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Effect of *ecd*^{1} mutation on the expression of genes mapped at the *Drosophila melanogaster* 3C11-12 intermoult puff

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Summary

The *Drosophila melanogaster ecd*^{1} 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*^{1} 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*^{1} 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.

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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)*^{ts67g} 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 glue 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*^{1} 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*^{1} 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*^{1} 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 *dunce*, 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*^{1} 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*^{1} 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*^{1} (Garen *et al.* 1977) was utilized for the experiments described in the text. Batches of thoroughly synchronized *ecd*^{1} larvae were obtained from embryos collected after an egg laying period of 2 h. At the chosen developmental stages, *ecd*^{1} 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 *EcoR* I/*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 SC1 *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 digesta fractionated in low melting temperature agarose and labelled to a specific activity of at least 5×10^8 cpm μg^{-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*^{1} 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*^{1} 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*^{1} *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*^{1} 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*^{1} larvae cultured at 21 °C show the same *Sgs-4* developmental expression profile as wild-type strains. When *ecd*^{1} 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,

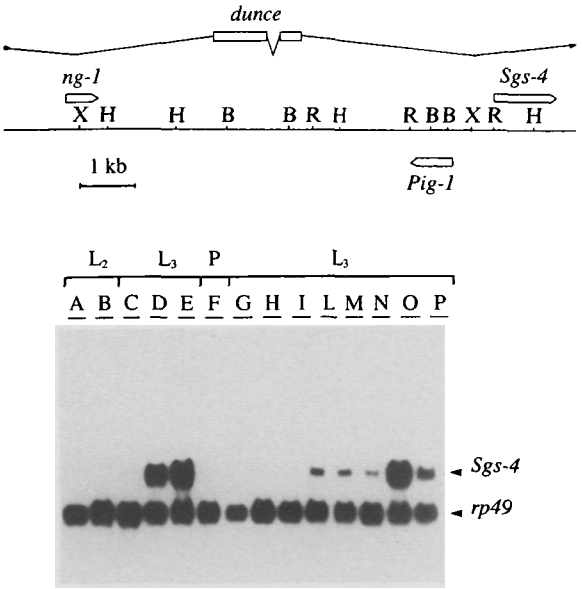

Fig. 1. Effect of the *ecd*^{1} mutation on the *Sgs-4* mRNA level. On the top is shown a restriction map of the genomic region, with the respective positions of the *Sgs-4*, *Pig-1* and *ng-1* genes and that of *dunce* exons 1 and 2. Total RNA was extracted at different developmental stages, indicated as: L_2, second larval instar; L_3, third larval instar; P, pupae. Lanes A–F: developmental expression profile of the *Sgs-4* gene in *ecd*^{1} 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*^{1} 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*^{1} 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*^{1} 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*^{1} 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.

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

Lane	*Sgs-4*	*rp49*	*Sgs-4/rp49*	Relative amount[b]
21 °C				
D	8·29	7·66	1·08	0·89
E	10·93	8·98	1·21	1
30 °C				
I	0·26	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

[a] 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.
[b] 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).

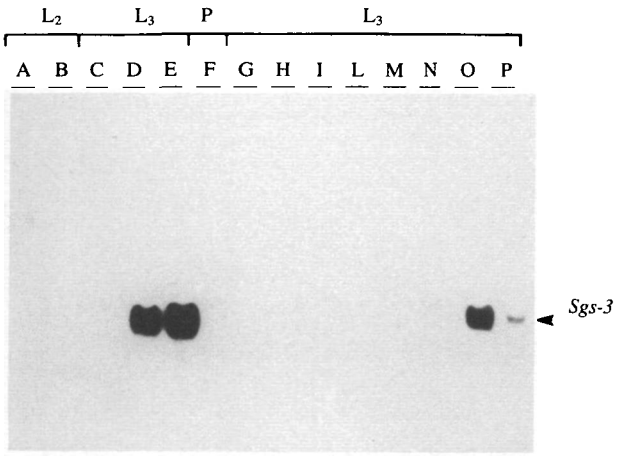

Fig. 2. Effect of the *ecd[1]* mutation on the *Sgs-3* mRNA level. The same filter shown in Fig. 1 was washed to eliminate both *Sgs-4* and *rp49* probes and then rehybridized to the *Sgs-3* specific probe. Lanes A–P as in Fig. 1.

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[1]+* 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 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[1]+* gene product. This finding suggests that the requirement for *ecd[1]+* 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[1]+* 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[1]* 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[1]* on the accumulation of both *Sgs-4* and *Sgs-3* RNA was directly correlated to ecdysone deficiency, *ecd[1]* 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[1]* 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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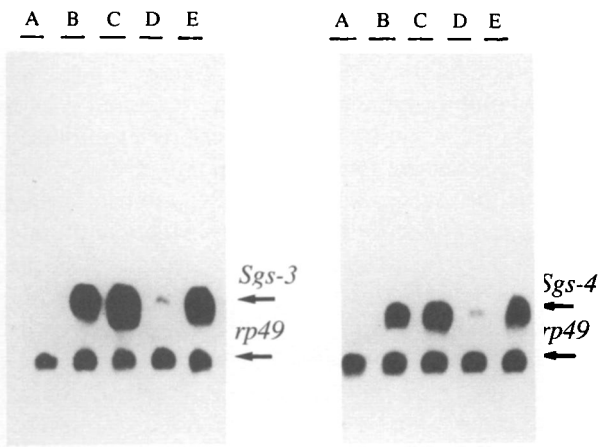

Fig. 3. Ecdysone rescue of the effect of *ecd*^{1} mutation on the accumulation of *Sgs-4* and *Sgs-3* mRNAs. Lanes A–C: RNA extracted from *ecd*^{1} larvae grown at 21 °C for 100, 140 and 164 h. Lanes D, E: RNA extracted from *ecd*^{1} larvae grown at 21 °C for 100 h and then shifted to 30 °C; after 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} ecdysterone (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*^{1} *mutation on the level of Pig-1 and ng-1 transcripts*

The *ecd*^{1} 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*^{1} 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*^{1} 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*^{1} 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

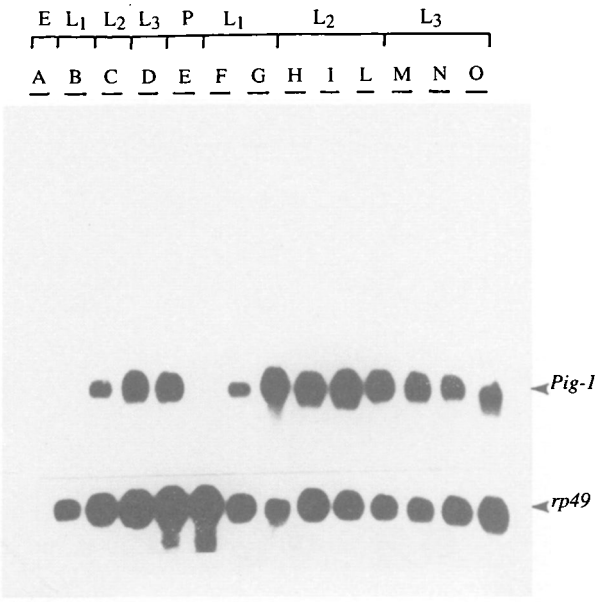

Fig. 4. Effect of the *ecd*^{1} mutation on the level of *Pig-1* transcript. Total RNA was extracted at different developmental stages, indicated on the top: E, embryos; L_1, first larval instar; L_2, second larval instar; L_3, third larval instar; P, pupae. Lanes A–E: developmental expression profile of *Pig-1* gene in *ecd*^{1} strain cultured at 21 °C. The RNA was extracted respectively at 0–2, 50, 76, 116 and 190 h after egg laying. Lanes F, G: RNA extracted from *ecd*^{1} larvae obtained from 0–2 h embryos shifted to 30 °C and maintained at this temperature for additional 24 (lane F) or 48 h (lane G). Lanes H, I: RNA extracted from *ecd*^{1} larvae grown at 21 °C for 50 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane H) or 48 h (lane I). Lanes L, M: RNA extracted from *ecd*^{1} larvae grown at 21 °C for 76 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane L) or 48 h (lane M). Lanes N, O: RNA extracted from *ecd*^{1} larvae grown at 21 °C for 116 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane N) or 48 h (lane O). 10 μg of total RNA was applied in each lane.

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*^{1} *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*^{1} 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

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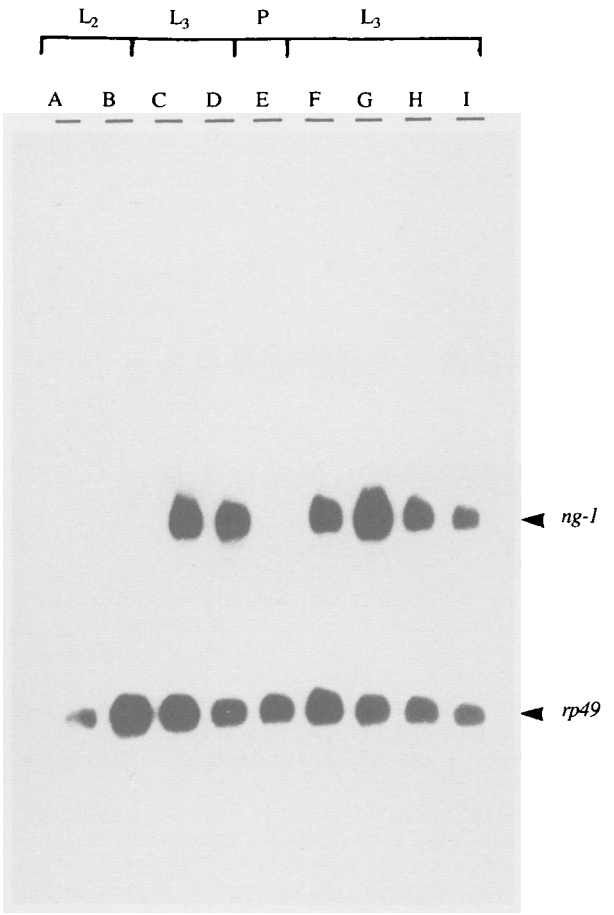

Fig. 5. Effect of the *ecd*^{1} mutation on the level of *ng-1* transcript. Total RNA was extracted at the developmental stages indicated on the top: L_2, second larval instar; L_3, third larval instar; P, pupae. Lanes A–E: developmental expression profile of *ng-1* gene in *ecd*^{1} larvae cultured at 21 °C. The RNA was extracted respectively at 88, 100, 116, 140 and 190 h after egg laying. Lanes F, G: RNA extracted from *ecd*^{1} larvae grown at 21 °C for 88 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane F) or 48 h (lane G). Lanes H, I: RNA extracted from *ecd*^{1} larvae grown at 21 °C for 100 h and then shifted to 30 °C and maintained at this temperature for additional 24 (lane H) or 48 h (lane I). 10 μg of total RNA was applied in each lane.

dissected and squashed and the polytene chromosomes observed after orcein staining. A prominent puff is present at the 3C11-12 polytene bands at both 21 and 30 °C, that is at both normal and reduced ecdysone levels; the width of the puff does not significantly change between the two temperatures (data not shown). This demonstrates that a normal-sized 3C11-12 puff can be formed in conditions in which *Sgs-4* transcription is drastically reduced (see Fig. 1), indicating that puff size is not simply related to *Sgs-4* high level expression.

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*^{1} 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*^{1} 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

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formation might be induced by the binding of trans-acting factors at the *Sgs-4* upstream regulatory region, the sequence of which would be required for normal-sized 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 the 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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