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A Transcriptional Switch between the *Pig-1* and *Sgs-4* Genes of *Drosophila melanogaster*

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***Pig-1* and *Sgs-4* are a pair of closely linked and divergently transcribed *Drosophila melanogaster* genes, which are both expressed in larval salivary glands but at different times during development. While *Sgs-4* is expressed at high levels only at the end of the third instar, *Pig-1* exhibits a major peak of expression during late second and early third instar. Thus, *Pig-1* expression declines as *Sgs-4* expression is induced. In this paper, we show that three adjacent elements located within the short region between these genes can account for the switch from *Pig-1* to *Sgs-4* expression. A 170-bp segment acts as an enhancer to direct *Sgs-4* expression in late-third-instar salivary glands. A 64-bp sequence located just upstream from the enhancer can modify its temporal specificity so that it works throughout the third instar. Expression induced at mid-third instar by a combination of these two elements can be repressed by a negative regulatory sequence located still further upstream. We present evidence suggesting that the changing interactions between these regulatory elements and the *Sgs-4* and *Pig-1* promoters lead to the correct pattern of expression of the two genes.**

The development of multicellular organisms requires the expression of both structural and regulatory genes at the correct time and in the correct cell type. Regulation of this expression is at least partially achieved at the level of transcription and involves *cis*-acting sequences with positive or negative effects on gene expression. Although a large number of these sequences have been shown to induce gene expression (reviewed in reference 1) or restrict it to a given subset of tissues (7, 38), only a few sequences mediating temporal regulation have been found (32, 44, 50, 53). To identify such elements, we chose to study the 832-bp intergenic region of the two *Drosophila melanogaster* genes *Sgs-4* and *Pig-1* (see Fig. 1A). These two genes exhibit the same spatial pattern of expression, both being expressed slightly in the larval proventriculus and abundantly in the salivary glands (3), but have different temporal patterns. During third instar, high-level expression switches from *Pig-1* to *Sgs-4* (see Fig. 1B) (3, 9, 27). Since these genes are divergently transcribed and since regulatory sequences controlling both tissue specificity and high-level expression had been identified in the region between them (3, 27, 28, 34, 48), it seemed likely that further dissection of this region would identify some of the temporal regulators responsible for the switch.

Sgs-4 is one of seven glue protein genes which are expressed in the salivary glands at the end of the third instar, in response to the steroid hormone ecdysone (6, 25, 29). This response appears to be mediated, at least in part, by the broad-complex (*BR-C*), an ecdysone-responsive locus involved in pupariation and metamorphosis. The *BR-C* encodes a family of zinc-finger-containing proteins with the potential to activate transcription by directly binding DNA (14). In larvae that are mutant for the *BR-C*, the glue genes are expressed at very low levels (13, 24). The products of the glue genes are glycoproteins which are secreted into the lumen of the salivary glands and then expelled from the mouth of the larva to attach the newly-formed pupal case to a dry surface (19). The *Sgs-4* gene is transcribed at very low levels throughout early larval development (3). Beginning in the middle of the third instar, *Sgs-4* transcription is induced dramatically so that by late third instar, the level of *Sgs-4* transcripts has increased 3,000- to 5,000-fold (3), thereby accounting for the large amount of *Sgs-4* protein present in the glue. Just before pupariation, transcription of the gene decreases and its expression is very low in pupae and adults. *Pig-1*, whose function is currently unknown, is expressed at high levels in the salivary glands but with a developmental pattern of expression different from that of *Sgs-4*. *Pig-1* RNA is expressed throughout early larval development, with a major peak occurring in late second and early third instar (9, 27, 43). We show that during the third instar, *Pig-1* RNA levels decline progressively, suggesting that transcription has been turned off.

A critical regulatory region which appears to carry all the information for salivary gland-specific expression has been previously localized between 567 and 149 nucleotides 5′ to the *Sgs-4* transcription initiation site (48). Using a series of truncations in this sequence, Jongens and colleagues identified three separate elements, referred to as I, II, and III, which interacted to direct expression to late-third-instar salivary glands (28). However, these experiments used a transient expression assay (33), which monitors tissue-specific expression only and cannot reveal temporal or quantitative regulation.

We have now examined whether these elements carry any

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timing control sequences that might account for the differential developmental expression of *Pig-1* and *Sgs-4*. For this purpose, various combinations of elements I, II, and III, as well as an adjacent sequence which we call element IV (see Fig. 1A), were tested by germ line transformation for their ability to direct salivary gland-specific gene expression at several times during the third instar, when high-level expression switches from *Pig-1* to *Sgs-4*. Our results indicate that elements I, II, and III carry out different, temporally distinct functions but do not suggest a role for element IV in the switch. Element III, located between −434 and −264 with respect to the *Sgs-4* transcription initiation site, acts as a classical enhancer, is necessary for *Pig-1* expression, and can by itself specify the correct *Sgs-4* pattern of expression. Element II, located just upstream of element III (between −498 and −434), is able to modify the element III enhancer to make it active at earlier developmental times. Element I (−567 to −498) carries a negative regulatory sequence which blocks the enhancer activity of the II/III combination during the second half of third instar. Since element III carries by itself all the information for the normal late-third-instar expression of *Sgs-4*, we suggest that the addition of elements I and II serves to direct element III activity to the *Pig-1* gene at early developmental times and then switches it back to *Sgs-4* during late third instar.

MATERIALS AND METHODS

Fly strains and germ line transformation. The *Adh* null stock (ACR: Adh^{fn6}, *cn*; ry^{506}) used for injections and the balancer stocks used in the production of homozygous transformants were described previously (48). P element-mediated transformation (45) was carried out as described previously (48).

Developmental staging. Embryos were collected from minicage stocks (100 to 200 flies) for 2-h periods on grape medium supplemented with yeast (15). After 20 h, the newly-hatched first-instar larvae were transferred to cornmeal plates and grown at 25°C. At approximately 72 h postovoposition, late-second-instar larvae were selected and transferred to yeasted grape plates. Two hours later, newly molted, 0- to 2-h-old third instar larvae were selected by the diagnostic finger-like projections of their anterior spiracles and transferred back to yeasted cornmeal medium plates. Larvae were then collected at 14 ± 2 or 24 ± 2 h after the second molt. At 40 h, the larvae are less synchronous and only the wandering animals were collected; at 50 h, the larvae were selected on the basis of the bloated morphology of their salivary glands.

DNA constructions. The *Pig-1/lacZ* fusion was constructed starting with the plasmid pC4βgal (49a), which contains an AUG-less and promoterless *Escherichia coli lacZ* gene fused to splicing and polyadenylation sequences from simian virus 40 t antigen. This plasmid was linearized by partial *Eco*RI digestion at the fourth codon of the *lacZ* gene, and the ends were filled in with the Klenow fragment of DNA polymerase I. p4.6 (43) contains DNA corresponding to the *Pig-1* coding and upstream sequences, from +3 to −1527 (relative to *Sgs-4*). Digestion of p4.6 with *Hin*cII yields an 873-bp fragment containing the intergenic region, the *Pig-1* promoter, and the first three codons of the *Pig-1* gene. This fragment was ligated into the digested and filled pC4βgal. This resulted in pPigβgal, a translational fusion of the first three codons of *Pig-1* fused to the *lacZ* gene and polyadenylation site. An *Eco*RI fragment from pPigβgal extending from +3 (the *Sgs-4* start site) to the 3′ side of the polyadenylation signal was excised, filled in, and cloned into the *Sma*I site of pUC119Δ*lac* (43), which is a pUC119 derivative lacking the *lacZ* gene. The resulting plasmid is p*lacZ*/+3, which was used to generate the truncation constructs. Constructs p*lacZ*/−221 and p*lacZ*/−265 were constructed by *Bal* 31 exonuclease treatment of p*lacZ*/+3 starting at the *Eco*RI site at +3. Construct p*lacZ*/−388 was made by truncating p*lacZ*/+3 at the *Xho*I site at −388. Construct p*lacZ*/−504 was made by *Bal* 31 deletion starting at the *Xho*I site. All constructs were then cloned into the P element transformation vector Carnegie 20 (45a) for injection as described below.

The several combinations of elements I, II, III, and IV were excised from the corresponding pAF plasmids (28) and inserted either into the unique *Sal*I site or into the unique *Xba*I site of the Carnegie 20-derived vector, pG0S. This plasmid carries the *Hpa*I-*Eco*RI fragment of Adh^{F} (42), which includes the *Adh* structural gene along with 380 nucleotides of upstream sequence and 640 nucleotides of the 3′ sequence; in this construct, the *Adh* gene is driven by its own proximal promoter. The ry^{+} gene, which serves as a dominant selective marker, is transcribed in the same direction as the *Adh* gene.

The −838/*Adh* construct was previously described as pGARP838 (3). The −567/*Adh* construct, a truncated version of −838/*Adh*, was obtained by a two-step procedure. First, the *Sal*I (−838)-*Xho*I (−391) fragment of pGAfun (3) was replaced with the *Sal*I (−567)-*Xho*I (−391) fragment of pAF −567:−274 (28); then, the *Sal*I-*Xba*I fragment of the resulting plasmid was used to replace the *Sal*I-*Xba*I fragment of −838/*Adh*. The −498/*Adh* construct was prepared by the same strategy, but by using the *Sal*I (−498)-*Xho*I (−391) fragment of pAF −498:−274 (28). The −445/*Adh* construct was obtained by replacing the *Sgs-4 Sal*I-*Xba*I fragment from −838/*Adh* with the *Sal*I-*Xba*I fragment of pUC −445:+1. This latter plasmid was constructed by replacing the *Sal*I-*Eco*RI fragment of pGAfun (3) with the *Sau*3AI (−443)-*Eco*RI (+1) fragment of the *Pig-1/Sgs-4* intergenic region; this fragment was first made blunt-ended by filling in the 5′ overhangs in a Klenow reaction, and then *Sal*I linkers were added and the resulting fragment was finally cut out with both *Eco*RI and *Sal*I.

The pGEM-SGS5 plasmid carries a 600-nucleotide *Eco*RI-*Hin*dIII fragment isolated from the *Sgs-5* subclone paDm1687 (49) and cloned into the *Eco*RI-*Hin*dIII sites of pGEM2 (Promega). The pGEM-PIG1D plasmid was constructed by cloning a 640-nucleotide *Hpa*II fragment (−1002 to −363 of the *Sgs-4* transcription initiation site) into the *Acc*I site of pGEM1 (Promega). pGEM-SGS4 was obtained by cloning the *Eco*RI (−3)-*Hin*dIII (+738) fragment of *Sgs-4* into the *Eco*RI-*Hin*dIII sites of pGEM1 (Promega).

Antibody staining for *lacZ* expression. Larvae carrying *Pig-1/lacZ* reporter constructs were hand-dissected and stained as described previously (41). Tissues were incubated overnight with a commercial rabbit anti-β-galactosidase antibody (5 prime-3 prime, inc.) and then with horseradish peroxidase-conjugated goat anti-rabbit antibody.

Histochemical staining for *Adh* expression. Larvae were hand-dissected and stained for *Adh* as described previously (28, 52).

RNA preparation and RNase protection assay. RNA was extracted from 10 to 15 animals by using the procedure described previously (3). Antisense RNA probes were prepared as described previously (35). Before transcription, the SP6-MEL DNA (17) was digested with *Hin*dIII, resulting in an ~600-nucleotide probe, and pGEM-SGS5 was linearized

with *Hin*dIII, resulting in an ~600-nucleotide probe. pGEM-PIG1D was digested with *Hin*cII, which cuts the *Pig-1* sequences 171 nucleotides after the transcription initiation site, resulting in an ~700-nucleotide probe, and pGEM-SGS4 was digested with *Dde*I, resulting in an ~235-nucleotide probe. The transcription reactions contained 100 μCi of [^{32}P]CTP for the *Adh*, *Sgs-4*, and *Pig-1* probes and 10 μCi of [^{32}P]CTP for the *Sgs-5* probe. The ribonuclease protection assay was performed as described previously (2), with the following modifications: the samples were not preheated at 85°C, and all RNase digestions were done at 37°C for 15 min. Protected RNA fragments were separated on a 6% polyacrylamide gel in 1× TBE (Tris-borate-EDTA buffer). ^{32}P-labeled DNA fragments from the 1-kb DNA ladder (Bethesda Research Laboratories) were used as molecular weight markers.

RESULTS

The transcriptional switch from *Pig-1* to *Sgs-4* occurs in mid-third instar. To determine when high-level expression shifts from *Pig-1* to *Sgs-4* (3, 9), RNA levels for both genes were measured throughout the third instar. Synchronized larvae were collected 14, 24, 40, and 50 h after the second molt, and their salivary gland RNA was analyzed for the presence of *Pig-1* and *Sgs-4* transcripts. As shown in Fig. 1B, *Pig-1* is expressed at high levels in early third instar (14 h after the second molt); then, its expression decreases progressively until pupation (50 h). In contrast, high levels of *Sgs-4* RNA can only be detected during the second half of the third instar (Fig. 1B) (3, 39). Thus, *Pig-1* expression appears to be turned down just before the appearance of high levels of *Sgs-4* RNA, suggesting that a transcriptional switch between *Pig-1* and *Sgs-4* occurs in the middle of the third instar.

The intergenic region contains the essential regulatory sequences for both *Pig-1* and *Sgs-4*. Previous studies have shown that the intergenic region between *Pig-1* and *Sgs-4* (−838 to +1) is sufficient to direct transcription from the *Sgs-4* promoter in late-third-instar salivary glands (3, 34). To localize the essential *Pig-1* regulatory sequences, the left end of the intergenic region (−838) was fused to the *E. coli lacZ* gene. As shown in Fig. 2A, the intergenic region was then truncated from its right end so that the minimal requirements for *Pig-1* expression could be determined. Both anti-β-galactosidase antibodies (Fig. 2B) and histochemical staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (data not shown) were used to assay expression of the reporter gene. As shown in Fig. 2B, a construct (*lacZ*/+3) containing the entire intergenic region is correctly expressed in early-third-instar salivary glands. Removal of the sequences near the *Sgs-4* promoter (construct *lacZ*/−221) does not affect expression. Construct *lacZ*/−264, which removes element IV but leaves element III intact, is also sufficient for expression. However, animals carrying construct *lacZ*/−388, which removes part of element III, do not express β-galactosidase in early-third-instar salivary glands. Removal of both elements II and III, as in construct *lacZ*/−504, has the same effect on expression. These results indicate that the essential *Pig-1* regulatory sequences are indeed contained in the intergenic region and that an intact element III is required for transcription of *Pig-1*, as it is for *Sgs-4*.

Combinations of elements I, II, and III can have either positive or negative effects on salivary gland gene expression in third-instar larvae. Since *Pig-1* and *Sgs-4* share regulatory sequences but differ in their timing of expression, we wanted to localize the sequences responsible for temporal regulation of the two genes. To assay for temporal specificity, various combinations of the *Sgs-4* enhancer elements were inserted either 5′ or 3′ to the alcohol dehydrogenase reporter gene (*Adh*), driven by its own proximal promoter (Fig. 3A). On its own, this promoter directs *Adh* expression to larval fat bodies and anterior midgut, the two tissues in which *Adh* is normally transcribed (52), but is not able to direct expression in salivary glands. Therefore, with this series of constructs we have assayed the ability of the *Sgs-4* sequences to add salivary gland expression to the basal fat body-anterior midgut activity of the *Adh* sequences. The chimeric *Adh*/*Sgs-4* constructs were subcloned into a P element-derived vector (Fig. 3A) and inserted into the genome of *Adh* mutant flies via P element-mediated germ line transformation (45). Using an RNase protection assay, we assayed stable transformants for *Adh* RNA expression in salivary glands that were dissected free of adhering fat body.

In the first series of experiments, *Adh* expressed from the recombinant constructs was measured at the end of the third instar, when *Sgs-4* is transcribed at high levels. RNA extracted from salivary glands was hybridized to two probes, *Adh* and *Sgs-5*. As *Sgs-4* and *Sgs-5* are induced and re-

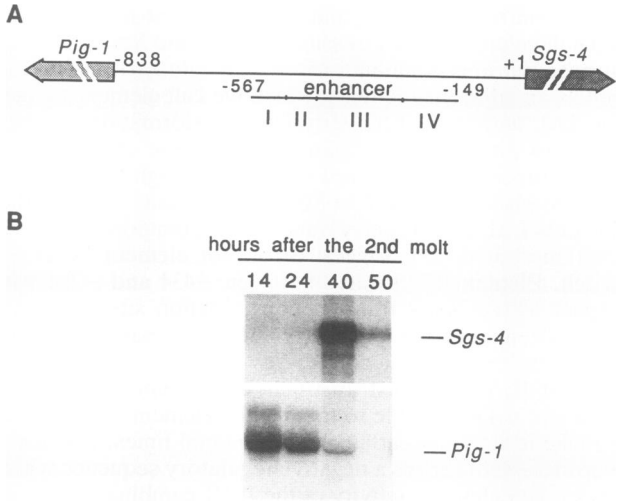

FIG. 1. *Pig-1* and *Sgs-4* are divergently transcribed genes whose expression changes reciprocally during the third instar. (A) The *Pig-1*/*Sgs-4* intergenic region. Arrows indicate the transcription units, with 3′ ends marked by the arrowheads. The position of the regulatory sequence (enhancer) previously defined (48) is indicated, as well as the three salivary gland-specific elements I, II, and III (described in reference 28). Also indicated is element IV, which corresponds to the remaining part of the regulatory region (48). +1 is the *Sgs-4* transcription initiation site. (B) *Sgs-4* and *Pig-1* RNA levels during the third instar, as measured by RNase protection. Larvae were staged and collected as described in Materials and Methods, and their salivary glands were carefully dissected. Since the amount of DNA per salivary gland increases during the third instar (about 1.5 DNA replication cycles are observed during this period [46]), the amount of RNA loaded per lane was adjusted to the amount of DNA. Thus, we used RNA extracted from six salivary glands at 14 h after the second molt, four salivary glands at 24 h, and two salivary glands each at 40 and 50 h. Each RNA sample was hybridized to two probes, pGEM-SGS4 and pGEM-PIG1D (see Materials and Methods), which were radioactively labeled to the same specific activity. The *Sgs-4* transcript protects a 228-nucleotide fragment of the pGEM-SGS4 probe, and *Pig-1* RNA protects 125 nucleotides of the pGEM-PIG1D probe.

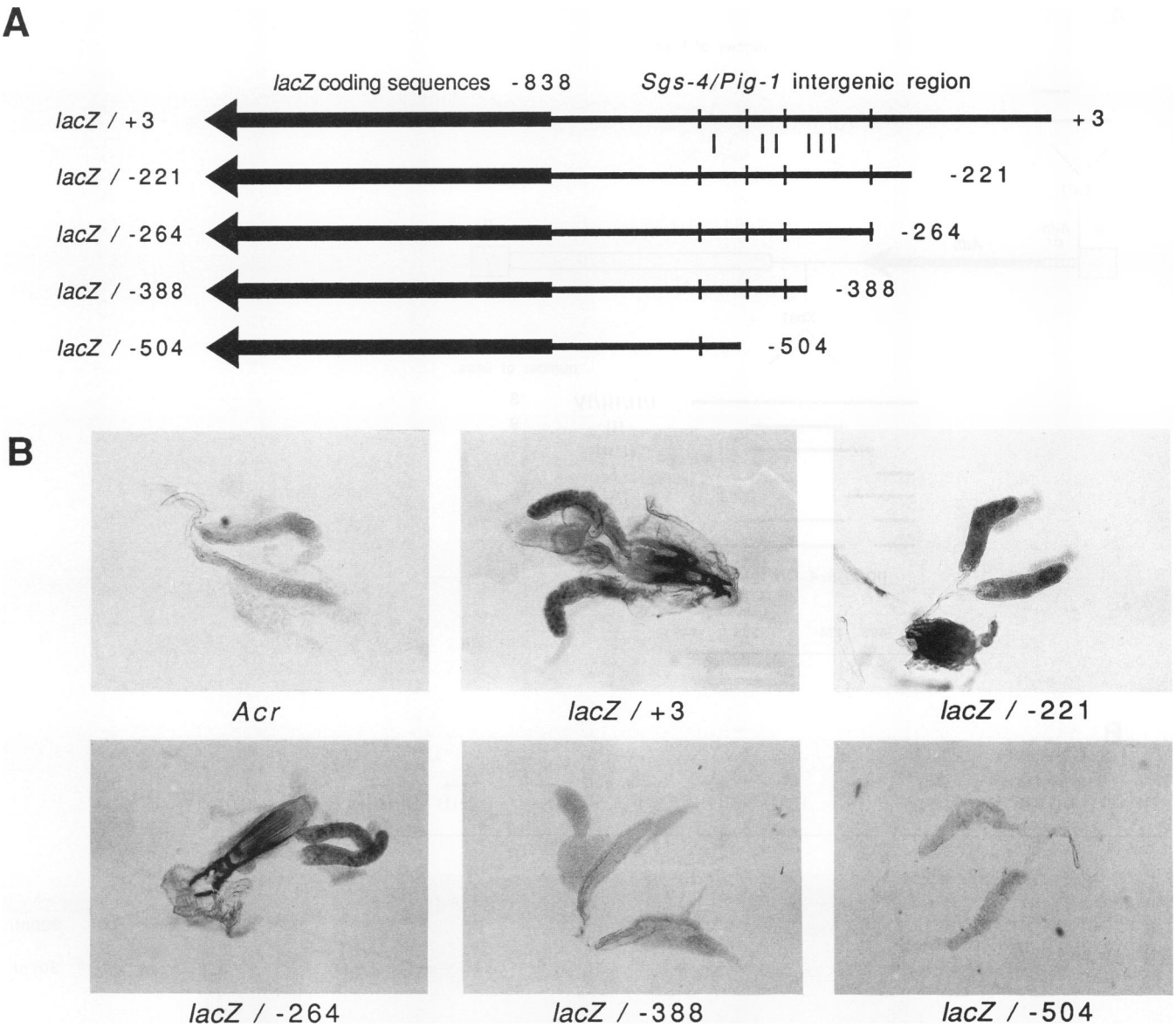

FIG. 2. Regulatory sequences required for *Pig-1* expression. (A) Constructs used to delimit the essential *Pig-1* regulatory sequences. The transcribed *Pig-1/lacZ* fusion is represented by a thick arrow, and the *Pig-1* upstream sequences are represented by thin lines. (B) Dissected salivary glands from larvae carrying the indicated constructs, stained with an antibody against the β-galactosidase protein. For each construct, two independent lines were tested.

pressed at the same times during the third instar, *Sgs-5* serves as a loading and timing control for the *Sgs-4/Adh* constructs. As shown in Fig. 3B, only three constructs, I/II/III/IV, II/III, and III are able to direct *Adh* expression to late-third-instar salivary glands. These results are somewhat different from those previously obtained with the transient assay (28). In the current experiments, I/II, I/III, and I/III/IV show little or no expression although they all were scored as positive in the transient assay (28). Such discrepancies between the results from transient and stable transformation have been reported a number of times, in both cultured cells and transgenic animals (23, 37, 40, 51). In transient experiments, the number of copies per cell of the gene to be studied cannot be controlled. Cells scored as positive might thus contain multiple copies of a construct expressed at very low levels. Moreover, a fraction of the DNA copies might be sufficient to bind all molecules of a scarce regulatory protein, thereby allowing artifactual expression from the remaining DNA copies. Since in stably transformed lines the gene of interest is present at only one copy per haploid genome and is integrated in the cellular chromatin as endogenous genes are, we believe that the stably transformed lines are more likely to reflect normal regulatory interactions.

Since all of the positive constructs contain element III and one of them carries only element III, this sequence, located between 434 and 264 nucleotides from the *Sgs-4* transcription initiation site, appears to carry all the information needed for expression in late-third-instar salivary glands. Levels of *Adh* expression in transformants with element III alone are similar to those obtained with elements I/II/III/IV

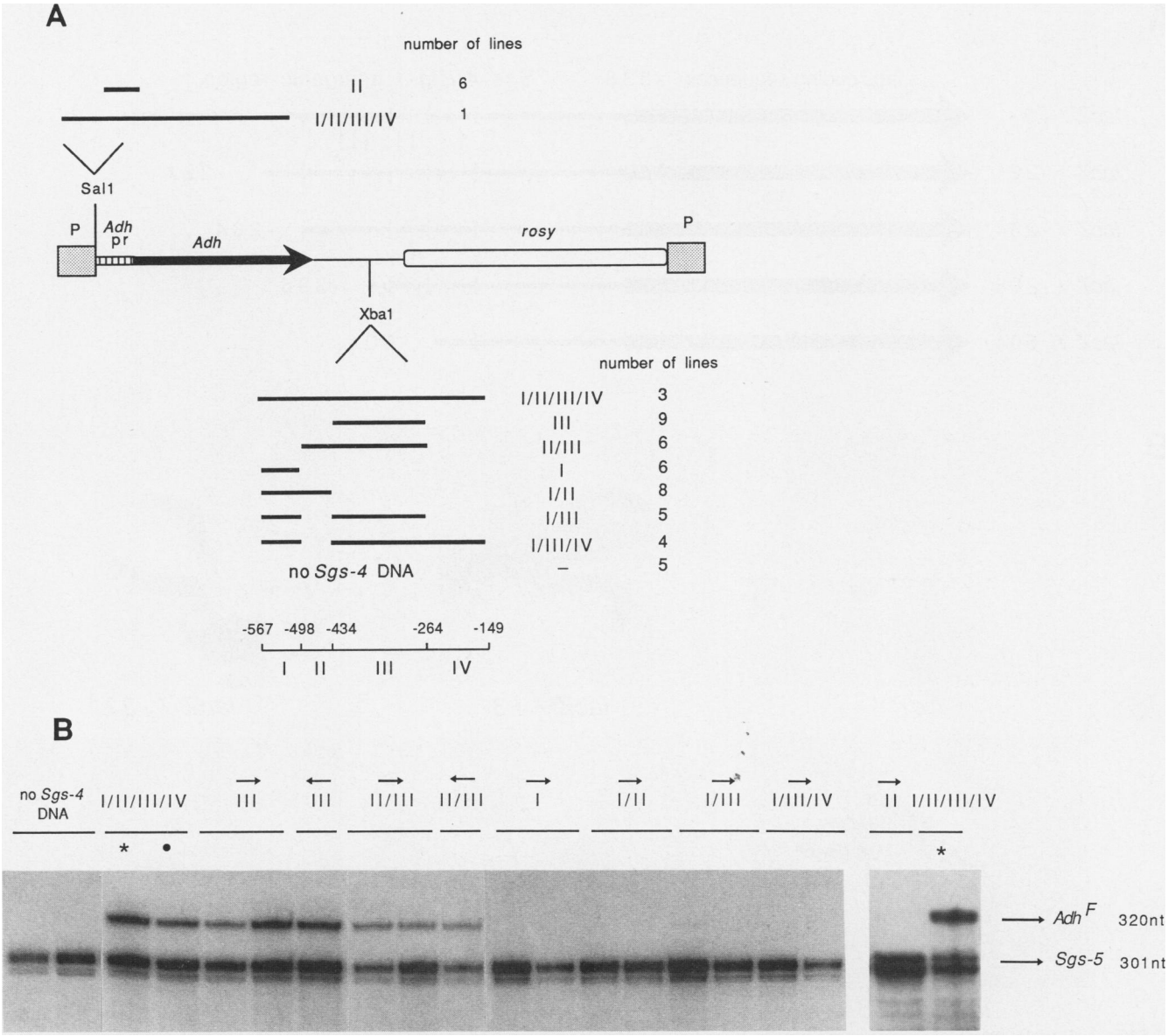

FIG. 3. Regulatory elements required for late-third-instar expression. (A) Constructs used to examine the substructure of the *Sgs-4* regulatory region. For each construct, the combination of Sgs-4 enhancer elements is indicated along with the number of independent transformant lines which have been tested. The numbers on the bottom line indicate the limits of the four elements, with +1 representing the *Sgs-4* transcription initiation site. The transcribed *Adh* sequence (thick arrow), the *Adh* promoter (hatched bar), the *Adh* 3′ sequences (thin lines), the P element transposon ends (stippled boxes), the *rosy* gene (open bar), and *Sgs-4* sequences which have been inserted either 5′ or 3′ to *Adh* (thick lines) are shown. (B) *Adh* transcripts in salivary glands of late-third-instar larvae stably transformed with the various constructs. RNA prepared from dissected salivary glands was assayed for *Adh* transcripts by RNase protection. Each lane represents an independent transformed line. For each line, RNA extracted from three salivary glands was hybridized to the ^{32}P-labeled probes SP6-MEL, complementary to the *Adh* transcript (16), and pGEM-SGS5, complementary to the *Sgs-5* transcript (see Materials and Methods). A protected RNA fragment (320 nucleotides) was diagnostic for the *Adh* transcripts; the protected *Sgs-5* fragment (301 nucleotides) provides an internal loading control. Elements of the *Sgs-4* regulatory region included in each construct are indicated above the appropriate lanes, along with an arrow indicating their orientation relative to the *Adh* transcription unit. ⋆, transformant line tGARP212, which carries the four elements (I, II, III, and IV) inserted 5′ to *Adh* (48); ●, transformant line which carries the four elements inserted 3′ to *Adh* (48).

combined. Moreover, element III has enhancer activity since it is functional in both orientations and when located 3′ of the reporter gene.

To verify that element III is specific for expression in salivary glands as opposed to other tissues, dissected larvae from transformants carrying the vector alone and those carrying the vector plus element III were stained for *Adh* activity (Fig. 4). In both groups, the *Adh* promoter caused expression in the fat body and anterior midgut. Salivary glands were the only additional tissue that could be seen to express *Adh* when element III was included in the construct (proventriculus was difficult to score in these experiments because of normal high-level *Adh* expression in the anterior midgut). Taken together, these results establish that, at the

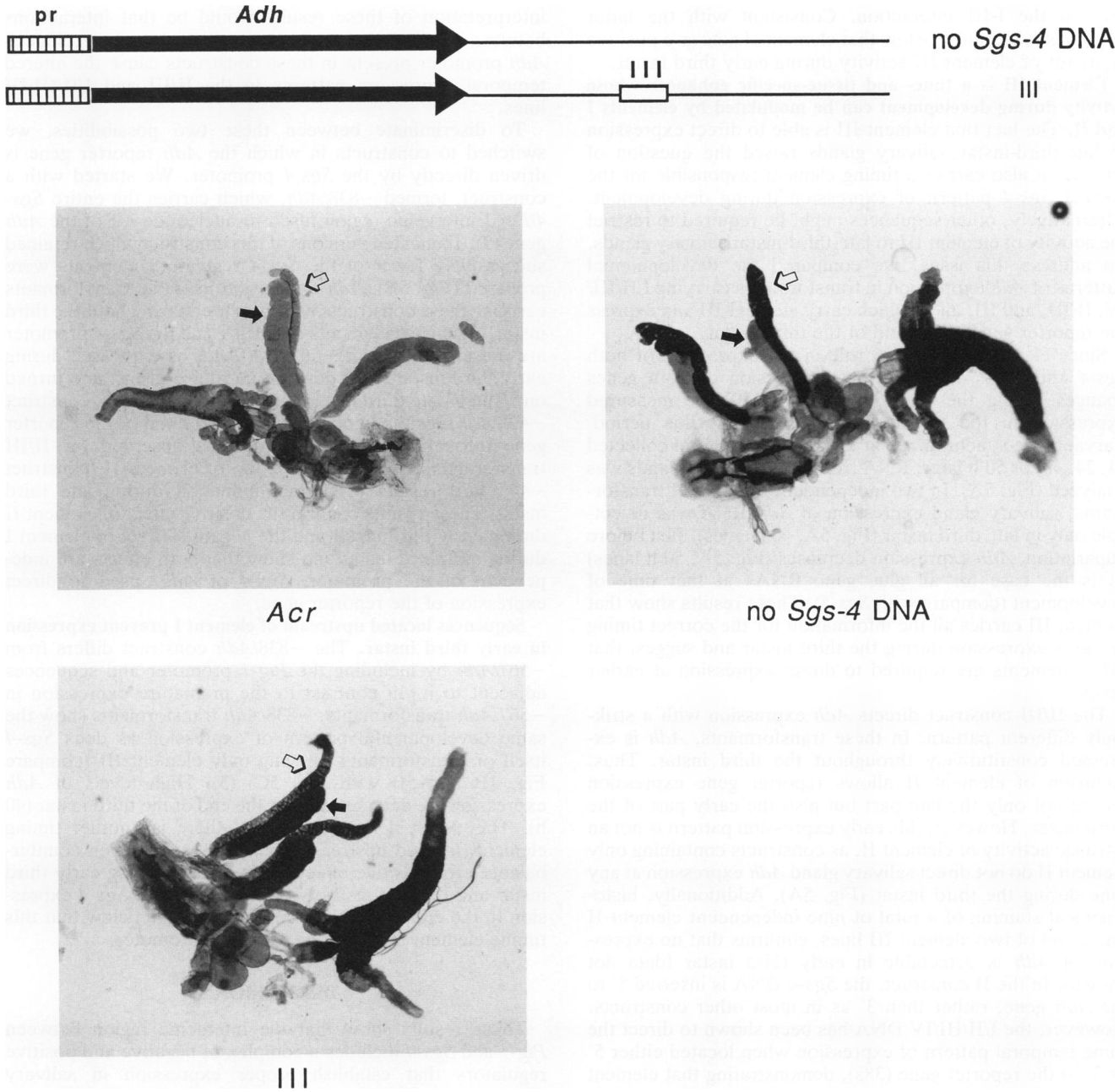

FIG. 4. *Adh* expression patterns in late-third-instar larvae. The relevant constructs are shown at the top of the figure. Late-third-instar larvae were dissected and stained for *Adh* activity, and the patterns of expression for the injection strain or transformant larvae are shown in the lower panels. *Acr*, injection strain (no *Adh* activity is apparent); no *Sgs-4* DNA, *Adh* expression driven by the promoter in the basic construct (note midgut and fat body expression); III, *Adh* expression driven by the basic construct plus element III (note the addition of salivary gland expression to the basal pattern). Salivary glands are indicated by filled arrows, and fat body is indicated by open arrows. Note that although the head skeleton appears black in these photos, this can be easily distinguished from the dark-blue staining seen in transformants and does not represent authentic *Adh* expression.

end of the third instar, element III is, by itself, a salivary gland-specific regulator of *Sgs-4*.

Despite this conclusion, the constructs I/III and I/III/IV, both of which include element III, do not direct *Adh* expression to late-third-instar salivary glands (Fig. 3B). This unexpected result suggests that, since both of these constructs include element I, this element acts negatively to block the action of element III.

Moreover, since the I/II/III/IV combination is active, element II must be able to interfere with the negative action of element I. Element II might act as a spacer to physically separate I and III, or it might bind one or more proteins that

prevent the I-III interaction. Consistent with the latter hypothesis, we show below that element II acts as a positive regulator of element III activity during early third instar.

Element III is a time- and tissue-specific enhancer whose activity during development can be modulated by elements I and II. The fact that element III is able to direct expression to late-third-instar salivary glands raised the question of whether it also carries a timing element responsible for the correct *Sgs-4* pattern of expression during development. Alternatively, other sequences might be required to restrict the activity of element III to late-third-instar salivary glands. To address this issue, we compared the developmental patterns of *Adh* expression in transformants carrying I/II/III/IV, II/III, and III, all of which carry element III and express the reporter gene at the end of the third instar.

Since element III plays a role in the expression of both *Sgs-4* and *Pig-1* and since the expression of both genes changes during the third instar (Fig. 1B), we measured expression in the transformants throughout this period. Larvae were synchronized at the second molt and collected 14, 24, 40, or 50 h later; RNA from their salivary glands was analyzed (Fig. 5A). In two independent element III transformants, salivary gland expression of *Adh* RNA was detectable only in late third instar (Fig. 5A, 40 h lanes). Just before pupariation, *Adh* expression decreases (Fig. 5A, 50 h lanes) as is the case for all glue gene RNAs at that time of development (compare with *Sgs-5*). These results show that element III carries all the information for the correct timing of *Sgs-4* expression during the third instar and suggest that other elements are required to direct expression at earlier times.

The II/III construct directs *Adh* expression with a strikingly different pattern. In these transformants, *Adh* is expressed constitutively throughout the third instar. Thus, inclusion of element II allows reporter gene expression during not only the late part but also the early part of the third instar. However, this early expression pattern is not an intrinsic activity of element II, as constructs containing only element II do not direct salivary gland *Adh* expression at any time during the third instar (Fig. 5A). Additionally, histochemical staining of a total of nine independent element II lines, and of two element III lines, confirms that no expression of *Adh* is detectable in early third instar (data not shown). In the II construct, the *Sgs-4* DNA is inserted 5′ to the *Adh* gene, rather than 3′ as in most other constructs. However, the I/II/III/IV DNA has been shown to direct the same temporal pattern of expression when located either 5′ or 3′ to the reporter gene (38a), demonstrating that element II is active in either position and that the inactivity of the II construct is not due to location of the *Sgs-4* DNA. Thus, the activity seen at early and mid-third instar in the II/III transformant lines results from the combination of elements II and III.

I/II/III/IV transformant lines show a third pattern of expression. *Adh* is expressed in early third instar (14 h), repressed in the middle of the third instar, and induced again at the end of third instar (Fig. 5A). We believe that the mid-third-instar repression is due to the presence of element I rather than element IV, since, as previously noted, element I by itself can repress the activity of element III in late third instar (Fig. 3B).

From these results, we infer that element II carries a positive regulatory sequence which modifies the properties of the element III enhancer during early third instar. Moreover, element I and/or IV can suppress the positive effect of element II in the middle of the third instar. An alternative interpretation of these results would be that interactions between the *Sgs-4* enhancer elements and the heterologous *Adh* promoter present in these constructs cause the altered temporal expression patterns in the II/III and I/II/III/IV lines.

To discriminate between these two possibilities, we switched to constructs in which the *Adh* reporter gene is driven directly by the *Sgs-4* promoter. We started with a construct, termed −838/*Adh*, which carries the entire *Sgs-4*/*Pig-1* intergenic region fused to nucleotide +1 of the *Adh* gene (3). Truncated versions of this construct which retained successively fewer of the *Sgs-4* regulatory elements were prepared (Fig. 5B). *Adh* expression in stable transformants carrying these constructs was measured throughout the third instar. When just elements I/II/III/IV and the *Sgs-4* promoter are present (construct −567/*Adh*), *Adh* is expressed during early third instar, shut down at mid-third instar, and turned on again in late third instar. Removal of element I (construct −498/*Adh*) results in constitutive expression of the reporter gene throughout the third instar, as observed for II/III transformants. Additional deletion of element II (construct −445/*Adh*) results in normal induction during late third instar. These results confirm the positive effect of element II during early third instar and the negative effect of element I during mid-third instar and show that both effects are independent of the promoter, *Sgs-4* or *Adh*, used to direct expression of the reporter gene.

Sequences located upstream of element I prevent expression in early third instar. The −838/*Adh* construct differs from −567/*Adh* by including the *Pig-1* promoter and sequences adjacent to it. In contrast to the premature expression in −567/*Adh* transformants, −838/*Adh* transformants show the same developmental pattern of expression as does *Sgs-4* itself or transformants carrying only element III (compare Fig. 1B and 5B with Fig. 5C) (3). High levels of *Adh* expression are detected only at the end of the third instar (40 h). This result demonstrates that there is another timing element, located upstream from element I, which counterbalances the positive effect of element II during early third instar and thereby restricts the high levels of *Sgs-4* expression to the end of the third instar. We argue below that this timing element may well be the *Pig-1* promoter.

DISCUSSION

These results show that the intergenic region between *Pig-1* and *Sgs-4* includes a complex of negative and positive regulators that establish proper expression in salivary glands. In the sections that follow, we discuss separately each of the three characterized regulatory elements and then propose a model for their interactions during the third instar.

Element III: an enhancer with both spatial and temporal specificity. Element III directs the expression of the reporter gene to salivary glands, with a developmental pattern similar to that of the intact *Sgs-4* gene. High levels of expression are detected only at the end of the third instar, indicating that element III carries all the information for induction in late third instar and repression before pupariation. Acting in concert with element II, element III is also a required enhancer element for the *Pig-1* gene. Like the sevenless, *Adh*, and chorion gene enhancers previously described for *D. melanogaster* (5, 11, 32), element III carries information for both tissue specificity and correct timing during development. Whether these two different functions can be separated is currently under study, but several lines of evidence already suggest that the coordinate action of several *trans*-

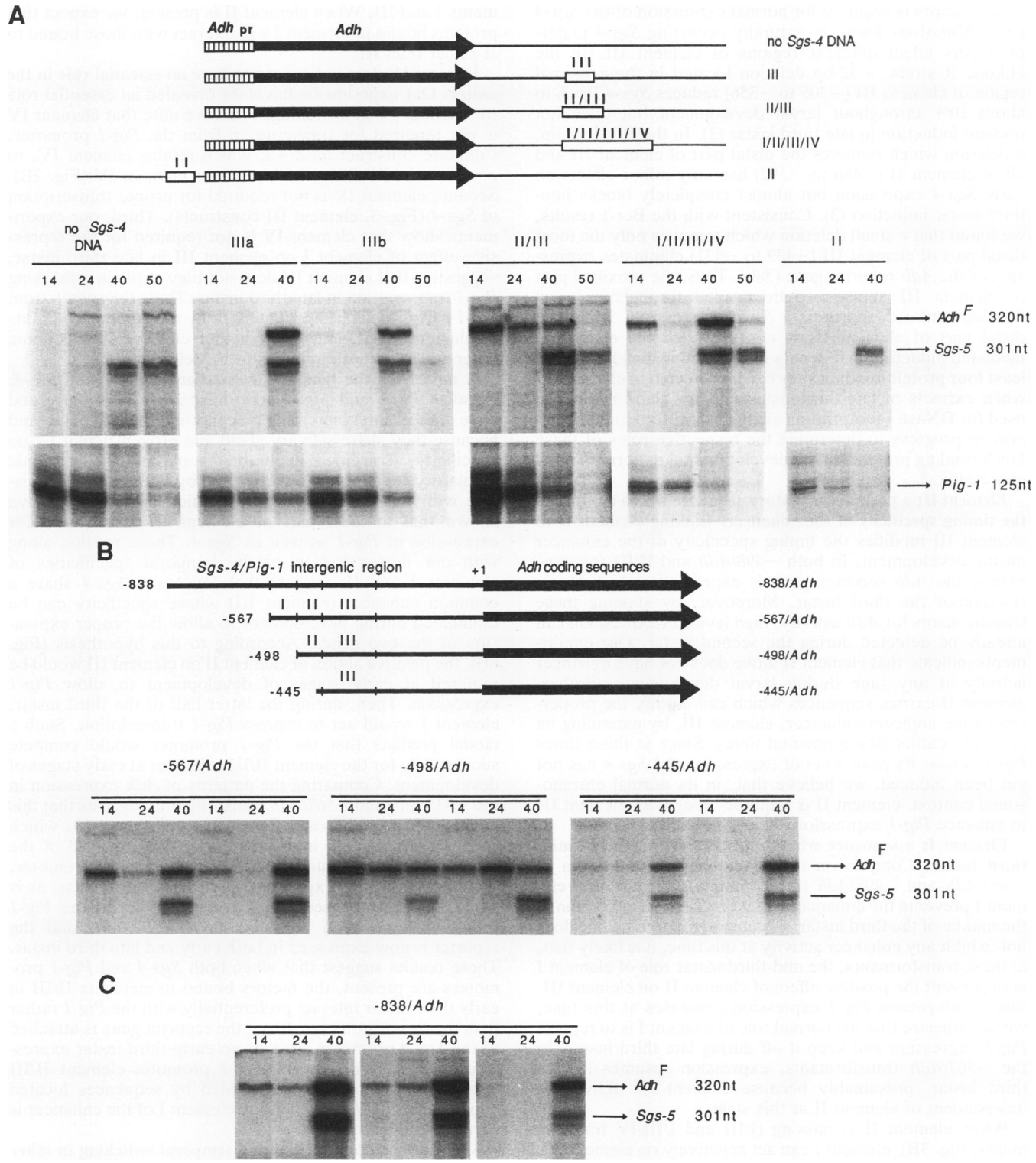

FIG. 5. Expression throughout the third instar of the *Adh* reporter gene in the salivary glands of transformed larvae. Larvae were staged and collected as described in Materials and Methods, and their salivary glands were carefully dissected. As in Fig. 1B, the amount of RNA loaded per lane was adjusted to the amount of DNA; we thus used RNA extracted from six salivary glands at 14 h after the second molt, from four salivary glands at 24 h, and two salivary glands each at 40 and 50 h. Each RNA preparation was assayed by RNase protection after hybridization to three probes, SP6-MEL (for the *Adh* transcripts), pGEM-SGS5 (for *Sgs-5*), and pGEM-PIG1D (for *Pig-1*). The last two probes were used as controls for correct timing. The *Pig-1* data are shown only for panel A. (A) Transformants in which *Adh* is driven by its own promoter. Transformed lines carrying the *Adh* gene either without any *Sgs-4* sequences or with the *Sgs-4* elements III, II/III, I/II/III/IV, and II were analyzed; IIIa and IIIb represent two independent lines carrying the III construct. The expression pattern shown is representative of the several lines tested for each construct. For a summary, see Fig. 6A. (B) Transformants in which *Adh* is driven by the *Sgs-4* promoter: truncated derivatives of −838/*Adh*. Two independent lines are shown for each construct. (C) Three independent lines carrying the −838/*Adh* construct.

acting factors is required for normal expression of the *Sgs-4* gene. Mutations found in naturally occurring *Sgs-4* underproducers affect different regions of element III. In the Hikone R strain, a 52-bp deletion located in the proximal region of element III (−305 to −356) reduces *Sgs-4* RNA to about 10% throughout larval development but does not prevent induction in late third instar (3). In the Ber-1 strain, a deletion which removes the distal part of element III and all of element II (−486 to −392) has only minor effects on early *Sgs-4* expression but almost completely blocks late-third-instar induction (3). Consistent with the Ber-1 results, we found that a small deletion which removes only the most distal part of element III (−439 to −421) eliminates expression of the *Adh* reporter gene (38b). Thus, the proximal part of element III appears to be needed throughout larval development for appropriate *Sgs-4* expression, while the distal part of element III is necessary for the ecdysone-mediated induction that occurs in the third instar. Finally, at least four protein-binding sites can be detected in element III when extracts of late-third-instar salivary gland nuclei are used for DNase I footprinting analysis (54). Experiments are now in progress to determine the respective roles of these DNA-binding proteins in the developmental pattern of *Sgs-4* expression.

Element II: a positive regulatory sequence which modulates the timing specificity of the enhancer. Linking element II to element III modifies the timing specificity of the enhancer during development. In both −498/*Adh* and II/III transformants, the *Adh* reporter gene is expressed at high levels throughout the third instar. Moreover, by staining these transformants for *Adh* activity, high levels of expression can already be detected during the second instar. Our experiments indicate that element II alone does not have enhancer activity at any time during larval development. Rather, element II carries sequences which can modify the properties of the adjacent enhancer, element III, by extending its action to earlier developmental times. Since at these times *Pig-1* is near its peak level of expression and *Sgs-4* has not yet been induced, we believe that, in its normal chromosomal context, element II is required along with element III to enhance *Pig-1* expression.

Element I: a sequence which represses expression in mid-third instar. Comparison of the pattern of expression in −567/*Adh* and −498/*Adh* transformants indicates that element I prevents the enhancer effect of a II/III combination in the middle of the third instar. Because element III alone does not exhibit any enhancer activity at this time, it is likely that, in these transformants, the mid-third-instar role of element I is to prevent the positive effect of element II on element III. Since endogenous *Pig-1* expression decreases at this time, we hypothesize that the normal role of element I is to repress *Pig-1* expression and keep it off during late third instar. In the −567/*Adh* transformants, expression resumes in late third instar, presumably because element III activity is independent of element II at this stage.

When element II is missing (I/III and I/III/IV transformants; Fig. 3B), element I can act negatively on element III. One possible explanation for this result would be that the abnormal juxtaposition of I and III allows a protein bound to element I to physically occlude the binding of a crucial factor to element III. However, we do not think this explanation is correct, because when extracts of late-third-instar salivary glands are used to footprint either I/III/IV or I/II/III/IV, the same footprints are seen in element III (55). Thus, the negative effect of element I is more likely to occur by interactions between proteins bound independently to elements I and III. When element II is present, we expect that proteins bound to element I will interact with those bound to II rather than III.

Element IV does not appear to have an essential role in the switch. Our experiments have not revealed an essential role for element IV in switching. First, we note that element IV is not required for transcription from the *Pig-1* promoter. Compare construct *lacZ*/+3, which retains element IV, to construct *lacZ*/−264, which removes element IV (Fig. 2B). Second, element IV is not required for proper transcription of *Sgs-4* (Fig. 3, element III constructs). Third, our experiments show that element IV is not required for the repressive effect of element I on element III in late third instar, suggesting that element IV does not play a role in repressing *Pig-1* transcription in late third instar. Thus, the switch from *Pig-1* to *Sgs-4* can be accounted for by the combined actions of elements I, II, and III. Whether element IV has some other regulatory role remains to be determined.

A model for the temporal regulation of *Pig-1* and *Sgs-4*. Because *Pig-1* and *Sgs-4* are transcribed divergently and have a short intergenic region separating their 5′ ends, and because they share salivary gland and proventricular tissue specificity, it seemed likely that some of the intergenic regulatory sequences would be used by both genes. Consistent with this idea, our transformation experiments have shown that at least part of element III is required for expression of *Pig-1* as well as *Sgs-4*. These results, along with our demonstration of the temporal specificities of elements I and II, suggest that *Pig-1* and *Sgs-4* share a common enhancer (element III) whose specificity can be modulated during development to allow the proper expression of the two genes. According to this hypothesis (Fig. 6B), the positive action of element II on element III would be required at early stages of development to allow *Pig-1* expression. Then, during the latter half of the third instar, element I would act to repress *Pig-1* transcription. Such a model predicts that the *Pig-1* promoter would compete successfully for the element II/III enhancer at early stages of development. Comparing the patterns of *Adh* expression in the −838/*Adh* and −567/*Adh* transformants suggests that this might indeed be the case. In a −838/*Adh* construct, which carries the complete intergenic region and thus all of the *Pig-1* upstream regulatory sequences including its promoter, the reporter gene is expressed only in late third instar, as is *Sgs-4*. In contrast, sequences corresponding to the *Pig-1* promoter have been removed from −567/*Adh*, and the reporter is now expressed in both early and late third instar. These results suggest that when both *Sgs-4* and *Pig-1* promoters are present, the factors bound to elements II/III in early third instar interact preferentially with the *Pig-1* rather than the *Sgs-4* promoter. Since the reporter gene is attached to the *Sgs-4* promoter, little or no early-third-instar expression is seen. Whether the *Pig-1* promoter–element II/III interaction is direct or is mediated by sequences located between the *Pig-1* promoter and element I of the enhancer is currently under study.

Shared regulatory regions and temporal switching in other genes. A variety of regulatory strategies have been adopted by pairs or groups of genes that are near each other and that share some aspect of regulation, such as tissue specificity. There are several cases of adjacent genes that share a common regulatory region. For two pairs of divergently transcribed *Drosophila* genes, the yolk protein genes *yp-1* and *yp-2* and the glue genes *Sgs-7* and *Sgs-8*, both genes in the pair are expressed in the same tissues and at the same times during development (4, 8, 20–22, 26, 36). In these

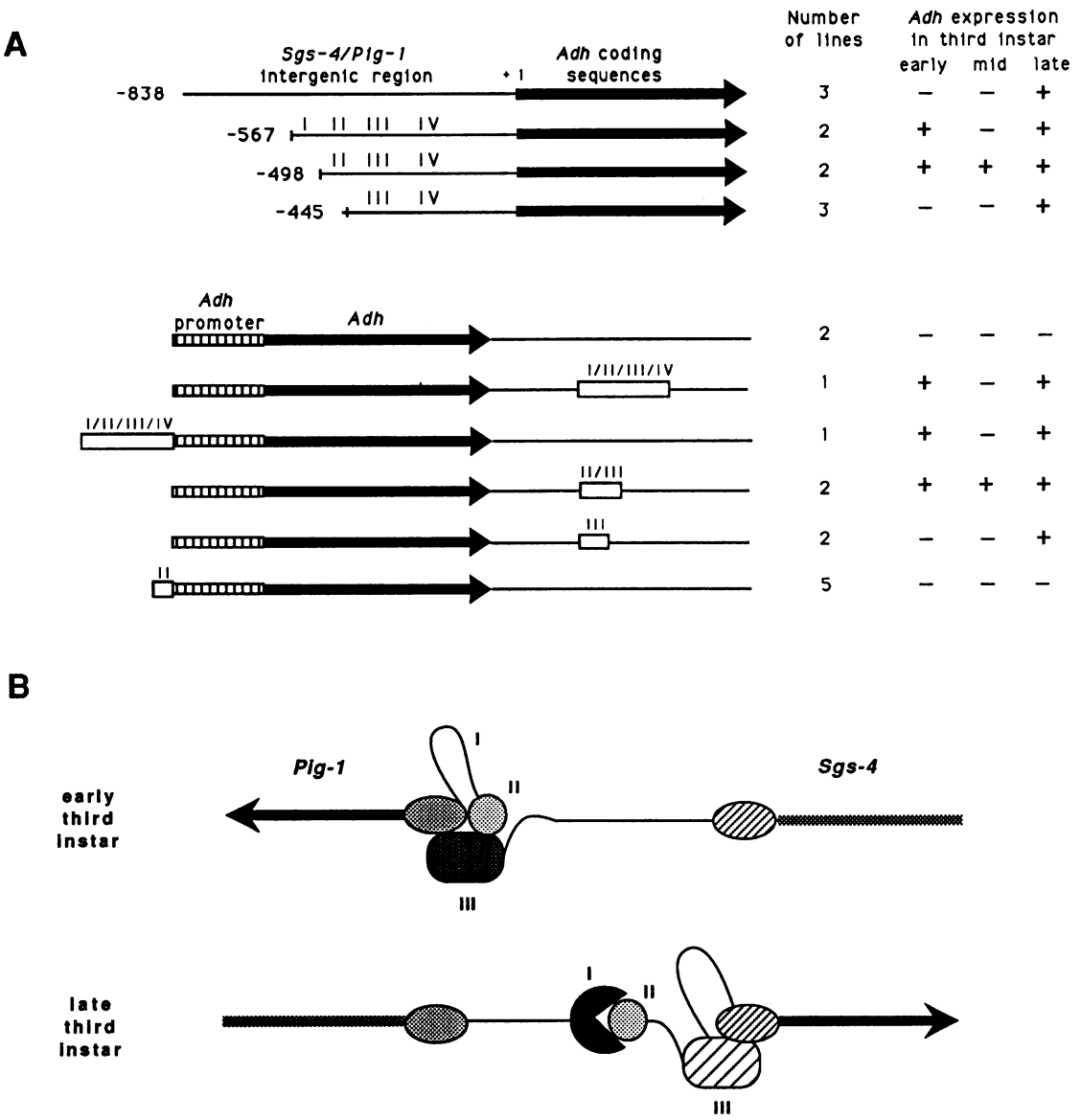

FIG. 6. Temporal regulation of *Pig-1* and *Sgs-4*. (A) Summary of the *Adh* pattern of expression throughout the third instar in the various transformed lines. Early, mid, and late correspond to 14, 24, and 40 h, respectively, after the second molt. Expression of the *Adh* reporter gene at high levels (+) or at very low or undetectable levels (−) is indicated. (B) Model for the switch between *Pig-1* and *Sgs-4* expression during the third instar. Proteins bound to elements I, II, and III are indicated by Roman numerals, and the *Pig-1* and *Sgs-4* promoters are indicated by ovals. We propose that during early third instar, proteins bound to elements II and III interact with proteins bound to the *Pig-1* promoter to direct *Pig-1* expression. Although the II/III protein complex can also interact with the *Sgs-4* promoter, the interaction is strongly biased towards *Pig-1*. However, if the *Pig-1* promoter is missing, as it is in many of our constructs, the II/III complex can interact productively with the *Sgs-4* promoter. Halfway through the third instar, one or more proteins bind to element I and prevent elements II and III from activating the *Pig-1* promoter. Since element III is required for late-third-instar expression of *Sgs-4* and since its function is independent of element II at this time, we propose that a I/II complex forms and releases element III so that it can interact with and activate the *Sgs-4* promoter. In addition, some of the factors binding to element III must change at this stage because constructs that have only this element show proper *Sgs-4* timing. Since we know that repressor proteins are still bound to element I at the end of the third instar (a I/III construct is not expressed), it is likely that these proteins are still required at that time to prevent *Pig-1* transcription.

cases, the genes react similarly to enhancers located between them, even when, for *yp-1/yp-2*, the intergenic region includes multiple enhancers with different specificities (30, 31).

The *Drosophila* chorion genes represent a different case: tightly clustered genes that have identical tissue specificity but are nonetheless independently regulated. These genes are tandemly, rather than divergently, arranged, and for those that have been characterized (*S36* and *S15*), each has its own tissue-specificity and timing regulators near the 5′ end of the transcript (32, 44). Interestingly, temporal restriction of *S15* expression to the final stages of choriogenesis occurs by a combination of a negative regulator that keeps it off early and a positive regulator that turns it on late (32).

For the *Drosophila Adh* gene, there is a pair of differentially regulated promoters, rather than differentially regu-

lated genes. Because of differential splicing, RNAs originating from the two promoters encode the same protein. As for *Pig-1* and *Sgs-4*, there is a switch between the two promoters in mid-third instar. The more proximal promoter is used during early larval stages, whereas the distal promoter is used during late third instar and in adults (47). However, the mechanism of the *Adh* promoter switch, transcriptional interference, is quite different from that used by *Pig-1*/*Sgs-4*. In mid-third instar, the distal promoter becomes active, apparently by the stage-specific activity of transcription factors that bind to it or to its enhancer (11). The proximal promoter is then repressed by transcriptional read-through from the distal promoter (12).

Regulation of the transition from embryonic to adult β-globin expression in chickens resembles the *Pig-1*/*Sgs-4* transition more closely. The adult β- and embryonic ε-globin genes are adjacent, transcribed from the same strand, and share an enhancer located approximately equidistant from their promoters. The transition from ε to β occurs by a competition between the two promoters for the enhancer element. At early stages, the ε promoter interacts preferentially with the enhancer, leading to ε-globin expression. After birth, the preference shifts to the β form. The sequence necessary for this transition, known as the stage selector element (10), maps to the β-globin promoter. Several transcription factor-binding sites have been identified within the stage selector element, and mutations in some of these sites both reduce β-globin expression and increase ε-globin expression (18). Thus, it is likely that at the time of the switch a protein binding to the stage selector element appears and increases the affinity of the β-globin promoter for the enhancer. As a result, this promoter wins the competition and β-globin expression ensues.

The *Pig-1* and *Sgs-4* promoters seem to be involved in a similar competition during early third instar. As shown by the −567/*Adh* and −498/*Adh* constructs in which the *Pig-1* promoter is absent, the *Sgs-4* promoter can be activated at this early time. However, when the *Pig-1* promoter is present, as in the normal context or in the −838/*Adh* construct, only *Pig-1* undergoes high-level expression. The fact that *Sgs-4* is expressed at a low level during embryonic and early larval development (3) may indicate that the II/III enhancer occasionally interacts productively with the *Sgs-4* promoter.

In the middle of the third instar, two changes which shift the competition in favor of *Sgs-4* expression occur in the regulatory machinery. First, the interaction of element III and the *Sgs-4* promoter becomes much more effective, leading to *Sgs-4* induction. This change is seen most clearly in transformants III and −445/*Adh*, in which *Sgs-4* is induced normally even though elements I and II are absent. Since the same induction is seen with two different promoters (*Sgs-4* and *Adh*), we think that, in contrast to the β-globin switch, there are changes in the proteins bound to the enhancer rather than in those bound to the promoters. A product of the *BR-C*, which appears in salivary glands at the time of the switch, has recently been found to bind some of the sites in element III (54). Second, element I function changes, leading to a negative interaction between I and II. This change destroys the active II/III combination that had been directing *Pig-1* expression. As a result, *Pig-1* transcription stops as *Sgs-4* is induced.

In summary, the developmental switch between *Pig-1* and *Sgs-4* expression appears to use a dual mechanism, one part of which disrupts a previously productive transcription complex while the other establishes a new active complex. To understand this mechanism more thoroughly, we must now identify and characterize the proteins that flip the switch.

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