble those detected in glands from $npr1^3$ mutants, as well as those from non-expressing tissues and stages. Weak binding to Site 1 and the TATA domain are observed, but characteristic protections and enhancements for Site 2 binding are not obtained. Therefore, binding to Site 1, which contains homology to the ecdysterone responsive element of *hsp23* (Mestril et al. 1986), is not eliminated despite the severe reduction in ecdysterone titers in these larvae. However, binding to Site 2 is affected by the decrease in hormone titer.

Discussion

We have used the PCR amplification technique of in vivo DMS footprinting (Mueller and Wold 1989) to analyze interactions between nuclear proteins and the DNA sequences in the proximal regulatory element immediately upstream of the *Sgs-3* glue gene. Our application of this technique to interactions within dissected tissues represents a significant step for the study of developmental gene regulation. The *Sgs-3* proximal element contains regulatory sequences sufficient for directing tissue- and stage-specific expression of this 68C glue gene (Vijay Raghavan et al. 1986). By comparing DMS footprinting patterns from salivary gland DNA methylated in vivo and in vitro, we are able to define three separate binding domains in the proximal region. These three domains fall with clusters of sequence homology upstream of the *Sgs-3* homologs in several related *Drosophila* species (Martin et al. 1988), thereby showing evolutionary conservation. Binding to Site 1 and the TATA domain occurs not only in expressing salivary glands, but is also observed to a weaker extent at stages and in tissues when *Sgs-3* is not transcribed. In addition, weak binding to these regions is seen in *trans*-acting mutant backgrounds which fail to express the 68C glue genes. In contrast, binding to the region defined as Site 2 occurs only in mid-third instar salivary glands, implicating this region in governing the temporal and spatial specification exhibited by the proximal element. The absence of binding to Site 2 in the *trans*-acting mutants suggests that these genetic lesions prevent a transcription activator from binding the upstream sequences of *Sgs-3*.

The results point to three important regions involved in protein:DNA interactions. Deletion or replacement studies (Martin et al. 1989; Roark et al., in press; Todo et al., accompanying report) and point mutagenesis (Giangrande et al. 1989; Todo et al., accompanying report) have shown the functional importance of each of these three regions for in vivo *Sgs-3* expression. Our findings would suggest that each of these three regions contributes separate functional components to the transcriptional activation process. Martin

et al. (1989), however, concluded that each of two regions of redundant function, roughly corresponding to Sites 1 and 2, was sufficient for tissue- and stage-specific regulation. These conclusions may be complicated by the findings of Roark et al. (in press), which show that the distal element around -600 bp is also capable of conferring tissue and stage specificity. Since this distal region was present in the deletion studies of Martin et al. (1989), it is possible that the deletion of Site 2 sequences, which we find to be responsible for developmental specificity, is replaced functionally in their constructs by elements at the -600 site. Indeed, Todo et al. (accompanying report) have found that sequences homologous to the functionally important bases in the proximal element are found at the distal element in a region which is necessary for full level expression (Ramain et al. 1988). Based on these homologies, we speculate that footprinting patterns in the distal element may show some similarity to those we present for the proximal element.

By mutating nucleotide -53, Martin et al. (1989) find aberrant expression extending well past the normal repression stage for *Sgs-3*. They conclude from this finding that a specific site which mediates repression is altered by this mutation. Because we fail to see binding in this region in late third instar salivary glands and because the altered base falls within the Site 2 domain, we instead propose that the -53 mutation alters the binding capabilities of the Site 2-binding protein, creating a protein:DNA complex which is inaccessible to repressive factors. In addition, we see a smaller footprint around the TATA region than found by Giangrande et al. (1989). This difference may be explained by the use of DMS in our studies versus DNase I for footprinting. The smaller DMS molecule would be better able to access regions of DNA which are covered by protein but not tightly bound. Also, the G-specific reaction used in our study limits the number of bases we can analyze for interactions. Therefore, the TATA domains presented in Figures 2 through 5 are minimal estimates based on our results of the region covered by the TATA-binding factor.

The apparent sightings of sequence homologies in the *Sgs-3* and other glue gene promoter regions are numerous (Shermoen and Beckendorf 1982; Hofmann and Corces 1986; Mestril et al. 1986; Jongens et al. 1988 as examples) and for this reason we cautiously approach some interesting homologies. Mestril et al. (1986) present a detailed deletion series of the ecdysterone inducible *hsp23* promoter and conclude that two closely spaced regions, showing some inverted repeat homology, are responsible for the ecdysterone responsiveness. They also present rough homologies to this sequence in other ecdysterone-inducible promoters (although the functional orientation of several of these putative "half-sites" is curiously inverted). We have searched for a seven nucleotide consensus of these sequences (TTT[G/C]CAT) in the 68C glue gene cluster (P.M., unpublished). Homology to this sequence, with an adjacent inverted repeat, was seen in five regions of the cluster. One of these repeats appears at Site 1 and is highlighted in Figure 4. Another repeat falls between the *Sgs-7* and *Sgs-8* genes in a region required for *Sgs-8* expression and shown to have enhancement capabilities (A. Hofmann, M. Garfinkel and E. Meyerowitz, in preparation). Homology is also seen in the *Sgs-4* glue gene, in a region known to be required for proper expression (Hofmann and Korge 1987; Jongens et al. 1988). Strong homology, however, is not seen at the -600 distal element of *Sgs-3*. This consensus also shows homology to the octamer motif (ATTTGCAT) found to bind the Oct family of transcription activators (see Schöler et al. 1989). Todo et al. (accompanying report) have found that four of the seven bases within the left half of this repeat in the *Sgs-3* proximal element are required for significant levels of expression. In agreement with this, we find the upstream half of Site 1 shows much stronger protection from DMS than the downstream half (see Figures 3 and 4).

The conclusion of Mestril et al. (1986) that their sequence is responsible for ecdysterone responsiveness is not clearly supported by our findings. If the factor binding to Site 1 is the same factor responsible for the activation of *hsp23*, one would expect that the binding to Site 1 would show ecdysterone dependence. On the contrary, binding to

Site 1 is still observed (although at reduced levels) in the hormone deficient, *l(1)su(f)^{ts67g}* mutant larvae. Indeed, it is unlikely that this sequence motif is responsible for binding the ecdysterone-receptor complex. Studies by Riddihough and Pelham (1987) report a different 23 bp dyad sequence capable of ecdysterone inducibility and protein binding, and several groups are currently working to determine whether a factor which binds this dyad is the ecdysterone receptor. This does not rule out the importance of the *hsp23* sequence motif in ecdysterone induction, however. Potential binding of a transcriptional activator which could then interact through protein-protein contacts with an ecdysterone-inducible product or the receptor complex could still suggest a key role for the *hsp23* inverted repeat and would fit well into a model for *Sgs-3* induction. In addition, potential cooperative binding and/or functional synergism may occur between Sites 1 and 2, such as those seen in other inducible promoter systems (Strähle et al. 1988; see Robertson et al. 1988; Tsai et al. 1989; LeBowitz et al. 1989).

Interaction at Site 2 appears to be a key in the developmental regulation of *Sgs-3* initiation. Binding to this region is the only detected interaction absolutely correlated with *Sgs-3* transcription, showing both temporal and spatial specificity. The weak dyad symmetry around Site 2 (see Figure 4) may represent a recognition sequence for the Site 2-binding factor, but we are unable to discern this without further study. Binding in this region is also influenced by the *l(1)su(f)^{ts67g}* and *npr1^{3}* mutations. The former of these findings points out that ecdysterone is necessary for either activation or synthesis of the Site 2-binding factor. The latter of these findings suggests that a product of the *BR-C* is responsible for regulation of protein binding in this region or for binding itself. If we are allowed to speculate, these two phenomena could be unified by considering that the 2B5 puff (Ashburner 1972) and transcripts from the region (Chao and Guild 1986) are known to be inducible upon increasing hormone titers late in third instar. While this ecdysterone induction corresponds to "early" puff responses (which despite its name occurs directly after glue synthesis is halted), the *BR-C* is known to be active earlier (Ashburner and

Berendes 1978; Crowley et al. 1984). It is conceivable that ecdysterone levels in early third instar larvae act to stimulate *BR-C* transcription, the product(s) of which is directly or indirectly responsible for Site 2 binding and *Sgs-3* transcription.

The transcriptional regulation for full level expression of the *Sgs-3* gene is a complex process. Regulatory elements have been found in the proximal element, the -600 distal element, further upstream sites which have modest enhancement effects (Giangrande et al. 1987) and possibly even the element between *Sgs-7* and *Sgs-8*, which has been shown to be capable of enhancing an isolated *Sgs-3* proximal element (A. Hofmann, M. Garfinkel and E. Meyerowitz, in preparation). At least two of these regions are known to include controls for temporal and spatial regulation. These two regions are also involved in changes in chromatin structure which correlate with active expression (Ramain et al. 1986, 1988). These results extend our knowledge of the proximal promoter region of *Sgs-3*. In conjunction with the deletion and point mutation studies mentioned above (and accompanying report), they allow a clearer understanding of the developmental expression of this gene, and may help in the isolation of the transcription factors responsible for its regulation.

Materials and Methods

Drosophila *culture conditions and strains*

Drosophila cultures were maintained on standard cornmeal-sucrose-agar medium and raised at 22°C, with the exception of *l(1)su(f)^{ts67g}* mutant larvae, which were maintained at 25°C until shifted to the non-permissive 30°C temperature at 60 hr post-oviposition. The wild-type stock used (OR16f) was made isogenic for the third chromosome and originates from an Oregon-R strain (Meyerowitz and Hogness 1982). For analysis of mutant phenotypes, the *y npr1^{3} Sgs-4^{BER}/Binsn* and the *car l(1)su(f)^{ts67g}* ; OR16f third chromosome stocks were used (P.M., unpublished). The deficiency stocks (chromosomes *Df(1)S39* and *Dp(1;Y)Sz280*) were provided by Dr. Istvan Kiss (Szeged, Hungary). A stock carrying the *npr1^{3}* mutation was provided by Dr. James Fristrom (California-Berkeley). Larvae were staged by their overall size and specifically by the size and morphology of the salivary glands.

Methylation and purification of DNA samples

Larvae were dissected in drops of MOPS buffer (10 mM morpholinopropane sulfonic acid [pH 7], 80 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$) and the tissue of interest transferred to 50 µl of fresh buffer in siliconized microfuge tubes. Salivary glands and dissected carcass were used at 2 animals worth per lane, while fat body and gut lanes utilized either 2 (Figure 5) or 10 animals worth. Dimethylsulfate (DMS) was added at 0.5%, the mixture vortexed gently and incubated for 2 min at 22°C. Tissue was washed twice with 1 ml each of ice cold MOPS buffer, after pelleting at lowest speed for 10 sec in a variable speed microfuge. DNA was purified as described by Crowley et al. (1984) for unlabeled RNA, except as follows: tissue was transferred to 100 µl lysis buffer with

10 µg yeast tRNA; two phenol:Sevag extractions and one Sevag extraction were performed, using 200 µl and 100 µl respectively; the ether extraction was omitted. The nucleic acids were precipitated with ethanol, lyophilized dry and resuspended in 20 µl 1M piperidine. Piperidine cleavage was performed for 30 min at 90°C. After lyophilization, DNA was resuspended in 25 µl ddH_2O and lyophilized again. In vitro samples of DNA were obtained from salivary glands at mid-third instar and treated similarly to in vivo samples, delaying the DMS treatment. DNA (~50 to 100 ng) was purified from these glands and resuspended in 200 µl MOPS buffer. DMS was added at 0.25% and subsequent steps followed the procedure of Maniatis et al. (1982) for a G-specific sequencing ladder.

In vivo PCR footprinting procedure

The protocol used for in vivo footprinting was modified slightly from that of Mueller and Wold (1989). Briefly, the procedure is as follows (also see Figure 1). Piperidine-cleaved DNA was denatured and hybridized with 0.3 pmol of either gene-specific primer TS1 (5'-TACCGAAAACAAGAGCATTC - nucleotides +89 to +70) or BS1 (5'-CTTGAACT-AGCTAAGTAACG - nucleotides -209 to -190) at 50°C for 30 min. Nucleotide numbering is relative to the start site of transcription, which is position 4457 by the numbering of Garfinkel et al. (1983). These primers were extended to form flush ends at the G-specific cleavage end using Sequenase (USBiochemicals). After heat killing the Sequenase, two complementary, staggered linkers were ligated to this blunt-end. The linkers used were a 25-mer and an 11-mer (see Figure 1B), the same as those used by Mueller and Wold (1989). After heat inactivation of the ligase reaction, DNA was ethanol precipitated using 10 µg of tRNA as carrier and prepared for PCR amplification. The PCR primers included the 25-mer linker and either of two nested primers depending on the strand extended in the Sequenase step. TS2 (5'-ACCTAGGGCGGTAGCAATGGTCAGC), which corresponds

to nucleotides +59 to +35, or BS2 (5'-CGGGTATCTGTTAGTCTCGTTAGCG), which corresponds to nucleotides -191 to -167, were used as nested primers along with the 25-mer linker at a concentration of 1 pmol per 100 µl reaction. Amplification using Taq DNA polymerase (Cetus) was processed through 20 cycles of 1 min at 94°C, 2 min at 62°C and 3 min at 76°C on a Coy TempCycler, under 60 µl of mineral oil. The first denaturation step was extended to 2 min at 94°C to ensure enough time for the reaction to reach temperature. After amplification, 1 pmol of ^{32}P-end-labeled primer (either TS2 or BS2, respectively) was added to the reaction along with fresh nucleotides and enzyme. Unlike Mueller and Wold (1989), the same primers were used for both amplification and labeling. The mixture was denatured 2 min at 94°C, followed by 2 min at 62°C and 10 min at 76°C. After phenol/Sevag extraction, the DNA was precipitated with ethanol and 10 µg of tRNA and lyophilized. Samples were resuspended in 10 µl of loading dye, denatured and run over a 6% polyacrylamide sequencing gel (80 cm x 0.56 mm thick) at 40-60 W. The xylene cyanol dye was run to the 50 cm mark. Gels were transferred to Whattman paper, wrapped and exposed without intensifying screen at -80°C for various times using Kodak XAR-5 film. Signal could usually be detected within 2-5 hr. Each strand was analyzed 7 to 9 times for mid-third instar salivary glands and naked DNA. Non-expressing tissues and stages were examined 2 to 4 times, while mutant samples were measured 4 to 6 times.

Quantification of autoradiographic exposures

Individual lanes were scanned using an LKB UltraScan densitometer and the LKB Gelscan XL integration program (version 1.20), kindly loaned to us by Dr. Peter Dervan. Exposures were shown to be in the linear range of the film before quantification began. Quantification was performed to compare wild-type salivary glands methylated in vivo to in vitro DNA controls. Multiple scans of a lane were performed (usually 5) and integrated using the Gaussian option of the Gelscan XL program. Areas for each band were averaged

for the five integration data points. Similar exposures from two sets of in vivo and in vitro treated samples were compared and a ratio for each band established (see Figure 3). The ratio of a band showing no footprint should be approximately equal to 1. To correct for slight differences in autoradiographic exposures, bands showing no detectable footprint were used to obtain a correction factor. This factor was used to adjust the in vivo to in vitro ratios. Individual band ratios consistently below 0.7 (30% protection) or above 1.7 (1.7-fold enhancement) were considered strongest (see Figures 3 and 4). Tight doublets containing bands of greatly differing intensities, such as positions -33/-32 and -23/-22, the string of four G's at -106 to -103, and extremely light bands such as -101 are difficult to quantify accurately and were therefore not included in the analysis.

Acknowledgements

We are indebted to Paul Mueller and Dr. Barbara Wold for sharing the PCR in vivo footprinting protocol prior to publication, for advice and reagents in applying the protocol to *Drosophila* tissue and for helpful discussions. Our thanks to Drs. Istvan Kiss and James Fristrom for *Drosophila* stocks. We also express our appreciation to Drs. Peter Dervan, for use of the LKB scanner and integration software, and Eric Davidson, for computer facilities. Our thanks to the Microchemical Facility at Caltech for furnishing oligonucleotides. We appreciate the helpful discussions from members of our lab, especially Bruce Hamilton, Glen Morangie and Drs. Margaret Roark and K. Vijay Raghavan. This work was supported by a grant (number GM28075) to E.M.M. from the Institute for General Medical Sciences, National Institutes of Health. P.H.M. was supported during part of this work by a National Research Service Award (GM07616).

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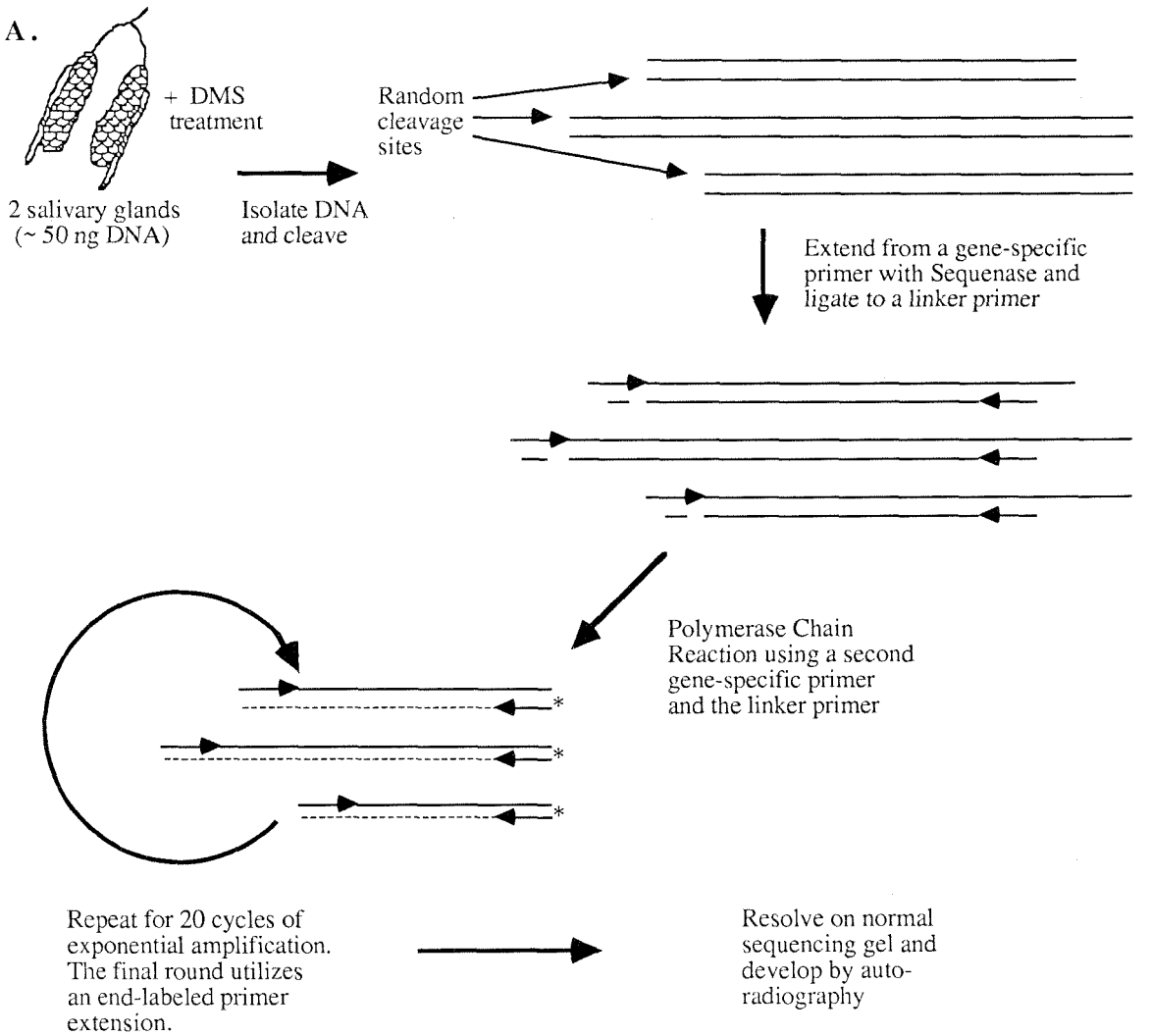
Figure Legends

Figure 1. (*A*) Strategy of the PCR in vivo footprinting protocol. After methylation and purification, the DNA is cleaved with piperidine to give a G-specific ladder. Extension of a gene-specific primer serves to create a blunt end at the cleavage site, to which a PCR linker is ligated (see *B*). PCR amplification between a nested gene-specific primer and the 25-mer linker is processed through 20 cycles. Addition of the nested primer, now ^{32}P-end-labeled, serves to label each amplified fragment in the final round of PCR. Fragments are resolved on a standard sequencing gel. (*B*) Structure of the linker primers used in the ligation step (Mueller and Wold 1989).

Salivary Gland Footprinting Strategy using PCR Amplification



B.

```
5'-OH-GCGGTGACCCGGGAGATCTGAATTC-OH-3'
3'-OH-CTAGACTTAAG-OH-5'
```

25-mer ligation and PCR linker

11-mer ligation linker

Figure 2. In vivo DMS footprints of the proximal promoter element of *Sgs-3*. Each lane represents the DNA content of the salivary glands from two mid-third instar larvae. DNA was methylated under in vitro or in vivo conditions as indicated in Materials and Methods. Top and bottom strands, as shown in Figure 4, are presented with each band numbered according to its relation to the RNA start site. Bold lines represent the observed limits of protein:DNA interactions discerned by comparing in vivo and in vitro samples.

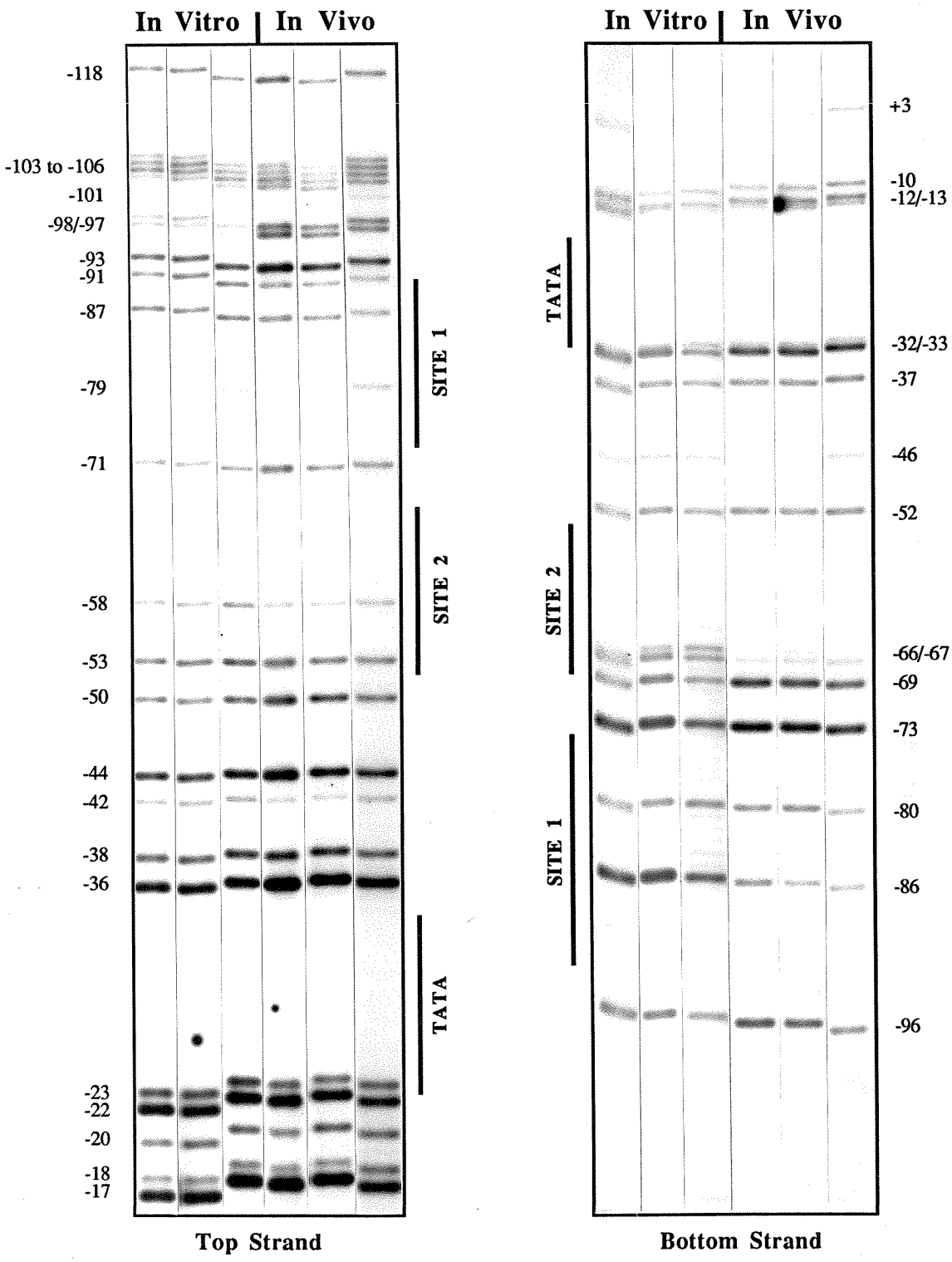

In Vitro
In Vivo
-118
-103 to -106
-101
-98/-97
-93
-91
-87
-79
-71
-58
-53
-50
-44
-42
-38
-36
-23
-22
-20
-18
-17
SITE 1
SITE 2
TATA
Top Strand
In Vitro
In Vivo
+3
-10
-12/-13
-32/-33
-37
-46
-52
-66/-67
-69
-73
-80
-86
-96
TATA
SITE 2
SITE 1
Bottom Strand

Figure 3. Quantification of footprint interactions. Gel lanes from in vivo and in vitro methylated DNA samples were analyzed by densitometric scan. The area under each peak was obtained by Gaussian integration and an average for each band obtained. The ratio for each band represents the average of two comparisons between separate in vivo and in vitro lanes, after these lanes have been standardized to compensate for slight differences in autoradiographic exposures. A baseline of 1.0 should represent no interactions, with protections falling below this line and enhancements above. Protections of less than 15% (0.85-1.0) were considered insignificant, except for base -79 which consistently shows protection in other samples. Protections of more than 30% were considered strongest, while enhancements of >1.7-fold were rated as strongest. Enhancement below the 1.2-fold level was considered to be insignificant. Apparent protections at -42, -10 and +2 were discounted, lacking reproducibility between samples, and appear to be a coincidence of the samples chosen, as were apparent enhancements at -44 and -37. In addition, we have reclassified the apparently strong enhancement at -96 as weak, as additional samples not used for quantification would suggest. All G-residues between -125 and +2 are included in this figure, except bases -106 to -103, -101, -33/-32 and -13/-12, which could not be quantified accurately because of closely spaced bands or weak DMS reactivity (see Materials and Methods). Boxed regions represent approximate limits of protein:DNA interactions.

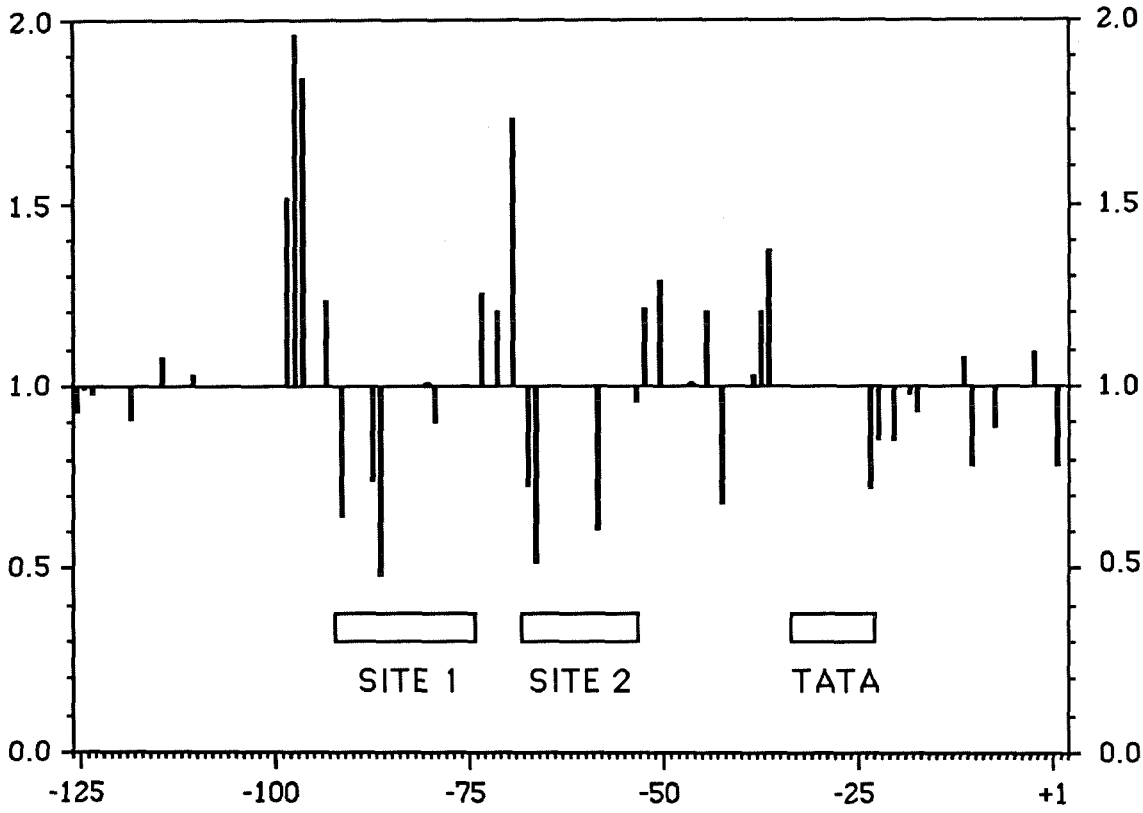

2.0
1.5
1.0
0.5
0.0
-125
-100
-75
-50
-25
+1
SITE 1
SITE 2
TATA

Figure 4. Sequence of the proximal promoter region of *Sgs-3*. Changes in DMS reactivity observed consistently in multiple comparisons between in vivo and in vitro DMS-treated DNA samples are presented. Protections are distinguished between strong (⇓) and weak (⇓) from the results in Figure 3, as were enhancements (⇑ and ↑, respectively). Interactions at bases -33 and -32 were classified as weak based on visual analysis since accurate quantification of these bands is difficult due to their close spacing and uneven abundance. Approximate boundaries of binding domains are bracketed and homology to the ecdysterone responsive element of *hsp23* is denoted by a heavy bar between the sequences. The weak dyad symmetry surrounding Site 2 is noted by underlining. Nucleotides which have been shown by point mutagenesis (Todo et al., accompanying report) to be required for proper expression are boxed.

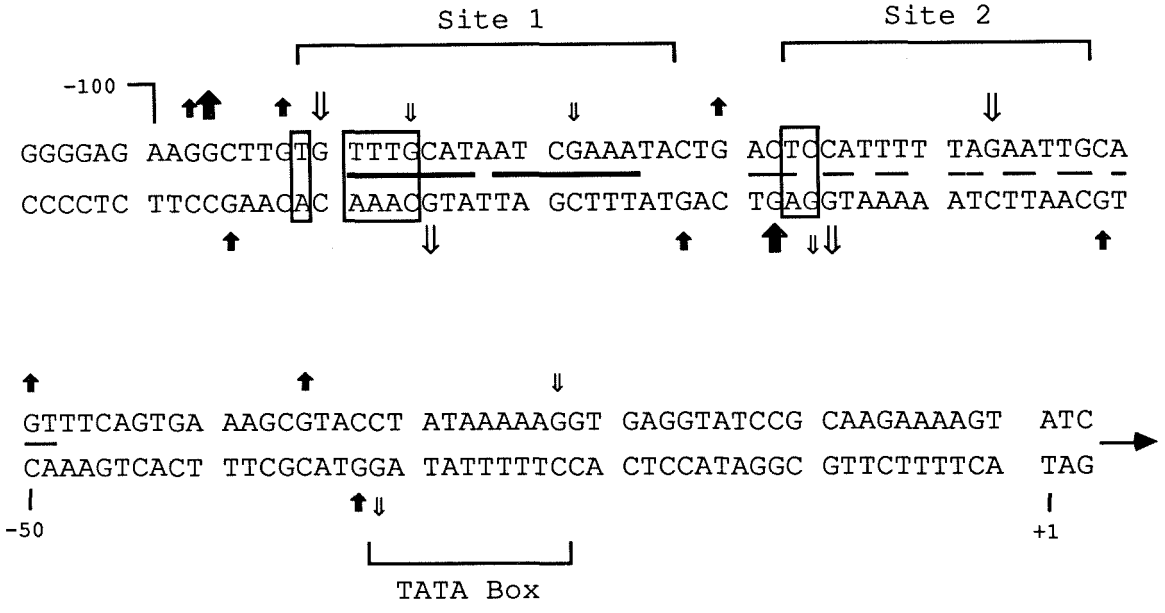
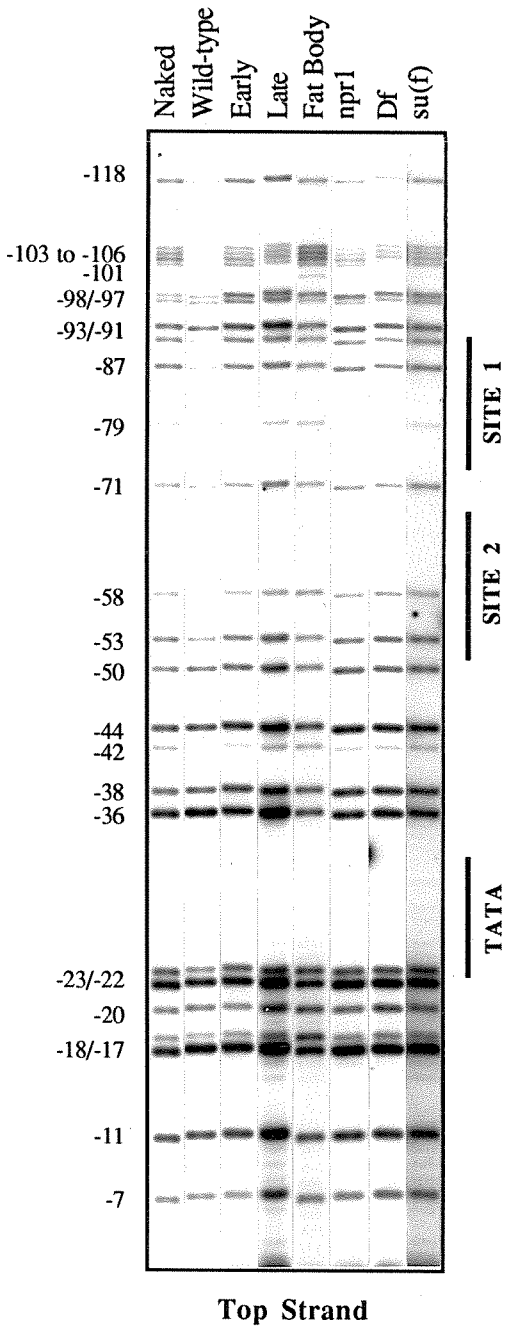
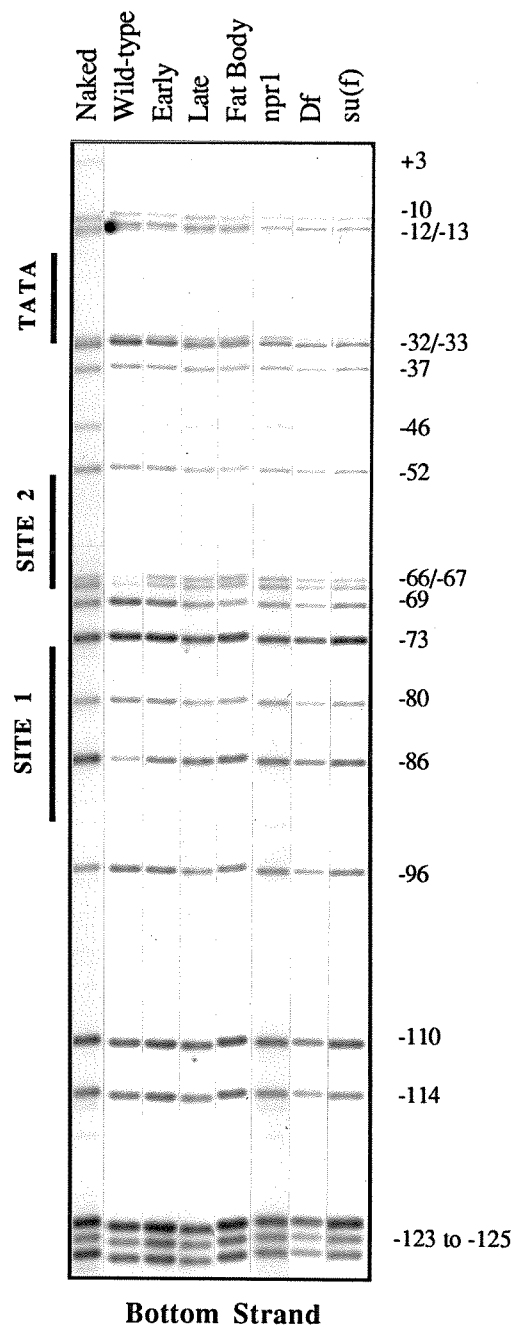

Site 1
Site 2
-100
GGGGAG AAGGCTTGTG TTTGCATAAT CGAAATACTG ACTCCATTTT TAGAATTGCA
CCCCTC TTCCGAACAC AAACGTATTA GCTTTATGAC TGAGGTAAAA ATCTTAACGT
GTTTCAGTGA AAGCGTACCT ATAAAAAGGT GAGGTATCCG CAAGAAAAGT ATC
CAAAGTCACT TTCGCATGGA TATTTTTCCA CTCCATAGGC GTTCTTTTCA TAG
-50
+1
TATA Box

Figure 5. In vivo DMS footprinting of the *Sgs-3* promoter in non-expressing stages and tissues and in salivary glands of *trans*-acting mutants. G-specific footprint ladders of top and bottom strands of the proximal element are presented as in Figure 2. Lanes represent amplified DNA from in vitro methylated samples (Naked), mid-third instar salivary glands (Wild-type), early third instar salivary glands (Early), late third instar salivary glands (Late), mid-third instar dissected fat body (Fat Body), and salivary glands from $npr1^3$ larvae (npr1), from *Df(1)S39/Dp(1;Y)Sz280* larvae (Df) and from $l(1)su(f)^{ts67g}$ larvae shifted to non-permissive temperature at 60 hr post-oviposition (su(f)). Numbering and binding domains are indicated as in Figure 2.



Top Strand



Bottom Strand

Figure 6. Summation of footprint pattern under non-expressing conditions. A diagram is presented of interactions usually observed in non-expressing salivary glands and other larval tissues, as well as in salivary glands from larvae carrying the *npr1*^{3} mutation, a deficiency for the *BR-C*, and the *l(1)su(f)*^{ts67g} mutation. Annotations are those explained for Figure 2. All interactions were qualitatively determined to be weak, as quantification was not performed.

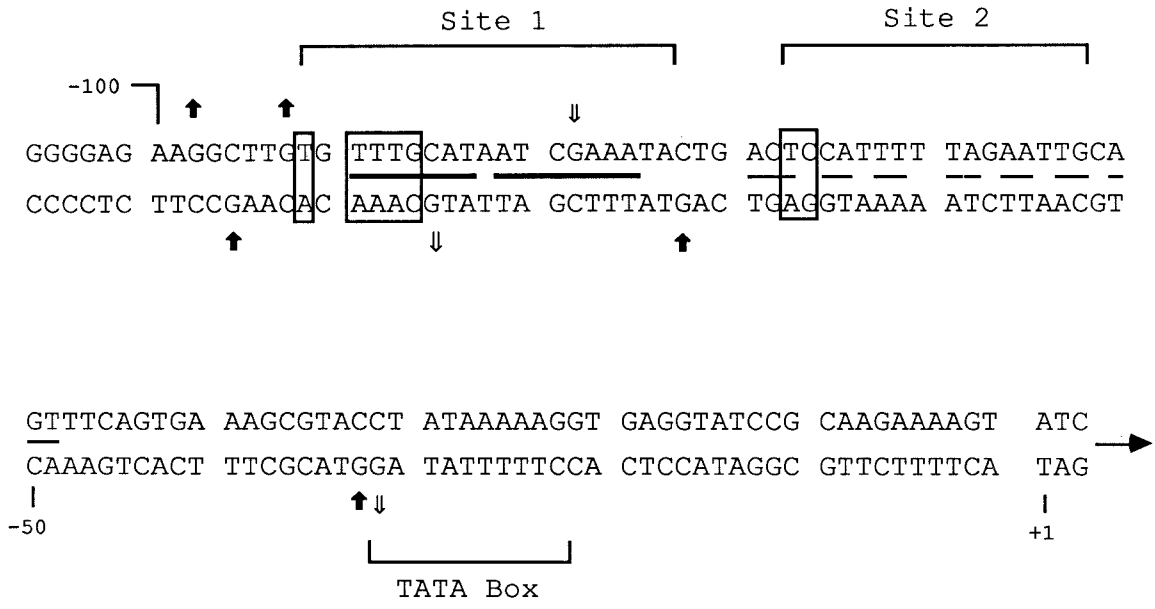

Site 1
Site 2
-100
GGGGAG AAGGCTTGTG TTTGCATAAT CGAAATACTG ACTCCATTTT TAGAATTGCA
CCCCTC TTCCGAACAC AAACGTATTA GCTTTATGAC TGAGGTAAAA ATCTTAACGT
GTTTCAGTGA AAGCGTACCT ATAAAAAGGT GAGGTATCCG CAAGAAAAGT ATC
CAAAGTCACT TTCGCATGGA TATTTTTCCA CTCCATAGGC GTTCTTTTCA TAG
-50
+1
TATA Box

Figure 7. Compilation of protein:DNA interaction patterns under expressing and non-expressing conditions. Binding domains are those presented in Figure 4. Strong contacts are observed only in expressing tissue and define the binding domains (signified by ++), while weak contacts (+) or no interactions (-) are seen in non-expressing tissue.

Mid-third instar salivary glands	++	++	++
Early third instar salivary glands	+	−	+
Bloated salivary glands	+	−	+
Dissected carcass Fat body or Gut	+	−	+
Mid-third $npr1^{3}$ salivary glands	+	−	+
Mid-third 2B5 Df salivary glands	+	−	+
$l(1)su(f)^{ts67g}$ salivary glands at non-permissive temp.	+	−	+

Chapter 4:

An Element Required for Formation of a Puff at 68C in *Drosophila*

An Element Required for Formation of a Puff at 68C in *Drosophila*

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Running Title: 68C puffing determinants

Summary

Abundant expression of genes in the salivary gland polytene chromosomes of Dipterans is often associated with localized chromosomal decondensation (chromosome puffing). We have utilized P-element transformation of DNA from the 68C glue gene region of *Drosophila* to analyze the *cis*-acting elements required for the developmentally specific puffing associated with the activity of the 68C *Sgs* glue genes. A small (152 basepair) DNA sequence from the region between the glue genes *Sgs-7* and *Sgs-8* is necessary, but not sufficient, for puffing. This sequence alone does not induce puffing, but when associated with any of several tested active transcription units, will do so. Further, we find that the level of transcription in a region, and the transcription unit length, are not decisive factors in determining whether or not the region will puff. We also find that two *trans*-acting genetic factors are required for the formation of the intermolt puff at 68C; they are specified by the *Broad-Complex* and *suppressor of forked* loci. Finally, we use our findings to support a two-step model for puff formation.

Introduction

Balbiani (1881) described the larval salivary gland chromosomes of *Chironomus*, including the chromosomal decondensations we now recognize as Balbiani Rings. While Bridges (1935, 1938) referred to the 2B region on the *Drosophila* polytene chromosomes as the "puff," Poulson and Metz (1938), using salivary gland chromosomes from *Sciara ocellaris*, appear to be the first to recognize that particular regions change from a banded pattern to a decondensed "puff" or "bulb." The subsequent literature concerning the study of the giant chromosomes of Dipterans has been frequently reviewed (Ashburner 1977; Ashburner and Berendes 1978; Zhimulev et al. 1981; Korge 1987). Specific to our interests are the studies on the control of chromosome puffing and its correlation with transcription. Beermann (1952, 1956) and Pavan and Breuer (1952) proposed that puffs represent a visible sign of gene activity, a theory supported by the finding that incorporation of ^{3}H-uridine into chromosomal RNA was most prevalent at puffed regions (Pelling 1959, 1964). The induction of puffing with external stimuli such as ecdysone (Clever and Karlson 1960) and heat shock (Ritossa 1962) has allowed dissection of the puffing process at both cytological and molecular levels (see above mentioned reviews).

The molecular characterization of puffing has advanced significantly since the advent of methods for germline transformation of *Drosophila* (Spradling and Rubin 1982; Rubin and Spradling 1982). The ability of transformed segments of DNA to create specific puffs at new chromosomal locations shows that decondensation is not limited to specific chromosomal environments and, therefore, allows manipulation of puff-inducing segments for further analysis (Lis et al. 1983; Bonner et al. 1984; Cohen and Meselson 1984; Dudler and Travers 1984; Crosby and Meyerowitz 1986; Semeshin et al. 1986; Hofmann et al. 1987; Semeshin et al. 1989). Studies have suggested that transcription unit length and promoter strength are important factors in determining the size of a puff (Simon et al. 1985b) and that puffing alone is insufficient to promote transcription (Simon et al. 1985a).

Similar studies have shown that puffing is not a prerequisite for high-level transcription, as certain reintroduced DNA fragments show abundant transcription without formation of their normally associated puffs (Richards et al. 1983; Hoffman and Corces 1984; Crosby and Meyerowitz 1986; McNabb and Beckendorf 1986). A different type of uncoupling of the puff-transcription relation, puffs observed without detectable transcription of the genes thought to cause them, has also been reported (Hansson et al. 1981; Hansson and Lambertsson 1983; Crowley et al. 1984; Korge 1987).

We have chosen to study the developmentally regulated puffing at chromosomal location 68C in *Drosophila melanogaster*. The 68C puff has been identified in association with the genes, *Sgs-3*, *Sgs-7*, and *Sgs-8* (Korge 1975; Akam et al. 1978; Crowley et al. 1983), which code for protein components of the larval salivary gland secretion involved in affixing pupae to a solid substrate during metamorphosis (Fraenkel and Brookes 1953). These 68C glue genes are coordinately expressed during the third larval instar in salivary glands, under the control of the steroid hormone ecdysterone. As a member of the intermolt class of puffs, 68C is decondensed at puff stage 1 (PS1; Ashburner 1972b; Ashburner and Berendes 1978) and regresses between PS2 and PS3, as a result of an increase in the titer of ecdysterone (Ashburner 1973; Crowley and Meyerowitz 1984). Expression of the three glue genes at 68C is prevented by mutations at either of two loci, *suppressor of forked* (*su(f)*; Hansson and Lambertsson 1983) and the *Broad-Complex* (*BR-C*, Crowley et al. 1984; also known as 2B5 and npr$^+$). The *l(1)su(f)^{ts67g}* mutation appears to severely reduce levels of the steroid hormone ecdysterone and is temperature-sensitive (Klose et al. 1980; Hansson and Lambertsson 1983). The *npr1^3* mutation of the *BR-C* (also known as *l(1)npr-1*; Kiss et al., 1978, 1988; Lindsley and Zimm 1986) acts on 68C glue gene expression in a cell-autonomous fashion (Vijay Raghavan et al. 1988). Although 68C glue RNAs are undetectable in larvae hemizygous for either of these mutations, the 68C region still appears to puff normally in these mutant larvae (Hansson et al. 1981; Belyaeva et al. 1981; Crowley et al. 1984).

Crosby and Meyerowitz (1986) analyzed two segments of 68C DNA reintroduced into the chromosome by germline transformation. Both were found to express *Sgs-3* at abundant levels, but only the larger construction shows puffing at new sites of insertion. We report results from puffing studies on numerous additional constructions derived from DNA of the 68C region. Our results define an element of 152 basepairs (bp) in the *Sgs-7* and *Sgs-8* intragenic region which is necessary, but not sufficient, for chromosomal puffing. Detectable puffing requires both this element and an adjacent functional transcription unit. In addition, we conclude from our findings that a strong promoter and long transcription unit are not sufficient for puff formation. Finally, we analyze the ability of transformed lines to produce puffs in the $l(1)su(f)^{ts67g}$ and $npr1^{3}$ mutant backgrounds, and find that the puffs caused by insertion of 68C DNA into new locations are not present in larvae carrying these mutations. The puffs seen at 68C in these mutants are thus not equivalent to the 68C intermolt puff associated with glue gene expression.

Materials and Methods

Drosophila cultures were kept on standard cornmeal-sucrose-agar food at 22°C, except the stock carrying the *l(1)su(f)^{ts67g}* mutation. Eggs from this stock were collected at 1 hr intervals and raised at 25°C for 60 hr, at which time they were shifted to the non-permissive 30°C for 40 hr prior to dissection. Control larvae were raised at 22°C or 25°C. The *car l(1)su(f)^{ts67g}* and *y npr1^{3} Sgs-4^{BER}/Binsn* stocks were used to analyze puffing of regions transformed with 68C DNA under conditions which prevent 68C glue gene expression. In situ hybridizations and orcein squashes were performed essentially as described by Crosby and Meyerowitz (1986). Puffs and bands were located according to Lefevre's revisions of Bridges' chromosome maps (Lefevre 1976). In situ localization utilized the following ^{35}S-labeled plasmids: for *Adh*-containing fragments, nDm9035; for *LacZ*-containing fragments, either pOX10 or kEc001; for the GXX0.19 transformed lines, Carnegie 20 (Car 20) or aDm2029. Orcein squashes were performed for puff determination on larvae at puff stage 1 or 2, when 68C is maximally puffed (Ashburner 1972b; Ashburner and Berendes 1978). Puff stage is not a relevant term in the chromosomes from mutant larvae, due to the disruption in the developmental process (Belyaeva et al. 1981). Analysis in the mutant lines was, therefore, performed when the 68C puff is observed. The ability of a fragment to induce puff formation was determined by a consistent increase in diameter (puffing) or thorough decondensation of a band in the region corresponding to the site of insertion, when compared to non-transformed sibling lines. Formation of micropuffs (visible only in the electron microscope, Semeshin et al. 1986) in the lines scored as negative remains a possibility. Puffing normally includes decondensation, whereas decondensation can occur without visible puffing. Over 20 nuclei, representing several animals, were examined for each transformed line to assess puffing. Puffs were also tested for intermolt specificity by their presence at puff stages 1 or 2, followed by regression at subsequent stages.

P-element germline transformation followed the technique of Spradling and Rubin (1982), with modifications described by Crosby and Meyerowitz (1986). Nomenclature of transformants follows the convention of Crosby and Meyerowitz (1986), with *Tf* standing for transformant, followed by the chromosome of the insertion; G stands for glue, A for *Adh*, L for *LacZ*, X for *Xdh* and O for synthetic oligonucleotide sequences, with the amount of 68C sequence in kilobases (kb) following to define the construction. Several of these constructions have been described earlier or will be presented elsewhere, as follows: GA8.1-3 and GA6.0 (Crosby and Meyerowitz 1986); GOAX2.76 and GOAX0.98 (Roark et al., in press); GLAX1.0, GAX0.26, GAX0.24, GAX0.12, GLX0.68 and GLX0.66 (Garfinkel 1988; A. Hofmann, M. Garfinkel and E. Meyerowitz, in preparation); GLX3.3 and GLX1.34 (Vijay Raghavan et al. 1986); GLX1.1 and GLX0.8 (E.M.M., unpublished; Meyerowitz et al. 1985); GGAX-1 (A. Hofmann et al., in preparation). The GXX0.19 construction contains the 250 bp EcoRI-SalI intragenic enhancer element from nDm9053 and a 4.8 kb SalI-EcoRI fragment of the *Xdh* (*rosy*) coding region, inserted into the Car 20 transformation vector (Rubin and Spradling 1983). The extra *rosy* sequences were added such that the intragenic element is flanked on both sides to create a buffer against position effects on the puff-inducing capacity of the element. A schematic diagram of the DNA transformation fragments is presented in Figure 1. Nucleotide numbering is derived from the published sequence of the 68C glue gene cluster (Garfinkel et al. 1983).

Results

Analysis of puffing in transformed lines containing Sgs-3 *sequences*

Richards et al. (1983) found that DNA containing the *Sgs-3* and *Sgs-7* genes is able to express *Sgs-3* RNA at high levels when reintegrated into *Drosophila* chromosomes, but found no evidence for associated puffing (but see Semeshin et al. 1986 for electron microscopic analysis showing what the authors term "micropuff" formation). Similarly, Crosby and Meyerowitz (1986) found that DNA containing the *Sgs-3* glue gene with 2.3 kilobases (kb) of upstream sequence is capable of high level expression of the *Sgs-3* gene, but incapable of inducing puff formation. When they analyzed a construction which possesses all three glue genes and included a total of 8.1 kb of 68C DNA, puffing was observed at the time of expression of the 68C glue genes. To further delimit the sequences at 68C required for chromosomal decondensation and to further characterize the factors involved in puffing of developmentally regulated loci, we examined additional transformations of DNA from the 68C region. A large number of lines transformed with various constructions derived from 68C sequences have been previously analyzed for their transcriptional activity (Crosby and Meyerowitz 1986; Vijay Raghavan et al. 1986; Roark et al., in press; Garfinkel 1988; A. Hofmann, M. Garfinkel and E. Meyerowitz, in preparation). These transcriptional studies have utilized two reporter genes for analyzing the abundance and pattern of expression from transformed DNA. The *Drosophila Adh* gene has been fused to the *Sgs-3* gene at position +12 basepairs (bp) relative to the transcription start site (Roark et al., in press), and has also been fused to *Sgs-7* at position +25 bp (Garfinkel 1988). In addition, the *E. coli LacZ* gene has been fused in frame to the *Sgs-3* gene at +948 bp (Vijay Raghavan et al. 1986) and to the *Sgs-8* gene at +245 bp (E.M.M., unpublished; described in Garfinkel 1988). A schematic diagram of the introduced DNA fragments analyzed or referred to in this study is presented in Figure 1.

An analysis of puffing in two similar constructions, GOAX2.76 and GOAX0.98, reveals several interesting points. The lines analyzed, their expression levels, their sites of insertion and a summary of their puffing capability are summarized in Table 1. The three GOAX2.76 lines analyzed each show distinctive intermolt puffs which are not present in non-transformed sibling lines (Figure 2). On the other hand, none of the three GOAX0.98 lines analyzed shows detectable levels of decondensation when compared to siblings. Therefore, as little as 2.76 kb of 68C sequence is sufficient to cause an intermolt puff when attached to a reporter gene, and the DNA from the *Adh* reporter gene is not sufficient to cause a puff on its own. The report of a developmentally specific puff in the region of the *Adh* gene (Visa et al. 1988) makes this an important control. Roark et al. (in press) report that the level of expression of the *Sgs-3/Adh* fusion gene in the GOAX2.76 lines is high. Based on the findings of Richards et al. (1983) and Crosby and Meyerowitz (1986), the GOAX2.76 lines should be active to a level approximately equal to that expected from the endogenous *Sgs-3* gene. In comparison, expression from GOAX0.98 is reduced to an average of 8% of full-level expression (Roark et al., in press). The two DNA insertions, therefore, differ in two important ways: the level of expression and the presence of additional sequences, including the *Sgs-7* gene, in the GOAX2.76 construction.

Two similar constructions utilizing the *Sgs-3/LacZ* fusion gene were also analyzed. GLX3.3 contains 2.3 kb of sequence upstream from *Sgs-3*, while GLX1.34 has only 130 bp of 5' sequence. Both of these sequences direct transcription in the salivary gland during third instar, with the GLX3.3 lines showing abundant expression, while the GLX1.34 construction is weakly expressed (Vijay Raghavan et al. 1986). When analyzed for puff formation, neither the GLX3.3 (Figure 2) nor the GLX1.34 (Figure 3) lines show detectable levels of decondensation. Analysis of the GLX3.3 lines is complicated by two factors. First, the insertion *Tf(3)GLX3.3-1* occurs at position 82E on the third chromosome, in a region which in non-transformed lines is larger than the surrounding chromosome. Because this insertion causes no increase in the degree of puffing in this

region, we score it as negative for puffing, although slight changes in chromosome diameter might be undiscernible. The second complication comes in the analysis of the *Tf(2)GLX3.3-2* and *-4* lines, which are both homozygous lethal. Puffing was analyzed in synapsed chromosomes which are heterozygous for the insertion event. Therefore, we cannot eliminate the possibility that some new phenomenon of synapsis-dependent inhibition of puffing is occurring. The GA6.0 construction (Crosby and Meyerowitz 1986 and see Figure 1), however, does not induce puffing, and it shares the same 5'-end point with the GLX3.3 construction. Therefore, the abundant expression of a 4.4 kb RNA from the *Sgs-3/LacZ* gene is insufficient for chromosome decondensation to a level distinguishable in the light microscope. The failure of GLX3.3 to induce puffing, while fusions with the *Adh* reporter gene (which produces transcripts of about 1.1 kb, Benyajati et al. 1983) are able to cause puff formation, suggests that transcript length alone is not a sufficient determinant in puff induction. Comparison of these constructions with GOAX2.76 suggests that the region around or within the *Sgs-7* gene may be required for puff formation.

Analysis of puffing in constructions containing Sgs-7 *and* Sgs-8 *sequences*

In an effort to determine if *Sgs-7* expression or sequences in the region of this gene were responsible for inducing puffing, transformants representing a 5'-deletion series of the *Sgs-7* promoter (Garfinkel 1988) were analyzed for their ability to puff at the insertion site. The GLAX1.0 construction (see Figure 1) contains the 467 bp intragenic region between *Sgs-7* and *Sgs-8*, and upon chromosomal insertion, abundantly expresses the *Sgs-7/Adh* and *Sgs-8/LacZ* gene fusions (Garfinkel 1988). This DNA is also capable of directing puff formation (Figure 3). Determination of puffing in the *Tf(3)GLAX1.0-7* line is difficult due to the normally enlarged diameter of the 82 region. Insertion of this construction into the 82D region does appear to increase the diameter of the chromosome, however, and is

scored as capable of puffing. Deletions which retain 236 bp (GAX0.26) and 211 bp (GAX0.24) of *Sgs-7* upstream sequence are still capable of causing puff formation (Figures 3 and 4, respectively). These constructions also retain promoter activity, as the *Sgs-7/Adh* gene is abundantly expressed in both cases (Garfinkel 1988). A further deletion to position -92 of *Sgs-7* (GAX0.12) results both in a loss of expression (Garfinkel 1988) and a lack of chromosomal decondensation (Figure 4).

A deletion series of the *Sgs-8* promoter region allows us to determine whether puff formation is dependent on sequences within the *Sgs-7* gene or within the intragenic region. The GLX1.1 and GLX0.8 constructions contain 554 bp 5' of the *Sgs-8* RNA start site, with the upstream breakpoint within the intron of the *Sgs-7* gene. GLX1.1 and GLX0.8 differ only in the presence of a 268 bp fragment containing the *Sgs-8* 3'-untranslated region and polyadenylation site at the 3'-end of the *LacZ* reporter gene in the GLX1.1 construction (see Figure 1). Transformed lines carrying either construction express the *Sgs-8/LacZ* fusion RNA and produce β-galactosidase in the salivary glands (Meyerowitz et al. 1985). In addition, larvae of both types of transformed lines form puffs at the insertion site (Figures 4 and 5). Analysis in the *Tf(2)GLX1.1-1* line is complicated by insertion into the 56E region, which normally shows decondensation in non-transformed controls. As seen in Figure 4, transformation into this region does cause changes in chromosome structure and decondensation of the 56E1 band. On this evidence, the *Tf(2)GLX1.1-1* line is scored as positive for puffing. The *Tf(2)GLX0.8-1* insertion occurs within or adjacent to the thick band constituting the 50BC border. Inspection of this region in *Tf(2)GLX0.8-1* reveals a strong decondensation of this thick band, which is associated with either a small increase in chromosome diameter or no increase at all. Figure 5 represents one of the better examples of this puff. Despite the lack of a large increase in diameter, the *Tf(2)GLX0.8-1* line is scored as capable of puffing based on the strong decondensation in the region (Table 1).

Because one end of the 68C DNA in these constructions falls within the *Sgs-7* gene, activity from the *Sgs-7* promoter is expected in these lines. In order to eliminate activity

from this promoter as a possible requirement in puff formation, additional constructions with deletion of the *Sgs-7* TATA domain were analyzed. The GLX0.68 construction contains 432 bp of sequence upstream of the *Sgs-8* gene, the right end of which corresponds to position -43 bp relative to the *Sgs-7* RNA start site. GLX0.66 contains 415 bp of *Sgs-8* upstream sequence, corresponding to position -60 bp upstream of the *Sgs-7* RNA start site. Transformed lines for both of these constructions show chromosomal decondensation at the sites of insertion in PS1-2 nuclei, as shown in Figure 5. A high level of introduced gene activity in the GLX0.68 lines has been observed by measuring β-galactosidase activity in soluble extracts of transformed salivary glands. The activity from GLX0.66 is reduced, however, to an average level below 10% that of the GLX0.68 lines (Garfinkel 1988; A. Hofmann et al., in preparation). In comparison to the results of the GA6.0 and GLX3.3 lines which fail to puff despite high level expression, the GLX0.66 lines show puffing activity even with low levels of expression. Reasoning from the results obtained with the GAX0.24 lines and the GLX0.66 lines, an intragenic element of no more than 152 bp is necessary for the formation of this intermolt puff; any 68C construction that does not contain this DNA does not puff. This element is located between -211 bp and -60 bp upstream of the *Sgs-7* gene (corresponding to nucleotides 1903 to 2054 in the published sequence of the 68C region, Garfinkel et al. 1983). This falls within an element that contains a transcriptional enhancer (A. Hofmann et al., in preparation).

As a further test of the necessity of the intragenic element for puff formation, we added it to sequences which do not cause puffing on their own. GGAX-1 contains the intragenic enhancer element fused to an *Sgs-3/Adh* fusion gene which contains only 130 bp of *Sgs-3* upstream sequence (A. Hofmann et al., in preparation). The fusion gene in the GGAX-1 construction shares similarity to the GOAX0.98 construction (which does not puff), with a deletion of sequences between -130 bp and -980 bp in the GGAX-1 lines (Figure 1). Therefore, the presence of the fusion gene alone should not be sufficient for puff formation. As shown in Figure 6, GGAX-1 induces puffing.

Is the intragenic element sufficient for puffing?

While the intragenic element may be necessary for puff formation, the preceding experiments do not address whether this element is sufficient on its own to induce puffing. In order to test this possibility, the element was isolated from the transcriptional activity of *Sgs-7* and *Sgs-8* by cloning the enhancer element (194 bp corresponding to nucleotides 1878 to 2071) into the Car 20 transformation vector along with a 4.8 kb fragment of the *rosy* gene, designed to act as a flanking buffer. The *rosy* fragment represents coding and 3'-flanking sequences, lacking both controlling elements and transcription start sequences (Lee et al. 1987; Keith et al. 1987). In addition, these *rosy* sequences are not sufficient to cause puff induction (see for example GAX0.12, GLX3.3 or GOAX0.98 lines). The resulting construction (GXX0.19) was introduced into the germline of flies, and six independent homozygous lines were analyzed for puff induction (Table 1 and Figure 6). No puff formation or significant band decondensation is observed at the site of insertion in these six lines. Therefore, the intragenic element is insufficient for puff formation.

Analysis of transformed lines in the background of trans-*acting mutants*

The results presented to this point suggest that in order for puffing to be observed at the light microscopic level, the intragenic puff element must be present, with concomitant transcription taking place nearby. This is an apparent contradiction of some findings from several laboratories, including our own. In mutants hemizygous for $l(1)su(f)^{ts67g}$ (Hansson et al. 1981; Hansson and Lambertsson 1983) or $npr1^{3}$ (Belyaeva et al. 1981; Crowley et al. 1984), puffing in the 68C region is observed, but RNA accumulation from the glue genes is not. To further analyze this apparent contradiction, several transformed lines containing DNA segments capable of inducing puffs were analyzed in the salivary glands of larvae carrying either the $l(1)su(f)^{ts67g}$ or $npr1^{3}$ mutation. The *Tf(3)GA8.1-3*

line (Crosby and Meyerowitz 1986) was chosen as capable of inducing puff formation and containing the largest stretch of 68C sequence. Using appropriate balancer stocks, the *Tf(3)GA8.1-3* insertion was crossed into each of the mutant stocks for analysis. As shown in Figure 7, the *Tf(3)GA8.1-3* insertion at the 97F/98A border causes significant puffs in control larvae of the genotype *Binsn;Tf(3)GA8.1-3*. When analyzed in $npr1^3$; *Tf(3)GA8.1-3* larvae, the 97F/98A puff is not formed (Figure 7A). We should note that in 1 of more than 50 nuclei analyzed, a puff at 97F/98A was observed in the $npr1^3$ background. We cannot explain this aberrant result, but have not seen it again, and consequently disregard it in what follows. In addition, larvae of the genotype $l(1)su(f)^{ts67g}$; *Tf(3)GA8.1-3* were analyzed at both permissive (22-25°C) and restrictive (30°C) temperatures. The restrictive temperature is sufficient to prevent 68C glue gene RNA accumulation (Hansson and Lambertsson 1983). Puff formation was only observed at permissive temperature (Figure 7B).

In addition, the puffs seen in the transformed lines *TfGOAX2.76-1* and *-5*, *TfGLAX1.0-2* and *-3*, *TfGLX1.1-1* and *-6*, and *TfGLX0.8-1* and *-4* are not observed when these insertions are analyzed (as heterozygotes) in an $npr1^3$ hemizygous mutant background (data not presented). This suggests that the puff at 68C in the $l(1)su(f)^{ts67g}$ and $npr1^3$ larval mutants does not represent the same puff as that resulting from 68C intermolt gene expression. In an attempt to identify transcripts produced in the 68C puff region in the salivary glands of $npr1^3$ mutant larvae, an RNA gel blot was probed successively with three ^{32}P-labeled λ clones representing over 37 kb from the 68C region (Meyerowitz and Hogness 1982), surrounding and including the glue gene cluster. No detectable signal was observed (data not presented), suggesting that if the puffing at 68C in the $npr1^3$ mutant is the result of expression of an undiscovered gene, either the coding sequence is outside the region covered by the probes used or the transcript from this putative gene accumulates to levels below our limits of detection. As a consequence of these studies, we can conclude that the products of the *BR-C* and *su(f)* loci are required for

the formation of that 68C puff which results from *Sgs-3, Sgs-7* and *Sgs-8* gene expression. In addition, the puff seen at 68C in these mutant backgrounds is not due to the same sequences as the glue gene puff.

Discussion

We have analyzed chromosome puffing by transforming fragments from the 68C puff region to new locations. Our results lead us to conclude that a specific element, found in the intragenic region between *Sgs-7* and *Sgs-8*, is an important component of the puff induction process. The inability of this intragenic element to induce puff formation on its own implies that additional factors, possibly the promoter elements associated with nearby transcription units, are necessary for visible decondensation and puffing. In the following discussion, we address data concerning the relationship between puffing and transcription, and discuss a two-step model for puff formation, with protein binding at the intragenic puff element and nearby promoter element(s) possibly leading to the initial decondensation event.

Transcription has long been thought to be associated with puffing, and the cause-and-effect nature of this relationship has recently been examined (see Simon et al. 1985a). Experiments on the incorporation of ^{3}H-uridine into the salivary gland chromosomal RNA have shown the active nature of puffed regions in the synthesis of RNAs, correlating the size of a puff with the rate of incorporation (Pelling 1959, 1964; Berendes 1968; Zhimulev and Belyaeva 1975; McKenzie et al. 1975; Belyaeva and Zhimulev 1976; but, see Bonner and Pardue 1977). Several previous lines of evidence, however, led to the interpretation that transcription is not necessary for puffing. The ability of two mutations, *l(1)su(f)^{ts67g}* (Hansson et al. 1981; Hansson and Lambertsson 1983) and *npr1^{3}* (Crowley et al. 1984), to eliminate the expression of the 68C glue genes without removing the 68C puff were taken to mean that puffing at 68C is independent of the transcription of the *Sgs-3*, *Sgs-7* and *Sgs-8* genes. A surprising result of this study is that 68C sequences transformed into these mutant backgrounds do not induce puffs. This forces us to postulate some other mechanism for the formation of a puff at 68C in these mutants. Perhaps puffing at 68C in the mutants results from the activity of an undetected gene closely linked to the glue genes.

Whatever is proposed, the puffing at 68C in the presence of these mutations can no longer be taken to indicate the existence of puffing without transcription. Thus, we conclude that the normal process of puffing associated with the *Sgs-3, Sgs-7* and *Sgs-8* genes requires the products of the *su(f)* and *BR-C* loci, while the puff at 68C in the presence of either mutation is a different puff than the 68C intermolt puff.

Two types of experiments still provide support for the notion of puffing without concomitant transcription. In the first, Korge (1987; Korge et al. 1990) found that a fusion between the *Sgs-4* promoter region and a *v-mil-myc* transcription unit was capable of puff formation, despite the inability to detect any transcript on RNA gel blots. The argument against transcription occurring was made stronger by the failure to detect with antibodies the presence of RNA polymerase II at the newly puffed location. Although the authors are uncertain why the gene fusion is incapable of RNA synthesis, it appears that transcription is not a necessary component of this puff formation. A similar conclusion can be reached from the studies on puff induction in the presence of inhibitors of RNA synthesis, such as actinomycin D. In these experiments, uridine incorporation (and presumably transcription) can be effectively stopped, and yet, puffing is still induced to low levels (Berendes 1968; Ashburner 1972a) or even high levels (Barettino et al. 1982).

Recent studies on puff formation utilizing transformation have led to a number of conclusions concerning additional determinants of puffing. For instance, Simon et al. (1985b) concluded from studies with the heat-inducible promoter of the *hsp70* gene of *Drosophila* that both high levels of transcription and a large transcription unit are necessary for the formation of a large puff. By increasing the size of the transcription unit, they were able to increase the area of decondensation in the inserted region. Semeshin et al. (1986, 1989) have verified this length-to-size correlation and have also shown that these P-element insertions occur primarily in interband regions with even small "micropuffs" detectable under electron microscopy. In addition, they conclude a minimum gene length requirement of 1 kb for the formation of a detectable puff. This minimum requirement could explain a

discrepancy in puffing seen between two similar constructions. Richards et al. (1983) showed the inability of SGS3F.1 (also known as g1) lines to puff visibly under light microscopy. Conversely, we find puffing associated with the GOAX2.76 construction. These two constructions share a common 5'-end point at -2.75 kb, which includes the intragenic element required for puffing, but differ in the type and length of transcript. The g1 construction contains the *Sgs-3*^{Formosa} allele which produces a primary transcript of about 900 nucleotides (Richards et al. 1983), whereas the GOAX2.76 construction produces an *Sgs-3/Adh* fusion transcript which is probably closer to 1.25 kilobases in length before processing (Benyajati et al. 1983). This 1 kb minimum does not, however, hold for formation of the endogenous puff at position 3C, which is correlated with expression of the *Sgs-4* glue gene (with a transcript of about 950 nucleotides; Korge 1975, 1977; Muskavitch and Hogness 1980). If a threshold value for size does exist, the cut-off appears to be sharp, since one experiment indicates that 900 nucleotides may not be sufficient. Furthermore, our findings show that neither a large transcription unit (4.4 kb in GLX3.3) nor abundant expression (GA6.0 and GLX3.3) are able to stimulate puff formation, while shorter transcription units (GOAX2.76 and the GAX series) and low level transcription (GLX0.66) are associated with a puff when the tested DNA includes the intragenic element. We should point out, though, that our analysis looked at the ability to form a puff, whereas the conclusions on the importance of promoter strength and transcription length were based on the effect on size of the puff produced. Therefore, these conclusions and our findings are not necessarily in conflict, and suggest at least two distinct processes in puff formation. These processes, puff formation and puff size determination, are the framework for the two-step model for puffing discussed below.

The involvement of promoter strength in the ability of a gene to form a puff has recently been studied by Korge et al. (1990). In their analysis of the *Sgs-4* promoter and its puffing capacity, they conclude that the promoter strength of the *Sgs-4* glue gene upstream sequences (from -840 to -1 bp) is the important determinant in the puffing of transformed

fragments. Their results are complicated, however, by the findings of McNabb and Beckendorf (1986) in which transformants of DNA from the *Sgs-4* region, containing 840 bp of upstream sequence from a full-strength promoter allele, failed to induce puffing upon insertion. The constructions tested by Korge et al. (1990) contained 2.5 kb of upstream sequence with variation in the region from -840 to -1 bp. An alternative explanation to the conclusions of Korge et al. (1990) might involve an element in the region from -2.5 kb to -840 bp, similar to the intragenic puff element found at 68C. In the presence of this element, a normally active promoter would be able to induce puffing (as is seen in Hofmann et al. 1987; Korge 1987; Korge et al. 1990), while deletion of this element would eliminate puffing without reducing transcription (McNabb and Beckendorf 1986). Even with this element present, however, the presence of functional promoter elements would be necessary to produce a visible puff, which might explain why disruption of sequences in the -340 bp region has effects on both transcription and puffing (Hofmann et al. 1987; Korge et al. 1990).

A two-step model for puffing

A conclusion that puffing can occur in the absence of transcription conflicts with our conclusions from the GXX0.19 transformants. The failure of this intragenic element to induce puffing unless adjacent to a transcription unit implies a necessary reliance for puffing on sequences associated with transcription. From the failure of highly expressed genes to puff (for example, GA6.0 and GLX3.3), it is obvious that transcription alone is insufficient to cause puffing. A two-step model for puff formation could explain the data, and has been proposed (Pelling 1966; Berendes 1968; Barettino et al. 1982; Daneholt et al. 1982). This two-step process involves initial decondensation caused by protein:DNA interactions, followed by an increase in puff size due to the transcription process, and RNA storage and processing. Indeed, Berendes (1968) postulated that the puffing seen in the

presence of RNA inhibitors could be due to the accumulation of acidic proteins (presumably transcription factors), which may account for minor increases in chromosome diameter. There is also structural evidence to support a two-step model for puff induction. Daneholt et al. (1982) found three distinct levels of DNA compaction associated with the 75 S RNA gene of *Chironomus* under electron microscopic analysis. The highest level of DNA packing was found in non-expressing tissue, while the lowest level of compaction was achieved only upon transcriptional activation. They propose that the intermediate compaction state represents initial puff formation. The results presented in this study suggest that any accumulation of protein into the 68C region capable of inducing a puff would require interaction with both the intragenic puff element and transcriptional promoter elements. Potential interactions between these two elements, possibly by DNA looping (Hochschild and Ptashne 1986), may help to facilitate this decondensation process. If the initial decondensation does involve promoter elements, this could explain the requirement for products of the *su(f)* and *BR-C* loci, as mutations in these loci have been shown to affect protein binding to an important regulatory domain in the proximal promoter of the *Sgs-3* gene (Mathers and Meyerowitz, submitted). Although this model is speculative, the absence of interaction between the intragenic and promoter elements could explain the failure of the GXX0.19 series of insertions to puff. Such a model could also account for puff formation without detectable transcription, or in the presence of RNA synthesis inhibitors. Further work is necessary to verify the existence of these proposed interactions.

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Table Legend

Table 1. Characteristics of transformed strains. The constructions used for P-element transformation are listed, along with the abundance of expression observed from these constructions. High level expression from these constructions is denoted ++, along with lower level (+) and no detectable (-) transcription, and these results were obtained from other studies (see Materials and Methods). The GXX0.19 lines were not analyzed for expression (ND). In addition, the number of the transformed lines analyzed, the site of chromosomal insertion and the result of puffing examination are presented. A # is used to define information obtained from Crosby and Meyerowitz (1986; see also Figure 7). The * signifies transformed lines that were analyzed as heterozygous insertions. A § denotes a case where a + for puffing is based primarily on decondensation, rather than an increase in chromosomal diameter. Finally, information marked † is from unpublished findings of A. Hofmann and G. Korge (personal communication).

TABLE 1

CHARACTERISTICS OF TRANSFORMED STRAINS

CONSTRUCT	EXPRESSION	INSERT NO.	CYTOLOGICAL LOCATION	PUFFING
GA8.1	++[#]	3	97F/98A[#]	+[#]
GA6.0	++[#]	-	-	-[#]
GOAX2.76	++	1	56F/57A	+
		2	91C	+
		5	70B	+
GOAX0.98	+	4	62A	-
		5	87E	-
		6	46F	-
GLX3.3	++	1	82E	-
		2	59C	-[*]
		4	41F	-[*]
GLX1.34	+	1	86CD	-
		2	77DE	-
GLAX1.0	++	2	60EF	+
		3	99C	+
		7	82D	+
GAX0.26	++	1	39BC	+
		2	77F	+
		3	59AB	+
GAX0.24	++	1	49BC	+
		2	85D3	+
		4	86E	+
GAX0.12	-	1	48AB	-
		2	38F	-
		3	62B1-3	-
GLX1.1	++	1	56E	+
		6	60B	+
GLX0.8	++	1	50BC	+[§]
		2	23B	+
		4	69B	+
GLX0.68	++	1	85D	+
		2	23D	+
		5	95AB	+
GLX0.66	+	1	73DE	+
		3	30B	+
		4	64D	+
GXX0.19	ND	1	60CD	-
		2	91A	-
		3	53C	-
		6	58AB	-
		7	21BC	-
		8	62A	-
GGAX-1	++[†]	1	95AB[†]	+
		2	45D[†]	+
		3	96C1-3[†]	+

Figure Legends

Figure 1. Diagram of constructions analyzed. A schematic representation of the fragments used for transformation is presented. Transformation vector sequences have been omitted. All fragments were inserted into the SalI restriction site of the Car 20 vector (Rubin and Spradling 1983), except the GA8.1 and GA6.0 constructions which utilized the pAP5 vector (see Crosby and Meyerowitz 1986). Although GA6.0 was not analyzed in this study, it is diagrammed here for reference. Sequence composition of various fragments are pattern coded as explained in the key. Transcription direction is noted by arrows at the end of the fragment, or in cases where multiple transcription units are present, by white arrows within the fragment. The numbered line represents coordinates from the sequence of the 68C glue gene cluster (Garfinkel et al. 1983). Sequences from the 68C region (black shading) are placed under their respective position along the 68C map, with the exception of the polyadenylation site from the *Sgs-8* region in the GLX series of constructions. The position and transcription direction of the endogenous glue genes is also presented.

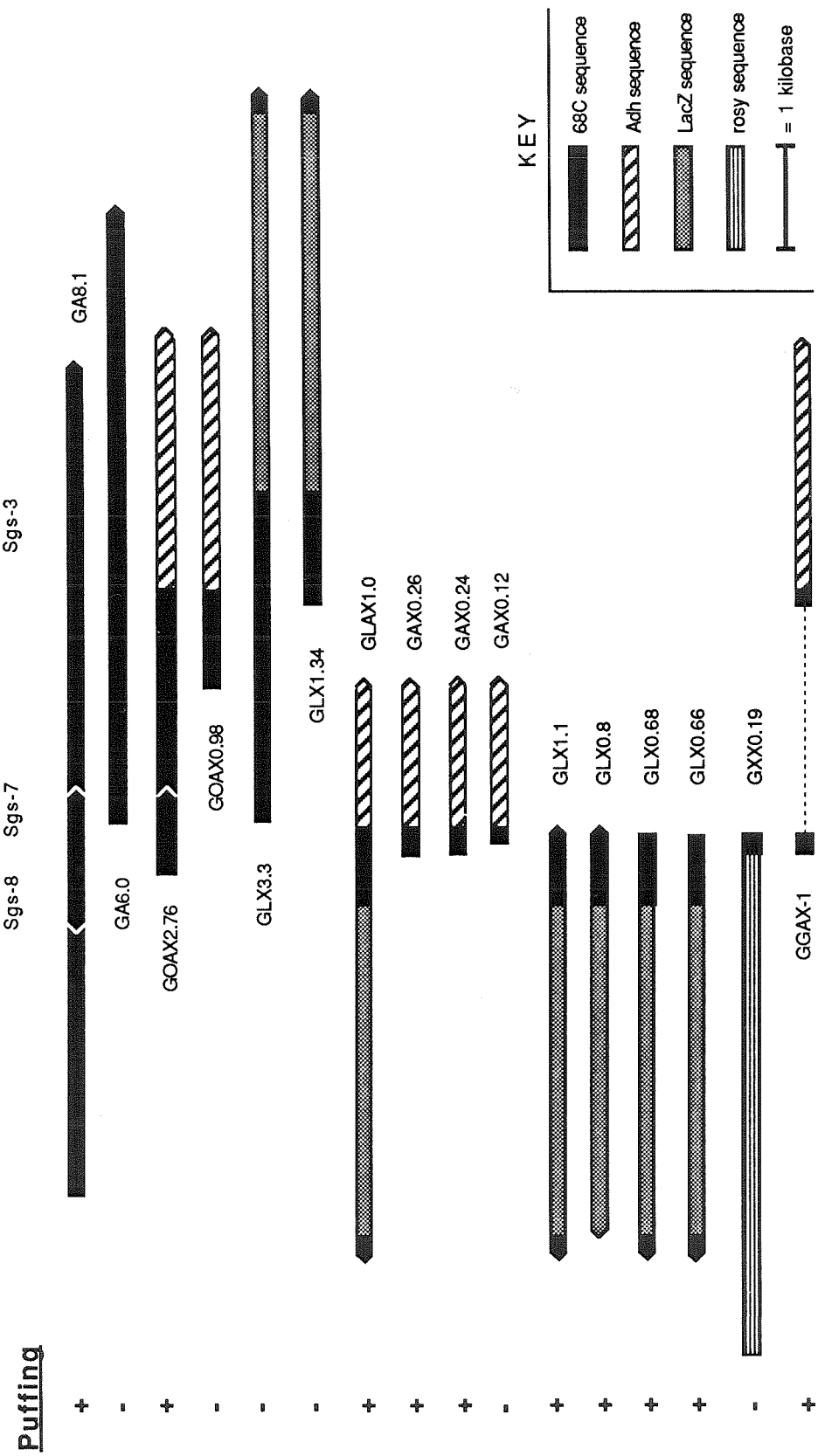

Puffing
0
1000
2000
3000
4000
5000
6000 bp
Sgs-8
Sgs-7
Sgs-3
GA8.1
GA6.0
GOAX2.76
GOAX0.98
GLX3.3
GLX1.34
GLAX1.0
GAX0.26
GAX0.24
GAX0.12
GLX1.1
GLX0.8
GLX0.68
GLX0.66
GXX0.19
GGAX-1
KEY
68C sequence
Adh sequence
LacZ sequence
rosy sequence
= 1 kilobase

Figure 2. Puffing analysis of GOAX2.76, GOAX0.98 and GLX3.3 transformed lines. Insertion sites (Table 1) were examined in transformed (Tf) and non-transformed sibling lines (n-Tf) at puff stage 1-2. Arrowheads point to the site of insertion as determined by in situ hybridization or the corresponding site in non-transformed controls. The *Tf(2)GLX3.3-2* and *-4* lines are homozygous lethals and were analyzed as heterozygous inserts over the *CyO* balancer chromosome.

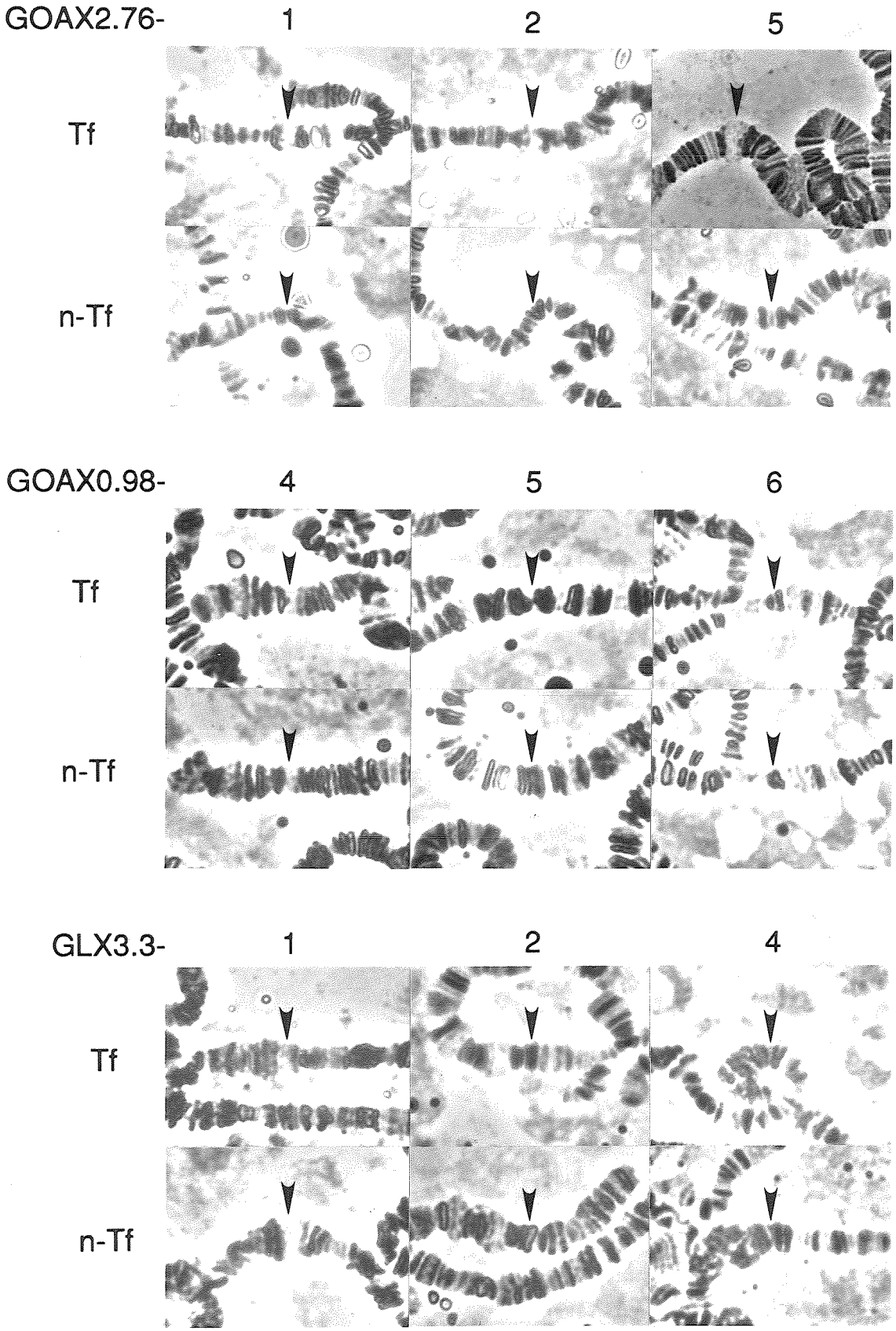

GOAX2.76-
1
2
5
Tf
n-Tf
GOAX0.98-
4
5
6
Tf
n-Tf
GLX3.3-
1
2
4
Tf
n-Tf

Figure 3. Puffing analysis of GLX1.34, GLAX1.0 and GAX0.26 transformed lines. Examination was performed as described in Figure 2.

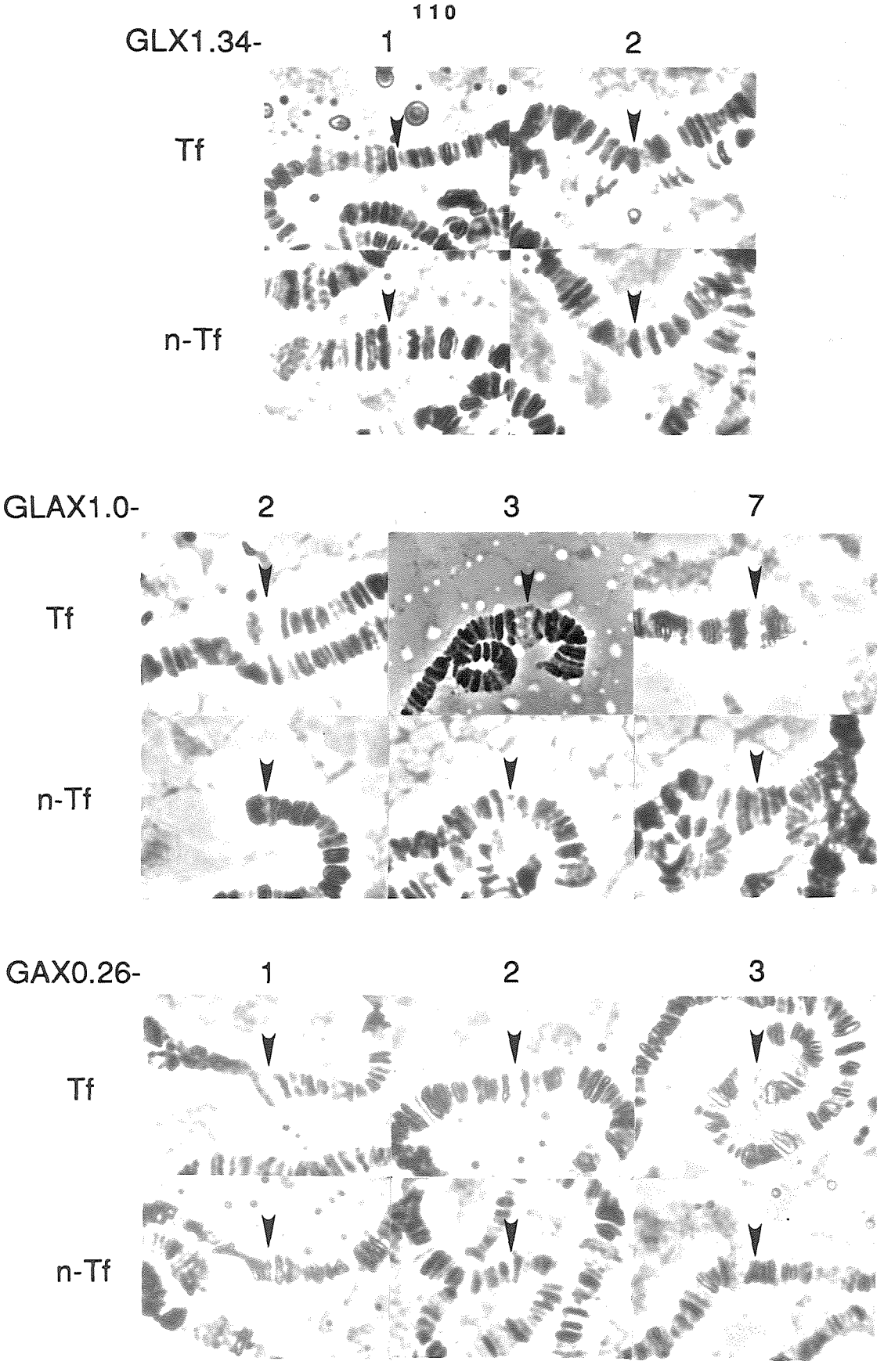
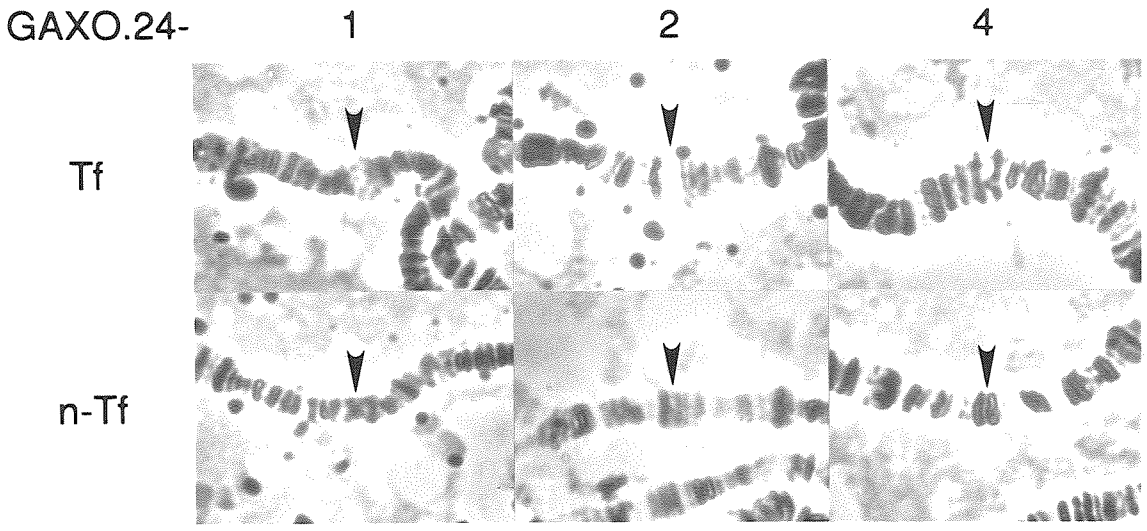
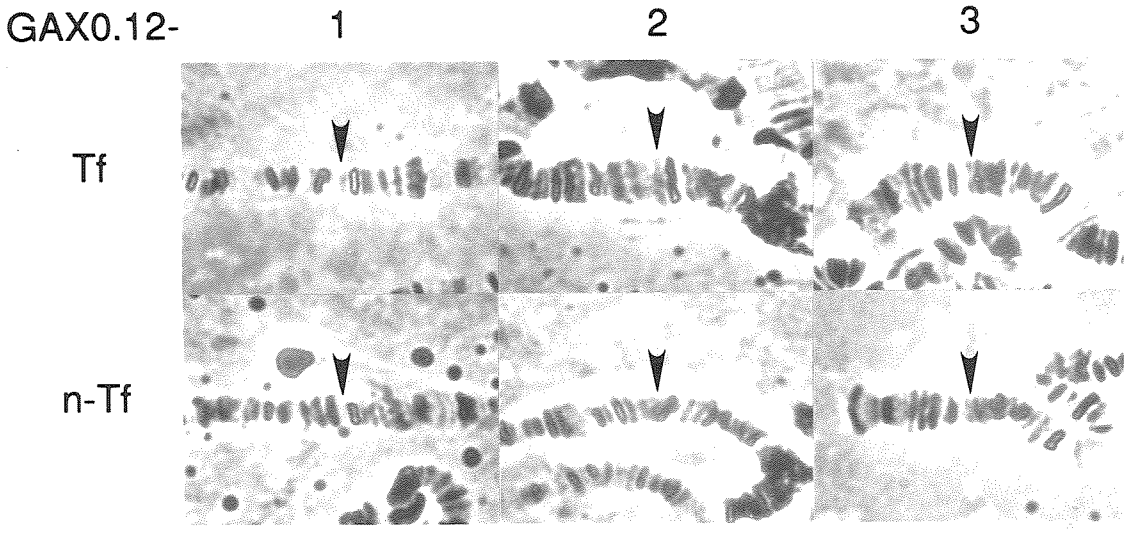

GLX1.34-
1
2
Tf
n-Tf
GLAX1.0-
2
3
7
Tf
n-Tf
GAX0.26-
1
2
3
Tf
n-Tf

Figure 4. Puffing analysis of GAX0.24, GAX0.12 and GLX1.1 transformed lines. Examination was performed as described in Figure 2.


GAXO.24-
1
2
4
Tf
n-Tf


GAX0.12-
1
2
3
Tf
n-Tf

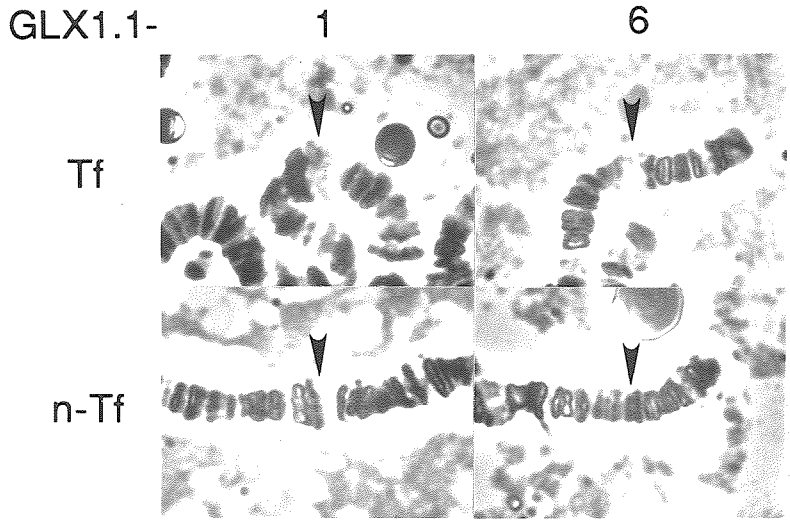

GLX1.1-
1
6
Tf
n-Tf

Figure 5. Puffing analysis of GLX0.8, GLX0.68 and GLX0.66 transformed lines. Examination was performed as described in Figure 2.

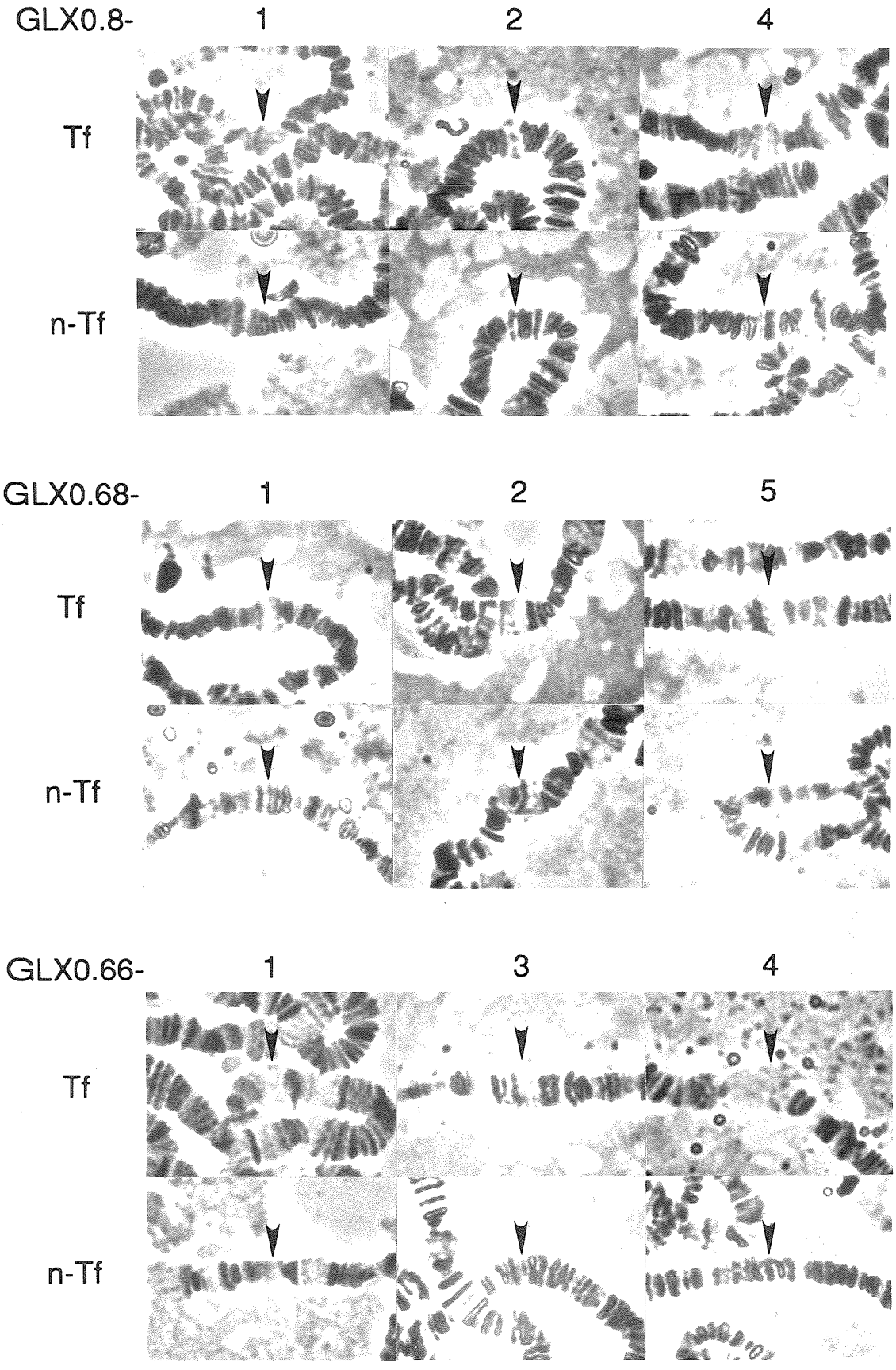
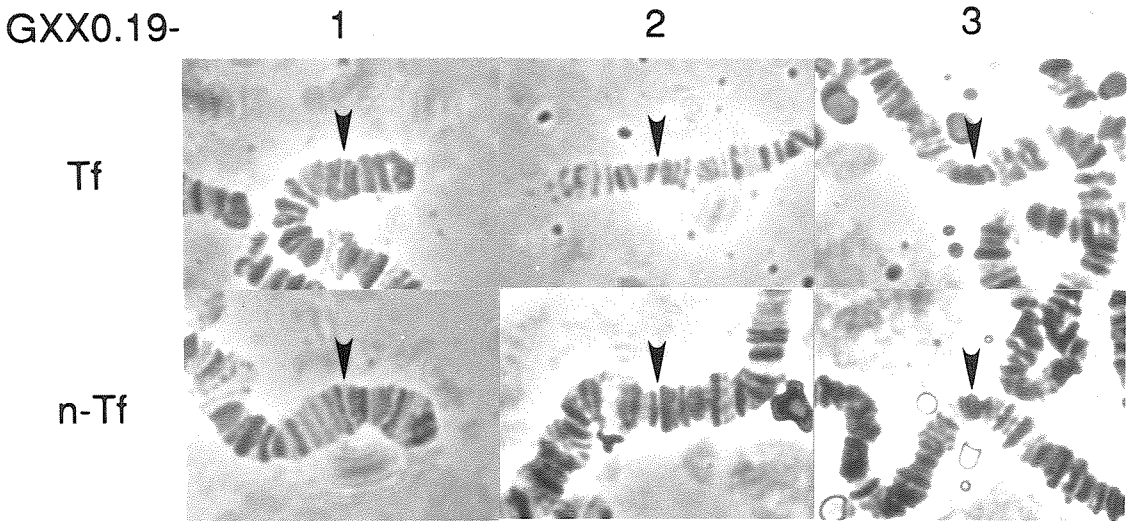
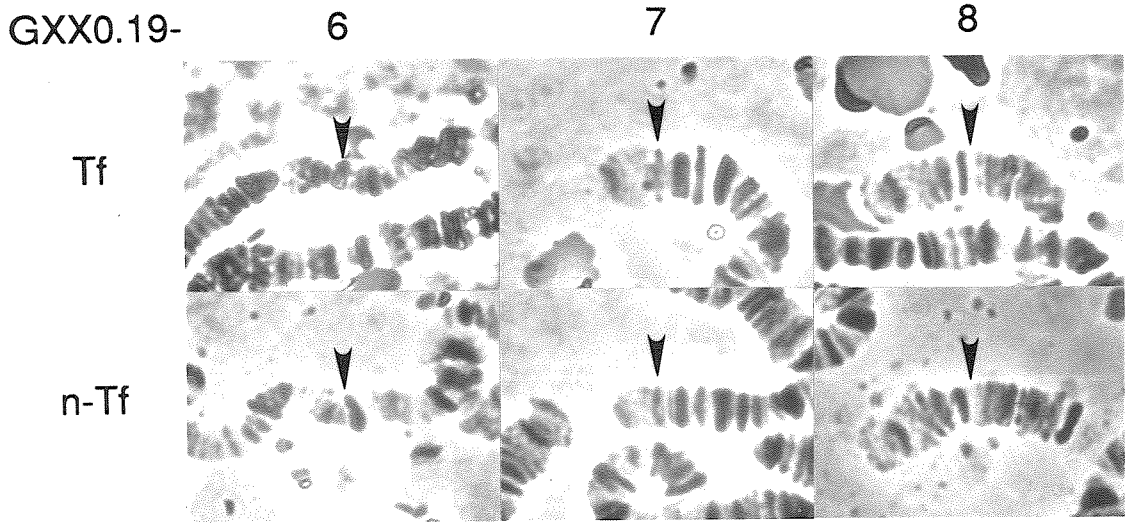

GLX0.8-
1
2
4
Tf
n-Tf
GLX0.68-
1
2
5
Tf
n-Tf
GLX0.66-
1
3
4
Tf
n-Tf

Figure 6. Puffing analysis of GXX0.19 and GGAX-1 transformed lines. Examination was performed as described in Figure 2.


GXX0.19-
1
2
3
Tf
n-Tf


GXX0.19-
6
7
8
Tf
n-Tf

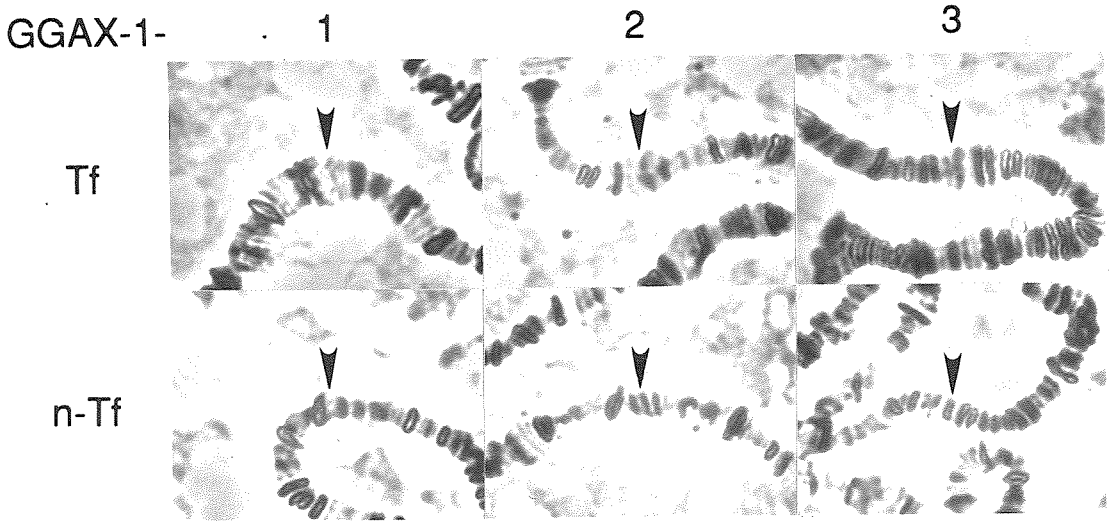
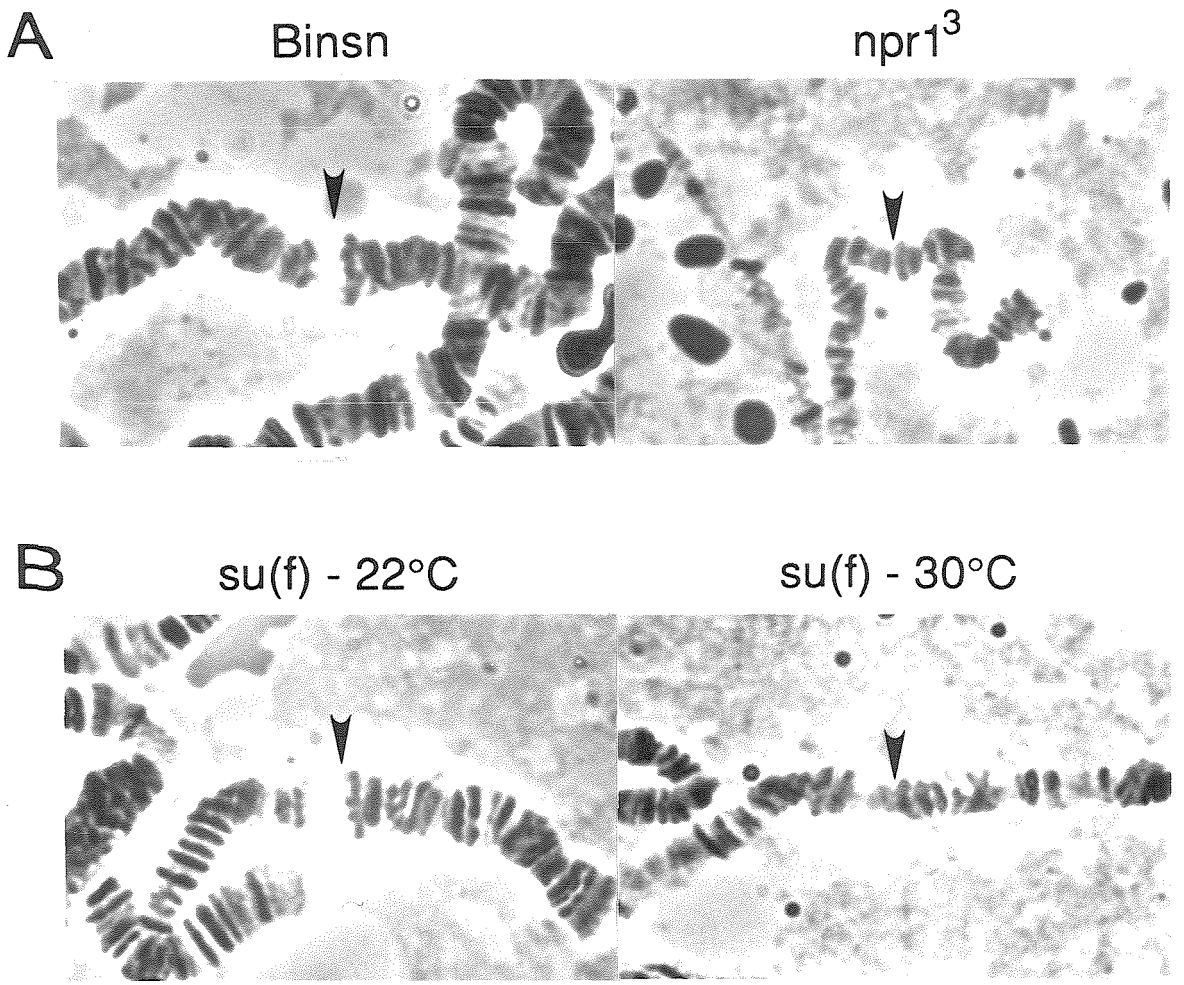

GGAX-1-
1
2
3
Tf
n-Tf

Figure 7. Puffing analysis of the *Tf(3)GA8.1-3* transformed chromosome in the background of *trans*-acting mutations. A. The insertion site at the 97F/98A border region was analyzed as a homozygous insert in males hemizygous for the X balancer chromosome *Binsn* (which carries a wild-type allele of the *Broad-Complex*) or the *y npr1*^{3} *Sgs-4*^{BER} chromosome. B. The insertion site was analyzed in larvae carrying the *car l(1)su(f)*^{ts67g} chromosome at either permissive temperature (22°C) or restrictive temperature (30°C).


A
Binsn
npr1^3
B
su(f) - 22°C
su(f) - 30°C

Appendix I:

Sequences Sufficient for Correct Regulation of *Sgs-3* Lie Close to or Within the Gene

Explanation of contribution:

The following paper is included as an appendix to this thesis to provide continuity and easy reference for the appendix to follow and to signify my contribution to its contents. The analysis of expression of the constructs mentioned within this paper in the *npr1*^{3} (or *l(1)npr-1*) mutant background were performed by me. In addition, a contribution was made in the writing and editing of the manuscript.

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Sequences sufficient for correct regulation of *Sgs-3* lie close to or within the gene

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The *Drosophila melanogaster* 68C chromosomal locus is the site of a prominent polytene chromosome puff that harbors the genes *Sgs-3, Sgs-7* and *Sgs-8*. These genes code for proteins that are part of the salivary glue that *Drosophila* larvae secrete as a means of fixing themselves to an external substrate for the duration of the pre-pupal and pupal period. The 68C glue genes are regulated by the steroid hormone ecdysterone, with the hormone required for both initiation and cessation of gene expression during the third larval instar. Previous work has defined sequences sufficient for expression of abundant levels of *Sgs-3* mRNA at the correct time and in the correct tissue. We show here that sequences sufficient for normal tissue- and stage-specific accumulation of *Sgs-3* RNA, but adequate only for low levels of expression, lie within 130 bp of the 5′ end of the gene, or within the gene.

Key words: Drosophila/glue/salivary glands/developmental regulation/ gene expression

Introduction

The pattern of gene expression in the larval salivary glands of *Drosophila melanogaster* is precisely regulated during development. In the third larval instar several dispersed genes that encode components of a proteinaceous glue are transcribed. These genes are expressed solely in the salivary glands. The glue, which consists of at least eight polypeptides, is glycosylated, secreted into the lumen of the glands, and then expelled to the outside surface of the larva (Fraenkel and Brookes, 1953; Korge, 1975, 1977a; Beckendorf and Kafatos, 1976). The structural genes for several of the glue polypeptides have been localized in the polytene chromosomes and their sites are characterized by the presence of prominent puffs, or decondensation of the chromatin, during the period when they are heavily transcribed (Korge, 1975, 1977b; Akam *et al.*, 1978; Muskavitch and Hogness, 1980; Velissariou and Ashburner, 1980, 1981; Crowley *et al.*, 1983; Guild and Shore, 1984). The gene for the sgs-3 glue polypeptide has been shown by genetic experiments (Akam *et al.*, 1978) to map to the left arm of the third chromosome to the 68C locus. A molecular analysis of the region has demonstrated that it is the site of three closely linked and co-ordinately expressed genes that encode the glue polypeptides sgs-3, sgs-7 and sgs-8 (Meyerowitz and Hogness, 1982; Crowley *et al.*, 1983). The DNA sequence of the region that includes the three genes and the size and the direction of the transcription units have all been determined (Garfinkel *et al.*, 1983).

The hormone ecdysterone affects the expression of the 68C glue gene cluster in two ways. First, there is evidence that ecdysterone is required for the initiation of RNA accumulation at the 68C puff. In the temperature-sensitive X-chromosomal mutant $l(1)su(f)^{ts67g}$ the 68C RNAs are present when homozygous or hemizygous animals are reared at 22°C. At 30°C, the mutant larvae appear to be deficient in ecdysterone. When mutant larvae are raised to the restrictive temperature near the time of the second to third larval instar molt, the 68C RNAs fail to accumulate in the salivary glands. When such larvae are fed the hormone the RNAs are observed to accumulate (Hansson and Lambertsson, 1983). Ecdysterone also acts to stop transcription of the three 68C glue genes. Toward the end of the third larval instar, the regression of the 68C puff and the cessation of accumulation of the 68C RNAs coincides with an increase in the hemolymph titer of the hormone (Becker, 1962; Crowley and Meyerowitz, 1983). When ecdysterone is added to *in vitro* cultured salivary glands the intermolt puffs rapidly regress (Ashburner, 1973; Ashburner and Richards, 1976) even when cyclohexamide is present in the medium (Ashburner, 1974), indicating the action of the hormone is not through the induction of synthesis of a regulatory protein. RNA pulse-labelling experiments have shown that the effect of high hormone titers *in vitro* is to very rapidly stop the accumulation of freshly synthesized RNA (Crowley and Meyerowitz, 1983). The effect of the hormone on the 68C locus could well be direct: immunofluorescence experiments have localized ecdysterone to several of the intermolt puffs during puff regression (Gronemeyer and Pongs, 1980; Dworniczak *et al.*, 1983).

Another *trans*-acting factor that affects the expression of the 68C glue RNAs has been identified. This is a product of the 2B5 locus as defined by the mutation *l(1)npr-1* (Kiss *et al.*, 1978). Animals that are homozygous or hemizygous for this mutation die as late larval lethals. The 68C RNAs are absent in mutant animals and hybrid selection of pulse-labelled RNA has demonstrated that there is no accumulation of freshly synthesized RNA from *Sgs-3, Sgs-7* and *Sgs-8* (Crowley *et al.*, 1984).

The 68C glue genes are thus a useful system for the study of hormonally regulated tissue- and stage-specific gene expression. Experiments on one of the genes, *Sgs-3*, have defined the sequences in the region sufficient for correct developmental accumulation of high levels of RNA (Richards *et al.*, 1983; Bourouis and Richards, 1985; Meyerowitz *et al.*, 1985; Crosby and Meyerowitz, 1986).

In this study we have examined constructs with only 130 bp of DNA upstream of the *Sgs-3* mRNA transcription start site (Garfinkel *et al.*, 1983) using two different vectors to introduce the gene into the germline of flies. We show that these constructs carry sequences sufficient for correct tissue- and stage-specific expression of RNA, but at levels that are much less than that from the native gene. In addition, we have constructed and introduced into flies an *Sgs-3* gene fused in frame to the gene for *Escherichia coli* β-galactosidase. We demonstrate that this fusion gene, in the presence of 2270 bp of 5′ flanking sequence, expresses an enzymatically active fusion protein in the salivary

glands of third instar larvae. When only 130 bp of 5′ sequence are present, the sgs-3−lacZ fusion protein is expressed at lower levels but tissue and stage specificity is retained.

Results

Plasmids and germline transformation

Four different plasmids were introduced into the *D. melanogaster* genome by P-element-mediated germline transformation (Spradling and Rubin, 1982; Rubin and Spradling, 1982) (Figure 1). The first two contain the *Sgs-3* gene with 130 bp of 68C sequence upstream of the mRNA coding region and 1100 bp downstream. One, pGA2.4, has this DNA inserted into the P-element transformation vector pAP2 (Goldberg *et al.*, 1983), allowing selection for alcohol dehydrogenase activity as an indicator of transformation. The second, pGX2.4, has the same sequences from the 68C region, but inserted into the Carnegie 20 P-element vector (Spradling and Rubin, 1983), which contains a xanthine dehydrogenase (*rosy*) gene as a transformation marker. Two different vectors were used to assure that the results obtained were not influenced by the sequences surrounding the tested DNA. The remaining two transformation plasmids, pGLX3.3 and pGLX1.34, contain 2270 and 130 bp, respectively, of upstream *Sgs-3* sequence as well as the first 948 nucleotide pairs of the *Sgs-3* gene fused in-frame to the *E. coli lacZ* (β-galactosidase)

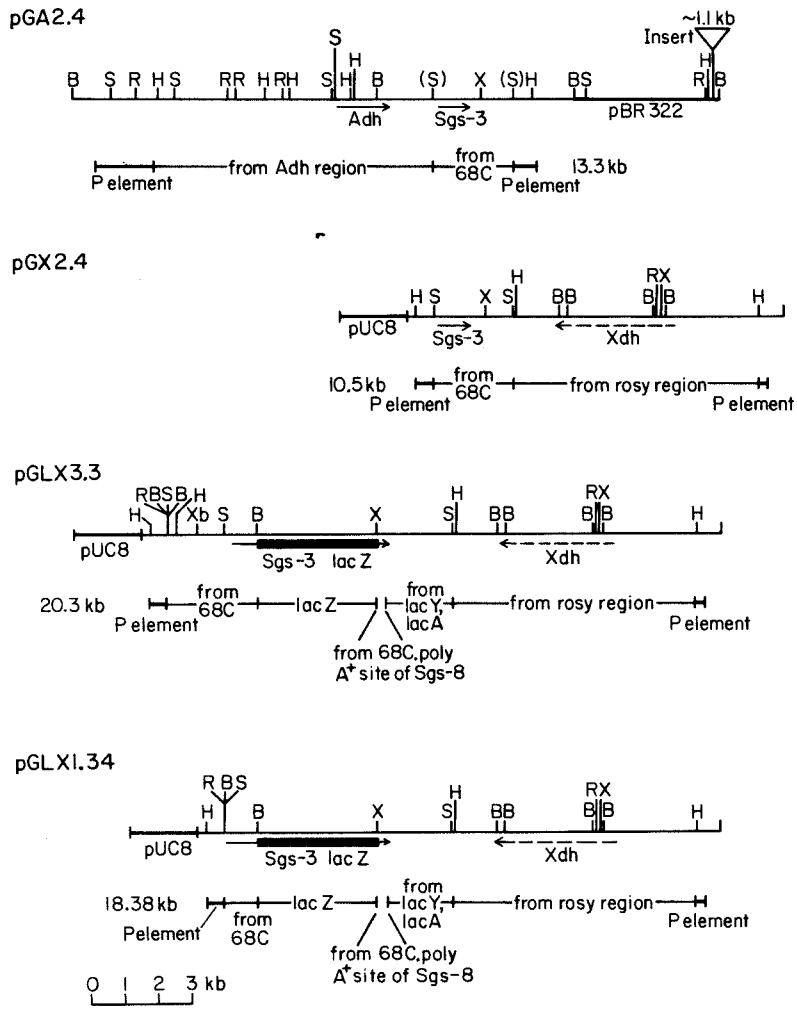

Fig. 1. Restriction maps of the four plasmids used in the transformation experiments discussed in the text. The nomenclature used for the plasmids is: G for glue, A for alcohol dehydrogenase (*Adh*), L for *lacZ* and X for xanthine dehydrogenase (*Xdh*). The number in the plasmid name denotes the amount, in kilobase pairs, of 68C sequence present in the construct. pGA2.4 therefore has *Adh* as the independent selectable marker used in the transformation experiments and 2.4 kb of 68C sequence. As shown above pGX2.4 and pGA2.4 have the same 68C insert but in different P-element vectors. These two plasmids and pGLX1.34 have identical sequences (130 bp) upstream of the mRNA start site of *Sgs-3*, the difference being that in pGLX1.34 the *Sgs-3* gene is fused in frame to an *E. coli lacZ* gene to produce an sgs-3/β-galactosidase hybrid protein. pGLX3.3 makes the same fusion RNA and protein as pGLX1.34 but has a larger (2270 bp) region from the 68C locus upstream of the *Sgs-3* mRNA start site.

gene (Casadaban *et al.*, 1981). These sequences include the small intron located near the 5′ end of the *Sgs-3* gene. At the 3′ end of the *Sgs-3*−*lacZ* fusion gene the poly(A) addition site from the *Drosophila Sgs-8* gene is inserted. These constructions were used to allow a histochemical assay of *Sgs-3* regulatory sequences as an independent check of results of the RNA blot hybridization assays used for the first two constructions, and to permit convenient examination of gene expression at the level of individual cells. The pGLX3.3 transformation vector is a control, since our previous work has shown that 2270 bp of 5′ flanking 68C sequence is sufficient for normal quantity, tissue and time of expression of the *Sgs-3* gene (Crosby and Meyerowitz, 1986).

Four independent fly strains transformed with pGA2.4, six with pGX2.4, three with pGLX3.3 and three with pGLX1.34 were analyzed (Table I).

RNA blot analyses

The activity of the introduced *Sgs-3* genes in the pGA2.4 and pGX2.4 lines were assayed by RNA blot hybridization: the introduced genes are from a wild-type *D. melanogaster* strain, Oregon-R, that makes a 1120-base long *Sgs-3* mRNA (Garfinkel *et al.*, 1983) and are in a genome in which the endogenous *Sgs-3* gene codes for a 820-base long message (the Formosa strain, Mettling *et al.*, 1985). In all 10 of these lines the introduced genes are expressed in the salivary glands of third instar larvae, at the same time as the endogenous genes (Figures 2A and 3, data for two pGA2.4 and six pGX2.4 transformants are shown).

For the pGA2.4 and the pGX2.4 transformant lines the relative levels of accumulation of the RNA from the introduced and endogenous genes were measured by scintillation spectrometry of bands cut from RNA gel blots. For the introduced genes, RNA accumulation between 2.5 and 11.1% of that from the endogenous genes was observed (Table I), calculated as described by Crosby and Meyerowitz (1986). In that paper, it was demonstrated that *Sgs-3* constructs with >4 kb pairs of upstream 68C sequence accumulate between 80 and 98% of endogenous levels of RNA transcripts. Thus, while 130 bp of 5′ flanking sequence (and the gene) are sufficient for normal tissue and time of *Sgs-3* mRNA accumulation, they are insufficient for accumulation of high levels of the message.

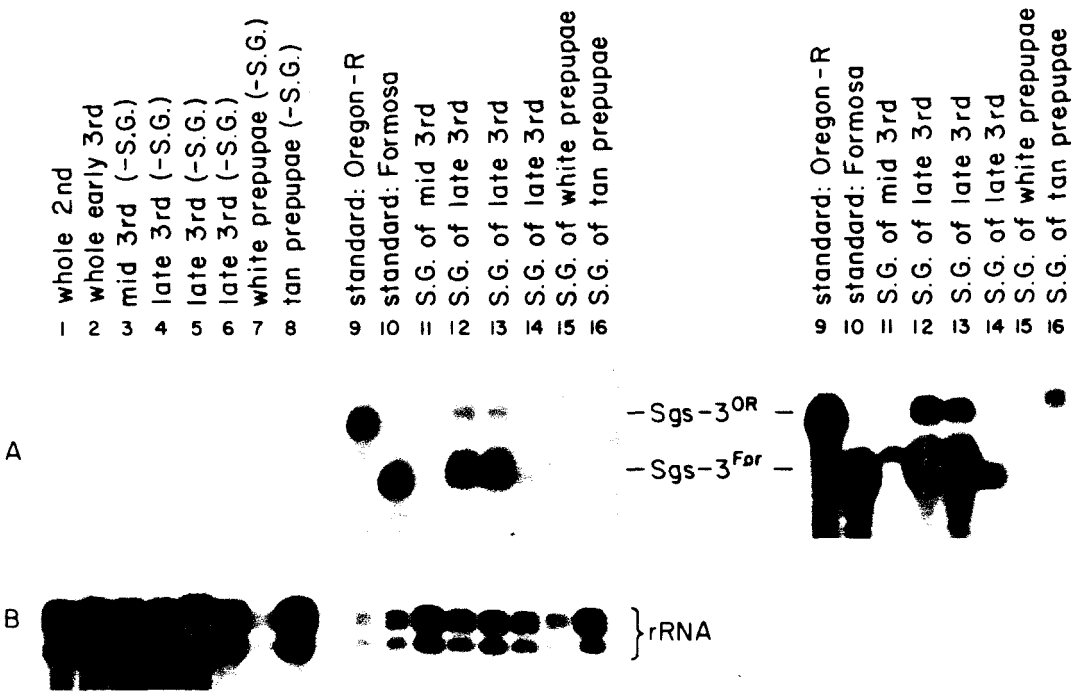

Fig. 2. RNA blot analysis of Tf(2)GA2.4-1 transformants at different developmental stages. RNA isolated from the salivary glands and carcasses, after removal of salivary glands, of homozygous Tf(2)GX2.4-1 animals was fractionated by size on a 1.5% agarose formaldehyde gel. RNA from two animals was used for each lane, except when otherwise indicated. Larval carcasses with the salivary glands removed were used as tissue-specific controls. The gel was blotted to a nitrocellulose filter and hybridized with ^{32}P-labelled probes. **Lane 1**: whole second instar larvae, 10 animals; **lane 2**: whole early third instar larvae, six animals; **lane 3**: carcasses of mid-third instar larvae, three animals; **lane 4**: carcasses of late third instar larvae (pre-climbing); **lane 5**: carcasses of late third instar larvae (early climbing); **lane 6**: carcasses of late third instar larvae (late climbing); **lane 7**: carcasses of white pre-pupae; **lane 8**: carcasses of tan pre-pupae; **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 pre-pupae; **lane 16**: salivary glands of tan pre-pupae. (**A**) The probe used was aDm2023 (Garfinkel *et al.*, 1983) which is homologous with *Sgs-3* RNA. Prior to polyadenylation the *Sgs-3*^{OR} RNA is 1120 nt long and the *Sgs-3*^{For} RNA is 820 nt long (Garfinkel *et al.*, 1983; Mettling *et al.*, 1985). A longer exposure of lanes 9−16 is shown to the right. (**B**) The probe was removed and the filter was rehybridized with λbDm103 which contains the *Drosophila* rRNA coding region. This control showed that the amount of RNA loaded in lane 7 was probably significantly lower than in the other lanes; a repeat experiment for this data point was therefore performed and showed no *Sgs-3* homologous RNA in the carcasses of white pre-pupae.

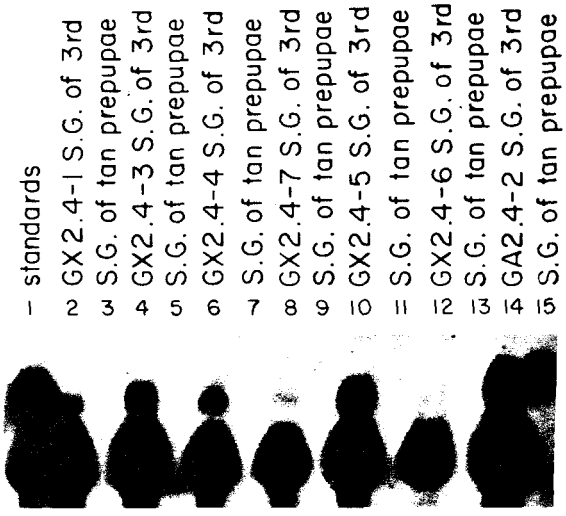

Fig. 3. RNA blots of GX2.4 transformant lines. The figure shows that the several independent transformants express the introduced *Sgs-3* gene correctly and that there is no aberrant expression at the tan pre-pupal stage as seen for the GA2.4 transformants. Total salivary gland RNA from two animals was loaded on each lane. **Lane 1**: standards, RNA from heterozygote $Sgs\text{-}3^{OR}/Sgs\text{-}3^{For}$. The upper band is the RNA from the Oregon-R size variant and the lower band that from the Formosa size variant. **Lanes 2–13** contained RNA from the salivary glands of different transformants at the third instar larval and the tan pre-pupal stage as shown labelled in the figure. **Lanes 14** and **15** contained salivary gland RNA from third instar larvae and tan pre-pupae, respectively, from Tf(2)GA2.4-2, showing expression of the introduced gene in the salivary glands of tan pre-pupae. This GA2.4 transformant is independent from that shown above in Figure 2. The tan pre-pupal lanes in the GX2.4 transformants do not show any hybridization to a probe homologous to *Sgs-3* RNA (aDm2023) while the third instar larval lanes show RNA of mobilities identical to RNA from both the introduced (the upper band) and endogenous genes (the lower band). The filter was washed off and reprobed with ^{32}P-labelled λbDm103 to show that RNA was present in the tan pre-pupal lanes (data not shown).

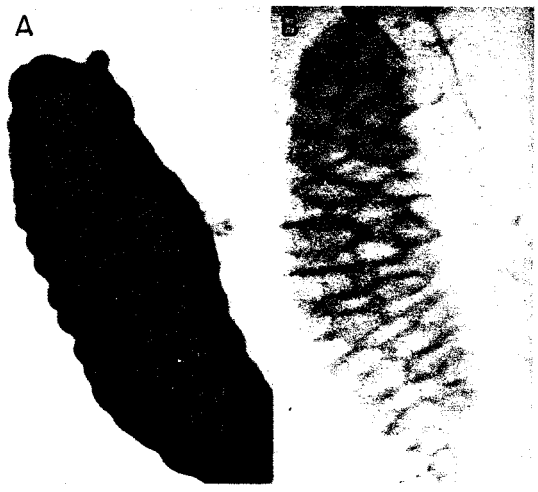

Fig. 4. β-Galactosidase activity in transformants carrying a *Sgs-3–lacZ* fusion gene. (**A**) Salivary gland lobe from Tf(3)GLX3.3-1 transformant (climbing third instar larva). The staining is a dark blue in ~5 h, though the preparations were usually examined after staining overnight. (**B**) In contrast, the staining of a lobe from a Tf(3)GLX1.34-2 transformant (same stage as above) is a lighter blue, even after a day. Salivary glands from non-transformed third instar larvae do not react with the galactosidase staining reagent.

Developmental expression of transformed Sgs-3 genes

RNA blot hybridization of the pGA2.4 transformants from different developmental stages showed that the expression of the introduced gene corresponds with that of the resident gene. The one exception was the tan pre-pupal stage of development. At this stage the introduced gene is expressed in the salivary glands (and not in any other tissue), at lower levels than at the third larval instar stage (Figure 2). This mis-regulation is observed in all the pGA2.4 transformants (two examples are shown, one in Figure 2A, lane 16 and the other in Figure 3, lane 15). The expression in tan pre-pupae is not a property of the host strain used as the introduced gene shows this aberrant expression both in the Hikone-R and Formosa wild-type backgrounds (data not shown). However, RNA accumulation from the introduced gene is not detectable at the tan pre-pupal stage in the pGX2.4 transformants (Figure 3). We therefore conclude that the expression seen in the pGA2.4 transformants at this stage is a vector-dependent phenomenon.

Developmental expression of Sgs-3–β-galactosidase fusion genes

To confirm the above results, and to allow analysis of the expression of the introduced genes in the different cell types of the larval salivary gland, transformed larvae homozygous or hemizygous for chromosomal integration of the pGLX3.3 and pGLX1.34 constructs were examined. Dissected climbing third instar larvae were assayed for *E. coli* β-galactosidase activity using the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at pH 8.0 (Singh and Knox, 1984), at which the *E. coli* enzyme, but not the endogenous *Drosophila* gut galactosidase, is active. The pGLX3.3 transformants show uniform dark blue staining of the secretory cells of the salivary gland and no staining of any other cell type including the neck cells and imaginal ring cells (Figure 4A). The staining pattern is thus restricted to exactly those cells in the salivary gland that normally synthesize the proteinaceous glue. The pGLX1.34 transformants show light blue staining of the secretory cells, with no other cell types stained (Figure 4B). Thus, the results from the pGX2.4 and pGA2.4 transformants, based upon RNA gel blots, are confirmed.

We had shown earlier that *Sgs-3* genes with 2270 nucleotides of 5′ flanking sequence were regulated correctly during development when introduced into flies (Crosby and Meyerowitz, 1986). pGLX3.3 transformants, which have 2270 bp of upstream sequence from *Sgs-3*, were examined for expression during development. A histochemical assay was used for *E. coli* β-galactosidase activity which allows the examination of expression at the cellular level. The stages tested were: whole embryos immediately after egg laying, first instar larvae, second instar larvae, third instar larvae, white pre-pupae, tan pre-pupae, pupae and adults. Transformant-specific galactosidase activity was observed in the salivary glands of third instar larvae and not in other tissues or stages. The fusion protein, which includes almost all of the sgs-3 protein coding sequences, behaves as a secretory protein. Late in the third larval instar the activity is detected in the lumen of the glands and in the salivary ducts (data not shown). Only rarely are we able to detect enzyme activity histochemically at the white and tan pre-pupal stages and the pupal case does

Table I. (A) A listing of the transformants studied. pGA2.4, pGX2.4, pGLX1.34 and pGLX3.3 were the plasmids used and the transformants obtained from their injection are respectively named Tf GA2.4, Tf GX2.4, Tf GLX1.34 and Tf GLX3.3 The number in parentheses indicates the chromosome of insertion for each transformation event. The asterisks denote the transformants that were examined when hemizygous for the X-linked mutation *l(1)npr-1*.
(B) The accumulated levels of RNA from four of the GA2.4 lines and two of the GX2.4 lines are shown. Quantitation was relative to steady state RNA levels from the endogenous gene and was carried out as described in Materials and methods.

(A) Transformants used in this study

Sgs-3 with 130 bp of 5′ seq		*Sgs-3–lacZ* fusions	
Adh vector	*rosy* vector	*rosy* vector 130 bp of 5′ seq	*rosy* vector 2270 bp of 5′ seq
*Tf(2)GA2.4-1	*Tf(2)GX2.4-1	Tf(3)GLX1.34-1	Tf(3)GLX3.3-1
*Tf(2)GA2.4-2	*Tf(3)GX2.4-3	Tf(3)GLX1.34-2	Tf(2)GLX3.3-3
*Tf(2)GA2.4-4	Tf(2)GX2.4-4	Tf(2)GLX1.34-3	Tf(2)GLX3.3-4
Tf(2)GA2.4-5	Tf(1)GX2.4-5		
	Tf(2)GX2.4-6		
	*Tf(2)GX2.4-7		

(B) Quantitation of RNA levels from the introduced genes

Tf(2)GA2.4-1	11.1% ± 1.5	$n = 5$
Tf(2)GA2.4-2	7.9% ± 0.9	$n = 3$
Tf(2)GA2.4-4	8.1% ± 1.4	$n = 3$
Tf(2)GA2.4-5	2.5% ± 0.7	$n = 7$
Tf(1)GX2.4-5	9.3% ± 1.2	$n = 3$
Tf(2)GX2.4-7	7.5% ± 2.1	$n = 3$

not stain at all. The interpretation is that the sgs-3–lacZ fusion protein behaves in a manner very similar to the native protein but probably loses its enzymatic activity as it is cross-linked with other components of the glue. The expression of the fusion protein in the pGLX1.34 transformants was examined in an identical way and no aberrant expression was detected.

Regulation by a trans-regulatory factor

The wild-type product of the X-chromosomal locus *l(1)npr-1* is required in *trans* for the accumulation of RNA from the *Sgs-3, Sgs-7* and *Sgs-8* genes (Crowley *et al.*, 1984). In the presence of the amorphic *l(1)npr-1* mutation, no RNA from these genes can be detected. A transformed line containing an *Sgs-3* gene with 2270 bp of 5′ *Sgs-3* sequence, and hemizygous for the *l(1)npr-1* mutation, shows no accumulation of RNA from the introduced gene. This indicates that the *trans*-acting regulatory factor coded by the *l(1)npr-1* gene interacts, directly or indirectly, with sequences 2270 nucleotides upstream of, within, or just downstream of the *Sgs-3* gene (Crowley *et al.*, 1984). To further delimit the *cis*-regulatory sequences with which this *trans*-acting product might act, the *l(1)npr-1* mutation was crossed into transformant lines containing the pGA2.4 and pGX2.4 constructs. Males of the transformant lines were crossed to females of the genotype *y l(1)npr-1 w mal / Binsn; Sgs-3*^{For} *ry*^{42}, and expression of the introduced *Sgs-3* gene was monitored by RNA blot hybridization in *l(1)npr-1* and *Binsn* male progeny. In each case the introduced gene (as well as the endogenous genes) was expressed in the control *Binsn* male larvae, but not in the *l(1)npr-1* male larvae. Thus, the low-level expression of the *Sgs-3* gene in the transformed lines requires the same *trans*-acting regulatory factor required by the endogenous gene, and the sequences that interact with the regulatory factor are shown to be very near, or within, the *Sgs-3* gene. Table I shows which of the transformants were analyzed in the *l(1)npr-1* background.

Discussion

We have shown that the *Sgs-3* gene with 130 bp of sequence upstream of the mRNA transcription start site is expressed in a developmentally regulated manner, qualitatively similar to the native gene, but at lower levels. This low-level expression depends on the same *trans*-acting regulator required by the normal gene. Our conclusions were drawn from testing expression in three different ways: an *Sgs-3* gene in a vector with alcohol dehydrogenase as the selectable marker, an *Sgs-3* gene in a vector with xanthine dehydrogenase as the selectable marker, both tested by RNA blot hybridization, and an *Sgs-3* gene fused in frame with *E. coli* β-galactosidase assayed histochemically. Our results contradict those reported by Bourouis and Richards (1985) with one vector, assayed by RNA blot hybridization. They were unable to consistently detect expression from *Sgs-3* genes that had upstream regions equivalent to those described here, and concluded that this 5′ DNA is insufficient for detectable expression. Thus they argue for a controlling element that acts over long distances to allow even low levels of expression. We believe that their failure to detect expression, which we measure as from 2.5 to 11.1% of normal, may be due to two reasons. First, they use RNA from whole larvae instead of from isolated salivary glands, which represent at most a few percent of larval tissue. Second, in their assay system RNA from the endogenous genes migrates more slowly than that from the introduced genes on RNA gels. This allows for the possibility that even slight degradation of the abundant RNA derived from the endogenous gene could obscure the low-level signal from the introduced gene. In our experiments the RNA from the introduced genes is larger than that transcribed from the endogenous genes.

By comparing the sequences upstream of *Sgs-4* with those flanking the 68C glue genes, Shermoen and Beckendorf (1982), suggested that sequences at −420 bp from *Sgs-3* could be necessary for its regulation. A similar analysis led Hoffman and Corces (1986) to suggest that sequences at −300 bp could have a role in regulation. Finally, Ramain *et al.* (1986) examined the DNase I hypersensitive sites upstream of *Sgs-3* and suggested that the ones at −600 bp and the one at −750 bp could be critical for developmental regulation. Our observations from three different constructions with 130 bp of upstream sequence, using two different expression assays, shows that the removal of sequences between −130 and −2270 bp does not result in a loss of correct tissue- or developmental stage-specific expression. Therefore, while tissue- and stage-specific elements may be present in the further upstream regions, their presence is not essential for proper tissue and stage control of *Sgs-3* expression.

Materials and methods

Transformed strains

P-element transformation experiments followed standard methods (Spradling and Rubin, 1982; Rubin and Spradling, 1982) and are described in detail separately (Crosby and Meyerowitz, 1986). In the case of the pGA2.4 plasmid, the host strain for the injections was *Adh*^{fn23} *pr cn; Sgs-3*^{HR} which carries the Hikone-R (HR) size variants for the *Sgs-3* mRNA [1000 nucleotides (nt) and 950 nt long prior to polyadenylation, there being two genes present as a duplication (Crosby and Meyerowitz, 1986)]. The *Sgs-3* gene in pGA2.4 is the Oregon-R (OR) size variant (1120 nt, Garfinkel *et al.*, 1983). The strong signal seen in RNA blots due to the message from the endogenous Hikone-R genes obscured low levels of signal from the RNA made from the introduced gene. This problem was solved by introducing the chromosome containing the transformed DNA into a strain which carries the Formosa (For) size variant (820 nt, Mettling *et al.*, 1985) for

Sgs-3 which is smaller than the Hikone-R variant and in which the message from the introduced and the endogenous genes are clearly resolved after agarose gel electrophoresis. pGA2.4 transformants were isolated by selecting for the ability of progeny of injected animals to survive in the presence of ethanol. The ethanol selections were as described (Crosby and Meyerowitz, 1986) following published methods (Goldberg *et al.*, 1983). pGX2.4 transformants were isolated by screening for ry^+ flies among the progeny of injected animals. The host strain for injections was ry^{42}. This strain carried an Oregon-R size variant for *Sgs-3*. After transformants were obtained the $Sgs\text{-}3^{OR}$ ry^{42} chromosome was replaced by the $Sgs\text{-}3^{For}$ ry^{42} chromosome by appropriate crosses. The pGLX3.3 and pGLX1.34 transformants were also isolated by screening for ry^+ animals; the host strain for injections and subsequent crosses were ry^{42} for the pGLX3.3 plasmid and ry^{506} for pGLX1.34. Several lines from each transformant were set up and the insertion mapped to specific chromosomes. Using appropriate balancer chromosomes the transformants were made homozygous or, in the case of homozygous lethal insertions, maintained over a balancer. *In situ* hybridization to the DNA on polytene chromosomes using a probe homologous to the introduced gene localized the insertion event and showed the absence of multiple insertions. Mapping of the insertion event and *in situ* chromosomal localization were the criteria used to determine that transformation was due to a single event. In addition, Southern blot hybridization (Southern, 1975) on the pGA2.4 transformants confirmed that only one insertion was present in each line.

RNA gel blots

RNA extraction, electrophoresis, blotting and probing were all done under standard conditions as described (Crosby and Meyerowitz, 1986). Probes were removed from filters for rehybridization by two 5-min washes in boiling 0.01 × SSPE, 0.1% SDS.

β-Galactosidase staining

Salivary glands and other tissues from transformants were stained for *E. coli* β-galactosidase activity using a modification of the method as described by Singh and Knox (1984). Animals were dissected in 10 mM phosphate buffer pH 8.0 and immersed in a drop of the staining solution (0.060 ml 5% X-gal; 0.020 ml 100 mM potassium ferrocyanide; 0.020 ml 100 mM potassium ferricyanide; 0.050 ml 1.0 M sodium phosphate pH 8.0 and 0.850 ml 35% Ficoll-400). Preparations were kept in a humid chamber to prevent the solution from drying and were usually analyzed after overnight staining.

Analysis of RNA in l(1)npr-1 mutants

Transformant males were crossed to females that were of the genotype *y l(1)npr-1 w mal/Binsn; Sgs-3*^{For} ry^{42}. Male larvae were identifiable on the basis of the larval phenotypic markers on the *l(1)npr-1*-bearing chromosome. Salivary gland RNA was examined on RNA blots from both mutant and control animals. The markers used have been described (Lindsley and Grell, 1968).

Quantitation of RNA levels

RNA isolated from mid-third instar larval salivary glands was fractionated on an agarose formaldehyde gel, blotted onto nitrocellulose and hybridized with a ^{32}P-labelled probe. After obtaining an autoradiogram to determine the positions of the RNA species, the corresponding positions of the nitrocellulose were cut out, submerged in Liquifluor scintillation fluid (NEN) diluted 15:1 with toluene and the number of counts per minute on each fragment of the filter determined in a scintillation counter. All pieces of nitrocellulose were of the same size. The level of background in each lane was determined by cutting out a piece of nitrocellulose of equal size from just above the largest RNA species. Multiple measurements were made for each transformant. Relative levels of expression are indicated as the mean ± the standard error of the mean. Transformants which have 2270 bp of sequence upstream of the mRNA transcription start site express high levels of RNA (71–86% of the endogenous gene) and have been described (Crosby and Meyerowitz, 1986).

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Appendix II:

Studies on the Regulatory Interaction Between the *Sgs-3* Glue Gene and the Product(s) of the *Broad-Complex* of *Drosophila*

In previous studies (Crowley et al. 1984 and Vijay Raghavan et al. 1986), we have analyzed the functional capacity of various deletion constructions of the *Sgs-3* promoter region after P-element transformation. Specifically important for this discussion was the analysis of two constructions in the background of a *trans*-acting mutation, *npr1*^{3} (also known as *l(1)npr-1*; Kiss et al. 1978). The *npr1*^{3} mutation affects the expression of all three 68C glue genes, causing a failure of RNA accumulation to levels detectable by standard assays. In addition, the *npr1*^{3} mutation inhibits the accumulation of RNAs from the two transformed genes, one carrying approximately 2270 basepairs (bp) of sequence upstream from the *Sgs-3* RNA start site (GA6.0 construction; Crowley et al. 1984), while the other contains only 130 bp upstream and is fused within the *Sgs-3* coding region to the *E. coli LacZ* gene (Vijay Raghavan et al. 1986). These findings show that the normal product(s) of the *Broad-Complex* (*BR-C*), where the *npr1*^{3} mutation occurs, is required to interact in some fashion, directly or indirectly, with sequences between 130 bp upstream of *Sgs-3* and the fusion point at position +948 bp in order to allow proper RNA accumulation to occur. Unfortunately, these results do not allow us to distinguish between transcriptional activation and post-transcriptional modification (e.g., RNA processing) as a means for *BR-C* regulation at *Sgs-3*.

In an effort to further delimit the sequences involved in the interaction between the *BR-C* and *Sgs-3* and to differentiate between the possible modes for *npr1*^{3} regulation of *Sgs-3*, I analyzed additional transformed lines utilizing both available and newly created gene-fusion constructions. The first such construction involves a gene fusion between the *Sgs-3* promoter and the *Drosophila* alcohol dehydrogenase (*Adh*) coding region. The fusion gene contains 2760 bp upstream of the *Sgs-3* RNA start site and 12 bp of 5'-untranslated sequence, and is joined to *Adh* at +10 from its start site (Figure 1). Transformants from injection of this construction, *Tf()GOAX2.76's* (Roark et al., submitted), were crossed to females of the genotype *y npr1*^{3}*/Binsn*, and male larvae were assayed for the ability to synthesize Adh in the salivary glands using a histochemical stain

(Roark et al., submitted). The results are presented in Figure 2. Only larvae carrying the *npr1*^{3} mutation, distinguished by their brown mouthhooks due to the *yellow* marker, failed to express Adh in the salivary glands to any detectable level. This signifies that sequences upstream of +12 in the *Sgs-3* gene are responsible for the regulatory control of *Sgs-3* expression by the *BR-C*. Taken together with the findings of Vijay Raghavan et al. (1986), this region of *BR-C* interaction should lie between -130 and +12 of the *Sgs-3* gene, in the proximal promoter region sufficient for spatial and temporal specificity (Vijay Raghavan et al. 1986).

This unfortunately does not distinguish between the two potential modes of regulation discussed earlier for the *BR-C* at 68C because we do not whether *BR-C* expression is required for stability of *Adh* transcripts. In an effort to make this distinction, fusions between *Sgs-3* and *Sgs-4* were produced. *Sgs-4* RNA accumulates in salivary glands of *npr1*^{3} mutant larvae, so this transcript does not require *BR-C* expression. This shows that the *Sgs-4* promoter is active in the *npr1*^{3} mutant background, but at reduced levels (Crowley et al. 1984), making it a good control promoter since the expression patterns closely mimic those of *Sgs-3*. Fortuitously, the *Sgs-4* gene has an EcoRI restriction site in the region of its RNA start site. In order to create these fusion genes, an EcoRI site was introduced into the RNA start site of the *Sgs-3* gene by oligonucleotide-mediated site-directed mutagenesis (Newman et al. 1983). The resulting M13mp10 plasmid (muDm7004) was sequenced by the standard dideoxy-chain termination method and determined to have the anticipated changes (see Figure 3). The SalI-XhoI fragment of muDm7004 was ligated to the BglII-SalI fragment upstream of *Sgs-3* (aDm7006) and eventually inserted into the Carnegie 20 (Car 20) vector (Rubin and Spradling 1983) for germ line transformation. The resulting construction is designated 3/3, having both upstream and downstream sequences from *Sgs-3*, but differing from the wild-type gene in the mutated bases at the RNA start site (Figure 2). The EcoRI-XhoI fragment from muDm7004 was added to the BamHI-EcoRI site of the *Sgs-4* promoter region, and this

fusion also inserted into Car 20. This construction is designated 4/3, having *Sgs-4* upstream sequence and the *Sgs-3* transcription unit (Figure 2). Finally, the BglII-EcoRI fragment from aDm7006 was ligated to the 1600 bp EcoRI fragment of *Sgs-4* and inserted into Car 20 to create the 3/4 fusion (Figure 2). A transformant of the *Sgs-4* region (t1.9A1; McNabb and Beckendorf 1986) was kindly provided by Dr. Steve Beckendorf (UC-Berkeley) as a control, and is called 4/4 here for consistency (Figure 2). The structure of the RNA start sites for these various constructions is presented in Figure 3.

Injection of each of these DNA fragments into pre-blastoderm embryos (using the phsπ helper plasmid) resulted in germ line transformants showing *rosy*$^+$ phenotypes, signifying xanthine dehydrogenase activity resulting from insertion of the Car 20-based transformation vectors. After preliminary analysis on multiple insertions of each construction, two homozygous viable lines for each fusion (except the 4/4 line) were crossed into the genotype, *y npr1*^{3} *Sgs-4*^{BER}*/Binsn; Sgs-3*^{For} *ry*^{42}, using appropriate balancer stocks. The *Sgs-4*^{BER} allele has extremely low levels of *Sgs-4* RNA and the *Sgs-3*^{For} allele has a ~200 bp deletion in the *Sgs-3* RNA, providing a size variant to distinguish between it and the full-sized Oregon-R allele used for transformation. Expression patterns were analyzed in salivary glands from both the mutant *npr1*^{3} larvae and *Binsn* male siblings as wild-type controls. Examples of these results are presented in Figures 4 and 5. The *Binsn* control lanes, containing the RNA from one salivary gland pair, show expression of each of the four constructions in transformed lines. Significantly, the expression of the Oregon-R allele of *Sgs-3*, which is 1120 nucleotides in length, is reduced in both the 3/3 and 4/3 transformants (Figure 4). The 3/3 construction is expressed at approximately 12% that of the endogenous Formosa allele, as measured by scintillation counting of bands from a filter blot (results not shown). Obviously, perturbation of the normal RNA start site in the 3/3 fusion affects the levels of RNA accumulation, presumably because of a change in either transcription initiation or RNA stability. The exchange of the *Sgs-3* promoter for the *Sgs-4* promoter in the 4/3

construction results in extremely low levels of accumulation, so low that proper quantitation was not obtained. Since *Sgs-4* is thought to have a weaker promoter strength than the 68C glue genes (unpublished observations), the reduction in the 4/3 lines could be caused by lower initiation frequency, and could be compounded by any RNA instability, proposed for the 3/3 reduction. The 3/4 constructions appear to be expressed at abundant levels (Figure 5).

The reduced expression of the 3/3 and 4/3 constructions in wild-type glands created problems when these transformants were analyzed in the *npr1*^{3} mutant background. As discussed earlier (and Crowley et al. 1984), the *Sgs-3* promoter produces no detectable transcript in *npr1*^{3} mutant larval salivary glands, and the *Sgs-4* transcripts accumulate to approximately 7% that of wild-type glands. Therefore, in order to increase any possible signals, poly-A^{+} RNA from 100-200 salivary gland pairs was collected and analyzed by RNA gel blot. In order to increase the sensitivity of these blots, single-stranded ^{32}P-labeled RNA probes of the *Sgs-3* or *Sgs-4* transcription units were utilized. The results are found in Figures 4 and 5. With *Sgs-3* probe, two weak bands appear in the 4/3 lane (*Tf(3)GX4/3-1*), with the lower band corresponding in length to the transformed Oregon-R sized allele (Figure 4). Significantly, no other bands appear in any of the other transformed constructions, nor in the non-transformed control. Despite successive probing of this blot with sequences upstream and downstream from the 4/3 transcription unit, the larger band in the *npr1*^{3}*;GX4/3-1* lane is not explained. It is not found at abundant levels in the *Binsn;GX4/3-1* lane, however.

Probing a similar gel blot with RNA probe for *Sgs-4* produced no transcript levels detectable above the background levels of the non-transformed parental stock. The parental stock carries the *Sgs-4*^{BER} allele, which expresses *Sgs-4* at extremely low levels because of a deletion in the upstream regulatory domain (Muskavitch and Hogness 1982). As shown in Figure 5, the levels of *Sgs-4* RNA accumulation are at equal or lower levels in both of the constructions tested to that of the transformed 4/3 line used as a control. These

levels are similar to those found for the non-transformed BER-1 wild-type stock (data not shown). The 4/4 construction expressed at levels consistent with a normal *Sgs-4* allele in the *npr1*^{3} mutant background (data not shown). Expression in the *npr1*^{3} mutant background for each of the constructions tested is summarized in Figure 2.

Taken together with the inability to detect expression from the 3/4 construction in salivary glands from *npr1*^{3} mutant larvae, the expression of RNA consistent in size with the transformed Oregon-R allele in the *npr1*^{3}*;GX4/3* lines establishes two significant points. First, the ability of the 4/3 construction to function in the *npr1*^{3} mutant background exhibits that no block to RNA accumulation occurs within the *Sgs-3* transcription unit. This suggests that the *BR-C* is not regulating *Sgs-3* expression post-transcriptionally, although this cannot be ruled out entirely since the perturbation of the RNA start site does create a slightly different transcript. More clear cut is the failure to detect transcription from the 3/4 construction in the *npr1*^{3} background above the levels of the parental stock. This demonstrates that sequences upstream of the RNA start site at *Sgs-3* are required for proper *BR-C* regulation, since the *Sgs-4* RNA is known to be able to accumulate in an *npr1*^{3} background. Together with the findings of Vijay Raghavan et al. (1986), this region is further delimited to between position -130 and the start site at +1. Since the proximal promoter element has since been shown to fall between -98 and -56 (Martin et al. 1989; Roark et al., submitted), we presume the *BR-C* exerts its regulatory influence within this region. Indeed, we have found altered protein:DNA interactions in this region in salivary glands from *npr1*^{3} mutant larvae upon in vivo DMS-footprinting analysis (Mathers and Meyerowitz, submitted and see Chapter 3). Interactions between the *BR-C* and the *Sgs-3* proximal promoter region could be direct, through DNA binding and transcription initiation, or indirect, possibly via protein-protein interaction or a regulatory cascade. The resolution of this interaction awaits the further characterization of the *BR-C* and its elaborate genetic and molecular structure.

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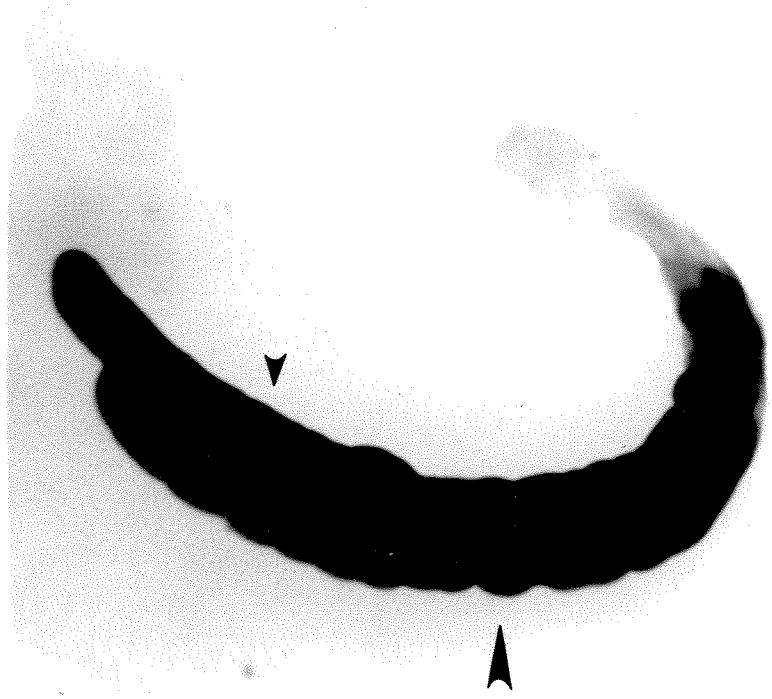
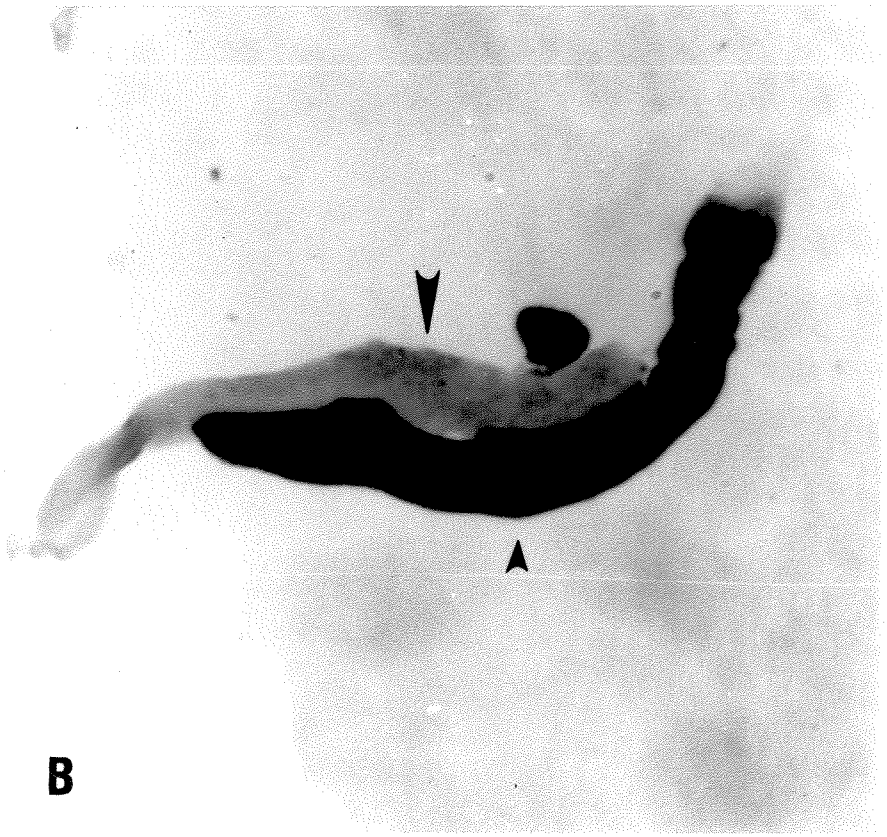
Figures

Figure 1. Transformed Adh enzyme activity in the salivary gland. (A) Expression of Adh activity in the salivary gland from a larva hemizygous for the ***Binsn*** X chromosome and heterozygous for *Tf(3)GOAX2.76-5*. Activity was histochemically assayed by the procedure set forth in Roark et al. (submitted). Both salivary gland (large arrowhead) and fat body (small arrowhead) expression are seen, the salivary gland expression resulting from the transformed construction and the fat body expression the result of endogenous activity introduced by the *npr1*^{3}*/Binsn* stock. (B) Salivary gland from a larva hemizygous for the *npr1*^{3} mutation and heterozygous for the *Tf(3)GOAX2.76-5* transformation. Adh enzyme activity is observed only in fat body cells (small arrowhead), which is the result of endogenous expression. Analysis was as in (A).



A



B

Figure 2. Schematic diagram of transformation inserts and summary of their expression in the *npr1*^{3} mutant background. Presented are the insertion fragments without the Carnegie 20 transformation vector, with fragment lengths in kilobases (except junction points which are in basepairs and are in parentheses) and landmark restriction sites noted. Sequences from the *Sgs-3* region are shaded, while those from *Sgs-4* are hatched and from *Adh* are hollow white. The type and location of the transcript expected from each is shown by a labeled arrow. Expression patterns in the *npr1*^{3} mutant larval salivary gland as judged by RNA gel blot or histochemical assay are given at the right. The GX2.4 construction has been previously published (Vijay Raghavan et al. 1986) and is presented here for reference.

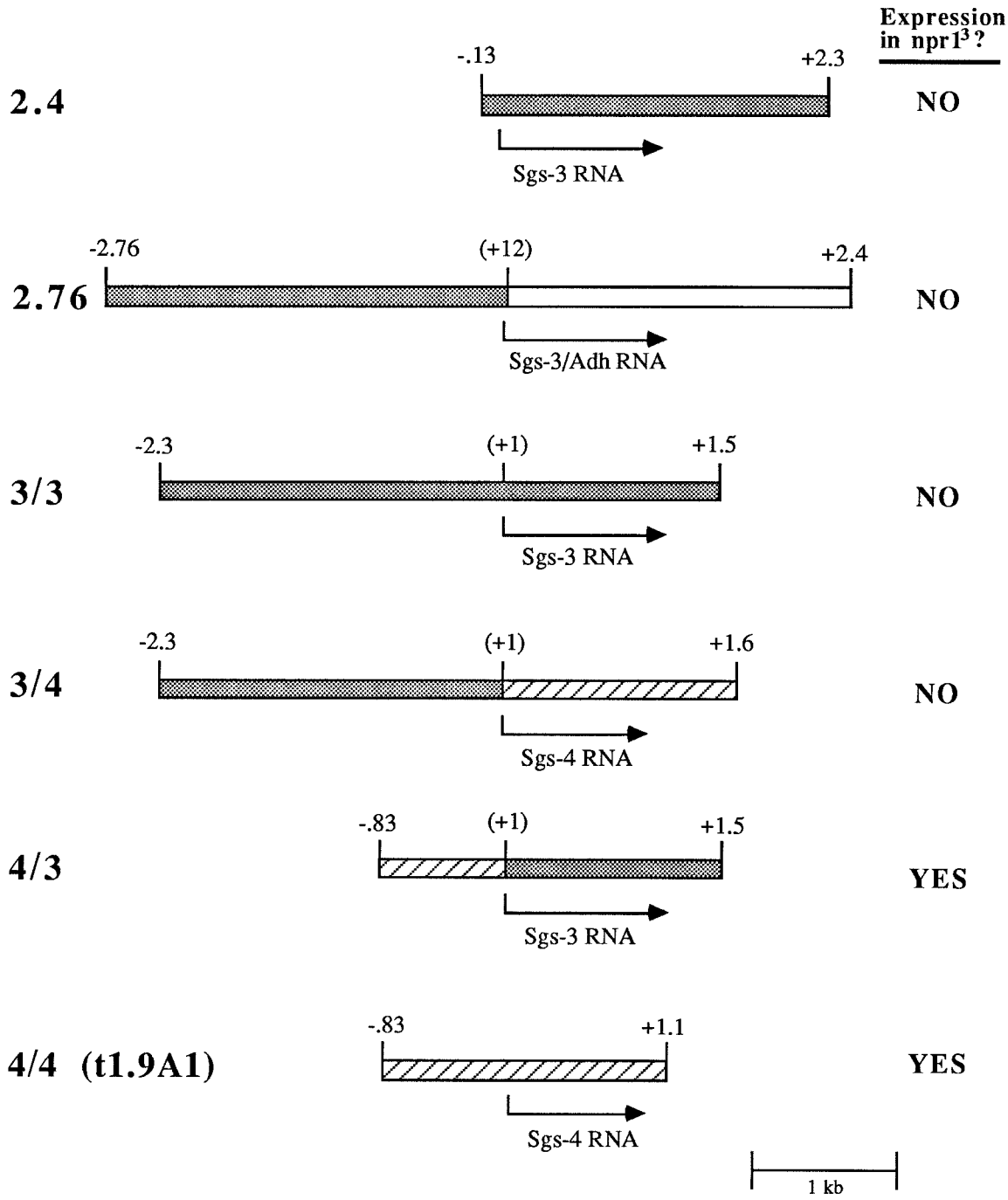

Expression in npr1[3]?
2.4
-.13
+2.3
NO
Sgs-3 RNA
2.76
-2.76
(+12)
+2.4
NO
Sgs-3/Adh RNA
3/3
-2.3
(+1)
+1.5
NO
Sgs-3 RNA
3/4
-2.3
(+1)
+1.6
NO
Sgs-4 RNA
4/3
-.83
(+1)
+1.5
YES
Sgs-3 RNA
4/4 (t1.9A1)
-.83
+1.1
YES
Sgs-4 RNA
1 kb

Figure 3. The transcription start sites and surrounding sequences from the naturally occurring promoters of *Sgs-3* and *Sgs-4*, as well as from the mutant 3/3 and gene-fusion 3/4 and 4/3 promoters. Known start sites for *Sgs-3* and *Sgs-4* are denoted by arrows. The EcoRI restriction sites which act as fusion junctions are bracketed.

Transcription Start Sites:

Figure 4. An RNA gel blot of fusion constructions probed with an *Sgs-3* RNA probe. The poly-A^+ RNA from 200 salivary glands (*npr1*^{3} mutant lanes) or total nucleic acids from one salivary gland pair (wild-type lanes) was separated by electrophoresis and transferred to nylon membrane. The blot was hybridized with ^{32}P-labeled RNA complementary to the *Sgs-3* mRNA and produced from the plasmid gDm7003 in vitro. The endogenous 900 nucleotide Formosa allele and the transformed 1120 nucleotide Oregon-R allele are noted. The npr BER signifies the *y npr1*^{3} *Sgs-4*^{BER} chromosome, while npr signifies the parental *y npr1*^{3} *w mal* chromosome. *Binsn* is the balancer chromosome in the *npr1*^{3} stock and BER is the X-chromosome from the BER-1 wild-type stock. The Oregon/Formosa lane represents one salivary gland pair from a cross between these two wild-type stocks.

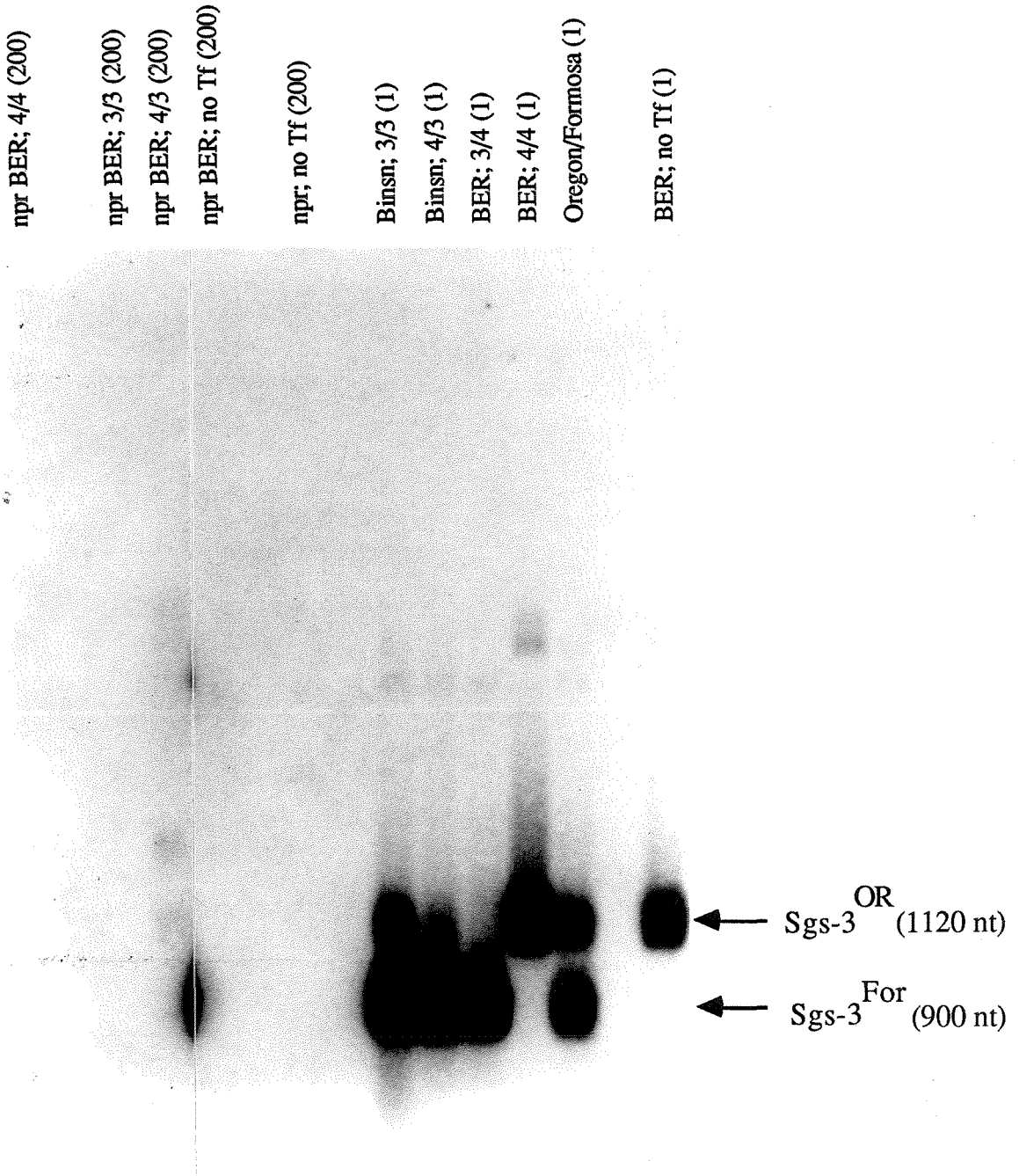
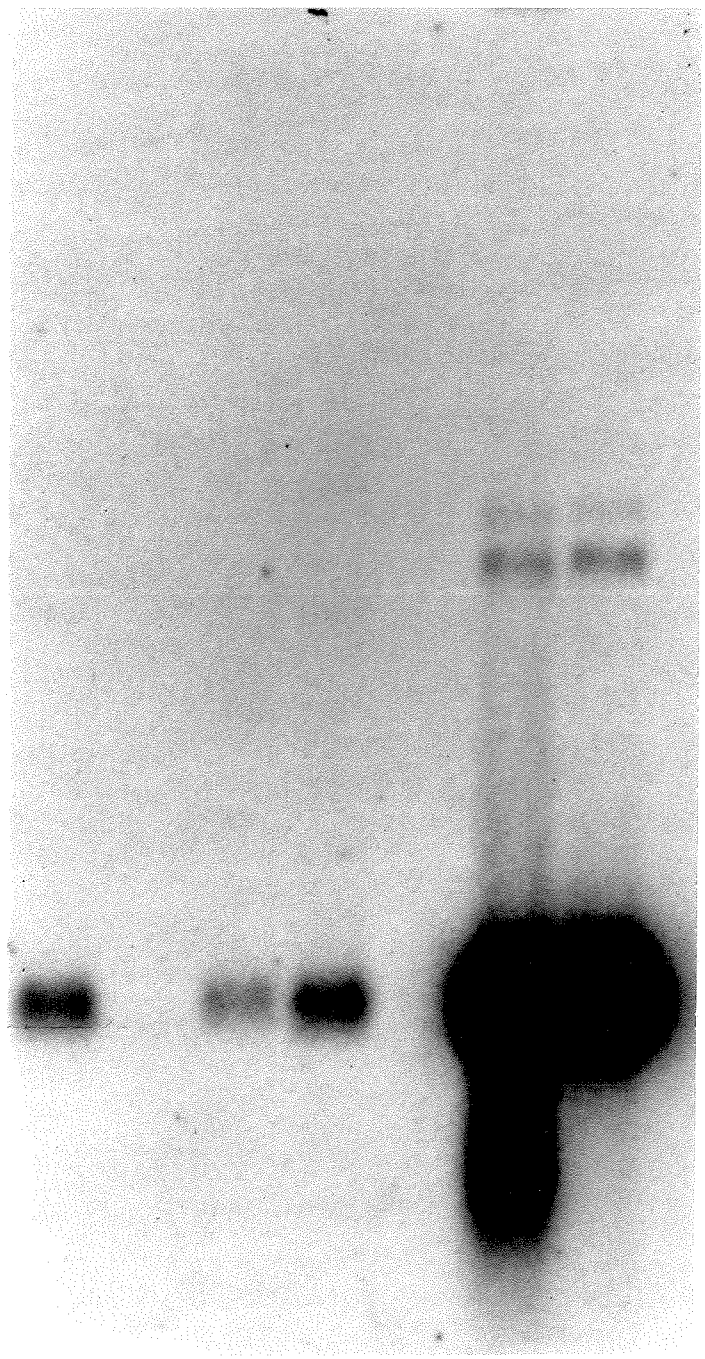

npr BER; 4/4 (200)
npr BER; 3/3 (200)
npr BER; 4/3 (200)
npr BER; no Tf (200)
npr; no Tf (200)
Binsn; 3/3 (1)
Binsn; 4/3 (1)
BER; 3/4 (1)
BER; 4/4 (1)
Oregon/Formosa (1)
BER; no Tf (1)
Sgs-3 OR (1120 nt)
Sgs-3 For (900 nt)

Figure 5. An RNA gel blot of fusion constructions probed with an *Sgs-4* RNA probe. The poly-A^+ RNA from 100 salivary glands (*npr1*^{3} mutant lanes) or total nucleic acids from one salivary gland pair (wild-type lanes) was separated and blotted as in Figure 4. Probe, however, was complementary to the *Sgs-4* mRNA and was synthesized in vitro from the hDm7012 plasmid. Abbreviations are those used in Figure 4.


npr BER; 4/3 (100)
npr BER; 3/4-1 (100)
npr BER; 3/4-2 (100)
Binsn; 4/3 (1)
BER; 3/4-2 (1)
Sgs-4