Developmental regulation by an enhancer from the *Sgs-4* gene of *Drosophila*

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Communicated by V. Pirrotta

A *cis* acting regulatory region has previously been identified 300–500 bp upstream of the *Drosophila* glue protein gene, *Sgs-4*. The functional capabilities of this region have now been examined by fusing it to the *Drosophila* *Adh* gene and determining the pattern of expression from the fused construct after transformation. The results show that the *Sgs-4* sequences between –150 and –568 are able to direct *Adh* expression in late third-instar salivary glands, the appropriate tissue and timing for *Sgs-4* expression. In addition, the *Sgs-4* sequence elevates *Adh* expression in the anterior midgut and fat body, despite the fact that *Sgs-4* is not normally expressed there. All three regulatory activities, tissue specificity, timing and enhancement, show the positional flexibility of enhancer elements. In addition, the *Sgs-4* and *Adh* regulatory elements combine to direct expression in novel spatial/temporal combinations in which neither would normally be expressed.

Key words: tissue specific expression/P-element transformation/*Adh* regulation

Introduction

In the salivary glands of late third-instar larvae, the *Sgs-4* gene is actively transcribed and five DNase I hypersensitive sites appear on its 5' side. Several lines of evidence indicate that the three farthest upstream sites delineate an essential regulatory region. Natural *Sgs-4* underproducers have either small deletions or sequence changes in this hypersensitive region (Muskavitch and Hogness, 1982; McGinnis *et al.*, 1983b). When the two most distal hypersensitive sites are missing due to a 95-bp natural deletion, no *Sgs-4* expression is detectable and the hypersensitive sites nearer the transcription start site fail to form, implying a mandatory interaction between the near and far sites to allow transcription (Shermoen and Beckendorf, 1982). Finally, *Sgs-4* transformants that, in addition to the gene, include 840 bp upstream are capable of normal *Sgs-4* expression, while those including only 392 bp upstream and therefore missing about half of the regulatory region are completely inactive (McNabb and Beckendorf, 1986).

The distance between this regulatory region and the structural gene it controls suggested that it might be acting as an enhancer element. This hypothesis was strengthened by analysis of a natural variant that had a 1.3-kb hobo transposable element inserted between the upstream regulatory region and the TATA box (McGinnis *et al.*, 1983a). In this variant, *Sgs-4* transcripts as well as novel transcripts that initiated within the hobo element were found only in late third-instar salivary glands. Thus, the *Sgs-4* sequences

appeared to be directing appropriate timing and tissue specificity even though all sequences upstream from TATA had been displaced by 1.3 kb.

To test whether the *Sgs-4* upstream region can indeed act as an enhancer and to examine its functional capabilities, we have placed it upstream or downstream from a truncated form of the *Drosophila* alcohol dehydrogenase gene (*Adh*). Because this trun-

Fig. 1. Plasmid constructions used for transformation. (a) The *Sgs-4* gene, donor of the regulatory region. The lollipops indicate the positions of the 5' DNase I hypersensitive sites (HSS) found in the chromatin of late third-instar salivary glands. Both the *Bam*HI/*Aha*III fragment and the *Dde*I/*Dde*I fragment were tested for their regulatory activity. Each included the sequences of the distal cluster of HSS, but not those over which the two proximal HSS form. (b) pARP-11, the parent plasmid for the construction of *Sgs-4*/*Adh* hybrid constructs. In addition to pUC9 sequences, this plasmid contains: the ends of the P element which allow insertion of the sequences between the *Sgs-4* and *Adh* genes; the *Adh^F* gene which facilitates identification of transformants, and the *Adh^F* gene. The two possible transcripts of *Adh^F* are diagrammed, with narrow lines indicating the introns. Prior to insertion of *Sgs-4* sequences, *Adh^F* was simplified by excision of the *Sal*I/*Hpa*I fragment. This digestion eliminates all *Adh* sequence more than 380 bp upstream from the proximal promoter. Therefore, the distal promoter is no longer present, and all *Adh* expression arises from the proximal promoter. The deleted construction serves as a control for the *Sgs-4*/*Adh* hybrids and is shown in (c) as pARP-9. (c) Plasmids tested for their *Adh* activity in transformed flies. In each case only the region immediately adjacent to the *Adh^F* gene is shown. pGARP-1. The 735-bp *Bam*HI/*Aha*III fragment from the *Sgs-4* upstream region was inserted between the *Sal*I and *Hpa*I sites of pARP-11 and in the same orientation as the *Adh* promoter. pGARP-212. The 419-bp *Dde*I fragment from *Sgs-4* was inserted between the same *Sal*I/*Hpa*I sites, but in the opposite orientation. pGARP-301. The *Dde*I fragment was inserted at the *Cl*aI site 1.8 kb 3' to the *Adh* promoter and in opposite orientation. The *Sal*I/*Hpa*I sites of pARP-11 were fused. pARP-9. This control construct contains no *Sgs-4* DNA and has the *Sal*I/*Hpa*I sites of pARP-11 fused to eliminate *Adh* sequences 5' to the *Hpa*I site.

Fig. 2. Tissues of transformed and control larvae stained for *Adh* activity. Salivary glands with their associated fat body (columns A and C) or midguts (columns B and D) were dissected out of second-instar larvae (A and B) or late third-instar larvae (C and D) and stained for *Adh* activity (Ursprung, 1970). The first row shows the lack of *Adh* activity in these tissues in larvae carrying the null, *Adh^{tr6}* allele. This stock was used as the recipient for transformation. The second row shows the *Adh⁺* phenotype in tissues from Oregon R larvae. Salivary glands are negative at both stages, while fat body and midgut stain intensely. tARP-9 shows a similar pattern except that fat body staining is reduced and often undetectable in second instar. The next five rows illustrate staining in transformed lines which have received the *Sgs-4/Adh* hybrid constructs. Tissues from three independent lines transformed with the pGARP-212 construct are shown along with one each for pGARP-1 and pGARP-301. As an aid in identification, labeled outlines of the tissues in panels B and C of the Oregon R row are shown above.

cated *Adh* gene is partially active by itself and retains its developmental specificity, we could assay changes in its activity resulting from addition of *Sgs-4* sequences. We chose *Adh* because it is easily assayed for enzymatic activity in both cell extracts and histological preparations. In addition, its tissue and temporal expression are different from *Sgs-4*. In larvae, 85% of *Adh* is found in the fat body and in the intestine, especially the anterior midgut and gastric caecae (Ursprung *et al.*, 1970; Maroni, 1978). The rest of normal *Adh* larval activity is distributed among somatic muscles, posterior midgut and Malpighian tubules, but is not found in salivary glands or in imaginal disks, brain or foregut.

The temporal pattern of *Adh* activity is more complex than the tissue distribution, in part because the gene has two differentially utilized promoters: a proximal promoter, used mainly in the larvae, and 710 bp upstream a distal promoter used mainly in adults (Benyajati *et al.*, 1983). However, in making the *Sgs-4/Adh* hybrid constructs described here, *Adh* promoter usage has been simplified. We have removed the distal promoter thereby forcing all *Adh* expression to come from the proximal promoter. This promoter is normally used from mid-embryonic stages through early third instar and then shuts off abruptly just as *Sgs-4* is turning on (Savakis *et al.*, 1986). It becomes active again at a low level in late pupae and in adults (Savakis *et al.*, 1986). Thus the tissues of *Sgs-4* and *Adh* expression are disjunct and temporally their expression overlaps only slightly during mid third instar. Because of these spatial and temporal differences, it was possible to evaluate separately the regulatory contributions of the *Sgs-4* and *Adh* sequences.

Sgs-4/Adh hybrid constructs have now been inserted into the genome of *Adh⁻* flies via P element-mediated germ line transformation and the effects of the *Sgs-4* sequence on *Adh* expression determined. The results indicate that the *Sgs-4* upstream region controls at least three regulatory activities, and that it is able to interact with *Adh* regulatory sequences to produce novel phenotypes.

Results

Sgs-4/Adh hybrid genes

To examine the regulatory functions of the *Sgs-4* control region, three *Sgs-4/Adh* hybrids were constructed (Figure 1). Each contained *Adh* DNA starting 380 bp 5' to the larval cap site, continuing through the *Adh* gene and ending at least 700 bp 3' to the gene. Fragments containing the *Sgs-4* upstream regulatory region were then added in either orientation upstream of *Adh* or in the inverted orientation downstream of *Adh*. The first construct, pGARP-1, contains the largest piece of *Sgs-4* DNA, a 735-bp fragment extending between 839 and 105 bp upstream from *Sgs-4*. In this case the *Sgs-4* DNA is in its normal orientation relative to the structural gene but is moved 275 bp further upstream. The two other constructs, pGARP-212 and pGARP-301, contain a shorter 419-bp fragment which includes *Sgs-4* sequence between -568 and -150. In these constructs the *Sgs-4* DNA is in the inverted orientation relative to the structural gene. In pGARP-212 the hypersensitive region is located about 100 bp further upstream than in intact *Sgs-4*, while in pGARP-301 it is located about 2 kb downstream from the cap site. Each of these hybrid genes was constructed by modifying pARP-11 (Figure 1b), a plasmid that in addition to the *Adh* gene also contains the ends of the P element, which mobilize the transposon during transformation, and the *rosy⁺* gene, which facilitates identification of transformants.

The plasmids were injected into *Adh^{tr6} cn; ry⁵⁰⁶* embryos along with the non-mobile P element pIT25.7wc (Karess and Rubin, 1984) which acts as a supplier of transposase activity. Transformants were selected as *ry⁺* G₁ larvae or flies and were subsequently tested for *Adh* activity. One transformant carrying the pGARP-1 construct, three with pGARP-212 and six with pGARP-301 were recovered and several of these were chosen for further analysis. The transformants are designated as tGARP lines to distinguish them from the pGARP plasmids with which

they are injected. That each transformant carried a single un-rearranged insert was demonstrated by Southern analysis of transformant DNA. Two results indicate that the transposons were not grossly rearranged. First, both the *Adh* and *ry* genes are functional in all cases. Second, the expected *Bam*HI fragments extending from the middle of the *Adh* gene into *ry* were found. Each transformant is the result of a single insertion as shown by a single, variably sized fragment extending from the 5' end of the *Adh* gene out into the adjacent genomic DNA.

Tissue-specific expression of the hybrid genes

Because *Adh* is expressed in different tissues and at different times from *Sgs-4*, we could learn something about the functions of the *Sgs-4* sequence by examining when and where the hybrid genes were expressed. As a first step larvae were dissected at two stages, late second instar or late third instar, and as shown in Figure 2 the tissues were stained to detect *Adh* activity (Ursprung *et al.*, 1970). For Oregon R, the *Adh*⁺ control, the staining was similar in second- and third-instar larvae. The fat body and anterior midgut stained intensely and accounted for most of the *Adh* activity of the larvae. Small amounts of activity were also present in the middle midgut and hindgut, in the Malpighian tubules, and in the muscles of the body wall (data not shown; see also Goldberg *et al.*, 1983; Ursprung *et al.*, 1970). The proventriculus, the bulbous structure at the junction of the midgut and foregut, occasionally showed a weak, median band of activity, especially in third instar. Several tissues were negative at both stages — salivary glands, imaginal disks and brain. The tARP-9 transformant, which includes the same *Adh* fragment as the tGARP strains but is lacking *Sgs-4* sequences, shows a similar pattern of staining as the *Adh*⁺ strain, but the intensity of staining is markedly reduced, consonant with its reduced level of *Adh* expression (J. Posakony and T. Maniatis, personal communication). This reduced staining is evident in the tARP-9 fat bodies shown in Figure 2. With shorter staining times it was also detectable in the midgut.

The tGARP transformants are distinctly different from tARP-9 at both second and third instars. All three types of transformants showed enhanced staining of midgut and fat body which were, in fact, indistinguishable from *Adh*⁺. In addition, the salivary glands of all three were now stained, showing that salivary gland tissue specificity was imparted by the *Sgs-4* DNA. Curiously, this expression was present not only in salivary glands from third-instar larvae, but also at a somewhat lower level in second-instar glands, a tissue in which neither *Sgs-4* nor *Adh* is normally active. The proventriculus also showed an unexpected pattern of expression. The tGARP-212 lines often showed activity throughout the proventriculus in second instar, but third instar proventriculi usually looked like *Adh*⁺ (for exception see tGARP-212b, Figure 2). The pattern in tGARP 301 was strikingly different. In both second and third instar the anterior half of the proventriculus was intensely stained while the posterior half remained unstained. The same pattern was seen in two other tGARP 301 lines, suggesting that the pattern depends on the structure of the transposon and not its chromosomal insertion point.

Temporal regulation and enhancer activity

To get a better idea of the changes in *Adh* expression during larval development of the transformants and to compare the expression of *Adh* in wild-type, tARP-9, tGARP-1 and tGARP-212, we measured *Adh* activity at frequent intervals in both whole larvae and separated tissues. In whole larvae of the Oregon R strain (*Adh*⁺), the *Adh* specific activity (units/ μ g protein) peaks near the end of second instar and again in mid third instar and then declines rapidly in wandering larvae that are about to pupariate

Fig. 3. Changes in *Adh* activity during development of wild-type and transformed larvae. Staged larvae from the *Adh*⁺ strain Oregon R and the tARP-9 and tGARP-212b transformed lines were spectrophotometrically assayed for *Adh* activity as described in Materials and methods.

(Figure 3). The tARP-9 and tGARP-212 transformants show a small peak of enzyme activity during third instar, but lack the rapid decline of wild-type. While it is apparent that the *Adh* gene in tGARP-212 functions at a lower level than the wild-type *Adh* gene, it is nevertheless more active than the *Adh* of tARP-9. This difference is particularly significant because the transposons in the two types of transformants differ only by the addition in tGARP-212 of sequences normally located upstream of *Sgs-4*. And this increased activity is evident not only in late third instar when *Sgs-4* is normally active, but throughout larval development. The effect of these upstream sequences can be seen even more clearly in measurements of *Adh* activity in individual tissues.

Figure 4 shows *Adh* enzymatic activity in the anterior midgut and the carcass (larvae with anterior midgut and salivary glands removed), assayed at 8-h intervals throughout third instar. Expression of the transformed gene in the carcass is maintained at a low level with the tGARP transformants higher than tARP-9 but well below the wild-type level. Additionally the wild-type gene shows the characteristic peak of specific activity at mid third instar, whereas the transformants do not.

A different profile of expression is seen in the anterior midgut. The peak of activity is again at mid third instar, but now it is present in the transformants as well as Oregon R. The three transformants differ from Oregon R by having a second rise at 40 h, a time at which Oregon R is experiencing a marked decline in its *Adh* activity. In fact during late third instar the tGARP transformants maintain similar or slightly higher *Adh* levels than Oregon R. Throughout third instar the *Adh* level in the tGARP transformants is elevated above that in tARP-9, by factors of 1.6–3.4.

Additional information about the enhancement activity of the *Sgs-4* sequences in several transformed lines came from measuring *Adh* activity in larvae that had just molted from second to third instar. This time was chosen because the larvae can be accurately staged by collecting them as they molt and because the activities in the transformants are not changing much at this stage (Figure 3). As shown in Table I the tGARP transformants show a rather uniform enhancement of 4- to 8-fold under these conditions.

To examine *Adh* expression in tGARP salivary glands, a comparison was made between midgut and salivary glands within tGARP-212. (Comparisons with Oregon R or tARP-9 are not relevant since these strains do not make detectable *Adh* in their

Fig. 4. *Adh* activity during third instar in the anterior midgut and in larval carcasses. Anterior midguts and carcasses (larvae from which the anterior midgut and salivary glands had been removed) were isolated from staged larvae and assayed for *Adh* activity (see Materials and methods). Approximately three quarters of the *Adh* activity in the carcass fraction comes from the fat body.

salivary glands.) As shown in Figure 5, the timing of *Adh* expression in the salivary glands is quite different from that in anterior midgut. Salivary gland expression is near background for the early parts of third instar and only increases detectably during the last 16 h. This is, in fact, the temporal pattern expected not of the *Adh* gene, but of *Sgs-4*.

Discussion

Three activities in the *Sgs-4* regulatory region

The DNase I hypersensitive region located upstream from *Sgs-4* had previously been implicated as a necessary regulator of *Sgs-4* expression (Shermoen and Beckendorf, 1982; Muskavitch and Hogness, 1982; McGinnis *et al.*, 1983b; McNabb and Beckendorf, 1986). The current experiments have allowed us to investigate the functions of this regulatory region. The results show that the *Sgs-4* DNA in the pGARP constructs specifies at least three regulatory activities: enhancement of the expression of an adjacent gene, salivary gland tissue specificity, and late third-instar temporal specificity.

The enhancement activity is evident in both major *Adh* tissues

Table I. *Adh* activity in newly molted third-instar larvae from transformed lines

Transformant	<i>Adh</i> activity (unit/μg protein)	Enhancement (transformant/tARP-9)
Homozygous lines		
tARP-9	1.3	1.0
tGARP-1	8.6	6.6
tGARP-212b	5.8	4.5
tGARP-301	10.0	7.8
Heterozygous lines		
tGARP-212a	3.8	2.9 (5.8)
tGARP-212c	2.6	2.0 (4.0)

Between 5 and 10 third-instar larvae, 0–2 h old were collected and assayed for *Adh* activity as described in Materials and methods. Two of the transformant lines were still heterozygous for the transposon when they were assayed. For these the enhancement that would be expected for homozygous lines is indicated in parentheses.

Fig. 5. *Adh* activity in the anterior midgut and salivary glands of tGARP-212b. Experimental conditions are the same as described in Figure 3. The data for activity in the anterior midgut of tGARP-212b appear quite different because here they are expressed as units/midgut or units/salivary gland rather than units/μg protein. This difference was necessitated because the salivary glands are so small, especially at the earlier time points, that measurements of protein in the sample were impractical.

as well as in whole larvae, ratios between the tGARP strains and tARP-9 varying between 1.6 and 8. These ratios represent minimal estimates of the true enhancer activity because the *Adh* gene in tARP-9 retains several regulatory sequences of its own (Posakony *et al.*, 1985; Heberlein *et al.*, 1985) and is substantially active without the added *Sgs-4* sequence.

The tissue specific activity of the *Sgs-4* sequences is shown by the appearance of salivary gland *Adh* expression in the tGARP strains. This salivary gland expression shows two phases. First, it is expressed in second- and early third-instar larvae, times at which *Sgs-4* expression is undetectable (Muskavitch and Hogness, 1983). Then at the end of third instar, salivary gland *Adh* activity increases as would be expected for normal *Sgs-4* expression. Both phases require the *Sgs-4* sequences. We conclude that a tissue specific element of these sequences is able to recognize salivary glands as its appropriate tissue at least as early as late second instar, more than a day before *Sgs-4* is normally expressed. This finding is reminiscent of the appearance of tissue specific DNase I hypersensitive sites near the chicken vitellogenin gene substantially before the developmental activation of this hormonally regulated gene (Burch and Weintraub, 1983). By analogy, it may be

that in second-instar salivary glands one or more of the *Sgs-4* hypersensitive sites has already formed, poisoning the gene for subsequent activation.

Since the *Sgs-4* sequences can recognise the salivary gland cells long before *Sgs-4* is expressed, there must be an additional *cis*-acting element involved in the timing of *Sgs-4* expression. That this element is included in the pGARP constructs is demonstrated by the increase in salivary gland *Adh* expression near the end of third instar. This timing element is apparently dependent on the action of the tissue specific regulator since no similar increase is detected in anterior midgut or in fat bodies at the end of third instar. Since the steroid hormone ecdysterone is required for the expression of *Sgs-4* as well as the other *Sgs* genes (Hansson and Lambertsson, 1983), and since a small peak in hemolymph ecdysterone concentration has been detected in early third instar (Berreuer *et al.*, 1979), it seems reasonable that this ecdysterone peak is, directly or indirectly, the timing signal for *Sgs-4* expression. This may, in fact, be a direct interaction since anti-ecdysterone antibodies bind to the intermolt puff which contains *Sgs-4* during the period in which the gene is expressed (Dworiczak *et al.*, 1983).

All three of these regulatory activities possess the positional flexibility that has been associated with enhancer elements: they can act in *cis* in either orientation and at variable distances upstream or downstream of the gene whose expression they regulate. We cannot yet distinguish whether these multiple activities reside in separable DNA sequences, but data from this and previous investigations of *Sgs-4* organization imply that the important sequences span a larger region than would usually be bound by a single protein. Transformation experiments with DNA which includes the *Sgs-4* structural gene and varying amount of flanking sequence have shown that there is a crucial sequence for *Sgs-4* expression between -840 and -392 (McNabb and Beckendorf, 1986). Since the *Sgs-4* sequence in pGARP-212 and pGARP-301 extends in the 5' direction only to -568, and is sufficient for normal developmental regulation, this crucial sequence probably lies between -568 and -392. Analysis of naturally occurring deletions (Muskavitch and Hogness, 1982) or point mutants (McGinnis *et al.*, 1983b) showed that there are quantitatively important though not absolutely required sequences between -356 and -305. Finally, *in vitro* insertion of 11 bp at -301 prevents *Sgs-4* expression following DNA transformation (T.Jongens, personal communication). These results suggest that the important sequences extend from -301 out past -392 and perhaps as far as -568. The pattern of DNase I hypersensitivity also implies the binding of multiple proteins, since there are three hypersensitive sites extending from -480 to -330 (Shermoen and Beckendorf, 1982). Multiple hypersensitive sites spread over 200-300 bp have been found for the hypersensitive regions overlying both cellular and viral enhancers (Saragosti *et al.*, 1980; Herbolme *et al.*, 1981; Wu and Gilbert, 1981). In addition, many enhancers, including those of SV40 (Herr and Clarke, 1986; Wildeman *et al.*, 1986), the immunoglobulin kappa and heavy chains (Banerji *et al.*, 1983; Gillies *et al.*, 1983) and cytomegalovirus (Boshart *et al.*, 1985), have been shown to include multiple, clustered sequence motifs, detected either as DNase I protected footprints, functionally required sequences, or consensus sequences found in several different enhancers (reviewed in Serfling *et al.*, 1985). Given this complexity, it seems quite reasonable for enhancers to have multiple functions. In fact, the other well characterized *Drosophila* enhancer, the fat body-specific enhancer of the yolk protein 1 gene, determines stage, sex and tissue specificity (Garabedian *et al.*, 1986). Experiments are currently under-

way to subdivide the *Sgs-4* region to see whether the three regulatory activities we have detected can be separated.

Adh regulatory sequences

Since a fly transformed with pARP-9 expresses *Adh* in its normal larval tissues, the *Adh* DNA in this transformant must contain at least one tissue specificity element. Temporal specificity appears to be more complicated. In third-instar anterior midgut, Oregon R and the transformants all showed the same mid third-instar peak in *Adh* activity, although the levels of activity were very different (Figure 4). In contrast, in the carcass none of the transformants showed the mid third-instar peak of activity found in Oregon R. These results suggest that an *Adh* temporal regulator which is normally active in fat body (the major *Adh*⁺ tissue in the carcass) is missing in the tARP-9 construct, while sequences governing the mid third-instar peak of midgut activity are retained. An additional difference between Oregon R and the transformants is seen in anterior midgut at 40 h. The transformants share a peak of *Adh* activity that is not found in Oregon R (Figure 4). The sharp decrease in Oregon R activity at this time probably reflects the rapid disappearance of *Adh* RNA at mid third instar (Savakis *et al.*, 1986). Then the late peak in the transformants implies that some *Adh* sequences are missing from pARP-9 that are normally involved in shutting *Adh* off.

Interaction of Sgs-4 and Adh regulators

The *Adh* regulatory activities present in tARP-9 are retained in the tGARP transformants and in two cases seem to interact with the regulatory activities brought in by the *Sgs-4* sequences. These cases of interaction were unexpected because one or both of the regulators must be active in the wrong tissue or at the wrong time.

In the first case, the *Sgs-4* enhancement activity is able to work in the midgut and fat body at a time and in a tissue in which *Sgs-4* itself is not expressed. This result implies that *trans*-acting factors necessary for activity of the *Sgs-4* enhancer are present in midgut and fat body. We cannot yet tell whether such factors normally interact with the enhancer in these tissues even though the *Sgs-4* gene does not become active, or whether in the tGARP constructs it is interaction with the *Adh* sequences that makes the *Sgs-4* enhancer accessible to the postulated *trans*-acting factors.

The second case of interaction between *Adh* and *Sgs-4* regulatory sequences is shown by the expression of the tGARPs in second-instar salivary glands, a tissue in which neither *Sgs-4* nor *Adh* expression has been detected. One interpretation of this result, that *Sgs-4* is normally expressed at a low and previously undetected level in second-instar salivary glands and that *Adh* simply provides a sensitive assay for this expression, appears to be ruled out. A construct in which the entire *Sgs-4* sequence from -840 to +1 is fused to the body of the *Adh* gene (from +2 onward), expresses *Adh* in third-instar salivary glands as well as do the tGARP transformants, but shows no detectable *Adh* in second-instar salivary glands (K.Flynn, unpublished results). Therefore, we favor an alternative explanation, that a temporal regulatory sequence which normally activates *Adh* during this time period combines with the *Sgs-4* tissue specificity sequence to give a low level of second-instar salivary gland expression.

Expression of the GARP constructs in the proventriculus also indicates an interaction between *Sgs-4* and *Adh* sequences. While slight amounts of *Adh* activity can be seen in a band around the broadest part of the proventriculus in Oregon R, more extensive staining is seen in the tGARP larvae, and the pattern of staining varies depending on the plasmid construct used. The tGARP-212 lines characteristically show *Adh* activity throughout the proven-

tricus in second instar but only rarely in third instar. The tGARP-301 lines, which contain the same *Sgs-4* sequence but in a different location, have a distinctly different pattern. They show discrete staining in the anterior half of the proventriculus at both second and third instars. The proventriculus from tGARP-1, however, stains very much like that of Oregon R at both stages. Thus, expression in the proventriculus shows a special kind of position effect. The spatial and temporal expression depends not on the chromosomal location of the inserted transposon, but on the position and perhaps orientation of the *Sgs-4* sequence relative to the *Adh* sequences within the transposon.

These cases of interaction demonstrate that the three regulatory activities of the *Sgs-4* enhancer need not act in concert. For example, in midgut or fat body the enhancement activity is independent of timing or tissue specificity. In second-instar salivary glands the tissue specifier can act without the timing element. We imagine that these results reflect the binding of individual proteins to the enhancer. To investigate this possibility further we have begun characterizing proteins that, *in vivo* or *in vitro*, bind to this region.

Materials and methods

Fly strains

The recipient strain used for injections was *Adh^{h6} cn; ry⁵⁰⁶*. *Adh^{h6}* is an *Adh* null which produces low levels of stable RNA, but no CRM⁺ (Benyajati *et al.*, 1982). *ry⁵⁰⁶* is a null-allele of *ry* (Cote *et al.*, 1986). Balancer stocks for the second chromosome, *CyO/nub b Sco II; stw³ ry⁵⁰⁶*, and the third chromosome, *ry⁵⁰⁶/In(3LR)TM3, Li p⁹ sep ry⁵⁰⁶ bx^{34c} Sb e⁺*, were gifts from B. Wakimoto. Mutations are as specified in Lindsley and Grell (1968), except for *ry⁵⁰⁶*, an EMS-induced mutation of *In (3LR)TM3* constructed by R. Karess.

DNA constructions

The initial plasmid into which *Sgs-4* sequences were inserted was pARP-11 which contains a 8.1-kb *SalI* fragment including the *ry⁺* gene and a 4.6-kb *EcoRI/XhoI* fragment containing the *Adh^F* gene. These two genes are inserted between P element ends (Figure 1). Before *Sgs-4* DNA was added, the 5' end of the *Adh* DNA was removed by digestion with *SalI* and *HpaI*. This removes 274 bp of the intron between the proximal and distal promoters and all *Adh* sequences further 5' including the distal promoter. Remaining are 380 bp upstream of the proximal promoter and the entire *Adh* structural gene. For pGARP-1 and pGARP-212 the *Sgs-4* DNA was inserted between these *SalI* and *HpaI* sites. To construct pGARP-301 and the control plasmid pARP-9, these sites were fused after filling in the *SalI* overhang. For pGARP-301, *Sgs-4* DNA was then inserted at the *Clal* site 1.8 kb downstream from the proximal cap site.

The *Sgs-4* DNA came from the plasmid pCS.85 which includes, from the Canton S strain, the 845-bp *BamHI/EcoRI* fragment flanking *Sgs-4* on its 5' side. The *BamHI* site was converted to a *SalI* site by attachment of a synthetic linker and the resultant *SalI/EcoRI* fragment was inserted in pBR322 to produce pCS.85. For pGARP-1 this *SalI* site was used to excise the *SalI/AhaIII* fragment (−839/−105 of *Sgs-4*), while for pGARP-212 and pGARP-301 a shorter *DdeI* fragment (−568/−150) was used. The resulting constructs are diagrammed in Figure 1.

Germ line transformation

Transformation was carried out essentially according to Rubin and Spradling (1982). Early embryos (0–90 min) from the *Adh^{h6} cn; ry⁵⁰⁶* strain were injected with the pGARP or pARP constructs at 50 µg/ml and the defective P element helper pII25.7wc (Karess and Rubin, 1984) at 100–200 µg/ml. Adults derived from injected embryos (GO) were mated individually to flies from the *Adh^{h6} cn; ry⁵⁰⁶* recipient strain, and *ry⁺ G1* flies were selected. These flies were mated individually to the recipient strain to determine whether the inserts were X-chromosomal or autosomal. X-chromosomal inserts were made homozygous by repeated pair matings. Autosomal inserts were made homozygous following crosses to the two balancer strains listed above.

Histochemical assay

Tissues were dissected in *Drosophila* Ringers (7.5 g/l NaCl, 0.35 g/l KCl, 0.28 g/l CaCl₂·2H₂O) and stored at 4°C for no more than 1 h. The tissue samples were then partially fixed in 1% glutaraldehyde (15 min) on ice, washed with ice cold Ringers (3×, 10 min), and then treated for 15 min at room temperature with *Adh*-specific stain (0.24 M Tris–HCl, pH 8.2, 0.5 mg/ml nitroblue tetrazolium, 0.02 mg/ml phenazine methosulfate, 0.5 mg/ml NAD⁺, 0.27 M 2-butanol; Ur-

sprung *et al.*, 1970). Control samples from which the 2-butanol substrate was omitted showed no staining.

Enzyme activity measurements

Enzyme activity measurements were done essentially as described by Maroni (1978). Larvae were staged by collecting embryos from a population cage (5000–10 000 flies) over 4 h into a hard grape medium (Elgin and Miller, 1978). The larvae were fed fresh yeast suspension and grown at 25°C, 80% humidity. First-instar larvae were collected at 36 h after oviposition. For other samples, the larvae were collected 72 h post-oviposition and late second-instar individuals were selected by the morphology of their anterior spiracles which had a fuzzy, fan-like appearance. Some of these were assayed for late second-instar time points while the remainder were returned to the grape medium with a drop of yeast and put at 25°C, 80% humidity. After 2 h, 0–2-h-old third-instar larvae were selected by the diagnostic finger-like projections of their anterior spiracles. For later time points third-instar larvae were aged 8–48 h on normal cornmeal and molasses food with a drop of yeast.

Enzyme measurements were made on larvae washed free of food and stored at 4°C (0–45 min). Five to ten whole larvae were homogenized in 500 µl of buffer (0.1 M Tris–HCl, pH 8.5, 1 mM EDTA, 7 mM βME). For dissected larvae, 5–15 anterior midguts or pairs of salivary glands were homogenized in 500 µl of buffer. The homogenate was left on ice for 30 min, then transferred to 1.5-ml Eppendorf tubes and the debris pelleted by a 15-min spin in an Eppendorf microfuge at 4°C. The supernatant was transferred to another tube and then assayed for enzyme activity at 25°C. All activity measurements were made on fresh tissue. The remaining supernatant was usually frozen for later protein analysis. Protein measurements were made by the Bradford microprotein assay (Bradford, 1976) with BSA as a standard. Protein reagent was purchased from BioRad.

Acknowledgements

We thank James Posakony for providing the pARP-11 plasmid, the tARP-9 line of transformed flies and the transformation recipient line *Adh^{h6} cn; ry⁵⁰⁶*. This work was supported by grants from the Department of Energy and the National Science Foundation. S.W.B. was supported by the Damon Runyon–Walter Winchell Cancer Fund.

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Received on 19 September 1986; revised on 29 October 1986