The promoter of DNA puff gene II/9-1 of *Sciara coprophila* is inducible by ecdysone in late prepupal salivary glands of *Drosophila melanogaster*

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DNA puffs occur in Sciarid salivary gland chromosomes; they are sites of DNA amplification and intense transcription and they appear to encode secreted structural proteins needed for pupation. In this report we have used P-element transformation of *Drosophila* to study regulation of a *Sciara* DNA puff gene. We found that a 718-bp promoter fragment of DNA puff gene II/9-1 from *Sciara coprophila* directs expression of the bacterial reporter gene CAT in late prepupal salivary glands of transgenic *Drosophila melanogaster*. The identical tissue and analogous stage specificity indicate that some aspects of the ecdysone response are evolutionarily conserved between *Drosophila* and *Sciara*. When transgenic salivary glands are cultured in vitro, CAT activity is rapidly induced by ecdysone, suggesting direct control of gene expression by the ecdysone receptor. Putative stage-specific factors limit expression of the chimeric *Sciara*-CAT gene in transgenic *Drosophila* to late prepupae but not to third instar larvae when ecdysone titers are also high.

Introduction

In many dipteran insects the polytene chromosomes of late larval and prepupal salivary glands undergo decondensation at a few distinct loci resulting in cytologically visible swellings or puffs that are tissue and stage specific (Beermann, 1952). Recently it was found that puffing and RNA transcription are independent events despite their association (Korge, 1987; Meyerowitz *et al.*, 1987). Sciarid flies are unique in that they also contain DNA puffs that are sites not only of RNA synthesis (Gabrusewycz-Garcia and Kleinfeld, 1966) but also DNA amplification (Rudkin and Corlette, 1957; Crouse and Keyl, 1968; Rasch, 1970; Glover *et al.*, 1982). Because

puffing is under strict developmental control, genes within puffs are favorable candidates to study tissue and stage-specific control of gene expression, and in the case of DNA puffs, gene amplification as well.

In *Drosophila*, the sequence of puffing observed in the salivary gland chromosomes of late third instar larvae and prepupae is divided into 21 puff stages (Ashburner, 1967, 1969) comprising six main groups: the intermolt and the early- and late-responding larval puffs and the mid-prepupal and the early- and late-responding late prepupal puffs (Figure 1). The small group of intermolt puffs is active during most of the third larval instar and contains the glue protein genes; most of the intermolt puffs regress when the ecdysone titer starts to rise near the end of the third larval instar. At that time, about half a dozen early-responding larval puffs appear and are followed by ~100 late-responding larval puffs. A second cycle of puffing, comparable with the larval cycle, begins a few hours after puparium formation. This prepupal puffing sequence starts with the small group of mid-prepupal puffs that, like the larval intermolt puffs, are active while ecdysone levels are low in the animal. A presumed rise in the ecdysone titer toward the end of the prepupal stage initiates puffing in late prepupae, first of the early-responding puffs followed by the late-responding puffs.

The entire sequence of larval puffing can be induced in vitro when *Drosophila* intermolt salivary glands are cultured in the presence of ecdysone (Ashburner, 1972–1974; Ashburner *et al.*, 1974; Ashburner and Richards, 1976). However, transition from the larval to the prepupal puff sequence does not occur in vitro unless salivary glands are placed in an ecdysone-free culture medium and allowed to carry out protein synthesis. During this period, the mid-prepupal puffs expand, and competence to respond to ecdysone is acquired. A subsequent exposure of competent salivary glands to ecdysone induces puffing of the early- and late-responding puffs of late prepupae (Richards, 1976a–c).

Figure 1. Schematic representation of the six groups of larval and prepupal RNA puffs in *Drosophila* and corresponding period in *Sciara*. (Top) Each bar indicates the developmental stage during which members of that group typically puff in *D. melanogaster* (modified after Richards, 1980). The developmental stages are indicated by hours with respect to puparium formation (PF) that occurs about 120 h at 25°C after fertilization (Fraenkel and Bhaskaran, 1973). Ap marks the beginning of larval/pupal apolysis and Ev marks head evagination. Hatched bars represent puffs that are active when ecdysone titers are presumed low (intermolt and mid-prepupal puffs); black bars represent puffs that are active when ecdysone titers are presumed high (early- and late-responding puffs of larvae and late prepupae). Dots on the left side of bar 1) and the right side of bar 6) indicate that these two groups of puffs may be active beyond the stages shown in the figure. (Bottom) The corresponding period is shown for *S. coprophila* (modified after Rasch, 1970). Eyespots, which are the anlage to the adult eye, appear toward the end of the fourth larval instar; E, M, and L represent early-, mid-, and late-eyespot, respectively. Ec denotes ecdysis. The fourth larval instar lasts 13 d at 17°C in females.

Further studies on cultured salivary glands led to a model of how ecdysone regulates puffing (Ashburner *et al.*, 1974). Because the early-responding larval and prepupal puffs are activated by ecdysone in a rapid and dosage-dependent fashion and without a need for protein synthesis, the model proposes that they are directly induced by the ecdysone receptor. It further postulates that the protein products of the early-responding puffs cause regression of their own puffs while the ecdysone titer is still high. Because hormonal induction of the late-responding puffs is delayed and requires protein synthesis, it is hypothesized that the protein products of the early-responding puffs are also needed to release the inhibitory effect by the ecdysone receptor on the late-responding puffs. The late-responding puffs are believed to encode protein products that allow metamorphosis to proceed.

The Ashburner model regained publicity (Ashburner, 1990) in recent reports describing the molecular cloning of DNA derived from three early-responding RNA puffs: 2B5, 74EF, and 75B (Chao and Guild, 1986; Burtis *et al.*, 1990 and Thummel *et al.*, 1990; Segraves and Hogness 1990; respectively). The genes located at these loci have a complex structure with multiple transcription units encoding different proteins, some of which appear to be DNA binding proteins with nuclear localization and the ability to bind to ecdysone-inducible puffs. For example, it has recently been shown that the E74A protein encoded by the 74EF puff binds to both early- and late-responding puffs (Urness and Thummel, 1990).

Many early-responding puffs appear both in late third instar larvae and late prepupae; however, there are only a few examples of early-responding puffs that are unique to prepupae and absent from larvae (e.g., 93F). It is hypothesized that a protein repressor restricts this category of early-responding puffs to just one developmental stage (Richards, 1976c). There have been no reports describing molecular cloning of early- or late-responding puffs that are unique to just late third instar larvae or to late prepupae of *Drosophila*; therefore, until now, it has not been possible to test the molecular basis for stage specificity of hormonal induction.

In this work we present evidence that in transgenic *Drosophila* ecdysone-regulated activation of the promoter of DNA puff gene II/9-1 of *Sciara coprophila* fused to the reporter gene CAT has the characteristics of an early-responding RNA puff of *Drosophila melanogaster* unique to the late prepupal stage. DNA puff II/9A of *Sciara coprophila*, the fungus fly, is one of the largest puffs, appearing earliest in the DNA puffing sequence that occurs just before pupation (Figure 1) (Gabrusewycz-Garcia, 1964; Rasch, 1970). Unlike the cloned *Drosophila* genes from early-responding larval and prepupal RNA puffs (2B5, 74EF, and 75B) that encode regulatory proteins, DNA puff gene II/9-1 seems to encode a structural protein. Isolation and characterization of DNA sequences derived from DNA puff locus II/9A demonstrated the presence of two genes, II/9-1 and II/9-2, with 85% sequence identity to each other (DiBartolomeis and Gerbi, 1989). Both genes have a simple overall structure; they are ~1.1 kb long, contain a short intron in the 5' part of the gene, and encode only one transcription unit. DNA sequence analysis suggests that they encode secretory proteins with α -helical coiled coil structures that may be

Figure 2. Schematic representation of the *Sciara*-CAT P-element construct. The *Sciara* promoter fragment (–616 to +102 relative to the cap site indicated by an open box) was linked to the SV40-CAT gene (the CAT gene, marked by a black box, is flanked by 5' and 3' untranslated sequences of SV40 marked by a solid line, and includes an intron). The P-element ends (P) are shown as vertically hatched boxes, and the selectable marker gene *rosy* is depicted by a diagonally hatched box. Directions of transcription of the *rosy* gene and the hybrid SV40-CAT gene are indicated by arrows. This schematic map is not drawn to scale.

needed for pupation (DiBartolomeis and Gerbi, 1989). During the late fourth larval instar, the two genes undergo an ~20-fold amplification before a burst in transcription in the salivary glands just before pupation resulting in massive amounts of puff-specific mRNA (DiBartolomeis, Wu, Liang, Smith, and Gerbi, unpublished data).

We report here that the promoter of *Sciara* DNA puff gene II/9-1 drives expression of the reporter gene CAT exclusively in late prepupal salivary glands of *Drosophila* P-element transformants. In vitro studies on isolated salivary glands from transgenic *Drosophila* show a rapid induction of CAT activity by ecdysone, suggesting direct control of gene expression by the ecdysone receptor. In addition, stage-specific trans-acting factors are proposed to restrict hormonal induction to the late prepupal stage in transgenic *Drosophila*. These stage-specific trans-acting factors as well as the ecdysone receptor of *Drosophila* must interact specifically with the 718-bp promoter fragment of *Sciara* DNA puff gene II/9-1, resulting in appropriate tissue and stage-specific expression in transgenic prepupae.

Results

The *Sciara* DNA puff promoter shows stage specificity in *Drosophila*

A 718-bp long promoter fragment of DNA puff gene II/9-1 from *Sciara coprophila* (–616 to +102, relative to the cap site) was linked to the bacterial reporter gene CAT and inserted into a P-element transformation vector (Figure 2). The recombinant plasmid was stably introduced into germ line chromosomes of *Drosophila melanogaster* by P-element transformation, and several homozygous transformed lines were established. All lines carried a single copy insertion as shown by Southern blot analysis of

genomic DNA (Figure 3, shown only for the A-line and C-line) and by in situ hybridization for one line (Figure 3, C-line).

To determine the time period during which the chimeric *Sciara*-CAT gene is expressed in *Drosophila*, CAT assays were carried out on whole transgenic animals at various developmental stages. Figure 4 shows that in two different lines (A-line and C-line) high levels of CAT activity first appear in 9-h prepupae (PP9) and continue at the same high levels throughout the late prepupal and early pupal stages (P15) until they decrease and disappear in 36- to 48-h pupae (P36 and P48). Data mentioned later suggest that the continued high levels of CAT activity after its induction at PP9 reflect CAT stability rather than de novo synthesis throughout this period. No CAT activity can be detected during the early larval stages (L1,2), the late pu-

Figure 3. Single insertion site of the *Sciara*-CAT P-element construct in transgenic *Drosophila*. Southern blot analysis (left panel) was carried out by hybridizing a labeled P-element probe (derived from the downstream P-element end, Figure 2) to *Xho* I digested genomic DNA. The DNA was prepared from 9-h prepupae (PP9) or adult flies (Adult) transformed with the *Sciara*-CAT P-element construct. The single hybridizing *Xho* I fragment is ~8 kb long for the C-line and 10 kb for the A-line, reflecting the location of the next *Xho* I site in the host's DNA beyond the insertion. The fragment sizes of *Hind*III digested λ DNA are shown on the left. The stronger signal for transgenic adult flies (A-line) is due to loading more genomic DNA rather than amplification of *Sciara* specific DNA in adult *Drosophila*. The right panel shows a representative in situ hybridization of polytene chromosomes of transgenic *Drosophila* (C-line) hybridized to a biotin-labeled 5.5-kb probe containing *Sciara* gene I and upstream sequences (see map in Figure 6). The site of insertion at locus 56 AB on chromosome II/R is indicated by an arrow.

Figure 4. Temporal specificity of gene expression in transgenic *Drosophila*. CAT assays are shown for two lines of transgenic *Drosophila* (A-line and C-line). CAT activity was determined in extracts prepared at various developmental stages each from 10 total animals: first and second instar larvae (L1, 2), late third instar larvae (L3, ~115 h old), prepupae (PP), pupae (P), and adults (A). The numbers for the prepupal and pupal stages represent hours after puparium formation (which occurs at PPO) at $25 \pm 1^\circ\text{C}$. Positions of unacetylated chloramphenicol (CAM) and its acetylated forms (ac-1 and ac-3) as well as the origin (ori) on the chromatogram are indicated on the right.

pal (P72), or adult stages (A). In the A-line, very low levels of CAT activity are seen during the late third larval instar and the early- and mid-prepupal stages (L3, ~115 h old; PP0 and PP6). In the C-line, this low level expression is only visible after longer exposure times.

The sharp increase in CAT activity in late prepupae of transgenic *Drosophila* coincides with the proposed increase in ecdysone levels during the prepupal period that induces the early-responding RNA puffs (Richards, 1981). This suggests a positive and stage-specific hormonal regulation of hybrid gene expression in *Drosophila*. As will be discussed later, the prepupal expression of the chimeric gene in *Drosophila* appears to be analogous with the temporal specificity in *Sciara* where there is a rapid increase in mRNA from DNA puff gene II/9-1 shortly before pupation (Figure 1) (DiBartolomeis, Wu, Liang, Smith, and Gerbi, unpublished data).

The *Sciara* DNA puff promoter shows tissue specificity in *Drosophila*

In *Sciara coprophila*, mRNA expression of DNA puff gene II/9-1 is seen only in salivary glands and not in the remaining carcasses (DiBartolomeis, Wu, Liang, Smith, and Gerbi, unpublished data). To test whether the chimeric *Sciara*-CAT gene is expressed with the same tissue specificity in transgenic *Drosophila* as in *Sciara*, CAT activity was determined in tissues dissected from 9-h prepupae, the stage when high levels of CAT gene expression first appear. Figure 5 shows that CAT activity is located in the salivary glands of 9-h prepupae in both transformed lines (A-line and C-line). CAT activity is not detectable in other tissues: cuticle structures and muscles, imaginal discs and brain, and fat body; very low levels of CAT activity were found in the Malpighian tubules and gut. CAT activity is also absent in the salivary glands of 9-h prepupae transformed with a promoterless CAT construct or a CAT construct driven by the promoter of the late chorion gene s15 of *Drosophila* (B. Bienz-Tadmor, Wu, Liang, Smith, and Gerbi, unpublished data). This indicates that CAT activity seen in transformed salivary glands is due to the presence of the *Sciara* DNA puff sequences rather than vector or host sequences. In 24-h pupae, the stage when histolysis of the salivary glands is completed (Bod-

Figure 5. Tissue specificity of gene expression in transgenic *Drosophila*. CAT assays were carried out on extracts prepared from various dissected tissues from 10 transgenic prepupae that were 9 h old. For the A-line, CAT activity was determined in tissues such as the cuticle structures and muscles (CM), Malpighian tubules and gut (MTG), imaginal discs and brain (IDB), fat body (FB), and salivary glands (SG) and in total animals (Tot). For the C-line, CAT activity was determined in carcasses (Ca), prepupae from which the salivary glands were removed, in the salivary glands (SG), and in total animals (Tot). CAT activity was also determined in salivary glands of prepupae transformed with a promoterless CAT construct (SGo) or with a CAT construct carrying a promoter of the chorion gene s15 of *Drosophila* (SGp).

enstein, 1965), CAT activity is associated with undefined cell masses and body fluids and could not be attributed to any of the tissues still discernable at this stage of general metamorphosis (data not shown). Hence, CAT activity seen in 24-h and older pupae is unlikely to be caused by de novo expression of the CAT gene in a novel tissue; rather, CAT activity in intact pupae can be explained by a high stability of the CAT protein before and during the final stages of histolysis of the salivary glands, as will be discussed below.

Therefore, it appears that hybrid gene expression in transgenic *Drosophila* is primarily restricted to the salivary glands, the same and only tissue in which DNA puff gene II/9-1 is expressed in *Sciara*. It should be pointed out that the *Sciara* DNA puff promoter retains the same tissue and analogous stage specificity in transgenic *Drosophila* as in *Sciara*, even though Southern blot analysis (Figure 6) and in situ hybridization (Figures 3 and 10) show no heterologous hybridization between *Sciara* DNA puff gene II/9-1 and the *Drosophila* genome. Nonetheless, hybrid gene expression driven by the *Sciara* DNA puff promoter in transgenic *Drosophila* coincides with the appearance of the early-responding RNA puffs of *Drosophila* during the late prepupal period (Richards, 1976c).

The stage and tissue specificity of hybrid gene expression illustrated for two lines of transgenic *Drosophila* is identical in all four lines that we analyzed (data shown only for the A-line and C-line in Figures 4 and 5), but the quantitative levels of CAT activity varied from line to line. All four lines have independent positions of autosomal integration. This suggests that the observed pattern of expression is due to *Sciara* DNA rather than flanking host sequences, although it appears that there is a position effect on the level of expression of the hybrid gene dependent on its site of chromosomal integration. A position effect on quantitative levels of gene expression has been reported for many other gene constructs in transgenic *Drosophila* (e.g., Mitsialis *et al.*, 1987).

To extend our analysis from the protein to the RNA level, Northern blot analysis and primer extension experiments were carried out. However, neither of the two techniques detected CAT-specific mRNA at any developmental stage (preliminary data not shown) including late prepupae (PP6–9), a time when high levels of CAT activity are present. The inability to detect CAT-specific mRNA in late prepupae may in part be due to a much higher sensitivity of the enzymatic assay for CAT as compared with the sen-

sitivity of the RNA detection experiments. Additionally, it may also indicate that CAT mRNA is expressed indeed only very briefly and hence is present only in a fraction of the 6- to 9-h prepupae from which the RNA was prepared.

DNA from Sciara DNA puff gene II/9-1 is not amplified in transgenic Drosophila

It is unknown where the amplification control elements reside in *Sciara* DNA puff II/9A. However, it seemed likely that the observed CAT activity in transgenic *Drosophila* was due to hybrid gene expression from nonamplified DNA. We have used quantitative Southern blots to show that in fact even the 8.8-kb region of *Sciara* DNA from which the 718-bp promoter fragment was derived does not become amplified in transgenic *Drosophila*. This 8.8-kb region contains DNA puff genes II/9-1 and II/9-2 and flanking sequences. As can be seen in Figure 6, there is no amplification of this DNA in salivary glands from 0- to 1-h prepupae (PP0–1) or from 8- to 9-h prepupae (PP8–9) as compared with carcasses of the corresponding stages or adult flies.

The Sciara DNA puff promoter shows a stage-specific response to ecdysone in vitro

To validate the notion that DNA puff gene II/9-1 of *Sciara* is active in the same manner as the late prepupal RNA puffs of *Drosophila*, its in vitro response to ecdysone was examined in cultured salivary glands of transgenic *Drosophila*. As mentioned earlier, it has been demonstrated that puffing of the late prepupal RNA puffs of *Drosophila* can be induced in competent prepupal salivary glands that are cultured in the presence of 20-OH ecdysone (Richards, 1976c), shown to be the most active form of ecdysone (Ashburner, 1971). Competence to respond to ecdysone is reached in vivo in the salivary glands of 6-h prepupae, a stage when ecdysone levels are deduced to be extremely low (Richards, 1976a). Competence to respond to ecdysone can also be reached in vitro if early prepupal salivary glands are incubated in an ecdysone-free medium before hormonal induction (Richards, 1976b,c).

To test the *Sciara* promoter for a stage-specific response to ecdysone in vitro, salivary glands of transgenic *Drosophila* (A-line) were isolated from several developmental stages and incubated for various periods in the presence (+E) or absence (–E) of 20-OH ecdysone. CAT activity was determined in these glands after the in vitro incubation was completed and com-

Figure 6. DNA from *Sciara* DNA puff II/9A does not amplify in transgenic *Drosophila*. A map of the 8.8-kb *Sciara* DNA (1.6-kb and 7.2-kb fragments) integrated into the *Drosophila* genome is shown at the top. The 8.8-kb *Sciara* DNA contains the DNA puff genes II/9-1 and II/9-2 (■) and flanking sequences (□). The labeled DNA probes are shown below the map (the dotted line represents *Sciara* genomic sequences present in the 5.5-kb probe but not included in the transgenic insertion). Southern blot analysis was carried out with *Xho* I digested genomic DNA from three transgenic *Drosophila* lines (A184, A200, S191) and nontransformed flies (cn,ry). The DNA was prepared from 0- to 1-h prepupal salivary glands and carcasses (PP0-1 SG and Ca) or 8- to 9-h prepupal salivary glands and carcasses (PP8-9 SG and Ca), as well as from adults of the same lines. Genomic DNA from lines A184 and A200 was hybridized to probes derived from gene 1 and gene 2. The hybridizing *Xho* I fragments are 2.4, 2.7, and 7.7 kb long, as indicated in the left-most lane by the hybridization pattern of the *Xho* I digested *Sciara*-P element construct and on the schematic map above (the *Xho* I site in parentheses was destroyed during cloning). For line S191 the 5.5-kb gene 1 fragment was used as a probe resulting in only one hybridizing 7.7-kb *Xho* I fragment (indicated by a dot). (In addition to this single band, two bands of λ DNA used as a carrier are also visible in the salivary gland sample.) A labeled probe derived from the *Drosophila* Alcohol dehydrogenase gene (*Adh*) was used to normalize the amount of loaded DNA; it detected an 8-kb *Xho* I fragment.

pared with the enzymatic activity of salivary glands taken directly from animals at the corresponding developmental stage. As shown in Figure 7, CAT activity can be induced by incubating competent salivary glands in a medium containing 5×10^{-6} M 20-OH ecdysone. Competence to respond to ecdysone was reached either in vivo or in vitro, but the induced levels

of CAT activity in 6-h prepupal salivary glands that reached competence in vivo (PP6, +E2 and +E4) are higher than in 3-h prepupal salivary glands that reached competence in vitro during a 3-h preincubation in ecdysone-free medium before induction (PP3, 3/+E2). Note that about the same levels of induced CAT activity are observed if competent salivary glands are incubated for 2 (PP6, +E2) or 4 h (PP6, +E4) in the presence of ecdysone. This indicates that steady-state levels of CAT activity have been reached essentially within the first 2 h of an in vitro exposure to ecdysone, as will be shown conclusively below.

In the absence of ecdysone, induction of CAT activity does not take place; the low levels of CAT activity found in salivary glands cultured without ecdysone (PP6, -E2 and PP3, 3/-E2) are not higher than the levels seen in salivary glands directly isolated from prepupae of the corresponding stages (PP6, salivary gland and PP3, salivary gland) that are before in vivo induction by ecdysone. In addition, CAT activity cannot be induced by ecdysone in salivary glands of ~ 110 -h third instar larvae (L3, +E4) or in incompetent salivary glands of 3-h prepupae (PP3, +E2). Taken together, the results from these in vitro studies indicate that the chimeric *Sciara*-CAT gene can be induced by 20-OH ecdysone in competent prepupal *Drosophila* salivary glands. This indicates a stage-specific hormonal induction restricted to the late prepupal period. Note that steady-state levels of

Figure 7. Stage specificity of hormonal induction in vitro. CAT assays were performed on extracts prepared from five pairs of transgenic *Drosophila* salivary glands (A-line) cultured in vitro in the presence (+E) or absence (-E) of ecdysone for 2 (+E2 or -E2) or 4 h (+E4 or -E4) and from salivary glands that were not cultured (SG). A 3-h preincubation in ecdysone-free medium before transfer in ecdysone or control medium is indicated by 3/-E2 or 3/+E2, respectively. The developmental stages at which the salivary glands were isolated are as follows: late third instar larvae (L3, ~ 110 h old), 3-h prepupae (PP3), 6-h prepupae (PP6), and 9-h prepupae (PP9).

protein activity (CAT) were measured rather than mRNA initiation because nuclear run-on experiments are not feasible in this system. However, hormonal induction seems to take place at the transcriptional level because in vitro induction of CAT activity in 6-h prepupal salivary glands does not take place in the presence of actinomycin D (data not shown).

The *Sciara* DNA puff promoter shows a quick response to ecdysone in vitro

As mentioned earlier, in *Drosophila* the larval and late prepupal RNA puffs are divided into early- and late-responding puffs. One difference between these groups of puffs is seen in the rapidity by which they respond to ecdysone in vitro. In late third instar larvae early-responding puffs respond to ecdysone within minutes with a maximal response between 1 and 4 h, whereas late-responding puffs show a delay in their response to ecdysone for ≥ 4 h (Ashburner, 1972). The puffing sequence in late prepupae in vivo is much shorter than in larvae, and although the early-responding prepupal puffs are induced quicker than the late-responding RNA puffs, the differences are less pronounced in late prepupae than in larvae (Richards, 1976c).

To determine the rapidity by which the *Sciara* DNA sequences respond to ecdysone in vitro, competent salivary glands of 6-h prepupae were cultured in the presence of ecdysone for various periods of time. As shown in Figure 8, a strong induction of CAT activity was detected in salivary glands that were cultured in ecdysone-containing medium for 1 h or longer. The amount of induced CAT activity is almost as high as that found in vivo for salivary glands of 9-h prepupae (PP9, salivary gland) and is essentially the same regardless of whether the salivary glands were incubated for 1 (PP6, +E1), 2 (PP6, +E2), or 6 h (PP6, +E6). However, shorter incubation periods of half an hour or less do not result in a significant induction of CAT activity; only low levels of CAT activity are found in salivary glands incubated for a few minutes (PP6, +E0; incubation shorter than 10 min) or half an hour (PP6, +E1/2). Similar low levels of CAT activity are also present in 6-h prepupal salivary glands that were either cultured for 6 h in the absence of ecdysone (PP6, -E6) or that were not cultured at all (PP6, salivary gland).

These results indicate that steady-state levels of induced CAT activity are reached between half an hour and 1 h of exposure to ecdysone in vitro, suggesting a rapid hormonal induction of the chimeric *Sciara*-CAT gene. Note that

Figure 8. Time-course of hormonal induction in vitro. CAT activity was determined in five pairs of transgenic *Drosophila* salivary glands isolated from 6-h prepupae (A-line). The salivary glands were cultured in the presence of ecdysone for various periods of time (PP6, +E0; +E1/2; +E1; +E2; +E6). The incubation time is given in hours with an accuracy of ± 10 min (except the incubation time for +E0 was 0–10 min). CAT activity was also determined in 6-h (PP6, SG) and 9-h (PP9, SG) prepupal salivary glands that were not cultured and in 6-h prepupal salivary glands that were cultured for 6 h in the absence of ecdysone (PP6, -E6).

protein levels, rather than mRNA levels or puff sizes, were determined in this experiment, so the presumed transcriptional response must have been even more rapid. Hence, the *Sciara* DNA sequences show a response to ecdysone in transgenic *Drosophila* like that expected for an early-responding gene of a *Drosophila* RNA puff.

Gene expression driven by the *Sciara* DNA puff promoter is terminated in vitro while still in the presence of ecdysone

In vitro studies on isolated *Drosophila* salivary glands showed that although ecdysone induces puffing of the early-responding larval or prepupal RNA puffs, these puffs typically regress rapidly while ecdysone is still present (Ashburner, 1972; Ashburner and Richards, 1976; Richards, 1976c). To test the behavior of the chimeric *Sciara*-CAT gene after high levels of CAT activity have been reached in vivo, salivary glands of 9-h prepupae were cultured for 12 h with and without ecdysone. As shown in Figure 9, levels of CAT activity are approximately the same during a 12-h incubation in the presence or absence of ecdysone (PP9, +E12 and PP9, -E12, respectively). Therefore, once CAT activity has been induced in vivo in 9-h prepupae, this activity is maintained for an additional 12 h even in the absence of ecdysone. This steady-state level of CAT activity for 12 h could reflect either (1) continued synthesis of CAT mRNA and/or

protein coupled with an equal rate of turnover or (2) no further synthesis of CAT mRNA and/or protein after the initial burst but a stable CAT product. To distinguish between these possibilities we did a 12-h incubation of 9-h prepupal salivary glands in the presence of actinomycin D (PP9, +Am12) or cycloheximide (PP9, +Ch12). As seen in Figure 9, neither drug significantly changes CAT activity levels from that already present in salivary glands of 9-h prepupae (PP9, salivary gland). This indicates that maintenance of CAT activity for an additional 12 h does not require *de novo* synthesis of either CAT mRNA or CAT protein, suggesting that the CAT protein has virtually no turnover in *Drosophila* salivary glands for ≥ 12 h.

When these findings are applied to the *in vivo* situation, they suggest that most expression of the CAT gene occurs only for a short period in late prepupae that are probably between 6 and 9 h old and that CAT activity seen thereafter primarily is due to high stability of the CAT protein rather than *de novo* expression of the CAT gene. Similarly, the early-responding RNA puffs of *Drosophila* larvae and late prepupae are only transiently induced by ecdysone, and they regress rapidly (Ashburner, 1972; Richards, 1976c).

DNA from *Sciara* DNA puff II/9A does not puff in transgenic *Drosophila* salivary gland chromosomes

Because the chimeric *Sciara*-CAT gene is expressed in late prepupal salivary glands of transgenic *Drosophila*, we wondered if expression of the inserted DNA is also associated with polytene chromosome puffing. It is not known where the sequences responsible for puffing occur in the *Sciara* II/9A locus, so rather than just testing the limited 718-bp promoter region, we tested the larger 8.8-kb genomic region containing it (see map in Figure 6). *In situ* hybridization of salivary gland chromosomes from *Drosophila* transformed with the 8.8-kb *Sciara* DNA (line A184) showed a single insertion site at locus 1C on the X chromosome (Figure 10, top). When this locus was examined in polytene chromosome squashes from 6- to 9-h (PP6–PP9) prepupal salivary glands, no puff was found at the site of the inserted *Sciara* DNA (Figure 10, middle). However, the early-responding RNA puffs of *Drosophila* late prepupae were clearly present (e.g., RNA puff 2B5). Because the expected time interval of transcriptional activity and hence potential puffing activity is only for ~ 1 h during this 3-h prepupal period (see pre-

Figure 9. Stability of CAT proteins in transgenic salivary glands *in vitro*. CAT activity was determined in five pairs of transgenic *Drosophila* salivary glands isolated from 9-h prepupae (PP9, A-line) that were not cultured (SG) or were cultured for 12 h in the presence (+E12) or absence of ecdysone (–E12) or cultured for 12 h in the presence of actinomycin D (+Am12) or cycloheximide (+Ch12). In this particular experiment, CAT activity of salivary glands that were not cultured (SG) was slightly lower than usual. As a control, both drugs were shown to be active because they inhibited the induction of CAT activity when added to the medium containing ecdysone for culture of 6-h prepupal salivary glands (data not shown).

ceding section), we also examined squashes of salivary gland chromosomes from competent prepupae after a 30- to 60-min *in vitro* incubation with ecdysone. Even in this case, no puff was apparent at the site of insertion (Figure 10, bottom), although other early-responding prepupal RNA puffs of *Drosophila* were induced *in vitro* by ecdysone (e.g., RNA puff 2B5).

Discussion

Expression of the *Sciara* DNA puff genes occurs in the absence of DNA amplification or puffing

In this work we present evidence that a 718-bp long promoter fragment of DNA puff gene II/9-1 of *Sciara coprophila* directs expression of the reporter gene CAT in transgenic *Drosophila* with correct tissue and analogous stage specificity. However, expression occurs in the absence of DNA amplification or puffing of an 8.8-kb fragment of *Sciara* DNA that contains the 718-bp promoter fragment. This indicates that neither amplification nor puffing is required for expression of the chimeric *Sciara*-CAT gene. Similar observations have been previously reported; expression of the *Drosophila* glue proteins does not require RNA puffing at the site of gene in-

tegration (Korge, 1987; Meyerowitz *et al.*, 1987), and correct temporal and tissue-specific expression of the *Drosophila* chorion genes does not require major amplification of the chorion locus (Orr *et al.*, 1984; Parks *et al.*, 1986; Wakimoto *et al.*, 1986). Future studies will be needed to define the positions of control elements for amplification and puffing in *Sciara* DNA puff II/9A. However, it is possible that these control elements are present in the tested 8.8-kb *Sciara* DNA but are not recognized or the physiological conditions (e.g., ecdysone levels) are not appropriate in transgenic *Drosophila*.

The Sciara DNA puff promoter retains tissue and stage specificity in Drosophila

Northern blots show that in *Sciara coprophila*, DNA puff gene II/9-1 is expressed only in salivary glands of fourth instar larvae (DiBartolomeis, Wu, Liang, Smith, and Gerbi, unpublished data). The experiments we report here demonstrate that this tissue specificity is retained in transgenic *Drosophila* in which the 718-bp-long promoter fragment of *Sciara* DNA puff gene II/9-1 directs expression of the reporter gene CAT exclusively in the late prepupal salivary glands (Figure 5). Sequences within the 718 bp of *Sciara* DNA responsible for this tissue specificity have not yet been identified.

The retention of stage specificity for expression of the chimeric gene in transgenic animals (Figure 4) is harder to ascertain because of developmental differences between *Sciara* and *Drosophila*, although both are holometabolous organisms with a complete metamorphosis. *Sciara* is a lower dipteran fly (suborder *Nematocera*) with four larval instars preceding pupation (Figure 1); each stage is demarcated by ecdysis, that is shedding of the cuticle, presumably triggered by an increase in ecdysone titer, though this has not been measured directly. *Drosophila*, instead, is a higher dipteran fly (suborder *Cyclorrapha*) that has evolved a puparium (Fraenkel and Bhaskaran, 1973). At the end of the third larval instar, the larval cuticle is not shed in response to the increased ecdysone titer (unlike the third/fourth larval instar ecdysis of *Sciara*) but instead forms the barrel-like puparium (PF in Figure 1). Four hours after puparium formation, larval/pupal apolysis begins (Ap in Figure 1) in which the puparium separates from the underlying epidermis. Twelve hours after puparium formation, the head evaginates (Ev in Figure 1); this event was suggested to be analogous to larval/pupal ecdysis in lower diptera and marks the onset of pupation (Fraen-

kel and Bhaskaran, 1973). There has been debate whether the term "molt" should refer to apolysis or ecdysis and which of these two processes should be used to define an instar (Hinton, 1976; Whitten, 1976). There also has been ambiguity in how to use the term "prepupa." In this study we use "prepupa" in the same way used by Ashburner and Richards to refer to the stage of *Drosophila* extending from puparium formation to head evagination (PP0-PP12; "prepupa" also used in this sense by Fraenkel and Bhaskaran, 1973; Bainbridge and Bownes, 1981, 1988). Our results therefore suggest that DNA puff gene II/9-1 sequences are active in both fly species (*Sciara* fourth larval instar or *Drosophila* prepupa) at the developmental stage preceding pupation.

The retention of tissue and analogous stage specificity of the *Sciara* DNA puff promoter in transgenic *Drosophila* is the first indication that some aspects of the mechanism by which ecdysone controls gene expression are evolutionarily conserved. The *Sciaridae* family belongs to the suborder *Nematocera* for which the fossil record dates back to the Triassic, 220 million y ago; the *Drosophilidae* family belongs to the suborder *Brachycera* for which the fossil record dates back to the Jurassic, 180 million y ago (Carpenter, 1991). Therefore, the taxonomic split between *Sciara* and *Drosophila* was quite ancient, having occurred >180 million y ago. The evolutionary conservation of the ecdysone response with respect to tissue and stage specificity between these dipteran flies is consistent with the conservation seen for several receptors of other steroid hormones (for reviews see Yamamoto, 1985; Evans, 1988; Green and Chambon, 1988; Beato, 1989).

Gene expression driven by the Sciara DNA puff promoter is inducible by ecdysone

A rise in salivary gland ecdysone titer is correlated with DNA puffing (Stocker *et al.*, 1984), and hormone injection and ligation experiments have shown that ecdysone controls DNA puff formation in *Sciarids* (Crouse, 1968; Stocker and Pavan, 1974; Amabis and Amabis, 1984a,b). We have now extended these cytological observations to the molecular level. Our *in vitro* experiments with cultured *Drosophila* salivary glands indicate that the chimeric *Sciara*-CAT gene is inducible by 20-OH ecdysone as it shows that a quick response to ecdysone and steady-state levels of CAT activity are reached between half an hour and 1 h of exposure to ecdysone. The rapid response of the hybrid gene parallels the

rapidity by which ecdysone induces the early-responding RNA puffs of *Drosophila*. These puffs respond to ecdysone within minutes and reach their maximal size between 1 and 4 h in larval salivary glands (Ashburner, 1972) and after ~ 1 h in prepupal salivary glands (Richards, 1976c). According to Ashburner's model, early-responding RNA puffs of *Drosophila* are directly regulated by the ecdysone receptor (Ashburner *et al.*, 1974). This model is supported by the example of puff 74EF, an early-responding RNA puff of *Drosophila*, to which a cloned ecdysone receptor is able to bind (cited in Burtis *et al.*, 1990).

Promoters of genes that are directly regulated by steroid receptors contain a hormone responsive element, which is a short palindromic sequence with a variable degree of conservation between different steroid hormones (Evans, 1988; Green and Chambon, 1988; Beato, 1989). The DNA binding site (EcRE) of the ecdysone receptor appears to be an imperfect palindrome, the more conserved right side of which has the consensus sequence of TGA A/C CY (Cherbas *et al.*, 1991). There are three sequences (starting at -500 , -421 , and -271) (DiBartolomeis and Gerbi, 1989) in the 718-bp promoter fragment of DNA puff gene II/9-1 identical to this putative EcRE consensus sequence except that Y is A/T in the *Sciara* sequences. This further agrees with the notion that the *Sciara* DNA puff promoter of gene II/9-1 is directly controlled by the ecdysone receptor. Sites in DNA puff gene II/9-1 that bind a labeled ecdysone receptor will be experimentally determined, but note that our present re-

Figure 10. DNA from *Sciara* DNA puff II/9A does not puff in transgenic *Drosophila* salivary gland chromosomes. The polytene chromosome squashes were prepared from prepupal salivary glands of *Drosophila* (line A184) transformed with 8.8 kb of *Sciara* genomic DNA (see map in Figure 6). (Top) In situ hybridization to salivary gland chromosomes from larvae just before puparium formation using a biotinylated probe of the 5.5-kb *EcoRI* fragment that contains *Sciara* DNA puff gene II/9-1 and upstream DNA. This probe binds only to locus 1C on the X chromosome (arrow). RNA puff 2B5 of *Drosophila* is nearby and is puffed at this stage (Ashburner, 1969). (Middle) Polytene chromosomes were squashed from salivary glands of 6- to 9-h transgenic prepupae (PP6–PP9). The arrow marks the locus of *Sciara* DNA insertion (1C) as determined by in situ hybridization (shown above); the open arrowhead indicates the early-responding RNA puff 2B5 of *Drosophila*. (Bottom) Polytene chromosomes were squashed from salivary glands isolated from transgenic 0 h prepupae (PP0). These salivary glands were preincubated for 6 h at $25 \pm 1^\circ\text{C}$ in Robbs' medium and incubated for 30–60 min in the presence of ecdysone. Arrows same as middle panel.

sults predict that at least some of the binding sites for the ecdysone receptor must reside within the 718-bp long promoter fragment of gene II/9-1 tested in transgenic *Drosophila*. Similarly, an ecdysone responsive element was identified in the 5' flanking promoter region of the *Drosophila* hsp27 gene (Riddihough and Pelham, 1987). However, the E74A gene of *Drosophila* seems instead to contain the ecdysone responsive element(s) within the first intron, ~8–14 kb downstream of the start site of transcription (cited in Burtis *et al.*, 1990).

Induction of gene expression is a stage-specific event

In vitro, in transgenic *Drosophila*, induction of the chimeric *Sciara*-CAT gene by ecdysone is a stage-specific event occurring only in competent prepupal salivary glands and not in larval or early prepupal salivary glands. In vivo, in transgenic *Drosophila*, a burst in CAT activity is seen only during the late prepupal and not during the late larval peak in the ecdysone titer. Because hormonal induction of the hybrid gene does not occur in transgenic salivary glands of late third instar larvae in vitro and in vivo, there must be either a repressor-like factor that prevents induction of hybrid gene expression in late third instar larvae or, alternatively, a protein factor that facilitates induction of hybrid gene expression in late prepupae. The latter possibility of a prepupal co-inducer may be suggested by the necessity of protein synthesis to achieve competence (Richards, 1976b,c). The former possibility of a larval repressor is supported by the observation that puffing of 93F, an early-responding puff found only in late prepupae of *Drosophila*, could be prematurely induced in larval salivary glands if they were preincubated in the presence of a protein synthesis inhibitor (Richards, 1976c). Similar experiments are difficult to carry out in our system: we have not been able to show a significant premature induction of CAT activity by preincubating larval salivary glands in the presence of cycloheximide even if the drug was rigorously washed out before hormonal induction (data not shown).

Regardless of the nature of such stage-specific regulatory proteins, they should show some degree of evolutionary conservation between the two fly species because the *Sciara* promoter of DNA puff gene II/9-1 was assayed in transgenic *Drosophila*. This evolutionary conservation is remarkable in light of the obvious morphological differences seen at the late larval, prepupal, and pupal stages between *Sciara* and

Drosophila. Our construct of the promoter from *Sciara coprophila* DNA puff gene II/9-1 is the first description of sequences that are significantly active only in *Drosophila* salivary glands from late prepupae but not from larvae, thereby allowing future studies in *Drosophila* on the molecular mechanism restricting the hormonal response to just one specific stage.

Materials and methods

Plasmid construction and P-element transformation

To study transcriptional control, a promoter fragment of *Sciara* DNA puff gene II/9-1 was linked to the reporter gene CAT (Figure 2). The *Sciara* DNA puff promoter was isolated as a 718-bp long *EcoRV*-*Bal* I fragment. It contains DNA sequences from -616 to +102 relative to the cap site of the gene (DiBartolomeis and Gerbi, 1989). This promoter fragment was ligated to the unique *Sal* I site (filled in by reverse transcriptase) of the polylinker of the P-element vector CarCAT-1 previously described by Mitsialis *et al.* (1987). The CarCAT-1 vector is composed of the reporter gene CAT flanked by SV40 sequences (providing RNA processing signals) that were inserted as a 1.7-kb *Bgl* I-*Bam* HI fragment into the *Hpa* I site of the P-element vector Carnegie 20 (Rubin and Spradling, 1983). The *Sciara* DNA fragment was linked to the reporter gene CAT within the 5' untranslated sequences of SV40. Sequencing the junction indicated a short open reading frame from the *Sciara* ATG before the ATG start codon for CAT protein which occurs 74 nucleotides further downstream. Carnegie 20 also contains a 7.2-kb *Drosophila* fragment with the rosy gene (Rubin and Spradling, 1983).

For studies on amplification or puffing, an 8.8-kb fragment of *Sciara* genomic DNA containing DNA puff genes II/9-1 and II/9-2 and flanking DNA was used (Figure 6). The 8.8-kb-long fragment of *Sciara* DNA puff II/9A was isolated as a 1.6-kb *Xho* I-*Sal* I fragment and a 7.2-kb *Sal* I-*Sal* I fragment (the downstream *Sal* I site comes from the λ vector) (DiBartolomeis and Gerbi, 1989). The 1.6-kb *Xho* I-*Sal* I fragment was ligated into the unique *Sal* I site of the Carnegie 20 vector (Rubin and Spradling, 1983). The unique *Sal* I site of this hybrid plasmid was used to insert the 7.2-kb *Sal* I-*Sal* I fragment resulting in a P-element vector carrying 8.8-kb *Sciara* DNA sequences.

The resulting constructs were injected into embryos of the strain *cn;ry⁴²* of *Drosophila melanogaster*, together with the helper plasmid *p π 25.7w.c.* (Karess and Rubin, 1984) at DNA concentrations of 0.5 and 0.1 mg/ml, respectively. P-element transformation was carried out as previously described (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Surviving *G*₀ flies were individually mated with the parental strain and individual *G*₁ transformants of separate *G*₀ crosses were used to establish homozygous lines.

Southern blot analysis

Genomic DNA was isolated from transgenic *Drosophila* essentially as described by Jowett (1986), restricted, and subjected to gel electrophoresis on 1% agarose gels run at 70 V in 40 mM Tris-acetate, 1 mM EDTA (TAE) buffer (Sambrook *et al.*, 1989). After this the DNA was transferred to nylon (Gene Screen Plus, New England Nuclear-DuPont, Boston, MA) or nitrocellulose filters (Sartorius, distributed by Vanguard Intl., Neptune, N.J.). Probes were labeled with α -³²P dATP (New England Nuclear-DuPont) using a random prim-

ing kit (Boehringer Mannheim, Indianapolis, IN). Hybridization to nitrocellulose filters followed Sambrook *et al.*, (1989) with hybridization at 65°C and the final posthybridization wash in 0.1X SSC at 61°C. Hybridization to nylon filters followed the manufacturer's preferred method (Sartorius). Subsequent autoradiography used XAR 5 X-ray film (Eastman Kodak, Rochester, NY) and Cronex Lightening-Plus intensifying screens (Eastman Kodak) at -70°C.

Polytene chromosome analysis

Details of in situ hybridization were basically as described by Engels *et al.* (1986). A 5.5-kb *EcoRI* fragment containing *Sciara* DNA puff gene II/9-1 and upstream DNA was labeled for use as a probe with biotin-16-dUTP (Boehringer Mannheim) using a random priming kit (Boehringer Mannheim). The probe was subsequently detected with streptavidin-alkaline phosphatase (Gibco, BRL, Gaithersburg, MD), staining with nitro-blue tetrazolium (NBT) and 5-bromo-4 chloro-3 indolyl phosphate (BCIP) as described by Engels *et al.* (1986). TMAX-100 film (Eastman Kodak) was used for photography.

For in vitro induction studies, salivary glands from 0-h prepupae (PP0) of transgenic *Drosophila* were isolated and incubated for 6 h at 25 ± 1°C in Robbs' tissue culture medium (Robbs, 1969) followed by a 30- to 60-min incubation in 400 µl Robbs' medium containing 1 µl of 1 mg/ml 20-OH ecdysone. Salivary glands were squashed in 45% acetic acid for polytene chromosome analysis.

CAT assays

Transformed animals were grouped into developmental stages according to Bodenstein (1965). For the larval stages, hours were counted from the time of oviposition. Well-conditioned flies were allowed to lay eggs for 1 h and then removed from the vials. Larvae were harvested after the time indicated for each experiment. For the prepupal and pupal stages, hours were counted from the time of puparium formation (Fraenkel and Bhaskaran, 1973; Bainbridge and Bownes, 1981). Positions of individual prepupae were marked on the food vials at the time of puparium formation, and prepupae or pupae were collected after the number of hours indicated for each experiment. All vials were incubated at 25 ± 1°C. Animals were arrested at the desired developmental stage by putting them on ice for up to several hours.

Whole animals or dissected tissues were used to prepare extracts for CAT assays. Tissues were dissected in an ice-cold TSEL buffer [0.25 M tris(hydroxymethyl)aminomethane-HCl, pH 7.8, 0.4 M sucrose, 5 mM EDTA, 0.5 mM leupeptin]. Typically, 10 whole animals or dissected tissues were homogenized in 150 µl TSEL buffer containing 1 mg/ml bovine serum albumin (BSA) to stabilize the CAT protein. The presence of BSA precluded measurement of the total protein amount in the homogenate. For each sample, 100 µl of homogenate were assayed for CAT activity in an overnight incubation at 37°C as described by Mitsialis *et al.* (1987).

In vitro experiments

Transformed animals were staged as described above, except that they were never held on ice. Salivary glands of three to five larvae or prepupae of the indicated developmental stage were dissected in a drop of Robbs' tissue culture medium (Robbs, 1969) and immediately transferred into dissection wells containing 400 µl of Robbs' medium with 1 µl of ethanol or 1 µl of 1 mg/ml 20-OH ecdysone (dissolved in ethanol; Sigma, St. Louis, MO) to result in a final ecdysone concentration in the medium of 5 × 10⁻⁶ M. The medium used for preincubation of the salivary glands did not contain ethanol or ecdysone. For the inhibitor studies, cycloheximide

(Sigma) and actinomycin D (Sigma) were dissolved in water and added to the medium in a volume of 4 µl to give final concentrations of 7 × 10⁻⁵ and 9 × 10⁻⁷ M, respectively. The salivary glands were incubated for the indicated amount of time (±10 min) at 25 ± 1°C by covering the dissection well with a microscope slide. After the incubation period, the glands were immediately transferred into 150 µl of TSEL buffer (Mitsialis *et al.*, 1987). The entire homogenate was assayed for CAT activity, as described above. Each in vitro experiment was repeated two to three times, and the results were reproducible in these independent experiments.

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