The EMBO Journal vol.5 no.12 pp.3321–3326, 1986

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

K.Vijay Raghavan, Madeline A.Crosby[1], Peter H.Mathers and Elliot M.Meyerowitz

Divison of Biology, California Institute of Technology, Pasadena, CA 91125, USA

[1]Present address: Department of Genetics, Cambridge University, Downing Street, Cambridge CB2 3EH, UK

Communicated by F.C.Kafatos

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)

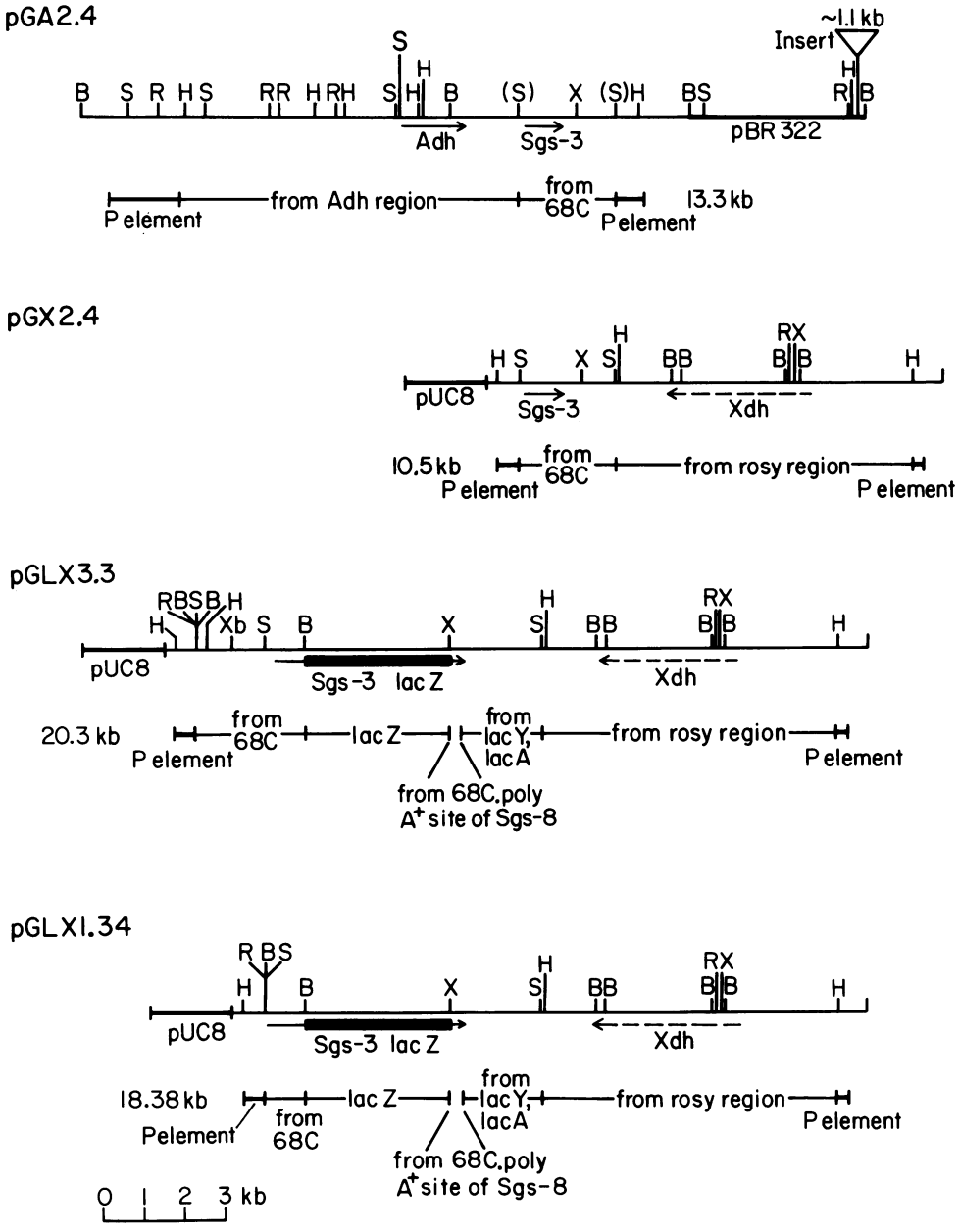

Fig. 1. Restriction maps of the four plasmids used in the transformation experiments discussed in the text. The nomenclature used for the plasmids is: G for glue, A for alcohol dehydrogenase (*Adh*), L for *lacZ* and X for xanthine dehydrogenase (*Xdh*). The number in the plasmid name denotes the amount, 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.

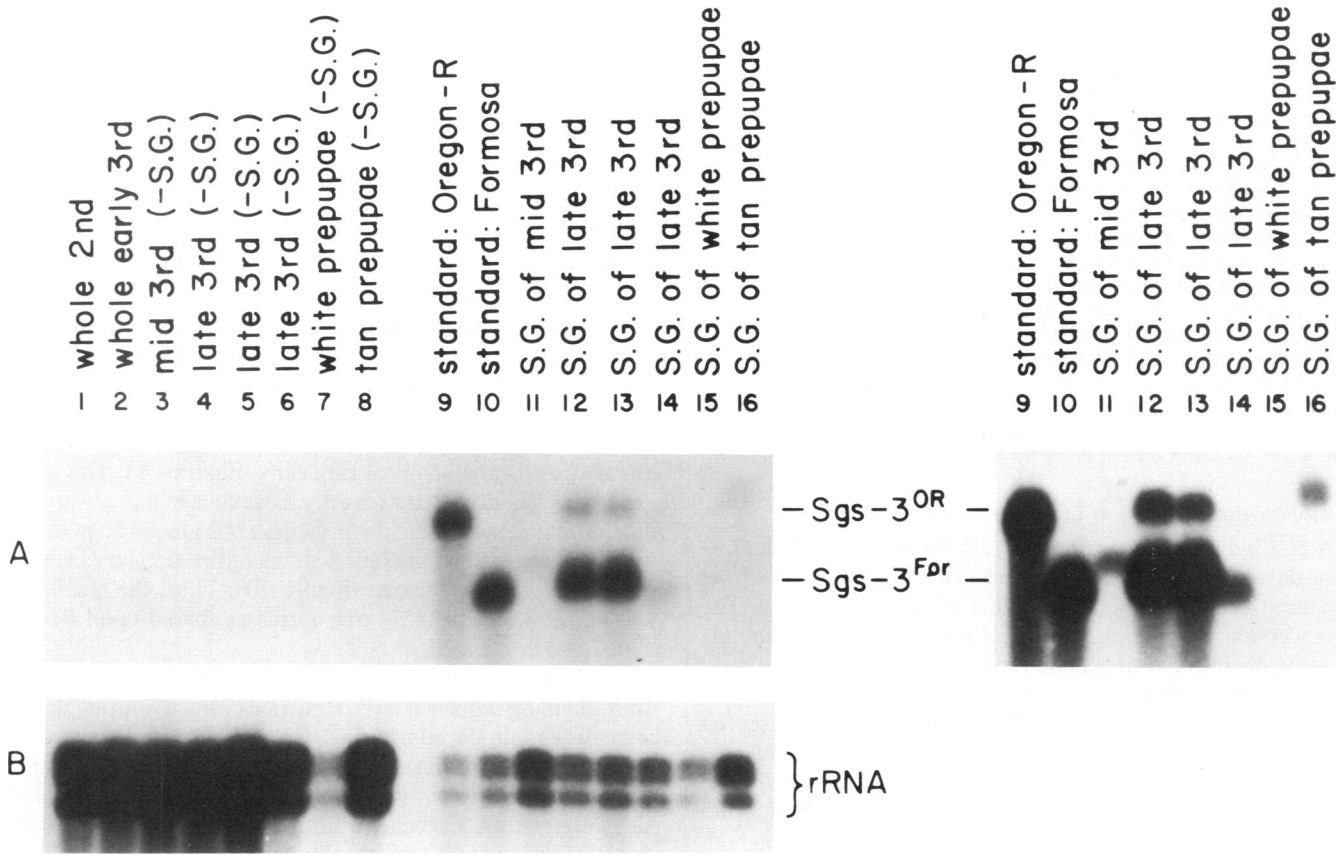

Fig. 2. RNA blot analysis of Tf(2)GA2.4-1 transformants at different developmental stages. RNA isolated from the salivary glands and carcasses, after removal of salivary glands, of homozygous Tf(2)GX2.4-1 animals was fractionated by size on a 1.5% agarose formaldehyde gel. RNA from two animals was used 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.

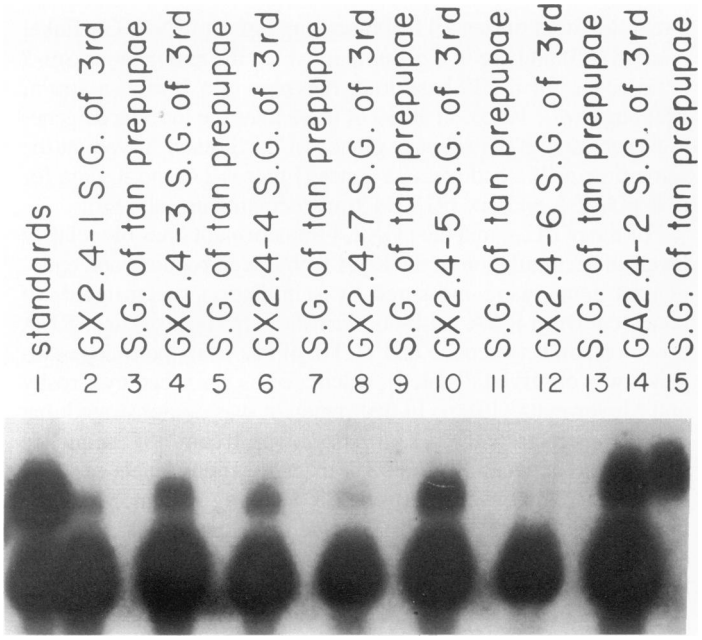

Fig. 3. RNA blots of GX2.4 transformant lines. The figure shows that the several independent transformants express the introduced *Sgs-3* gene correctly and that there is no aberrant expression at the tan pre-pupal stage as seen for the GA2.4 transformants. Total salivary gland RNA from two animals was 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).

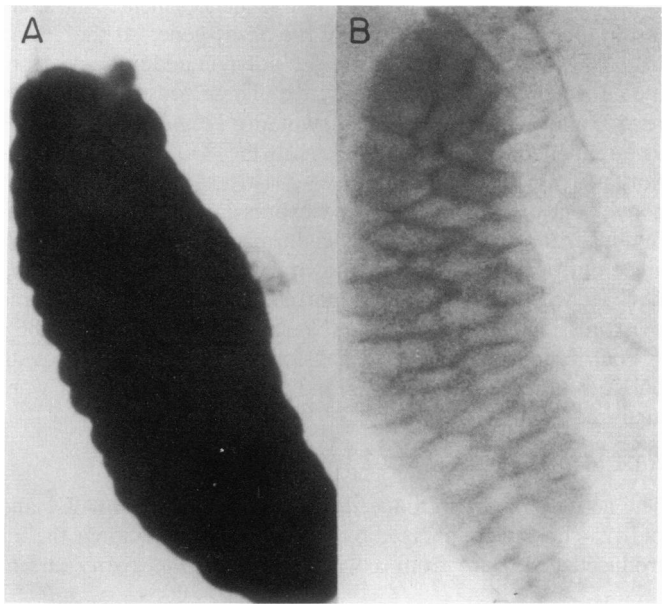

Fig. 4. β-Galactosidase activity in transformants carrying a *Sgs-3–lacZ* fusion gene. (**A**) Salivary gland lobe from Tf(3)GLX3.3-1 transformant (climbing third instar larva). The staining is a dark blue in ~5 h, though the preparations were usually examined after staining overnight. (**B**) In contrast, 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;* $Sgs\text{-}3^{For}$ 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).

Acknowledgements

We thank Liz Noell, Rita Mundy and Carol Mayeda for expert technical assistance and Mark Garfinkel for pGX2.4 DNA. Helpful comments from Karl Fryxell, Mark Garfinkel, Chris Martin and Margo Roark are acknowledged. This work was supported by grant GM28075 from the National Institutes of Health to E.M.M. M.A.C. and P.H.M. were supported by NIH grant 1 T32 GM07616-08 and K.V.R. was the recipient of a fellowship from Procter and Gamble.

References

Akam,M.E., Roberts,D.B., Richards,G.P. and Ashburner,M. (1978) *Cell*, **13**, 215–225.
Ashburner,M. (1973) *Dev. Biol.*, **34**, 47–61.
Ashburner,M. (1974) *Dev. Biol.*, **39**, 141–157.
Ashburner,M. and Richards,G. (1976) *Dev. Biol.*, **54**, 241–255.
Beckendorf,S.K. and Kafatos,F.C. (1976) *Cell*, **9**, 365–373.
Becker,J.H. (1962) *Chromosoma*, **13**, 341-384.
Bourouis,M. and Richards,G. (1985) *Cell*, **40**, 349–357.
Casadaban,M.J., Chou,J. and Cohen,S.N. (1981) *J. Bacteriol.*, **143**, 971–980.
Crosby,M.A. and Meyerowitz,E.M. (1986) *Dev. Biol.*, in press.
Crowley,T.E. and Meyerowitz,E.M. (1983) *Dev. Biol.*, **102**, 110–121.
Crowley,T.E., Bond,M.W. and Meyerowitz,E.M. (1983) *Mol. Cell. Biol.*, **3**, 623–634.
Crowley,T.E., Mathers,P.H. and Meyerowitz,E.M. (1984) *Cell*, **39**, 149–156.
Dworniczak,B., Siedel,R. and Pongs,O. (1983) *EMBO J.*, **2**, 1323–1330.
Fraenkel,G. and Brookes,V.J. (1953) *Biol. Bull.*, **105**, 442–449.
Garfinkel,M.D., Pruitt,R.E. and Meyerowitz,E.M. (1983) *J. Mol. Biol.*, **168**, 765–789.
Goldberg,D.A., Posakony,J.W. and Maniatis,T. (1983) *Cell*, **34**, 59–73.
Gronemeyer,H. and Pongs,O. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 2108–2112.
Guild,G.M. and Shore,E.M. (1984) *J. Mol. Biol.*, **179**,, 289–314.
Hansson,L. and Lambertsson,A. (1983) *Mol. Gen. Genet.*, **192**, 395–401.
Hoffman,E. and Corces,V. (1986) *Mol. Cell. Biol.*, **6**, 663–673.
Kiss,I., Szabad,J. and Major,J. (1978) *Mol. Gen. Genet.*, **164**, 77–83.
Korge,G. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4550–4554.
Korge,G. (1977a) *Dev. Biol.*, **58**, 339–355.
Korge,G. (1977b) *Chromosoma*, **62**, 155–174.
Lindsley,D.L. and Grell,E.H. (1968) *Genetic Variations of Drosophila melanogaster*. Carnegie Inst. of Washington Publication No. 627.
Mettling,C., Bourouis,M. and Richards,G. (1985) *Mol. Gen. Genet.*, **201**, 256–268.
Meyerowitz,E.M. and Hogness,D.S. (1982) *Cell*, **28**, 165–176.
Meyerowitz,E.M., Crosby,M.A., Garfinkel,M.D., Martin,C.M., Mathers,P.M. and Vijay Raghavan,K. (1985) *Cold Spring Harbor Symp. Quant. Biol.*, **50**, 347–353.
Muskavitch,M.A.T. and Hogness,D.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7362–7366.
Ramain,P., Bourouis,M., Dretzen,G., Richards,G., Sobkowiak,A. and Bellard,M. (1986) *Cell*, **45**, 545–553.
Richards,G., Cassab,A., Bourouis,M., Jarry,B. and Dissous,C. (1983) *EMBO J.*, **2**, 2137–2142.
Rubin,G.M. and Spradling,A.C. (1982) *Science*, **218**, 348–353.
Shermoen,A.W. and Beckendorf,S.K. (1982) *Cell*, **29**, 601–607.
Singh,M.B. and Knox,R.B. (1984) *Histochem. J.*, **16**, 1273–1296.
Spradling,A.C. and Rubin,G.M. (1982) *Science*, **218**, 341–347.
Spradling,A.C. and Rubin,G.M. (1983) *Cell*, **34**, 47–57.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Velissariou,V. and Ashburner,M. (1980) *Chromosoma*, **77**, 13–27.
Velissariou,V. and Ashburner,M. (1981) *Chromosoma*, **84**, 173–185.

Received on 2 September 1986