The action of the *l(1)npr-1*$^+$ locus on the *Drosophila* glue gene *Sgs-3* is cell-autonomous

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Abstract. The 2B5 chromosomal locus in *Drosophila* contains a gene, *l(1)npr-1*$^+$, whose product required in *trans* for the expression of the larval salivary gland-specific gene *Sgs-3*. We have addressed the question as to whether this factor acts in a cell-autonomous manner or not. This was made possible by the use of a transformant strain that makes an *Sgs-3–E. coli* β-galactosidase (lacZ) fusion protein, under the control of an *Sgs-3* promoter, allowing the cellular examination of gene expression by a histochemical assay for enzyme activity. Using genetic methods, larvae that were mosaic for the loss of function mutation *l(1)npr-1* were generated. The expression of the *Sgs-3–lacZ* fusion gene was assayed histochemically in such larvae. Our results strongly indicate a cell-autonomous requirement for the product of *l(1)npr-1*$^+$. This is in contrast to another factor, the hormone ecdysterone, which is also required for *Sgs-3* expression, but acts in a non-autonomous manner.

Keywords. *Drosophila*; *Sgs-3*; *l(1)npr-1*; *trans*-acting; glue; ecdysterone.

1. Introduction

Sgs-3 is one of three genes at the *Drosophila melanogaster* 68C locus that encode polypeptide components of a mucous secretion that allows the pupa to adhere to an external surface during metamorphosis (Meyerowitz and Hogness 1982; Crowley *et al* 1983; Garfinkel *et al* 1983). The 68C glue genes are transcribed only in the salivary glands of third instar larvae (Meyerowitz and Hogness 1982). Studies on the DNA sequences required for regulation of *Sgs-3* have shown that 130 base pairs (bp) of sequence 5′ of the mRNA start site are sufficient for correct developmental expression (Vijay Raghavan *et al* 1986). This expression, however, is at levels about ten-fold lower than normal. Constructs with 2·76 kilobase pairs (kb) of flanking DNA 5′ of *Sgs-3* show correct developmental expression at high levels (Bourouis and Richards 1985; Crosby and Meyerowitz 1986). These results were obtained by RNA blot analyses of *Sgs-3* transcripts and from the examination of β-galactosidase activity in germline transformants of *Sgs-3–lacZ* gene fusion constructs. The *Sgs-3–lacZ* constructs consist of the DNA coding for the *Sgs-3* protein fused in frame to the *E. coli–*β-galactosidase gene, with the fusion gene under the control of *Sgs-3* regulatory sequences. The assay of β-galactosidase during development shows that activity specific to the transformant is detectable only in the salivary glands of third instar larvae (Vijay Raghavan *et al* 1986): the pattern of expression of the *lacZ* gene in the fusion construct is the same as that of the native gene.

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The steroid hormone ecdysterone and a product of the 2B5 chromosomal locus, *l(1)npr-1*[+], are two of the factors that act in *trans* to regulate *Sgs-3* expression. Larvae that carry a temperature-sensitive mutation that does not allow the synthesis of ecdysterone, *l(1)su(f)*[ts67g] (Hansson and Lambertsson 1983), fail to accumulate the 68C RNA at non-permissive temperature. The hormone also functions to stop 68C gene activity later in the third larval instar (Crowley and Meyerowitz 1984).

The other *trans* acting factor that has been studied is defined by the lethal (1) nonpupariating-1 [*l(1)npr-1*] lesion (Kiss *et al* 1976a, 1978). Animals that are homozygous or hemizygous for this sex-linked mutation fail to pupariate and thus die. This phenotype is similar to that of larvae that do not synthesize ecdysterone. Further, as in the case of ecdysterone deficiency, *l(1)npr-1* larvae do not accumulate RNA from the 68C glue genes (Crowley *et al* 1984). While larvae deficient in ecdysterone do not accumulate 68C RNA, this phenotype can be rescued by addition of the hormone to the food (Hansson and Lambertsson 1983). During normal development the hormone is synthesized in the ring gland and not in the salivary gland (Becker 1962). This indicates that the hormone acts non-autonomously to regulate gene expression: each cell which requires the hormone does not have to synthesize it. The effect of the *l(1)npr-1* mutation on the 68C genes, while similar to that resulting from ecdysterone deficiency, cannot be rescued by exogenous ecdysterone (Belyaeva *et al* 1981).

While the phenotype of the *l(1)npr-1* mutation is consistent with the wild-type product being an ecdysterone receptor, there are other possibilities, too, which have not been tested: for example, the gene product could be involved in the modification of ecdysterone. If this were so, administration of ecdysterone would not rescue the l(1)npr-1 phenotype, but the action of the wild-type product would still be non-autonomous. On the other hand *l(1)npr-1*[+] could modify a receptor in the cells where the hormone–receptor complex acts: in this case its requirement would be cell-autonomous.

We describe below an experiment to test the autonomy of the *l(1)npr-1* mutation. This was done using genetic mosaics and *Sgs-3–lacZ* fusion transformants to assay for the effect of the absence of the *l(1)npr-1*[+] product in individual salivary gland cells. Our results indicate an autonomous role for this factor in the regulation of *Sgs-3*.

2. Materials and methods

2.1 *Drosophila stocks*

The Tf(3)GLX3·3-1 stock, which contains an *Sgs-3–lacZ* gene inserted on the third chromosome was constructed in our laboratory and has been described earlier (Vijay Raghavan *et al* 1986). Adult males bearing an X-chromosome that carried the *l(1)npr-1* mutation were viable because their Y-chromosome carried a duplication for the region. Such flies (figure 1) were the yellow-body-colored, white-eyed male progeny from the following cross: *y l(1)npr-1 w mal/Binsn × FM6/Y, Dp(1;Y)67g, y*[2]. The *l(1)npr-1* bearing stock from Dr Istvan Kiss of Szeged, Hungary, and the duplication stock from Dr Igor Zhimulev, Novosibirsk, USSR, have been studied for their effect on the 68C glue genes in our laboratory (Crowley

et al 1984). The stock *In(1)w^{vC}/yw spl; Tf(3)GLX 3·3-1* was constructed by standard methods. The ring-X bearing stock used for this cross was *In(1)w^{vC}/yw spl* and has been described (Lindsley and Grell 1968; Hall *et al* 1976). All marker mutations used are described in Lindsley and Grell (1968).

Eggs were collected in half-pint milk bottles for a day at 22°C and development was allowed to proceed at the same temperature. Third instar larvae crawling out of the food were dissected and their salivary glands stained as described below.

2.2 *β-galactosidase staining and viewing of stained glands*

Salivary glands were stained for *E. coli–β*-galactosidase activity using a modification of the method described by Singh and Knox (1984). Animals were dissected in 10 mM phosphate buffer pH 8 and immersed in a drop of the staining solution (0·06 ml 5% X-gal, 0·02 ml 100 mM potassium ferrocyanide, 0·02 ml 100 mM potassium ferricyanide, 0·05 ml 1 M sodium phosphate pH 8 and 0·85 ml 35% Ficoll-400). Preparations were kept in a humid chamber to prevent the solution from drying and were usually analyzed after overnight staining. The glands were then washed in 50% ethanol, mounted in an aqueous mounting medium (Hydramount, manufactured by Gurr), and viewed using a compound microscope.

2.3 *Controls*

Several kinds of controls established the validity of the mosaic pattern of staining observed. First, there were internal controls: secretory cells in the salivary glands of wandering third instar females and male larvae that did not inherit the ring-X chromosome always stained uniformly blue. Second, several hundred positive controls from the transformant stock Tf(3)GLX3·3-1 were also stained. For negative controls male larvae of the genotype *yl(1)npr-1 w mal; Tf(3)GLX 3·3-1/+* recognized by the *y* and *w* markers were picked from the progeny of the cross *yl(1)npr-1 w mal/Binsn; +/+ × +/Y; Tf(3) GLX 3·3-1/Tf(3) GLX 3·1-1.* Salivary glands from *l(1)npr-1* male third instar larvae are negative for *β*-galactosidase activity whereas glands from control *Binsn* male larvae always stain blue. Damaged salivary glands sometimes fail to stain evenly. When the glands are mounted on a slide and examined under the compound microscope such damage is evident. Care was therefore taken while handling the glands during removal from the larva, and stained glands were examined under the compound microscope to ensure that all the cells were intact.

Evidence that the *Sgs-3–lacZ* fusion protein can be used as a cell autonomous marker in the salivary glands comes from the following observations. When salivary glands from Tf(3)GLX3·3-1 third instar larvae are stained for *β*-galactosidase activity all the secretory cells in the lobes turn blue. The cells which do not turn blue are the neck cells at the proximal end of each lobe. These cells do not normally synthesize the glue either, as evidenced by their failure to produce any glycoprotein detectable by the periodic acid Schiff (PAS) reaction (Ashburner and Berendes 1978). The proximal non-staining cells lie adjacent to the cells that stain for *β*-galactosidase activity, yet there is no sign of diffusion of the stain. The *lacZ* fusion construct has a large part of the *Sgs-3* protein coding sequences including the signal sequences for correct secretion into the lumen of the glands. At the time of removal of the glands for staining, the fusion protein is still present inside the cells; secretion

into the lumen begins only at the end of the third larval instar (Vijay Raghavan *et al* 1986). In a transient assay system the *Sgs-3–lacZ* fusion gene with 2·76 kb of flanking sequence 5′ of the mRNA start site, in a plasmid vector, is injected into embryos and the salivary glands from the third instar larvae into which these embryos develop are stained for *β*-galactosidase activity. The *lacZ* fusion gene is expressed in the salivary glands in a subset of cells. In such transient assays (Martin *et al* 1986) single, stained cells with completely unstained neighboring cells are often observed (K Vijay Raghavan, M Roark, C Mayeda and E Meyerowitz, in preparation). This indicates that the stain does not diffuse into neighboring cells.

3. Results and discussion

Both ecdysterone and *l(1)npr-1* affect *Sgs-3–lacZ* gene fusions in the same manner as they affect the expression of the native *Sgs-3* gene. Salivary glands from third instar larvae that carry the insertion Tf(3)GLX3·3-1, an *Sgs-3–lacZ* fusion with 2·76 kb of flanking sequence 5′ of *Sgs-3* integrated in the third chromosome, stain strongly for *β*-galactosidase activity (Vijay Raghavan *et al* 1986). This activity is not seen in animals deficient in ecdysterone (Peter Mathers, personal communication) nor in *l(1)npr-1* animals (Vijay Raghavan *et al* 1986). Addition of ecdysterone to the food of hormone-deficient larvae at the correct stage restores *β*-galactosidase activity (Peter Mathers, personal communication). The *Sgs-3–lacZ* transformant Tf(3)GLX3·3-1 is therefore affected by the absence of these *trans*-acting factors in the same manner as the native *Sgs-3* gene.

   Using an unstable ring-X chromosome (Lindsley and Grell 1968; Hall *et al* 1976), gynandromorphs were generated that were mosaic for the *l(1)npr-1* mutation. The scheme used to generate mosaics is shown in figure 1. Females carrying an *Sgs-3 - lacZ* insertion on the third chromosome (Tf(3)GLX3·3-1) (Vijay Raghavan *et al* 1986) and one mitotically unstable ring-X chromosome were crossed to males carrying the *l(1)npr-1* mutation on their stable rod-X chromosome. Partial loss of the ring-X chromosome in some of the progeny of this cross, a phenomenon which generally occurs during the first few nuclear divisions, results in animals that are partly XO (phenotypically male) and partly XX (female). The loss of the ring-X chromosome is recognized by the presence of a mixture of male and female tissue and by the recessive markers on the *l(1)npr-1* chromosome, *y* (yellow), *w* (white), and *mal* (maroon-like) (Lindsley and Grell 1968). The marker *y* can be recognized in the external epidermis and the mouth hooks of the larva. The mutation *w* can be scored in the larval Malpighian tubules. The mutation *mal* does not confer a visible phenotype on the larva but results in an absence of aldehyde oxidase and pyridoxal oxidase activity in several larval tissues (Janning 1972; Cypher *et al* 1982). All the cells of the gynandromorphs carry the *Sgs-3–lacZ* fusion gene on the third chromosome. The female cells are, in addition, *l(1)npr-1*[+] since they have a wild-type copy of the 2B5 region on the ring-X chromosome. The male cells are *l(1)npr-1* since they have lost the ring-X chromosome.

   Six hundred and thirty-eight progeny of the cross outlined in figure 1 were scored in late third larval instar for external markers and sexual phenotype. The salivary glands, which consist of two lobes connected by a duct in the anterior of the animal, were dissected from all the 638 larvae and stained histochemically for *β*-galactosidase activity. A total of 43 gynandromorphs were identified. The basis for
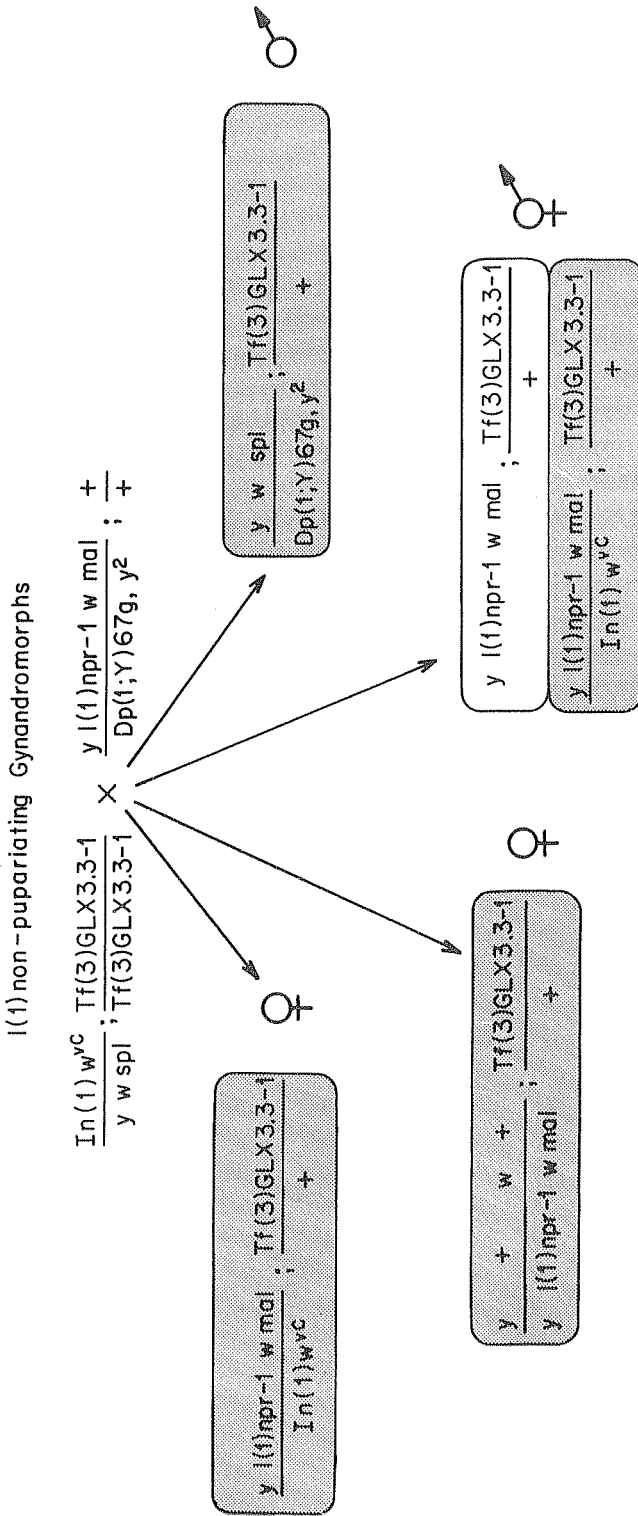
Figure 1. The cross used to generate $l(1)npr-1$ mosaics is shown at the top of the figure. The ring-X chromosome, $In(1)w^{vC}$, and the markers and chromosomes used have been described (Lindsley and Grell 1968; Hall *et al* 1976). Tf(3)GLX3-3-1 denotes the integration into the third chromosome of an *Sgs-3–lacZ* fusion gene, from which mRNA encoding *E. coli–β*-galactosidase is expressed under the control of the *Sgs-3* gene promoter (Vijay Raghavan *et al* 1986). The loss of the ring-X chromosome in a nucleus after one of the early nuclear divisions results in the formation of a gynandromorph. The male tissue in such an animal will express the recessive markers on the rod-X (the homologous X chromosome). In this case the visible markers in the larvae are y (yellow, scorable in the larval external epidermis and the mouth hooks) and w (white, scorable in the larval Malpighian tubules). In addition, since those cells which have lost the ring-X chromosome are phenotypically male, the sexually dimorphic gonads can be used as markers. In animals that are hemizygous for $l(1)npr-1$ and carry a copy of an *Sgs-3–lacZ* fusion gene transformed onto an autosome, the salivary glands do not stain for $β$-galactosidase activity. Salivary glands from control siblings that are $l(1)npr-1^+$ stain uniformly blue. Therefore, depending on the autonomy displayed by $l(1)npr-1^+$, gynandromorphs which have the mosaic line passing through a salivary gland lobe or between the two lobes may show a mosaic pattern of staining. The female progeny of the cross are represented by the two boxes on the left that are shaded to represent the fact that salivary glands from such third instar larvae always stain uniformly blue. Similarly, the shaded box on the top right of the figure denotes $l(1)npr-1^+$ male larvae. Gynandromorphs are represented by the bottom right double boxes, one box shaded to represent an $l(1)npr-1^+$ salivary gland cell and the other unshaded, representing a cell that has lost the ring-X chromosome.
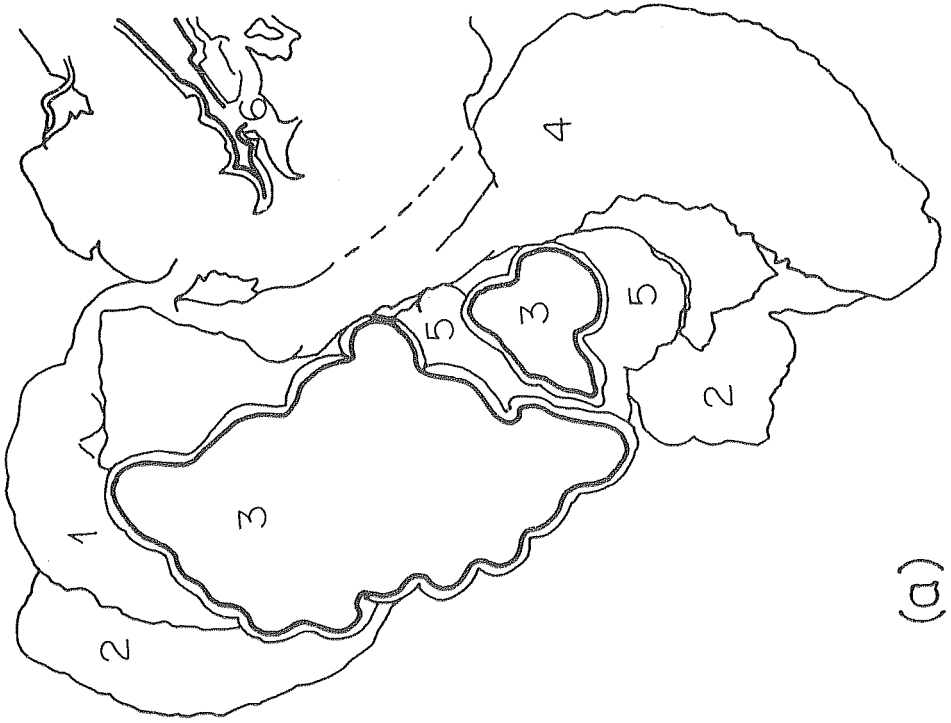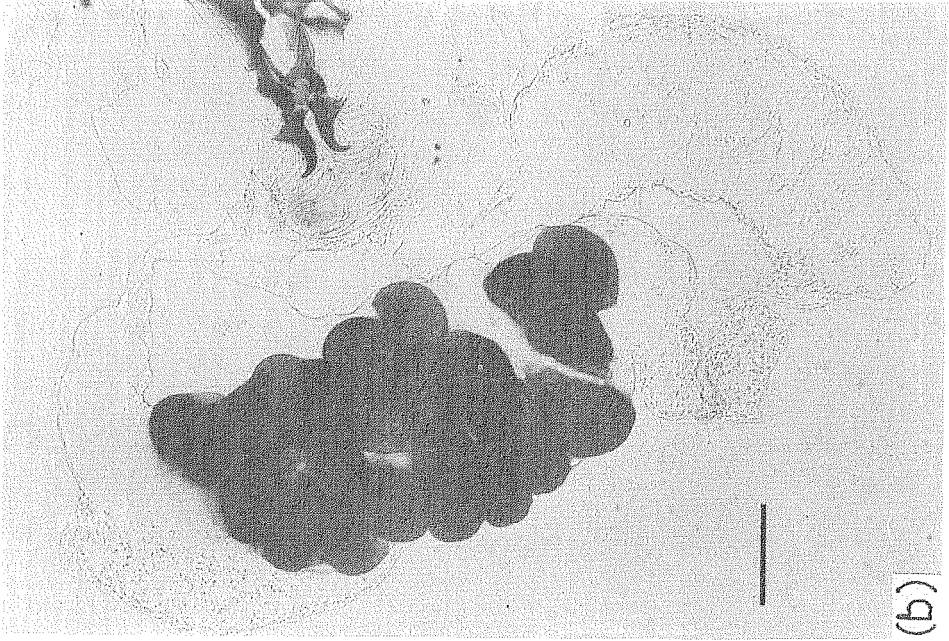
(b)

(a)

Figure 2. (a) A schematic representation of the gland in (b). The proximal cells of the gland (1) do not express the *Sgs-3* gene and also never stain positive for β-galactosidase expression. The fat body (2) is always unstained even when in close proximity to stained cells. One lobe has most cells staining positive (3) and the other lobe (4) is completely unstained. The stained lobe has a group of cells at the distal end (5) that do not stain, and stained and unstained cells lie next to each other. The mouth hooks in this larva were genetically mosaic (6), one being *y* and the other *y*$^+$.

(b) Salivary gland from a third instar larva, scored as a gynandromorph. The gland was stained for β-galactosidase activity and mounted. (In general salivary glands from all the progeny of a cross that developed to the third instar larval stage were examined irrespective of whether or not they were scored as gynandromorphs. This was an internal control. Details of these and other controls are given in §2).

In the pair of lobes pictured the one on the right is completely unstained while almost all of the second lobe is stained. The unstained cells are morphologically normal and the cell boundaries are not damaged as viewed under the compound microscope. Scale bar, 200 μm.

(c) A higher magnification view of the lower part of the stained lobe from (b) above. Note that the border between the stained and unstained cells is clearly demarcated. Scale bar. 50 μm.
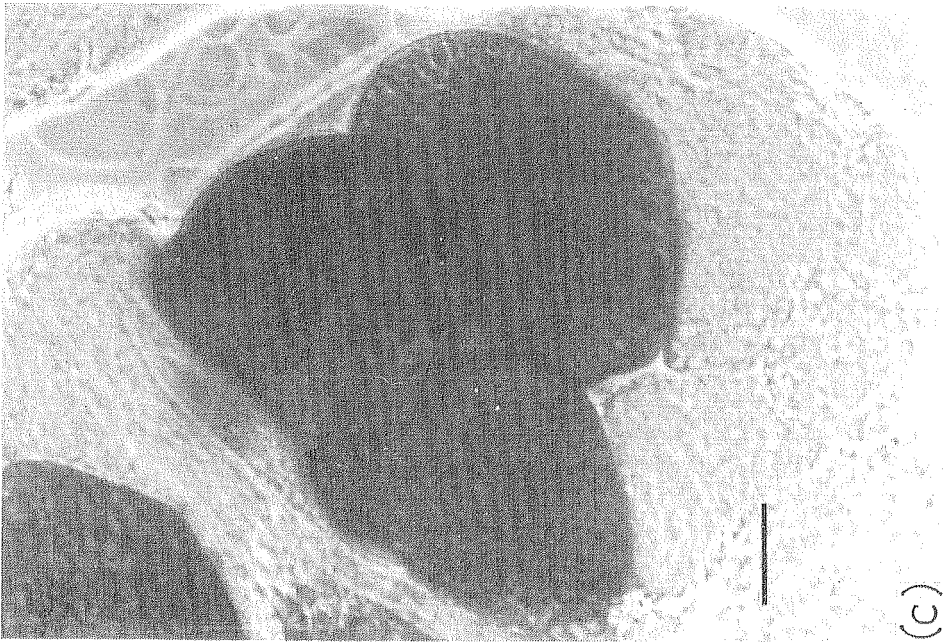
(c)

identifying an animal as a gynandromorph was mosaicism in at least one of the following: gonads, recessive markers, or salivary gland staining. Mosaicism that was restricted solely to salivary gland staining was seen only in 2 larvae. Several kinds of positive and negative controls established the validity of the staining data and these are described in §2. The most frequent type of gynandromorphs ($n = 29$) were those that showed a uniform absence or presence of staining within a lobe. This category consists of those gynandromorphs in which both lobes stained completely ($n = 12$), gynandromorphs in which both lobes were unstained ($n = 10$) and those in which one lobe was completely stained and the other completely unstained ($n = 7$). The other category ($n = 14$) consists of those gynandromorphs in which at least one salivary gland lobe showed a mosaic pattern of staining, with some cells in a lobe stained and the others unstained. The mosaic pattern of staining in the salivary glands demonstrates that an animal being partly $l(1)npr-1^+$ is not sufficient for an $l(1)npr-1^+$ phenotype in the salivary glands: the product of the 2B5 locus that acts to affect expression of *Sgs-3* is not freely diffusible through the hemolymph.

To rigorously prove that the action of the $l(1)npr-1^+$ product is cell-autonomous, an independent cell-autonomous marker is required to ascertain the genotype of each cell. We have attempted to use the maroon-like (*mal*) mutation, which results in the absence of aldehyde and pyridoxal oxidase activity (Janning 1972; Cypher *et al* 1982), as an autonomous marker of the $l(1)npr-1$ cells. Experiments on *mal* larvae showed that the background levels of staining were too high and variable for the reliable use of this marker in salivary gland cells. Another approach to learning the genotype of different cells was also tried: chromosome squashes of mosaic glands were made to examine individual cells for the presence or absence of the ring-X chromosome. This approach also was unsuccessful, as the chromosome structure was not preserved after several hours in the β-galactosidase staining solution.

Evidence for the cell-autonomous requirement for the $l(1)npr-1^+$ product thus comes from the examination of those gynandromorphs in which the mosaic boundary passes through a salivary gland lobe ($n = 14$). Figure 2 illustrates one example. The distribution of stained and unstained cells (regions 3 and 5 respectively in figure 2a) shows that unstained cells can surround a small group of stained cells in a lobe in which most cells are stained. The detection of gynandromorphs in which one lobe is stained while the other is not ($n = 7$) and the occurrence of unstained cells neighboring stained cells ($n = 14$) make a strong case for the cell-autonomous action of the product of $l(1)npr-1^+$ on *Sgs-3*. In the absence of a convenient independent autonomous cell marker the following arguments are pertinent to our view on the action of $l(1)npr-1^+$. First, under the conditions used, the β-galactosidase histochemical staining is not diffusible (see §2). Therefore, cells which stain positive do express the *Sgs-3–lacZ* fusion protein, and cells which do not stain do not. Second, if $l(1)npr-1^+$ acted non-autonomously, then one would not see a small group of unstained cells in a lobe with most of the cells stained (e.g. figure 2, b and c). Third, gynandromorphs with a mosaic pattern of staining within a lobe would be very rare or absent if the action of $l(1)npr-1^+$ was not cell-autonomous, but these constitute about 33% ($n = 14$, out of a total of 43 gynandromorphs scored) of the total.

One possible explanation of the mosaic lobes, other than cell autonomy of $l(1)npr-1^+$ action, is that the mosaicism results from a threshold level of a diffusible gene activator, which causes a random pattern of activation in some cells, but

not in others. If this were the case, however, the mosaics should always have each lobe showing a mixture of stained and unstained cells. In fact the majority of mosaic larvae show staining of all the secretory cells of one salivary gland lobe and, no staining in the contralateral lobe. The only result consistent with this result is cell-autonomy.

Genetic characterization of the 2B5 region shows it to be complex (Ashburner and Berendes 1978; Belyaeva *et al* 1981; Zhimulev *et al* 1982). The functions of the several transcripts that have been detected at the locus are as yet unknown (Chao and Guild 1986). It is possible that some of the effects of the absence of function of the 2B5 locus are direct while others result indirectly from the interaction of the locus with other genes or their products. Further, the 2B5 locus could act autonomously in one tissue and non-autonomously in another. For example, $l(1)npr-1^+$ function in one tissue could result in a product that is freely diffusible and has a different role from a non-diffusible product formed in another tissue. The $l(1)npr-1$ lesion affects the expression of genes in tissues other than the salivary glands (Kiss *et al* 1976a, b, 1978; Chao and Guild 1986), thus different modes of action are a possibility. Others have studied the non-pupariating phenotype of $l(1)npr-1$ in mosaics (Kiss *et al* 1976b, 1978, 1979) and found it to be autonomous. Our experiment tests the regulation of a specific gene in the salivary glands, at the cellular level, and provides evidence that the regulation of *Sgs-3* by $l(1)npr-1^+$ is cell-autonomous.

Acknowledgements

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