Effect of ecd\textsuperscript{l} mutation on the expression of genes mapped at the Drosophila melanogaster 3C11-12 intermoult puff

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Summary

The Drosophila melanogaster ecd\textsuperscript{l} mutation causes a severe temperature-sensitive deficiency in the titre of the steroid hormone ecdysone. This mutation was used to investigate the role of ecdysone in both the transcription of the genes mapped at the 3C11-12 intermoult puff region and the puff formation. Thoroughly synchronized ecd\textsuperscript{l} larvae were shifted to the non-permissive temperature at various times of the development; after 24 or 48 h, the levels of the transcripts derived from Sgs-4, Pig-1 and ng-1, the three genes located at the 3C11-12 polytene bands, were determined. The results showed that the levels of the transcripts encoded by Pig-1 and ng-1 are unaffected by the drop in the ecdysone titre occurring in non-permissive conditions whereas the amount of Sgs-4 mRNA is greatly reduced. These data clearly indicate that transcription of the three genes mapped within the puff region is affected differently by the hormone. Furthermore, ecd\textsuperscript{l} larvae cultured at the non-permissive temperature show a prominent puff at the 3C11-12 polytene bands, indicating that ecdysone is not essential for puff induction and that puff size is not simply correlated with high-level Sgs-4 transcription.

1. Introduction

It is well known that the steroid hormone ecdysone induces a complex pattern of polytene chromosome puffing within the salivary gland nuclei (reviewed by Ashburner & Berendes, 1978). The first set of developmentally-regulated puffs to be observed on the salivary gland chromosomes of Drosophila melanogaster third instar larvae are the so-called ‘intermoult puffs’. A few hours before puparium formation, in concomitance with a strong increase of the ecdysone concentration, the intermoult puffs regress and a small set of ‘early’ puffs is rapidly induced. The early puffs remain active until pupariation, when they regress and a large group of ‘late’ puffs is induced.

While knowledge of the molecular mechanisms responsible for the induction of both early and late puffs is rapidly increasing (Thummel et al. 1990; Burtis et al. 1990; Urness & Thummel, 1990; Segraves & Hogness, 1990), hormonal regulation of the intermoult puffs is far from clearly understood.

So far only two factors regulating puffing at the intermoult loci have been identified: ecdysone and the product of npr, an X-linked locus mapped to the site of the 2B5 early puff. Both factors are required for intermoult puff regression (Ashburner & Berendes, 1978; Belyaeva et al. 1981); however, no signal involved in the induction of this set of developmentally-regulated puffs has so far been clearly identified.

Several years ago, data based on the use of the l(1)su(f)\textsuperscript{1667y} mutation had suggested that ecdysone is required earlier during the development for an efficient RNA accumulation at intermoult puff loci. In fact, larvae carrying this mutation (originally isolated on the basis of its ability to suppress the forked bristle phenotype) are also ecdysone-deficient at the non-permissive temperature (Hansson et al. 1981) and fail to accumulate the transcripts encoded by the genes Sgs-4, Sgs-3, Sgs-7 and Sgs-8 (Hansson & Lambertsson, 1983), all of which map to intermoult puff sites (reviewed by Meyerowitz et al. 1987).

To further investigate the relationship between transcription, puffing and hormonal regulation of the intermoult puffs, we analyzed the effects of ecdysone...
deficiency on RNA accumulation and puffing at the 3C11-12 polytene bands, from which one of the most prominent intermoult puffs originates. The ecdysone deficiency was generated at various stages of larval development by means of the temperature-sensitive ecd' mutation, in which the ecdysone titre has been accurately determined during all larval stages at both permissive (21 °C) and non-permissive (30 °C) temperatures (Garen et al. 1977). When cultured at the restrictive temperature, ecd' larvae exhibit a severe deficiency of ecdysone, whose titre at the late third instar is only 5% that of wild-type strains (Garen et al. 1977). As a consequence of the lack of ecdysone, the larvae fail to pupariate but can remain viable at a stage corresponding to the late third instar for as long as 3 weeks.

In our experiments cloned probes corresponding to each of the three genes mapping within the 3C11-12 puff region, Sgs-4, Pig-1 and ng-1, were used to analyze the ecd' effect on the accumulation of the RNAs encoded by the region. Among the three genes examined, Sgs-4 is the most extensively characterized. It encodes a salivary gland secreted protein (Muskavitch & Hogness, 1980); the same function has been recently suggested for ng-1 (Furia et al. 1990), while Pig-1 function has not yet been clearly established. The three genes are located very close to each other within a DNA segment smaller than 10 kb, with Pig-1 mapping only 813 bp upstream Sgs-4 (Furia et al. 1991) and ng-1 mapping about 6 kb downstream Pig-1 (Furia et al. 1990). Sgs-4, Pig-1 and ng-1 share common structural and regulatory features, all being small uninterrupted genes that are specifically transcribed within the salivary glands during the larval period of the Drosophila life-cycle. Throughout the larval stages, however, each gene shows a particular developmental profile. Sgs-4 mRNA is detected at a high level only during the second half of the third instar (Muskavitch & Hogness, 1980) while ng-1 transcript is present at a high level during the entire third instar (Furia et al. 1990) and Pig-1 RNA is present at a high level throughout all the larval life, reaching a maximal level at the second instar (Chen et al. 1987; Hofmann & Korge, 1987).

It is interesting to note that these genes also share a peculiar localization, being nested within the transcription unit of dnce, a complex gene extending for more than 9 polytene bands and harbouring several genes within its introns (Chen et al. 1987; Furia et al. 1990).

In our experiments, we found that only the expression of Sgs-4 is severely reduced when ecd' larvae are shifted to the restrictive temperature; in contrast, the levels of the RNAs encoded by the neighbouring Pig-1 and ng-1 genes remain totally unaffected. Thus, the expression of the three genes located within the 3C11-12 puff region is not uniformly regulated by ecdysone. In addition, a prominent puff is present at the 3C11-12 region in ecd' larvae growth at non-permissive conditions, indicating that the hormone is not required for puff induction.

2. Materials and methods

(i) Fly stocks and growth of larvae

The temperature-sensitive ecdysone-deficient mutant ecd' (Garen et al. 1977) was utilized for the experiments described in the text. Batches of thoroughly synchronized ecd' larvae were obtained from embryos collected after an egg laying period of 2 h. At the chosen developmental stages, ecd' larvae were shifted from 21 °C (permissive temperature) to 30 °C (non-permissive temperature) and kept at this temperature for periods of 24 or 48 h.

(ii) Preparation and staining of salivary gland polytene chromosomes

Pairs of manually dissected salivary glands were stained in a 2% solution of lacto-aceto-orcein (Sigma) on a coverslip. After a few minutes, the glands were squashed and the stained chromosomes were examined and photographed using a phase contrast Zeiss photomicroscope.

(iii) Ecdysone induction test

Synchronously developing larvae were transferred to the non-permissive temperature (30 °C) 8 h before the time of the second larval moult and maintained at this temperature for 24 h. After a quick wash in Ringer’s medium, the larvae were transferred into Eppendorf tubes containing either a suspension of 50 mg dry baker’s yeast in 100 μl of 5% ethanol (control sample) or the same suspension supplemented with 10 mg ml⁻¹ 20-hydroxy-ecdysone (Sigma). After 6–8 h of further incubation at 30 °C the larvae were collected and the RNA extracted as described in (iv) below.

(iv) RNA isolation and Northern analyses

Total Drosophila RNA was extracted at different developmental times according to the guanidinium/ CsCl method (Chirgwin et al. 1979). For Northern analyses, the RNA was fractionated on 1:2% agarose formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham) and then hybridized and washed according to the protocols suggested by Amersham. Sgs-4 mRNA was detected using as a probe the 0.7 kb EcoRI/Hind III genomic fragment containing most of the Sgs-4 coding region (Muskavitch & Hogness, 1982), while to detect Pig-1 and ng-1 transcripts the 7a1 Pig-1 cDNA clone (Furia et al. 1991) and the SCI ng-1 cDNA clone (Furia et al. 1990) were used respectively. The genomic fragment
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aDm2023 (Garfinkel et al. 1983) was used as Sgs-3 specific probe.

(v) Standard techniques

Phage and plasmid DNAs were isolated as described by Maniatis et al. (1982). The probes consisted of DNA fragments isolated from plasmid digest fractionated in low melting temperature agarose and labelled to a specific activity of at least $5 \times 10^8$ cpm $\mu g^{-1}$ by the Multiprime labelling kit (Amersham).

(vi) Quantitative analysis

Amounts of RNA were measured by analysing the autoradiograms by means of a Zeineh video densitometer (Biomed Instruments Inc., Fullerton, CA).

3. Results

(i) General outline of the experiments

The effect of $ecd^{l}$ mutation on the expression of the three genes located at the 3C11-12 intermoult puff region was studied by means of Northern blot experiments, in which the levels of the transcripts encoded by the Sgs-4, Pig-1 and ng-1 genes were determined at various times of the development at both the permissive (21 °C) and non-permissive temperatures (30 °C). $ecd^{l}$ embryos were collected after an egg-laying period of 2 h and cultured at 21 °C for a given number of hours, corresponding to the chosen developmental stage. At this time, the larval population was divided into three groups: the first was immediately homogenized for RNA extraction; the second group, used as control, was maintained at 21 °C, while the third was shifted to the non-permissive temperature. After 24 or 48 h, the last two groups were homogenized and the RNA extracted and analysed by hybridization with the specific probes. The growth rate at the restrictive temperature is markedly higher, since the complete $Drosophila$ life-cycle is considerably shorter at 30 °C (8 days) than at 21 °C (14 days) (Ashburner & Thompson, 1978). Hence, larvae grown at permissive and non-permissive temperatures are best compared on the basis of the developmental stage achieved, rather than the number of hours following egg laying.

As an internal control of the amounts of RNA loaded in each lane, the filters were subsequently hybridized to the probe corresponding to the gene encoding $Drosophila$ ribosomal protein rp49, which is constitutively transcribed at a constant level throughout the life cycle (O’Connell & Rosbash, 1984).

(ii) Effect of $ecd^{l}$ mutation on the level of Sgs-4 mRNA

The accumulation of the mRNA encoded by Sgs-4, the glue gene mapped at the 3C11-12 intermoult puff, was first determined as an internal control. Since the transcription of the genes encoding the salivary gland secreted proteins (Sgs proteins) has been reported to be positively regulated by the ecdysone at the beginning of the third larval instar (Hansson & Lambertsson, 1983), the accumulation of Sgs-4 mRNA is expected to be strongly reduced when $ecd^{l}$ larvae are shifted to the restrictive temperature.

Sgs-4 encodes a single mRNA species, about 1-2 kb long, detected at a high level only during the second half of the third larval instar (Muskavitch & Hogness, 1980). As shown in Fig. 1 (lanes A–F), $ecd^{l}$ larvae cultured at 21 °C show the same Sgs-4 developmental expression profile as wild-type strains. When $ecd^{l}$ larvae were shifted from 21 to 30 °C at 88, 100 and 116 h after egg laying (corresponding, respectively, to the midpoint and the end of the second larval stage and the beginning of the third) and maintained at this temperature for a further 24 or 48 h (lanes G–H, I–L,

![Fig. 1. Effect of the $ecd^{l}$ mutation on the Sgs-4 mRNA level. On the top is shown a restriction map of the genomic region, with the respective positions of the Sgs-4, Pig-1 and ng-1 genes and that of dunce exons 1 and 2. Total RNA was extracted at different developmental stages, indicated as: L2, second larval instar; L3, third larval instar; P, pupae. Lanes A-F: developmental expression profile of the Sgs-4 gene in $ecd^{l}$ strain cultured at 21 °C. The RNA was extracted respectively at 88, 100, 116, 140, 164 and 190 h after egg laying. Lanes G, H: RNA extracted from $ecd^{l}$ larvae grown at 21 °C for 88 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane G) or 48 h (lane H). Lanes I, L: RNA extracted from $ecd^{l}$ larvae grown at 21 °C for 100 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane I) or 48 h (lane L). Lanes M, N: RNA extracted from $ecd^{l}$ larvae grown at 21 °C for 116 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane M) or 48 h (lane N). Lanes O, P: RNA extracted from $ecd^{l}$ larvae grown at 21 °C for 140 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane O) or 48 h (lane P). 10 μg of total RNA was applied in each lane.](https://www.cambridge.org/core/terms)


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Table 1. Quantitative analyses of Sgs-4 mRNA level in the ecd' strain cultured at permissive (21 °C) and non-permissive conditions (30 °C)

| Lane | Sgs-4 | rp49 | Sgs-4/rp49 | Relative amount² |
|------|-------|------|------------|-----------------|
| 21 °C |       |      |            |                 |
| D    | 2.29  | 7.66 | 0.08       | 0.89            |
| E    | 10.93 | 8.98 | 1.08       | 1               |
| 30 °C |       |      |            |                 |
| I    | 0.02  | 9.03 | 0.02       | 0.01            |
| L    | 2.71  | 8.20 | 0.33       | 0.27            |
| M    | 2.35  | 7.40 | 0.31       | 0.25            |
| N    | 1.03  | 7.43 | 0.13       | 0.10            |
| O    | 14.16 | 12.94| 1.09       | 0.90            |
| P    | 4.92  | 9.72 | 0.50       | 0.41            |

² The data derive from densitometric analysis of autoradiograms corresponding to the experiment described in Fig. 1. The filters have been overexposed to allow an estimation of the low Sgs-4 amount present in lane I.

The Sgs-4 relative amount is calculated as the ratio of the Sgs-4/rp49 value of the examined lane (representative of the accumulation of Sgs-4 mRNA at 30 °C) versus the Sgs-4/rp49 value of lane E (representative of the accumulation of Sgs-4 mRNA at 21 °C).

Fig. 2. Effect of the ecd' mutation on the Sgs-3 mRNA level. The same filter shown in Fig. 1 was washed to eliminate both Sgs-4 and rp49 probes and then rehybridized to the Sgs-3 specific probe. Lanes A–P as in Fig. 1.

M–N), the level of Sgs-4 mRNA was drastically reduced as compared to that of the controls (lanes D, E). The Sgs-4 mRNA is in fact nearly absent in most of the shifts made during the second instar (lanes G–H–I–L). However, a low transcript level can be detected in some of these lanes when the filters are overexposed (data not shown). Shifts made at the beginning of the third instar (lanes M, N) also cause a strong reduction in Sgs-4 mRNA level. No reduction was observed when the shifts were made at a successive developmental time (lane O), although the level of Sgs-4 mRNA became reproducibly lower when the larvae are kept at the restrictive temperature for a longer period (lane P). Densitometric analysis of the autoradiograms obtained after hybridization with Sgs-4 and rp49 specific probes allowed us to calculate for each lane the Sgs-4/rp49 ratio; these normalized values were then used to estimate the reduction of the Sgs-4 mRNA level at the restrictive temperature. The data obtained, shown in Table 1, indicated a sixfold mean reduction of Sgs-4 mRNA level when the shifts are made at the beginning of the third instar (lanes M, N) while a much greater reduction is observed when the ecdysone deficiency is established at earlier developmental stages (lanes G–H–I–L). All the shifts that precede Sgs-4 transcription, however, strongly affect the accumulation of Sgs-4 mRNA as compared to the controls indicating that ecd' + function is required to induce Sgs-4 transcription. In contrast, when the shifts are made after the onset of Sgs-4 transcription (lanes O, P), the level of Sgs-4 mRNA is not significantly affected. In these conditions Sgs-4 mRNA is also detected at a high level for a more prolonged period as compared to wild-type strains, probably because of the lack of an ecdysone increase at the restrictive temperature. Therefore, once Sgs-4 transcription has started, it proceeds at a high level even in the absence of the ecd' + gene product. This finding suggests that the requirement for ecd' + function is restricted to a defined developmental period, comprised between 100 and 140 h after egg laying at 21 °C. We found that the ecd' + function is also required, during the same period of development, for the induction of the Sgs-3 gene, one of the three glue genes mapped at the 68C intermoult puff region and that the reduction of the Sgs-3 mRNA level is even more extreme than that shown by Sgs-4 mRNA at the restrictive temperature (see Fig. 2).

Redfern & Bownes (1983) reported that the ecd' mutation causes a set of pleiotropic effects, including a reduction in the size of the salivary glands, probably due to cell death and/or inhibition of tissue growth. To check whether the effect of ecd' on the accumulation of both Sgs-4 and Sgs-3 RNA was directly correlated to ecdysone deficiency, ecd' larvae collected at 100 h after egg laying were transferred to the restrictive temperature; after 24 h the population was divided into two groups and kept at the non-permissive temperature for an additional period of 6–8 h. During this period, the first group was allowed to feed on a solution of 5% ethanol supplemented with yeast (control sample; Fig. 3, lane D) while the second was grown in the same medium supplemented with 10 mg ml⁻¹ of 20-hydroxy-ecdysone (Fig. 3, lane E). The addition of exogenous ecdysone to the growth medium fully reverses the effect of the ecd' mutation confirming that transcription of both the Sgs-4 and Sgs-3 glue genes is ecdysone-inducible. Note that in this experiment the amount of Sgs-3 mRNA in restrictive conditions (lane D) is higher than that usually obtained (see Fig. 2). This is probably due to the manipulation required by the experiment which
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Fig. 3. Ecdysone rescue of the effect of ecd' mutation on the accumulation of Sgs-4 and Sgs-3 mRNAs. Lanes A–E: RNA extracted from ecd' larvae grown at 21 °C for 100, 140 and 164 h. Lanes D, E: RNA extracted from ecd' larvae grown at 21 °C for 100 h and then shifted to 30 °C; after 24 h at the restrictive temperature, the larvae were divided in two groups maintained at 30 °C for further 6–8 h respectively on a growth medium with (lane E), or without 10 mg ml⁻¹ ecdysonesterone (lane D). 10 µg of total RNA was loaded in each lane.

exposes the larvae, even if for very short time, to the permissive temperature.

(iii) Effect of ecd' mutation on the level of Pig-1 and ng-1 transcripts

The ecd' mutation was used to analyse the role of ecdysone on the expression of Pig-1 and ng-1, the two other genes recently mapped within the genomic region required for the 3C11-12 puff formation. The effects of ecd' on the accumulation of Pig-1 and ng-1 transcripts are shown in Figs 4 and 5.

As mentioned in the introduction, Pig-1 high-level transcription starts during the first larval instar, thus preceding that of Sgs-4 and ng-1. Small peaks of ecdysone, however, have been described at each stage of the Drosophila life-cycle (Richards, 1981), including embryos and first instar larvae, and could therefore account for Pig-1 induction at these early stages. To check this hypothesis, ecd' embryos grown at the restrictive temperature for 24 or 48 h after egg laying (until they reach the first larval instar) were homogenized and the extracted RNA examined by Northern blot analysis (Fig. 4, lanes F, G). The effect of ecd' on the accumulation of Pig-1 transcript was also followed by means of a series of shifts made at successive developmental times, that is at the beginning of the first, second and third larval stages (Fig. 4, lanes H, I; L, M; N, O). The level of Pig-1 transcript was totally unaffected by the drop of ecdysone titre throughout the tested stages, indicating that gene expression is not induced by the hormone.

More surprisingly, similar results were also obtained for the ng-1 gene, whose transcript level is also totally unaffected by ecdysone deficiency occurring at the restrictive temperature (Fig. 5). This finding is quite unexpected. In fact, although the ng-1 high expression level slightly preceeds that of Sgs-4, both genes are abundantly transcribed only during the third instar, concomitantly with the appearance of the puff at 3C11-12.

(iv) Effect of ecd' on 3C11-12 puff formation

To further define the relationship between puff formation and active expression of the genes mapped within the corresponding polytene region, we checked for the presence and size of the 3C11-12 intermoult puff on salivary gland polytene chromosomes of third instar ecd' larvae, at both the permissive and non-permissive temperatures. To this goal, thoroughly synchronized larvae were transferred to the restrictive temperature at 116 h after egg laying at 21 °C (corresponding to a few hours after the second larval moult) and kept at this temperature for additional 48 h. At this point, the salivary glands were manually
4. Discussion

The 3C11-12 puff belongs to the intermoult puff set whose regression is known to be caused by the strong increase of the ecdysone titre occurring a few hours before pupariation. The puff region harbours three closely mapping genes, Sgs-4, Pig-1 and ng-1. Together with the puff originating from the polytene region 68C, 3C11-12 is so far the only intermoult puff where multiple genes have been mapped. Sgs-3, Sgs-7 and Sgs-8, the three genes mapped at 68C, are transcribed in an essentially coordinate fashion concomitantly with puff occurrence and their transcription has been proved to be ecdysone-inducible (Hansson & Lambertsson, 1983).

A more complicated situation emerges when analysing the 3C11-12 polytene region. Transcription of the three genes mapped at this region is not coordinate. Although transcription of Sgs-4, Pig-1 and ng-1 ceases simultaneously and at the time of the increase in ecdysone levels and the puff regression, it starts at different times during larval development. In addition, data presented in this paper indicate that these three genes are not similarly responsive to ecdysone concentration. In our experiments, the role of ecdysone on the induction of the genes mapped at the 3C11-12 puff site was investigated by means of the ecdysone-deficient ecd

mutation.

We show that while a wild-type ecdysone titre is required at the beginning of the third instar to induce Sgs-4 transcription, the levels of both Pig-1 and ng-1 transcripts are not influenced by reduced ecdysone concentrations at any of the developmental stages tested. This finding is quite surprising, especially for ng-1, since its developmental expression profile is very similar to that of Sgs-4; furthermore, there is evidence suggesting that, like Sgs-4, ng-1 belongs to the glue gene family (Furia et al. 1990; Furia, unpublished results), four members of which are induced by ecdysone (Hansson & Lambertsson, 1983). However, we cannot exclude the possibility that ng-1 is induced by an ecdysone concentration much lower than that required for induction of other glue genes.

As regards the relationship between puffing and transcription, many data indicate a direct correlation between the presence and size of the 3C11-12 puff and the level of Sgs-4 transcription. In fact, in several variant strains showing strongly reduced Sgs-4 mRNA level, the 3C11-12 puff is either absent or its size is drastically reduced (Korge, 1977; Muskavitch & Hogness, 1982). In contrast, the data reported in this paper indicate that a high level Sgs-4 transcription is not necessary for 3C11-12 puff formation. In fact, a large puff is present on third instar salivary gland chromosomes of ecd

larvae at the non-permissive temperature, a condition in which Sgs-4 transcription is reduced sixfold. This indicates that puffing is not directly dependent on high level-transcription of the corresponding polytene region.

Our results are in agreement with data recently reported by Korge et al. (1990) showing that developmentally regulated chromosomal puffing might occur without significant transcriptional activity within the puff region. These authors suggest that 3C11-12 puff
formation might be induced by the binding of transacting factors at the Sgs-4 upstream regulatory region, the sequence of which would be required for normalized puffing. The fact that variant strains exist in which Sgs-4 transcription and puff size are both strongly reduced in the presence of deletions or sequence alterations within this regulatory region (Muskavitch & Hogness, 1982) also supports this hypothesis.

The control of puffing at intermoult sites has also been discussed recently in a model proposed by Pongs to explain the hormonal regulation of the puffing cycle (Pongs, 1988). He has suggested that ecdysone regulates not only the regression but also the induction of the intermoult puffs. This is based on the observation that ecdysone binds sequentially to the sites of intermoult, early and late puffs (Dworniczak et al. 1983) at the time the puffs were active. The model suggests that the hormone plays the role of positive regulator for these three sets of developmentally-regulated puffs. According to the model, puffing at the intermoult sites is induced at a low ecdysone concentration, as occurs at the beginning of third larval instar (Berreur et al. 1979). This suggestion is stimulating but is not supported by experimental evidence.

The data reported in this paper do not favour this hypothesis but support previous observations indicating that puffing and transcription can be uncoupled.

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