Two-Step Regulation of Ecdysone-Inducible Late Puff Formation in *Drosophila* Salivary Glands

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ABSTRACT

The steroid hormone ecdysone directly induces sequential gene activations as it signals the onset of *Drosophila* metamorphosis. In the polytene chromosomes of larval salivary gland, sequential gene expressions are visible as the changes of puffed loci. Ecdysone primarily induces several early puff formation, which in turn sequentially induces more than 100 late puffs. A subset of late puffs can also be induced by a detergent digitonin in the absence of ecdysone, suggesting that these puff loci are repressed under a digitonin-sensitive suppression mechanism(s). In this study, digitonin-responsiveness of ecdysone-inducible puff loci was examined in larvae at various developmental stages and in salivary glands cultured *in vitro* to understand mechanisms under which ecdysone induces late puffs at proper timings.

Firstly, I treated salivary glands with digitonin at several stages in larval-white prepupal period and studied whether the digitonin-responsive stage was related to the stage of the beginning of developmentally programmed puffing at a locus. Digitonin induces different sets of developmentally regulated puffs depending on the stages of the treatment. Ecdysone-inducible early puff loci are always irresponsive to digitonin, while most late puff loci are activated by digitonin treatment only at the stage before their developmentally programmed puffing. This suggests a digitonin-sensitive suppression mechanism participates in halting late puff gene

activation until definite stages when puffing is programmed to start.

I found that several late puff loci respond to digitonin only in a short period immediately before beginning of developmentally programmed puffing. This finding has led me to infer that gene activation at these late puff loci is under two-step regulation. First, the digitonin-irresponsive state shifts to digitonin-responsive state and, second, the digitonin-responsive (but not yet ready to start puffing) state proceeds to the active puffing state.

As a next step, I examined whether ecdysone or early puff gene products participated in either of the two steps of late puff regulation, using an *in vitro* culture of salivary glands. Late puff formation had been previously shown to be regulated by protein products of early genes. The analysis revealed that (i) the acquisition of digitonin-responsiveness (the first step) can be induced *in vitro* by incubating salivary glands with ecdysone; (ii) the first step can also be attained by the inhibition of protein synthesis even in the absence of ecdysone; (iii) the transition from the first to the second step requires both ecdysone and protein synthesis unless salivary glands are treated with digitonin; and (iv) the first step, rather than the second step, determines the timing of normal puff formation in those late puff loci. These results suggest that ecdysone controls both steps through activating two types of early genes during normal development. The early genes of the first type, whose function can be mimicked by cycloheximide, render the loci responsive to digitonin. The early genes of second type, whose

function can be mimicked by digitonin, activate the loci to form puffs. Based on these findings I propose the presence of two types of early gene products with different functions and their participation in a system to control the sequential expression of late puffs in *Drosophila* salivary glands.

INTRODUCTION

Lipophilic thyroid and steroid hormones function as a temporal signal to coordinate multiple developmental events during metamorphosis in vertebrates and invertebrates (Scott, 1988; Riddiford, 1993, for review). Although these hormones have been well known to control transcription through a nuclear receptor that acts as ligand-regulated transcription factors (Evans, 1988; Beato, 1989; Koelle *et al.*, 1991; Beato *et al.*, 1995), it is relatively less understood how the transcription factors regulate gene expression which causes the dramatic changes associated with the hormonal activities. The fruit fly *Drosophila melanogaster* provides an ideal model system for understanding the mechanisms the hormone initiates metamorphosis in an intact animal. A surge of 20-hydroxy-ecdysone (hereafter referred to as ecdysone) at the end of the third larval instar triggers puparium formation and the onset of metamorphosis. During metamorphosis, larval tissues are eliminated or remodeled to adult forms, while imaginal cells recognize body plans then generate adult structures. Subsequent two moderate ecdysone surges at prepupal-pupal transition and during pupal development accompany the terminal differentiations into adult form (Handler, 1982; Sliter and Gilbert, 1992; Riddiford, 1993).

Insights into the mechanisms ecdysone initiates sequential gene expressions accompanied with metamorphosis have arisen from observations of the effects of the hormone on puffing pattern in the

larval-prepupal salivary gland polytene chromosomes of *Drosophila melanogaster*. The salivary gland polytene chromosomes attain their largest size at larval-prepupal period through multiple cycles of DNA replications without mitosis. These giant chromosomes provide with an opportunity to monitor gene activities under a light microscope; an actively transcribing gene is visualized as a change in chromosomal structures at a site in the chromosome, or a "puff", where chromosomal banding becomes unclear and chromosomal diameter increases (see Ashburner and Berendes, 1978, for review). In the salivary gland chromosomes, more than 125 specific puff loci have been reported to be involved in the gene activity during larval-prepupal metamorphosis (Ashburner, 1972a; 1975; Belyaeva *et al.*, 1974; Zhimulev, 1974). "Intermolt puff" loci (< 10 loci) are stably active until the ecdysone level starts to increase towards the end of the larval stage. The ecdysone surge induces "early puffs" (<10 puffs) within several minutes and renders the intermolt puff regression. Delaying for two or three hours, "late puffs" (>100 puffs) appear in a regular order over a 10 hour period (Ashburner, 1972b). The sequential changes can be induced in isolated larval salivary glands that have been cultured *in vitro* in the presence of ecdysone (Ashburner, 1972b). Using salivary glands cultured *in vitro*, Ashburner and his colleagues performed a series of experiments including ecdysone dose-response (Ashburner, 1973), hormone removal and readdition (Ashburner and Richards, 1976) and protein synthesis inhibition studies (Ashburner, 1974). Then they proposed

a model to explain the regulation of sequential gene activation by ecdysone during larval-prepupal metamorphosis (Ashburner *et al.*, 1974). Their model presumes that (i) regression of intermolt puffs and induction of early puffs are primary responses to ecdysone, while late puffs are activated by the protein products of the early puff genes as a secondary response to ecdysone; (ii) the protein products of the early genes cause their own gene expression to regress.

Tests of the Ashburner's model at molecular level have been achieved focusing on the early genes and their protein products. The results support the Ashburner's model. Not only is the transcription of a few early genes induced by ecdysone in a primary response and subsequently regressing in a secondary response, but these genes encode putative transcription factors (see Andres and Thummel, 1992, for review). Moreover, widespread expressions of both the early genes and the ecdysone receptor extend understanding of the model beyond its original confines of the larval salivary glands to the other ecdysone target tissues involved in the coordinated process of metamorphosis (Burtis *et al.*, 1990; Thummel *et al.*, 1990; Koelle *et al.*, 1991). However, the model does not explain how the strict temporal sequence of appearances and regressions of more than 100 late puffs are controlled.

A mild detergent, digitonin, can induce a large number of late puffs and nine heat shock puffs in the absence of ecdysone in larval salivary glands (Myohara and Okada, 1988a). This raises possibilities that gene expression at these late puff loci is suppressed

by a mechanism sensitive to digitonin and that digitonin could therefore be a useful agent for dissecting the control mechanism of late puff induction.

In the present study, digitonin treatment was applied to salivary glands at various stages from the late third instar larva to the white prepupa. The result revealed that digitonin induced different sets of late puffs depending on the stages of salivary glands at the treatment. Moreover, late puff loci were responsive to digitonin only at stages before, but not after, the initiation of their developmentally programmed puffing. I further disclosed in the present analysis that several late puff loci were responsive to digitonin only in a short period of time immediately before developmentally programmed puffing to start.

These findings led me to infer that gene activation at the late puff loci was accomplished through a two-step process; in the first step, the loci acquire digitonin-responsiveness and, in the second step, they are activated to start puffing. In salivary glands cultured *in vitro*, I showed that (i) acquisition of digitonin-responsiveness (the first step) requires either the presence of ecdysone or absence of protein synthesis; (ii) the second step requires the presence of both ecdysone and protein synthesis; (iii) the first step, rather than the second, is essential for determining the timing of normal puff formation in the loci.

Based on these results I discuss in this thesis that at least two sets of suppression mechanisms are involved in the regulation of

temporal gene activation at late puff loci: the first mechanism is cycloheximide-sensitive and this functions to prevent a subset of late puff loci from proceeding to digitonin-responsive (but not yet ready to start puffing) state from digitonin-irresponsive state, and the second mechanism is digitonin-sensitive and this functions at many late puff loci to halt gene activation until the stages of programmed puffing. Ecdysone controls derepression of both mechanisms through activating two types of early genes whose functions can be mimicked by cycloheximide and digitonin respectively.

MATERIALS AND METHODS

1. *Drosophila melanogaster* strain

Flies of a wild-type *Drosophila melanogaster* strain, Oregon-R, were used. Oregon-R flies were maintained at 25°C by daily passage of adults to 200 ml milk bottles containing cornmeal-agar medium (0.8% agar, 8% yeast extract, 8% corn meal, 6% sugar, 0.32% propionic acid, 0.24% n-butyl- p-hydroxybenzonate) seeded with dry active yeast.

2. Isolation and staging of salivary glands

a) Collection of salivary glands of each puff stage from PS1 to PS11

Salivary glands were dissected from late third instar larvae or newly formed white prepupae in a chemically defined medium, MTB1 containing 15mM K-phosphate, pH 7.0, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, and 1% PEG 6000 (Myohara and Okada, 1987). Sister lobes in each pair of salivary glands were divided. One lobe of paired salivary glands was immediately fixed, stained and squashed as described below and the puff stage was determined according to the puffing pattern as described by Ashburner (1972a). Briefly, PS1 (puff stage 1) was marked by three prominent intermolt puffs at 3C, 25AC and 68C with no detectable puffing at the early puff loci 74EF and 75B; PS2 was marked by appearance of small puffs at 47A, 47BC, 74EF, 85EF and 88EF; PS4-6 all showed prominent puffs at

63F, 74EF and 75B but puffs at 2B13-17, 2EF and 72D were observed only in PS4, and PS6 was marked by appearance of puffs at 55B and 93B; PS8 was marked by appearance of 63DE and regression of 63F; PS10-11 both showed prominent puffs at 62E and 63DE but puffs at 25D and 73B appeared only in PS11. The other lobe of the paired glands was used for experiments to examine digitonin-induced puffing pattern. Since sister lobes in each pair of glands exhibit an identical puffing pattern during normal development, the puff stage of the experimental gland lobes can be speculated by the normal puffing pattern of their sister lobes.

b) Selection of PS1 and PS8-9 salivary glands

For a series of experiments to compare digitonin-induced puffing patterns between PS1 (puff stage 1) and PS8-9, salivary glands were staged according to the behavioral characteristics of larvae and the morphological features of salivary glands as Myohara and Okada (1987) described. Briefly, PS1 salivary glands were obtained from third instar larvae wandering actively in the lower portion of the milk bottle walls, but only those exhibiting grape-like clusters of round cells were used as PS1 glands. PS8-9 salivary glands were obtained from larvae stationary in the upper portion of the bottle walls. Only salivary glands with an expanded lumen were selected as PS8-9 glands. Both PS1 and PS8-9 glands were dissected and collected in MTB1.

c) Mass collection of larval salivary glands for *in vitro* culture

To facilitate collection of salivary glands before the raise of the level of endogenous ecdysone (PS1 salivary glands), salivary glands were dissected from synchronized larvae. Oregon-R flies matured for 3-4 days after the eclosion were allowed to lay eggs at 25°C on grape juice/agar plates (50% grape juice, 2% agar, 1% ethanol, 1% acetic acid) seeded with active dry yeast. Eggs were collected at 1-hr intervals. The first two collections were discarded as prelays to condition the flies not to hold eggs. The plates of the third, fourth and fifth egg collections were put in vials containing cornmeal-agar medium seeded with dry yeast and the eggs were allowed to develop at 25°C. At 72 hr (±15 min) after egg laying (AEL), freshly molted third instar larvae were hand-picked and transferred to a small vial containing cornmeal-agar where incubated at 25°C. Larvae at 108 hr AEL were dissected in order to obtain salivary glands in Grace's medium (GIBCO) modified as described by Ashburner (1972b).

3. Puff induction by digitonin treatment

Individual salivary gland lobes were placed in 50 μl drops of MTB1 containing digitonin on siliconized depression slides (21-mm-diameter depressions), and kept there for 20 min at 25°C. They were then washed with four changes of MTB1 for 30 sec each, and incubated for 2 hr in a drop of MTB1 containing 0.6 mM each of ATP, CTP, GTP and UTP to allow the puffs to grow. Amount of the medium in a drop was adjusted in the range of 0.125-0.25 μl per

gland lobe (Myohara and Okada, 1988b).

4. Cytological analysis

Salivary glands were fixed for 3 min in 45% acetic acid containing 10% lactic acid, stained for 3 min with lactic-acetic-orcein (10:45:2), and squashed between a glass slide and a coverslip. Under a phase-contrast microscopy, polytene chromosomes were observed.

Puffing activities of individual loci on the polytene chromosomes were evaluated according to "puff size", the ratio of the maximum diameter of the puffed region to the diameter of an adjacent unpuffed reference band (Ashburner, 1972b). The diameters were measured on enlarged photoprints of the polytene chromosomes with the vernier calipers (MITUTOYO). In the series of experiments analyzing the response to digitonin in PS1-11 salivary glands *in vivo*, puff sizes were presented as the mean value with standard error of 6-33 measurements (15 on average) from 3-10 independent experiments (5 on average). In the series of experiments analyzing the response to digitonin in salivary glands cultured *in vitro*, the mean value and standard error of 20 measurements from 4-5 individual experiments were recorded. The measured puff loci and their respective reference bands are listed in Table 1.

5. Evaluation of the effects of digitonin

Effects of digitonin were determined by comparing the puff sizes of digitonin-treated glands with those of digitonin-untreated

controls. In the series of experiments analyzing the effects of digitonin in PS1-11 glands *in vivo*, two types of controls were used; (i) digitonin-untreated salivary glands which were fixed immediately after dissection, and (ii) digitonin-untreated glands which were incubated for two hours before fixation under the same condition as the digitonin-treated glands. A locus was regarded as digitonin responsive when the puff size of digitonin-treated glands was significantly ($P<0.05$) larger than the puff sizes of both the two controls. Significance of difference was statistically determined by T-test. Effects of incubation were determined by comparing digitonin-untreated, incubated glands with the sister lobes fixed immediately after dissection. In the series of experiments analyzing the response to digitonin in glands cultured *in vitro*, only one type of control, (ii), was used. In experiments shown in Fig. 10, the effects of digitonin on puffing activity are presented as a difference between puff sizes in digitonin-treated glands and those of digitonin-untreated controls. The standard error (SE) of the difference (Fig.10) was calculated as:

$$SE_{A\text{-}B}=\sqrt{SE_A^{\,2}+SE_B^{\,2}}$$

where A is the mean value of puff sizes in digitonin-treated glands and B is that of the digitonin-untreated controls.

6. *In vitro* culture of salivary glands

Salivary glands were collected in Grace's medium (GIBCO) that had been modified as described by Ashburner (1972b). Sister lobes of each pair of salivary glands were separated; one lobe was used for

experiment and the other was used for the control. Individual lobes were cultured separately in a 50 μl drop of the modified Grace's medium at 25°C. Ecdysone (20-hydroxyecdysone, Sigma) was applied at a concentration of 2.5 x 10^{-6} M (Ashburner, 1973). Before the addition of ecdysone, salivary glands were incubated in modified Grace's medium for 4 hr in order to remove endogenous ecdysone.

7. Evaluation of the effect of protein synthesis inhibitors

Cycloheximide (Sigma) was used at a concentration of 7.0 x 10^{-5} M (Ashburner, 1974). Efficacy of the inhibitor with respect to the inhibition of protein synthesis was double checked by lack of [^{3}H]-methionine incorporation and by inhibition of early puff regression at 74EF and 75B loci in treated salivary glands as described by Ashburner (1974).

RESULTS

1. Effects of digitonin on puff formation in salivary glands at different stages

I examined effects of digitonin on puff formation in salivary glands at various stages from the late third instar through the white prepupal. The developmental passage is divided into 11 puff stages (PS1 to PS11) according to the changes in sets of puffed loci, each of those stages has a unique puffing pattern (Ashburner, 1972a; 1975).

a) Selection of a digitonin concentration appropriate for puff induction

Since salivary glands change physiologically and morphologically as they proceed from PS1 to PS11, I determined the dose of digitonin that could induce a maximum number of puffs at several stages in the period. When salivary glands were treated with 0.005% digitonin, the number of digitonin-induced puffs reached maximum at all stages examined (Table 2). Furthermore, at this dose, all puffs that could be induced by digitonin were induced. The dose-dependent response of loci in the chromosome Ⅲ from PS8-9 glands is summarized in Table 3. Based on these results, I decided to use 0.005% digitonin in following analyses.

b) Stage-dependent puff induction by digitonin

To see whether digitonin induces different puffs depending on

the stage of salivary glands at the treatment, I compared digitonin-induced puffs in salivary glands at PS1 and PS8-9. Among 40 significantly induced puffs, 23 (2EF, 10E, 13A, 13E, 14BC, 18DE, 26B, 37E, 42A, 42C, 50C, 50D, 50F, 55B, 56DE, 63F, 67C, 70C, 84B, 88D, 88E, 93B and 96B) were observed only in PS1 glands (Figs. 1-2), four (7D, 30F, 55E and 73B) were exclusively in PS8-9 glands (Figs. 1-2), and the remaining 13 (33B, 45A, 63C, 64EF, 67B, 70A, 71DE, 84E, 87A, 87C, 93D, 95B and 95D) were at both stages. These observations indicate that some puff loci respond to digitonin only at specific stages in the larval development.

All of the nine heat shock puffs known in *Drosophila melanogaster* (33B, 63C, 64EF, 67B, 70A, 87A, 87C, 93D and 95D; see Ashburner and Bonner, 1979, for review) were among those induced by digitonin at the both stages. The other digitonin-activated loci observed here (except 37E, 63F and 96B) were all among late puff loci, which are all known to puff in a certain period(s) of time during normal development (Ashburner, 1972a; 1975; Belyaeva *et al.*, 1974; Zhimulev, 1974). Comparisons of the stage of developmentally programmed puffing and the digitonin-responsive stage of each locus were performed. The results revealed that, with a few exceptions, the loci which normally begin to puff during PS10-11 are responsive to digitonin at PS8-9 while the loci which normally begin to puff during PS2-8 were irresponsive to digitonin at PS8-9. These findings suggest that digitonin can activate a gene locus only before the beginning of its developmentally

programmed puffing. To know more about the correlation between the digitonin-responsive stage and the developmentally programmed normal puffing stage, I examined effects of digitonin on salivary glands at various stages from PS1 through white prepupa PS11.

c) Effects of digitonin on heat shock puff loci

It has been reported that puffing occurs at some of the heat shock puff loci (without a heat shock) during the larval-prepupal development (Ashburner, 1975). In fact, I observed puffing at five heat shock puff loci (33B, 63C, 67B, 87A and 93D) at respective regular developmental stages during the PS2-11 period in this study (see open bars in Fig. 3). However, digitonin activated all the heat shock puff loci at any stages examined, no matter whether they were puffing or not at the time of treatment, except at 33B and 93D at PS6 (Figs. 3-4). This stage independency of digitonin responsiveness is unique to heat shock puff loci, and thus suggests that the mechanism of digitonin puff induction in heat shock puff loci may be different from that in digitonin-responsive, developmentally regulated loci.

d) Effects of digitonin on developmentally regulated puff loci

Twenty-nine developmentally regulated puff loci at which prominent puffing is observed at a time during PS1-11 (Ashburner, 1975) were selected for analyses in detail of digitonin effects on salivary glands at various stages from PS1 through PS11. Of the 29 loci, 17 responded to digitonin (Figs. 5-6) while 12 did not (Fig. 8).

At the 17 loci responded to digitonin, puffs were induced only when the salivary glands were treated with digitonin before the stage of normal puffing (Figs. 5-6). Digitonin neither enhanced preexisting, developmentally programmed puffs (see, for example, 55B at PS6-8 in Figs. 5 and 7; the only exception was 63F at PS8), nor induced puffing at the loci after the programmed puffing ended (for example, 55B at PS10-11 in Figs. 5 and 7). At the loci where puffing is programmed twice in the PS1-11 period (10E, 49E, and 50F), digitonin induced the puff at stages not only before the first programmed puffing but also before the second puffing, except at PS8 at 50F (Fig. 5). These observations indicate that these 17 loci are responsive to digitonin before the initiation of developmentally programmed puffing, then become irresponsive during and after the programmed puffing.

The 12 loci which did not respond to digitonin in the PS1-11 period include four loci (2B5-6, 25AC, 58DE and 68C) where developmentally programmed puffing takes place at PS1 (Fig. 8). It is thus possible that these puff loci are responsive to digitonin before PS1, when the normal puffing has not begun as yet. The remaining eight loci (22B, 29F, 74EF, 75B, 78C, 82F, 85F and 88E), however, initiate developmentally programmed puff formation at or after PS2 (Fig. 8). It is thus evident that these loci are irresponsive to digitonin even at stages immediately before the normal gene expression.

The 29 developmentally regulated puff loci examined in the present study included all three categories established according to

their responses to ecdysone (Ashburner, 1972b): intermolt puffs (three loci), ecdysone-inducible early puffs (six loci) and ecdysone-inducible late puffs (20 loci) (Table 4). As summarized in Table 4, none of the intermolt and early puff loci (except 63F) were activated by digitonin in the PS1-11 period, while 16 out of the 20 late puff loci were activated by digitonin at stages before the developmentally programmed puffing begins. This shows that premature activation of at least 16 late puff loci and an early puff locus, 63F is suppressed at a digitonin-sensitive step in the process of gene activation.

Based on the stages when the loci are responsive to digitonin, the 17 digitonin-responsive puff loci can be classified into two groups (Table 4). Group A consisting of 11 loci (10E, 25D, 49E, 50C, 50D, 50F, 55B, 56DE, 63F, 84E, and 93B) is characterized as responsive to digitonin throughout the period of time until the first normal puffing after PS1 or after a previous puffing has ceased (Fig. 5), indicating that premature activation of these loci for the period are suppressed at digitonin-sensitive one-step process of gene activation. In contrast, the remaining six loci (2B13-17, 9E, 42C, 55E, 63DE and 71DE) comprising group B respond to digitonin only in a short period of time immediately before the initiation of normal puffing (Fig. 6). This indicates that gene activation at these loci is brought about with a two-step process; first, the digitonin-irresponsive state progresses to the digitonin-responsive state, and second, the digitonin-responsive (but not yet ready to start puffing) state progresses to active puffing state.

2. Effects of ecdysone and protein synthesis inhibition on the two steps needed for gene activation at group-B loci

The group-B loci are all among late puff loci, categorized as requiring protein products of ecdysone-inducible early puffs for puffing (Ashburner, 1972b). This led me to speculate that protein products of early genes may participate in either of the two steps of gene activation at the group-B loci. In the following series of experiments, I examined whether group-B loci require ecdysone or protein synthesis for proceeding through the two steps of gene activation. Using the *in vitro* culture method of salivary glands (Ashburner, 1972b), I asked whether ecdysone or protein synthesis inhibitor alters the reaction to digitonin with reference to the changes in puffing pattern observed during normal development. Although they are in group-B loci, 63DE and 71DE were omitted from the following analyses because their puffing behaviors were different from that of the other four loci; 63DE puff could be induced solely by inhibition of protein synthesis (Ashburner, 1974), 71DE showed low but significant puffing activity even before the surge of ecdysone (Ashburner, 1972b; Fig. 16).

a) Induction of digitonin-responsiveness by ecdysone in cultured salivary glands

To test whether the acquisition of digitonin-responsiveness (i.e., the first step of the gene activation) in four group-B loci can be reached *in vitro*, I applied digitonin to salivary glands that had been

cultured in the presence of ecdysone for various periods of time. All four loci examined (2B13-17, 9E, 42C and 55E) responded to digitonin by forming puffs only when salivary glands had been incubated with ecdysone for 2-4 hours before the treatment, while none responded to digitonin at the beginning of the culture period (Fig. 9, solid lines). Moreover, when ecdysone was omitted from the culture medium none of the loci responded to digitonin (Fig. 10, dotted lines with open circles). These results indicate that ecdysone is essential for these loci to become digitonin-responsive in cultured salivary glands.

As for many other developmentally regulated puff sites (Ashburner, 1972b), puffs were induced *in vitro* by the continuous culture of salivary glands with ecdysone (Fig. 9, dotted lines). Comparison of time points at which digitonin-responsiveness was acquired with time elements of ecdysone-induced puff formation reveals that, as seen in normal development (Fig. 6), digitonin-responsiveness is acquired immediately prior to the beginning of the ecdysone-induced puff formation (Fig. 9). In digitonin-untreated controls (Fig. 9, broken lines), the loci never formed puffs even though the glands had been cultured for 2 hours after the acquisition of digitonin-responsivenss in a buffer containing nucleoside triphosphate. It is thus concluded that the time lag between the acquisition of digitonin-responsivenss and ecdysone-induced puff formation is not due to the 2 hour incubation period after digitonin treatment, but reflects the fact that these loci are under two-step

regulation, the first step being the progression from the digitonin-irresponsive state to the digitonin-responsive state, and the second is the progression from the digitonin-responsive (but not yet ready to start puffing) state to the puff forming, active state (Fig. 9).

b) Effects of protein synthesis inhibition on the acquisition of digitonin-responsiveness

The abovementioned results show that the first step of gene activation at these four loci (i.e. the acquisition of digitonin-responsivenss) is reproducible *in vitro* by incubating salivary glands with ecdysone for 2-4 hours. To test whether protein synthesis is necessary for this step, I incubated salivary glands with ecdysone and a protein synthesis inhibitor, cycloheximide, for various periods of time and then tested the glands for digitonin-responsiveness. Sufficiency of the cycloheximide treatment upon inhibition of protein synthesis in cultured salivary glands was confirmed in [^{3}H]-methionine incorporation experiment (Table 5). The effect of cycloheximide was also evaluated by inhibition of early puff regressions at 74EF and 75B (Fig. 11), since these early puff regression at these loci has been shown to be induced by protein products of early genes (Ashburner, 1974).

As clearly shown in Fig. 10 (solid lines with filled diamonds), at none of the four loci was the acquisition of digitonin-responsiveness inhibited by cycloheximide. The result indicates that the four loci are able to acquire digitonin-responsiveness without protein

synthesis. Indeed, cycloheximide tended to enhance the response of these loci to digitonin; puffing occurred earlier and/or stronger in salivary glands incubated with ecdysone and cycloheximide (Fig. 10, solid lines with filled diamonds) than in those incubated with ecdysone alone (Fig. 10, solid lines with filled circles).

In order to examine the direct effects of protein synthesis inhibition on these loci, salivary glands were incubated with cycloheximide alone and then tested for digitonin-responsiveness. Despite the absence of ecdysone from the culture medium, all four loci became digitonin-responsive when salivary glands were incubated with cycloheximide for more than 2 hours (Fig. 10, dotted lines with open diamonds). The timing of obtaining digitonin-responsiveness was unaffected by omission of ecdysone from the culture medium (Fig. 10, graphs at the right). However, in the absence of both cycloheximide and ecdysone, loci did not become digitonin-responsive (Fig. 10, dotted lines with open circles). These results suggest that ecdysone effects a group-B locus rather indirectly to accomplish digitonin-responsiveness, for example by inactivating a repressor that prevents loci from responding to digitonin.

At 2B13-17, the size of the digitonin-induced puff was significantly larger in glands treated with ecdysone and cycloheximide than in those treated with cycloheximide alone (Fig. 10a, right). This suggests that cycloheximide treatment does not mimic all the effects of ecdysone in the 2B13-17 locus. Considering that cycloheximide inhibits the synthesis of early gene products, the

effects of ecdysone not mimicked by cycloheximide are probably due to a direct action of ecdysone at this locus.

c) Requirement of ecdysone in the second step

In order to investigate whether the transition from the digitonin-responsive but unpuffed state to the active puffing state (i.e., the second step of gene activation) requires ecdysone, I removed ecdysone from the culture medium immediately after the first step was completed. As shown in Fig. 12, puffing was aborted at all four loci following ecdysone removal. This suggests that ecdysone is necessary for the second step, and that these loci are early-late puffs rather than late-late puffs according to the definition of late puffs by Ashburner and Richards (1976). The response to ecdysone of these puff loci, i. e., forming puffs rather rapidly (in 4-6 hours), is consistent with that in early-late puffs (Fig. 9, dotted lines).

d) Requirement of protein synthesis for the second step of gene activation

In order to know whether the ecdysone-induced second step of gene activation requires protein synthesis, the effects of cycloheximide on puff induction by ecdysone were examined. When salivary glands were incubated with ecdysone and cycloheximide, none of the loci formed a puff (Fig. 13, solid lines). As the acquisition of digitonin-responsiveness (the first step) is not inhibited by cycloheximide (Fig. 10), the inhibitory effect of cycloheximide on

puffing is probably due to the prevention of the loci proceeding to the second step. The results indicate that *de novo* protein synthesis is necessary for the ecdysone-induced second step of gene activation at the four loci studied. This requirement is compatible with these puffs being categorized as ecdysone-inducible late puffs by Ashburner (1974).

As the incubation medium used for puff growth after digitonin treatment is a simple saline solution that contains nucleoside triphosphates but no amino acids, it is obvious that neither protein synthesis nor ecdysone is required for the second step of gene activation when it is induced by digitonin. In fact, cycloheximide did not inhibit digitonin-induced puff formation (Fig. 14).

e) Effects of protein synthesis inhibition on the ecdysone-induced puff formation at group B loci

As mentioned above, cycloheximide treatment of salivary glands promotes digitonin-responsiveness at the four group-B puff loci (Fig. 10). In order to test whether cycloheximide treatment also enhances response to ecdysone, salivary glands were incubated with cycloheximide for 2 hours, and then challenged with ecdysone. At the all four group-B loci examined (2B13-17, 9E, 42C and 55E), puffs were formed 2-4 hours earlier in cycloheximide-treated glands than in untreated glands (Fig. 15). The promotive effect of cycloheximide resulted in the simultaneous activation of these loci in treated glands (Fig. 15, solid lines), contrary to that these loci are

activated by ecdysone at different timings in culture (Figs. 9 and 15, dotted lines) and in the normal *in vivo* development (Fig. 6). Because incubation of glands with cycloheximide for 2 hours is sufficient for the loci to complete the first step (Fig. 10, dotted lines with open diamonds), the loci in cycloheximide-pretreated glands can be considered as having completed the first step of gene activation before they were challenged with ecdysone. The simultaneous puffing observed in the treated glands allows an assumption that the second step of gene activation is completed within 2 hours at any of the given loci (Fig. 15, solid lines). This assumption is competent to explain the fact that the time lag between the acquisition of digitonin-responsiveness and ecdysone-induced puffing was always 2 hours (Fig. 9). Taken together, these results suggested that it is the length of time required for a locus to proceed in normal development through the first step, rather than the second step, that determines the time at which puffs appear in the locus.

DISCUSSION

Heat shock puff induction by digitonin

Unlike developmentally regulated puffs, heat shock puffs were always induced by digitonin irrespective of the developmental stage of salivary glands. This suggests that heat shock puff loci differ from developmentally regulated puff loci in the molecular condition exposed to digitonin. The same inference has been drawn from a previous observation that digitonin activation of heat shock puffs, but not of other digitonin-responsive puffs, is repressed by preheat treatment of salivary glands (Myohara and Okada, 1988c). Mosser *et al.* (1990) reported that Nonidet P-40 and Triton X-100 activate DNA-binding of heat shock transcription factor (HSF) to bind to DNA by changing conformation of the factor. It is possible that digitonin activates heat shock genes through activation of HSF which probably exists always in salivary gland cells. It is also possible that digitonin activates heat shock genes by derepressing RNA polymerase II molecules poised at the 5' end of the genes in non-heat-shocked *Drosophila* cells (Gilmore and Lis, 1985; Rouguie and Lis, 1988; O'Brien and Lis, 1991; Weeks *et al.*, 1993).

Digitonin irresponsiveness

Failure in activation of the early puff loci suggests that digitonin cannot mimic the function of ecdysone to activate early puff loci. This is consistent with a previous observation that early puffs

are induced in digitonin-treated salivary glands only when the glands are incubated in the presence of ecdysone (Myohara and Okada, 1987, 1988b). The exceptional digitonin-responsiveness of an early puff locus, 63F, may be due to this locus being sensitive to heat shock (Bonner and Kerby, 1982). With the same logic the irresponsiveness of a late puff locus, 78C, is ascribable to its dependency on ecdysone for the activation (Stone and Thummel, 1993).

The digitonin irresponsiveness of intermolt and late puff loci after the stages of developmentally programmed puffing suggests occurrence of digitonin-resistant, self-regulation mechanism(s) which suppresses its own gene expression after the developmentally regulated puffing ceased. Self-regulation of puffing has been suggested in three cases. First, early puffs are no longer induced by ecdysone since normally expressed puffs have regressed (Richards, 1976). Since the regression of early puffs depends on *de novo* protein synthesis (Ashburner, 1974), the ecdysone-irresponsiveness of early puff loci after their normally scheduled expression can be explained by suppression effects of the accumulation of its own product. Second, heat shock genes are no longer activated by a temperature elevation when a significant amount of heat shock proteins (HSPs) has been accumulated in the cell (DiDomenico *et al.*, 1982; Myohara and Okada, 1988c). Moreover, accumulation of HSPs prevents heat shock puff loci from being activated by digitonin, suggesting that the self-regulation mechanism is digitonin resistant

(Myohara and Okada, 1988c). Third, endogenous expression of β FTZ-F1 gene, recognizable as a midprepupal puff 75CD, is repressed by its own product (Woodard *et al.*, 1994). It is thus reasonable to presume that intermolt puffs and late puffs are repressed by a digitonin-resistant, self-regulation mechanism after the normal expression.

Identification of digitonin-responsive puff loci

Since I located digitonin-responsive puff sites only by the light microscopic observation of salivary gland chromosomes, it could be argued that there is no molecular basis evidence for identification of the digitonin responsive loci as late puff loci. However, it is unlikely that digitonin activates unrelated genes just locating so close to a late puff site as inseparable under a light microscope. Because the developmental stage at which a puff locus can respond to digitonin is so well correlated with its developmentally programmed puffing stage. Additional puffs were never induced by digitonin at sites nearby a developmentally regulated puff. Moreover, digitonin-responsive puff sites could not be activated by ecdysone when a protein synthesis inhibitor, cycloheximide, was added to the culture medium (with four exceptions, 50C, 63DE, 63F and 71DE, Figs. 13 and 16). This indicates that at least 13 out of 17 digitonin-responsive puff loci are late puff loci, as the protein synthesis dependency on gene activation by ecdysone is an important character of late puffs for which it is distinguished from early puffs (Ashburner, 1974).

Mechanism of premature induction of late puffs by digitonin

Ashburner and Richards (1976) showed that some late puffs were induced prematurely by withdrawal of ecdysone from the culture medium of salivary glands, and proposed that the ecdysone-receptor complex is preventing these late puff loci from premature puffing. In my system, this was anticipated to occur in some of the digitonin-untreated controls where salivary glands were dissected out from larvae at stages of a high ecdysone titer and then incubated for two hours without ecdysone. Against the anticipation, the premature puffing was observed only at four loci (42C, 50F, 63DE, and 82F) out of 20 late puff loci examined (shadowed bars marked with an upward arrow in Figs. 5-6 and 8). This suggests that the premature late puff induction by digitonin is not due to washout of ecdysone-receptor complex that can occur during the detergent treatment.

An anionic detergent, sarkosyl, has been demonstrated to stimulate RNA synthesis in nuclei from chicken or mammalian cells (Gariglio *et al.*, 1974; 1981; Green *et al.*, 1975). This stimulatory effect of sarkosyl on transcription is explained by derepression of RNA polymerase II on DNA templates by removal of chromosomal proteins which prevent the enzyme from starting transcription (Green *et al.*, 1975). Engaged RNA polymerase II molecules have been shown to be present not only at the sites of genes transcribing at a given stage, but also at all sites of puffs which are formed in polytene chromosomes of *Chironomus* salivary glands throughout development

(Hameister, 1977; Sass, 1980). RNA polymerase II molecules have been shown to present at ecdysone-inducible puff loci on *Drosophila* salivary gland chromosomes even when neither puffing nor nascent RNA is found at the loci (Weeks *et al.*, 1993). Thus, a rational explanation for the premature late puff induction by digitonin is that the detergent removes repressor molecules from the late puff loci. Alternatively, the detergent may activate a preexisting positive factor by changing its conformation like the case of heat shock transcription factor (HSF) (Mosser *et al.*, 1990).

Regulation of digitonin-inducible step of late gene activation

Ashburner *et al.* (1974) have proposed that late puff loci are activated by protein products of early genes (see Introduction). As I mentioned above, protein synthesis is required to induce puffs by ecdysone at the digitonin-responsive late puff loci, suggesting the involvement of protein products of early genes in activation of these digitonin-responsive late puffs. Therefore the most simple explanation for the premature induction of late puffs by digitonin would be that digitonin mimics a function of protein products of early genes to remove repressors from late puff loci. The explanation is well applicable to the group A late puff loci, which respond to digitonin as early as at PS1 when no proteins from early puffs exist in the cells (for example, E74A and E75A, see Boyd *et al.*, 1991; Hill *et al.*, 1993). In contrast, the group-B late puff loci respond to digitonin only at stages later than PS1, after early puffs become

prominent. However, I have demonstrated that digitonin-inducible step (the second step) of gene activation at four group-B loci requires both ecdysone and protein synthesis unless digitonin is applied. This may support the idea that a protein product(s) of an ecdysone-inducible early gene(s) is involved in this process. Because the four group B loci respond to ecdysone rather rapidly (they form puffs in 4-6 hours after ecdysone treatment starts, see Fig. 9), it is unlikely that expression is induced by a product of another ecdysone-inducible late gene.

The first candidates for early gene products involved in gene activation at digitonin-responsive late puff loci are E74A protein and E75A protein, which have been demonstrated to be products of genes responsible for early puffs 74EF and 75B. These proteins were shown to be present at many puff loci in *Drosophila* larval salivary gland chromosomes (Urness and Thummel, 1990; Hill *et al.*, 1993), which I found in the present study to be digitonin-responsive. Although the function of these proteins in the process of late puff induction has not been shown, it may be similar to the one accomplished by digitonin.

Regulation of acquisition of digitonin-responsiveness at group-B late puff loci

My results suggest that gene activation at group-B loci follows the step to make the loci responsive to digitonin (the first step of gene activation) before the digitonin-inducible step (the second step). I

have demonstrated that the first step of gene activation can be induced *in vitro* by ecdysone at four different group-B loci (2B13-17, 9E, 42C and 55E). This induction was not inhibited by cycloheximide. However, this is not to imply that the first step is a primary response to ecdysone, because even cycloheximide alone (in the absence of ecdysone) can induce the first step of gene activation. A simple explanation for the results of the present study is that ecdysone acts indirectly by activating an early gene(s) whose product inactivates a repressor(s) that prevents these loci from responding to digitonin. One possibility is that cycloheximide mimics the action of the early gene product by inhibiting the synthesis of a hypothetical short-lived repressor. Another possible explanation is that the first action of ecdysone is to increase the concentration of a positive factor that acts in the same region as does the repressor (negative). Cycloheximide treatment may reduce the concentration of the repressor, making the concentration of the positive factor relatively higher, which alters the loci into their responsive states. However, the latter explanation is less reasonable, since the salivary glands used in the present study are unlikely to contain an early gene product at the beginning of any of the experiments (refer to Materials and Methods for the staging of the larvae).

The first candidate I propose for the hypothetical repressor that is capable of preventing the four loci from proceeding through the first step of gene activation is the product of an intermolt puff that is active in salivary glands before the surge of ecdysone and

regresses under the influence of the hormone (Ashburner, 1972b; 1973). However, it is unlikely that the repressor is a product of either 3C, 25AC or 68C loci, because these well-studied intermolt puffs regress even in the absence of ecdysone, albeit slowly in some cases (Ashburner, 1972b; 1974; Ashburner and Richards, 1976). Conversely, I was unable to induce digitonin-responsiveness at late puff loci in the absence of ecdysone unless protein synthesis was inhibited. Thus I presume a repressor encoded by an unknown gene that is active in larval salivary glands before the surge of ecdysone and that never regresses in the absence of ecdysone.

Models for the two-step regulation of late puffs

Based on the results I presented here, I propose two models for the two-step regulation of late puff activation (Fig. 17).

Model 1: The first step is put in motion when the ecdysone titer has reached high enough to activate an early gene (early gene 1) encoding a derepressor (derep. 1) that acts on the late puff locus to remove one of the two repressors (rep. 1) which hinders the locus to become digitonin-responsive. Rep. 1 is assumed to be short-lived. Cycloheximide can thus mimic the function of derep. 1 by cutting off the supply of rep. 1. In the second step, ecdysone activates another early gene (early gene 2) that encodes the other derepressor (derep. 2) that removes a cycloheximide-resistant (long-lived) repressor (rep. 2) from the locus. The locus is thus derepressed and starts puffing. Digitonin mimics the function of derep. 2 by removing rep. 2 from the

locus.

Model 2: The first step is principally the same as in the model 1. Only difference is that model 2 presumes a pre-existing dominant activator. In the second step, ecdysone activates a second early gene (early gene 2) encoding a modulator (mod.) that activates the pre-existing activator (act.) by changing its conformation. Digitonin mimics the function of the modulator by altering the conformation of the activator.

In both models, the loci go through a sequence of three states (inactive, digitonin-responsive and active state) in a step-wise fashion. Such a gene regulation system that involves two (or more) steps should be more effective than a system in which a simple switching event controls the complex changes in gene expression at more than 100 late puff loci during larval-prepupal metamorphosis in *Drosophila* salivary glands. Two groups of late puff loci of which existence the present study demonstrated can be characterized as being different in the step essential for determing the timing of gene activation; in group-B loci the first step, rather than the second step, determines the timing of developmental programmed puff formation, while in group-A loci the first step has defaultly been achieved, and only the second step is ready to function, waiting for early gene 2 or digitonin acts on them at proper stages of programmed puffing.

Ashburner (1972a) reported that there are more than 100 late puff loci in *Drosophila* salivary gland chromosomes. Among the late puff loci, I examined digitonin-responsiveness at 20 loci and found

16 digitonin-responsive loci. More detailed analyses may reveal larger number of late puff loci where the digitonin-inducible step takes part in temporal control of gene expression. For testing the two-step regulation model, further studies must be fullfiled at molecular level, for example isolating genes responsible for digitonin-responsive late puffs, and identifying proteins released from chromosomes by digitonin treatment. Such analyses at molecular level may also contribute to the understanding of the regulation of other hormonally controlled genes that are active in tissues where the puffing analysis is not feasible.

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TABLES

TABLE 1

List of Puff Sites Measured

Puff location	Reference band
2B5-6 2B13-17 2EF	3A
7D 9E 10E	8E
13A 13E 14BC	12A
18DE	18A
25AC 25D 26B	24A
22B	24D
29F 30F 33B	32A
37E	38C
42A 42C 45A 49E 50C 50D 50F	43A
55B 55E	54A
56DE 58DE	58A
63C 63DE 63F 64EF	63A
67B 67C 68C 70A 70C 71DE 73B 74EF 75B 78C	68A
82F 84B 84E 85F	85C
87A 87C 88D 88E	87F
93B 93D 95B 95D 96B	93F

TABLE 2.

Influence of the Digitonin Concentration on the Number of Puffs Induced*

	Digitonin Concentrations (%)					
Puff Stage	0.001	0.003	0.005	0.0075	0.0085	0.01
1	3	–	36	–	32	33
2	–	6	21	5	–	–
4	–	4	14	3	–	–
6	–	3	13	3	–	–
8-9	0	8	17	12	10	7
10	–	1	12	2	–	–
11	–	0	9	0	–	–

* The numbers of loci where digitonin induces puffing at indicated concentration are shown. The effects of digitonin on puff formation were evaluated by comparing puff sizes of digitonin-treated glands with digitonin-untreated controls. Puff sizes were as a rule measured at 38 puff sites presented in Table 4 at each of indicated stages, but at PS1 and PS8-9 puff sizes were measured at all of the loci where puffing was observed in digitonin-treated glands.

TABLE 3

Puff Induction by Digitonin in the
Chromosome Ⅲ in PS8-9 Salivary Glands

	Digitonin Concentrations (%)[1]					
Loci[2]	0.001	0.025	0.005	0.0075	0.0085	0.01
63C	−	+	+	+	−	+
63DE	−	+	+	+	+	−
63F	−	−	+	−	−	+
67B	−	−	+	−	−	+
70A	−	−	+	+	−	+
71DE	−	−	+	+	−	−
73B	−	−	+	+	−	−
84E	−	−	+	−	−	−
87A	−	+	+	−	+	+
87C	−	+	+	−	+	+
93D	−	+	+	+	+	−
95B	−	−	+	−	−	−
95D	−	+	+	+	+	+
96B	−	−	+	+	−	−

[1] It is shown whether indicated loci respond to digitonin to form puffs when salivary glands are treated at each of indicated concentration of digitonin for 20 min. The effect of digitonin on puffing at a locus was evaluated by comparing the mean value of puff sizes of digitonin-treated glands with those of digitonin-untreated controls.

[2] Indicated loci are all among the loci in the chromosome Ⅲ where digitonin induces puffing at PS8-9 .

TABLE 4

Digitonin-Response at Twenty-Nine Chromosomal Sites

	Digitonin responsive stage in PS1-11			
Category[1]	Always	From PS1 until DPPS[2]	Immediately before DPPS	Digitonin Irresponsive
Heat shock puff loci	33B 63C 64EF 67B 70A 87A 87C 93D 95D	–	–	–
Intermolt puff loci	–	–	–	25AC 58DE 68C
Early puff loci	–	63F	–	2B5-6 22B 74EF 75B 85F
Late puff loci	–	10E 25D 49E 50C 50D 50F 55B 56D 84E 93B	2B13-17 9E 42C 55E 63DE 71DE	29F 78C 82F 88E

[1] Puff loci were classified according to Ashburner and Bonner (1979) for heat shock puffs, and to Ashburner (1972b) for developmentally regulated puffs.

[2] DPPS is an abbreviation for developmentally programmed puff stage.

TABLE 5

Inhibition of [^{3}H]-Methionine Incorporation by Cycloheximide

Incubation time (hr)	Experiment No.	Control[1] (cpm)	with cycloheximide[2] (cpm)
0	1	1081	ND
	2	2810	ND
2	1	108308	2175
	2	59415	7272

[1, 2] Five lobes of salivary glands were incubated in the presence of ecdysone (2.5×10^{-6}M) with or without cycloheximide (7.0×10^{-5}M) for an indicated length of time. [^{3}H]-methionine was added to each incubation at a final concentration of 100 μCi/ml. After the incubation, the incorporation of [^{3}H]-methionine into the 10% TCA insoluble fraction was studied as described by Ashburner (1974).

FIGURES

Fig. 1 Puffing pattern of chromosomes from digitonin-treated salivary glands at PS8-9 (a), and PS1 (b). Salivary glands were treated with digitonin and then incubated in a buffer containing nucleoside triphosphate (MTB1 + NTPs). Arrows indicate puff loci which were activated by digitonin in PS8-9 salivary glands but not in PS1 glands; arrowheads indicate puff loci which were activated by digitonin at PS1 but not at PS8-9. Bar represents 20 μm.

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Fig. 2 Puffing pattern of chromosomes in digitonin-untreated salivary glands after the incubation in a buffer containing nucleoside triphosphate (MTB1 + NTPs). The puff loci analyzed are marked as in Fig. 1. (a) PS8-9; (b) PS1. Bar represents 20 μm.

salivary
e (MTB1
PS1. Bar

Fig. 3 Effects of digitonin on the puff size at heat shock puff loci. Puff sizes at the indicated loci were measured in digitonin-untreated salivary glands before (open bars) and after incubation in a buffer containing nucleoside triphosphate (MTB1 + NTPs) (shadowed bars) and in digitonin-treated salivary glands after incubation (solid bars). Asterisks indicate puffing induced by digitonin. Bold bars underneath abscissa of each histogram indicate the stages at which developmentally programmed puffing occurs, i.e. puff size in untreated, non-incubated glands is >1.15. Each vertical bar represents the mean value of 6-33 measurements (15 on average) with the standard error.

33B
63C
64EF
67B
70A
87A
87C
93D
95D
Puff size
Puff stage
1
2
4
6
8
10
11

Fig. 4 Puff induction by digitonin at heat shock puff loci, 33B (a, b, d, e) and 93D (c, f). (a-c) Salivary gland chromosomes from digitonin-untreated glands after incubation in a buffer containing nucleoside triphosphate (MTB1 + NTPs); (d-f) Salivary gland chromosomes from glands digitonin-treated, then incubated. Digitonin induced puffing at both stages before developmental programmed puffing begins (PS2, a, d) and after developmentally programmed puff regresses (PS11, b, e). At a stage during developmentally programmed puffing (PS4, c, f), developmental puffs were enlarged by digitonin treatment. Bar represents 5 μm.

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, c, f),
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Fig. 5 Effects of digitonin on the puff size at group-A loci among developmentally regulated puff loci. Upward and downward arrows respectively indicate the increase and decrease of puff sizes caused solely by incubation. For further explanations, see the legend for Fig. 3.

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Fig. 6 Effects of digitonin on the puff size at group-B loci among developmentally regulated puff loci. Upward arrows respectively indicate the increase of puff sizes caused solely by incubation. For further explanations, see the legend for Fig. 3.

2B13-17
9E
42C
55E
63DE
71DE
Puff size
Puff stage
1
2
4
6
8
10
11

Fig. 7 Puff induction by digitonin at a group-A locus, 55B and a group B locus, 55E. Micrographs show part of chromosomes from digitonin-untreated glands after incubation in a buffer including nucleoside triphosphate (MTB1 + NTPs) (a-c), and from digitonin-treated and incubated glands (d-f). (a, d) PS1; (b, e) PS8; (c, f) PS11. Note that 55B was induced by digitonin from PS1. In contrast, 55E was not induced at PS1 and induced only at PS8, that is the stage immediately before developmentally programmed puffing begins. Puffing activities at both loci were unaffected by digitonin during stages of their developmentally programmed puffing (55B at PS8, and 55E at PS11, respectively). After developmentally programmed puff regressed, digitonin failed to induce puffing (55B at PS11). Bar represents 5 μm.

a
55B
55E
d
b
55B
55E
e
c
55B
55E
f

Fig. 8 Digitonin irresponsiveness at 12 developmentally regulated puff loci including three intermolt (25AC, 58DE, 68C), five early (2B5-6, 22B, 74EF, 75B, 85F) and four late (29F, 78C, 82F, 88E) puff loci. Upward and downward arrows respectively indicate the increase and decrease of puff sizes caused solely by incubation. For further explanations, see the legend for Fig. 3.

2B5-6
22B
25AC
29F
58DE
68C
74EF
75B
78C
82F
85F
88E
Puff size
Puff stage
1
2
4
6
8
10
11

Fig. 9 Induction of digitonin-responsiveness by ecdysone in salivary glands cultured *in vitro*. Puff sizes at (a) 2B13-17, (b) 9E, (c) 42C and (d) 55E loci are plotted against time in hour of culture. Experimental designs are presented at the bottom, indicating the time of exposure to ecdysone (E; 0-10 hr), treatment with digitonin (D; 20 min) and incubation in a buffer containing nucleoside triphosphate (MTB1 NTPs) (□; 2hr). (◆) Salivary glands were cultured in modified Grace's medium with ecdysone for 0-10 hr. They were then treated with digitonin for 20 min and incubated in MTB1 + NTPs for 2 hr in order to grow puffs in the absence of ecdysone. (◇) Salivary glands were treated as in above, except the digitonin was omitted. (○) Salivary glands were fixed immediately after culture with ecdysone to see ecdysone-induced puf formation. The initiation of ecdysone-induced puff formation at each locus is pointed by a vertical arrow. Asterisks mark puffing induced by digitonin, determined by a comparison of results from salivary glands treated with and without digitonin. Horizontal arrows show the time period during which the loci are in the first step (1st), the second step (2nd) and the puffing state (puffing). During the puffing state (puffing), regression of ecdysone-induced puffs by continous 2 hr culture without ecdysone (◇) was evident. This is due to requirement of ecdysone for the loci to maintein puffing state (Ashburner and Richards, 1976).

(a)
(b)
(c)
(d)
1st
2nd
Puffing
Puff size
1.5
1.3
1.1
0.9
0
2
4
6
8
10
Time of culture with ecdysone (hr)
+E
+D
−D
20 min
2hr
0 -10 hr

Fig. 10 Effect of protein synthesis inhibition on the acquisition of digitonin-responsiveness in (a) 2B13-17, (b) 9E, (c) 42C and (d) 55E loci. As shown in the schematic diagram at the bottom salivary glands were cultured in modified Grace's medium under four different conditions: (●) with ecdysone (+E) alone; (○) without either ecdysone or cycloheximide; (◆) with both ecdysone and cycloheximide (+E+C); or (◇) with cycloheximide (+C) alone. Salivary glands were then incubated either with or without digitonin (+/−D) for 20 min and then incubated in MTB1 containing nucleoside triphosphate (MTB1 + NTPs) (□) for 2 hr in order to make puffs to grow. The effects of digitonin on puffing activity at different loci are plotted against the time of the culture in Grace's medium. The effects of digitonin are shown as differences (±SE) between puff sizes in digitonin-treated glands and those in digitonin-untreated controls. No puffing was observed without digitonin treatment under any culture condition except in the presence of ecdysone alone.

(a)
(b)
(c)
(d)
Digitonin effect
Culture time before digitonin treatment (hr)
+E
+/- D
+E+C
+C
0 -10 hr
20min 2 hr
0 - 6 hr

Fig. 11 Inhibition of early puff regression at (a) 74EF and (b) 75B loci by protein synthesis inhibition. As shown in the experimental design at the bottom, salivary glands were incubated with ecdysone and cycloheximide (●; E+C) or with ecdysone alone (○; +E) for 0-10 hr. Puff sizes at the two loci are plotted against culture time.

(a)
2.5
2.1
1.7
1.3
0.9
0
2
4
6
8
10
Puff size
(b)
2.9
2.5
2.1
1.7
1.3
0.9
0
2
4
6
8
10
Culture time (hr)
(—)
+E+C
(····)
+E
0 - 10 hr

Fig. 12 Requirement of ecdysone in the second step of gene activation. Puff sizes at (a) 2B13-17, (b) 9E, (c) 42C and (d) 55E loci are plotted against the total culture time. Experimental conditions are shown at the bottom. (●) Salivary glands were incubated in modified Grace's medium with ecdysone (+E) for 0-6 hr. (○) Salivary glands were incubated in medium with ecdysone (+E) for 2 or 4 hr, transferred to ecdysone-free medium (−E) and incubated for another 2 hr.

(a)
(b)
(c)
(d)
Puff size
1.5
1.3
1.1
0.9
1.4
1.2
1.0
0.8
0
2
4
6
Total culture time (hr)
(—)
+E
0-6 hr
(····)
+E
-E
2 or 4 hr
2 hr

Fig. 13 Requirement of protein synthesis for the second step of gene activation. As shown in the experimental design at the bottom, salivary glands were incubated with ecdysone and cycloheximide (●; E+C) or with ecdysone alone (○, +E) for 0-10 hr. Puff sizes at (a) 2B13-17, (b) 9E, (c) 42C and (d) 55E loci are plotted against culture time.

(a)
(c)
(b)
(d)
Puff size
Culture time (hr)
+E+C
+E
0-10 hr

Fig. 14 Induction of puff formation by digitonin in the absence of protein synthesis. As shown in the experimental design at the bottom, salivary glands were cultured in modified Grace's medium with ecdysone and cycloheximide for 0-6 hr. Salivary glands were then incubated either with cycloheximide and digitonin (●; +C+D) or with cycloheximide alone (○; +C−D) for 20 min and then incubated in MTB1 containing nucleoside triphosphates (MTB1+NTPs) (□) with cycloheximide (+C) for 2 hr in order to grow puffs. Puff sizes at (a) 2B13-17, (b) 9E, (c) 42C and (d) 55E loci are plotted against the time of culture with ecdysone.

(a)
(c)
(b)
(d)
Puff size
Time of culture with ecdysone (hr)
+E+C
+C+D
+C
+E+C
+C-D
+C
0 - 6 hr
20 min
2 hr

Fig. 15 Effects of protein synthesis inhibition on ecdysone-induced puff formation. Puff sizes at (a) 2B13-17, (b) 9E, (c) 42C and (d) 55E loci are plotted against the time of culture with ecdysone. Experimental conditions are shown at the bottom. Salivary glands were cultured in modified Grace's medium with (●; +C) or without (○) cycloheximide for 2 hr and then in medium with ecdysone (+E) for 0-10 hr.

(a)
(b)
(c)
(d)
Puff size
Time of culture with ecdysone (hr)
+C
+E
-C
+E
2 hr
0-10 hr

Fig. 16 Requirement of protein synthesis for ecdysone-induced puff formation at digitonin-responsive developmentally regulated puff loci. As shown in the diagrammatic design of experiments, salivary glands were incubated with ecdysone and cycloheximide (●; E+C) or with ecdysone alone (○; +E) for 0-8 hr. Puff sizes at 11 group A (10E, 25D, 49E, 50C, 50D, 50F, 55B, 56D, 63F, 84E, 93B) and two group B (63DE, 71DE) loci are plotted against culture time.

10E
25D
49E
50C
50D
50F
55B
56D
63DE
63F
71DE
84E
93B
Puff size
Culture time (hr)
(—) +E+C
(·····) +E
0 - 8 hr

Fig. 17 Two models proposed for explaining the two-step regulation of ecdysone-inducible late puffs. Ecdysone activates two types of early genes (early genes 1 and 2). The early gene 1 encodes a derepressor (derep. 1) removing a repressor of the late genes (rep. 1). When rep.1 is removed by derep.1 , the puff locus becomes digitonin-responsive. The early gene 2 encodes another derepressor (derep. 2) that removes repressor 2 (rep.2) from the locus and activates the locus to puff (Model 1). Alternatively, the early gene 2 encodes a modulator that activates a preexisting activator for the locus by changing its conformation (Model 2). See text for more details. act, activator; CHX, cycloheximide; derep., derepressor; mod, modulator; rep., repressor.

ecdysone
ecdysone
intermolt genes
early gene1
early gene2
CHX
rep.1
derep.1
derep.2
or
mod
inactive state
digitonin-responsive state
active state
model 1
first step
second step
derep.1
rep.1
derep.2
rep.2
rep.2
rep.1
rep.2
digitonin
OFF
OFF
ON
inactive state
digitonin-responsive state
active state
model 2
act.
first step
derep.1
act.
rep.1
second step
act.
mod
digitonin
rep.1
act.
OFF
OFF
ON

inches
1 2 3 4 5 6 7 8
cm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
C
Y
M
Kodak Gray Scale
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19