

EXPERIMENTAL INDUCTION OF GENE ACTIVITY
IN THE SALIVARY GLAND CHROMOSOMES
OF *TRICHOSIA PUBESCENS* (DIPTERA: SCIARIDAE)

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ABSTRACT

During the course of experiments with larvae of *Trichosia pubescens*, we have unexpectedly found that diethyl ether or chloroform anesthesia induces a large puff in a specific band in the polytene chromosomes of the salivary glands. This puff develops a few minutes after the treatment, attaining its maximum size after 60–100 min, and regresses completely 200 min after its activation. Through autoradiography, an intense incorporation of RNA precursors into that puff was observed. A few other smaller puffs are also induced by the treatment. The treatment with diethyl ether or chloroform does not induce puffing in the polytene cells of malpighian tubules and of midgut.

KEY WORDS gene activity · polytene chromosomes · RNA synthesis · diethyl ether · chloroform

Since the pioneer works on the puffing phenomenon in polytene chromosomes, a considerable amount of circumstantial evidence has supported the idea, first proposed by Beermann (2, 3) and by Breuer and Pavan (13, 14), that the puffs are the morphological expression of gene activity (for a review, see references 1 and 8).

Nowadays it is possible to characterize biochemically the products of a single puff (10, 11, 15, 20) and to gather information on puff ultrastructure (5, 16, 24). Thus, puffs offer unique opportunities for the investigation of the process of gene activation and deactivation in eucaryotes.

Of particular interest are the puffs that can be experimentally induced and/or repressed. They allow the study of factors involved in the regulation of the puffing phenomenon, as well as the establishment of correlations between individual

puffs and specific changes in cell metabolism. This is exemplified by recent studies on *Drosophila*, which have provided information about the nature of the RNA produced in specific puffs (9, 10, 12, 19, 22, 23) and the changes in cellular metabolism after puff induction (9, 17, 18, 25).

During the course of experiments with larvae of *T. pubescens*, we have unexpectedly found that diethyl ether or chloroform anesthesia induces, within a few minutes, the development of a large puff in a specific band of the salivary gland polytene chromosomes. This paper describes the kinetics of formation and regression of this puff. In addition, some features of RNA synthesis and nonhistone protein accumulation in the corresponding chromosome region, both in the unpuffed and in the puffed condition, are presented.

MATERIALS AND METHODS

Female larvae of *Trichosia pubescens* (Diptera, Sciaridae) at two stages of larval development were used in the experiments. Larvae from stage L₁ (midfourth instar)

were identified by their large size, the color of the fat body, and the absence of eye spots. Larvae at stage L_{3-6} (end of fourth instar) were recognized by the size of the eye spot regions and by their peculiar behavior in remaining under a layer of soil and starting to build the individual cocoon.

Puff Induction

The diethyl ether or chloroform treatments used to induce changes in the puffing pattern were carried out in vivo. A flow of air that had been bubbled through the anesthetic was applied to the larvae. The duration of the treatment was 2–5 min for diethyl ether and a few seconds for chloroform. The changes in the puffing pattern were studied in lactic-acetic orcein squashes at various time intervals after the end of the treatment. Three kinds of tissues were studied: the anterior portions of the salivary glands, the proximal portions of the malpighian tubules, and the midgut.

In each salivary gland preparation, five randomly selected X chromosomes were scored in each of five slides for each time interval. For the evaluation of size, the maximum diameter of the puff region (section X15E) was divided by the diameter of a particular nonpuffed band (section X17A) in the vicinity of the puff region.

To determine the band from which puff X15E originates, sections 15 and 16 of chromosome X were outlined with camera lucida in different phases of puff formation. From these outlines and from photographs, schematic drawings of sections 15 and 16, both in the unpuffed and in the puffed condition, were made.

Fast-Green Staining

Accumulation of nonhistone proteins in region 15E of chromosome X was investigated in salivary gland squashes double-stained with aceto-orcein and acidic fast green. The larvae were dissected in their own hemolymph, and the salivary glands were fixed for 2–5 min in acetic-ethanol (1:3), stained for 2–5 min in aceto-orcein (2% orcein in 70% acetic acid), washed in acetic acid 45%, stained for 1–2 min in acetic fast green (1% fast-green FCF in 45% acetic acid), and squashed in a drop of acetic acid 45%. After the squash, the cover slips were sealed and the slides were kept at -20°C for at least 24 h. After this treatment and after the preparations were frozen in liquid nitrogen, the cover slips were removed and the slides were immediately dipped into an *n*-butanol bath for 5 min. After this, the preparation was washed in absolute ethanol twice and then in a cedar oil-ethanol (1:1) mixture, in pure cedar oil and in xylol. Each of these baths took 5 min. The preparations were then mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.). The fast-green staining is improved in the permanent slides.

Autoradiography

Control larvae at periods L_1 and L_{3-6} were immobilized by cold, injected with $1\ \mu\text{l}$ of a concentrated [^3H]uridine ($\sim 4\ \mu\text{Ci}/\mu\text{l}$; sp act 40 mCi/mmol), and sacrificed 20 min later. The experimental larvae were treated the same way, 80 min after an exposure to diethyl ether for 5 min. The anterior portions of the salivary glands were fixed in acetic-ethanol (1:3) for 5–10 min and then squashed in a drop of 45% acetic acid. The coverslips were removed after freezing in liquid nitrogen, and the slides were air-dried after a bath in absolute ethanol. The preparations were washed in TCA 5%, at 4°C for 30 min, dehydrated in 70 and 95% ethanols, air-dried, covered with stripping film, and developed 15 days later in accordance with Kodak instructions. The autoradiographs were stained with 0.2% toluidine blue in 0.04 M acetate buffer at pH 5.2.

RESULTS

During the course of the experiments for a study of the effects of ecdysterone on the puffing pattern of the salivary gland chromosomes of *T. pubescens*, an unexpected observation was made. Larvae in period L_1 (midfourth instar) injected with the ecdysterone solutions, as well as controls injected only with the solvent, unlike untreated larvae, showed a large puff in section 15E of chromosome X. This puff is only sporadically observed in untreated larvae. On the other hand, injection of the solvent into larvae at the end of the fourth instar (after the induction of ecdysone-dependent puffs) did not bring about any alteration in the puffing pattern. After several tests, it became clear that the induction of this puff could be an effect of the diethyl ether anesthesia which was used in the experiments.

To test this hypothesis, the following experiments were carried out: Different groups of larvae at period L_1 were anesthetized with diethyl ether, CO_2 , and cold treatment. Larvae from each group were sacrificed at various time intervals after the end of the treatment, and the state of region 15E of chromosome X (puffed or unpuffed) was determined in squash preparations of the salivary glands. The analysis of the chromosomes of the treated larvae revealed that only the diethyl ether treatment resulted in the induction of puff X15E. Further experiments showed that chloroform treatment is also effective in the induction of this puff. The relative increase in the diameter of section X15E of the salivary gland chromosomes after diethyl ether and chloroform anesthesia is shown in Fig. 1.

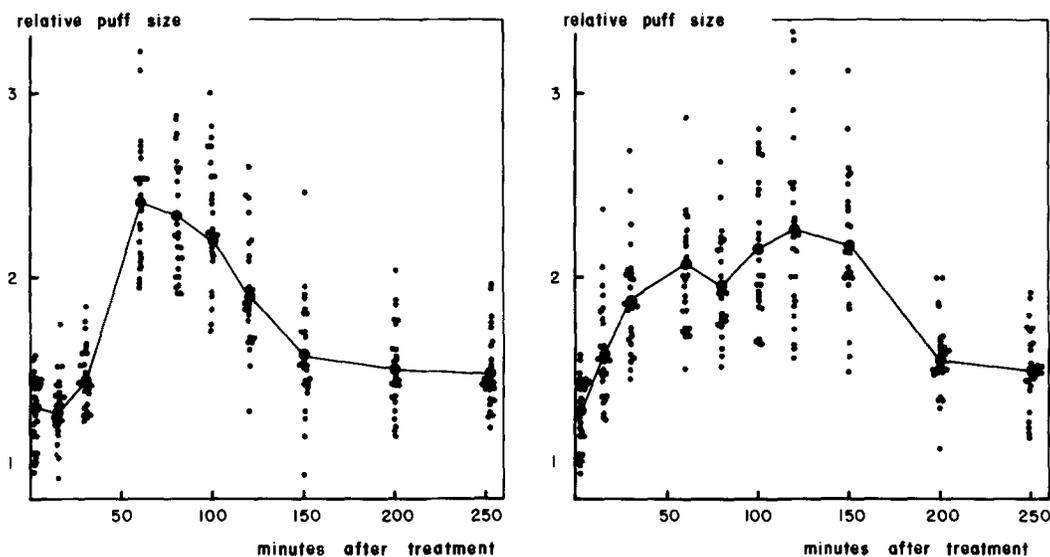


FIGURE 1 Relative increase in the diameter of section X15E of salivary gland chromosomes after anesthesia with diethyl ether (left) and chloroform (right). Each black dot represents the relative puff size of each chromosome analyzed. The straight line passes through the median value points.

As puff X15E has never been observed in old larvae, a test was run with them. When larvae at period L_{5-6} were anesthetized with diethyl ether or chloroform and their salivary gland chromosomes were analyzed at various time intervals after the treatment, no detectable morphological alterations could be observed in section X15E.

Analysis of the chromosomes from malpighian tubules and midgut cells of L_1 larvae, at various time intervals after the diethyl ether treatment, showed that puff X15E is not induced in these tissues (Fig. 2).

To determine the kinetics of formation and regression of puff X15E, salivary gland chromosomes of L_1 larvae treated with diethyl ether or chloroform were analyzed at various time intervals. As shown in Fig. 1, the puff attains its maximum size ~100 min after the treatment. 200 min after the treatment, the puff region is somewhat larger than in the controls; however, no typical puff can be seen on region X15E, showing that the induced puff has regressed by this time.

For the determination of the band(s) from which the puff originates, a careful analysis of sections 15 and 16 of chromosome X were carried out in chromosomes without the puff and at different stages of puff formation. In the unpuffed state, region X15E displays three dotted bands, named 1, 2, and 4, and a diffuse band or bands

(band 3) between bands 2 and 4 (Fig. 3). During the period after the diethyl ether or chloroform treatment, region X15E swells and attains, in some cases, twice the original diameter. In a fully expanded puff, bands 1, 2, 3, and 4 can no longer be seen. At one edge of the puff, one string of chromatin dots, probably resulting from the disruption of band 4, is seen. On the other edge, the dots form two strings, corresponding to bands 1 and 2. These observations suggest that the induced puff arises from band 3 (Fig. 3).

To verify whether region X15E synthesizes RNA, an autoradiographic study of the incorporation of tritiated uridine into chromosomes from treated larvae and from controls was carried out. Puff X15E becomes strongly labeled with a 20-min pulse of [3 H]uridine in vivo, given 80 min after the end of the diethyl ether treatment. With the same pulse, section 15E of chromosome X from control larvae showed a much lower incorporation of [3 H]uridine (Fig. 4).

Acidic fast-green staining revealed that in L_1 larvae, as well as in L_{5-6} larvae, a very sharp-stained band is present in the unpuffed section X15E (Fig. 5). An increase in the accumulation of nonhistone proteins at this region occurs simultaneously with the increase in puff diameter. When the puff is at its maximum expansion, a large amount of nonhistone proteins can be de-

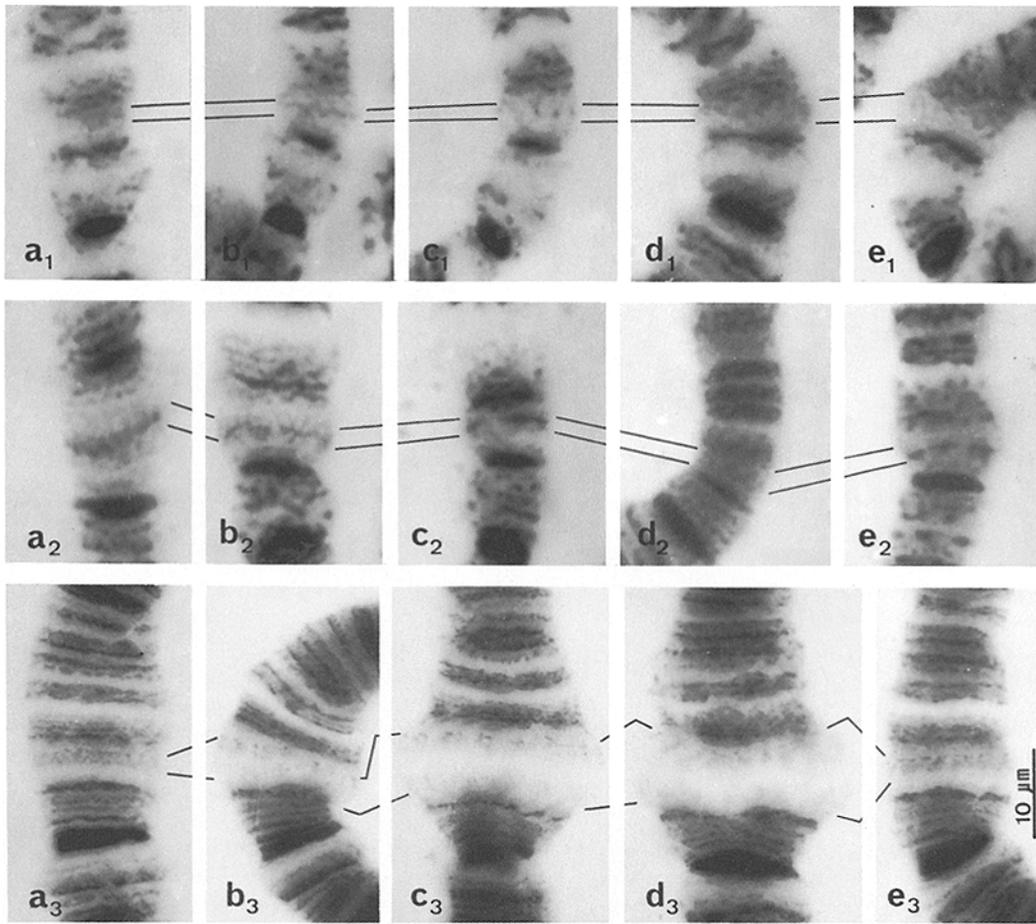


FIGURE 2 Chromosomes from malpighian tubules (1), midgut (2), and salivary glands (3). Chromosomes from control larva and from larva sacrificed 60, 90, 140, and 220 min after diethyl ether treatment are indicated, respectively, by letters *a*, *b*, *c*, *d*, and *e*. Straight lines indicate the location of section X15E \times 1,200.

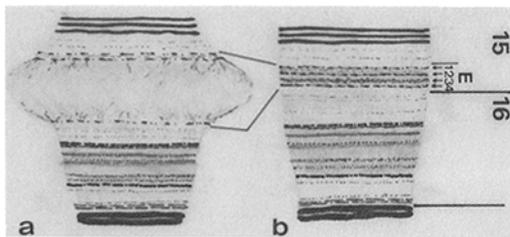


FIGURE 3 Diagrammatic representation of section 15E-16 of chromosome X in the puffed (*a*) and in the unpuffed condition (*b*).

tected over the expanded chromosome region (Fig. 5).

Analysis of the four polytene chromosomes of the salivary gland of *L*₁ larvae of *T. pubescens*

revealed that, in addition to puff X15E, other puffs of smaller sizes, namely, puffs X18D, X11D, C6C, B4E, and B10G-H, are also induced by diethyl ether or chloroform treatments (Fig. 6). As with puff X15E, these puffs are not induced in older larvae. They have not been analyzed in malpighian tubule and midgut polytene chromosomes.

DISCUSSION

The swelling induced by diethyl ether or chloroform in region 15E of the X chromosome can be recognized as a typical puff due to the increase in chromosomal diameter, accumulation of nonhistone proteins, and high uptake capacity for [³H]uridine. The latter characteristic reflects local-

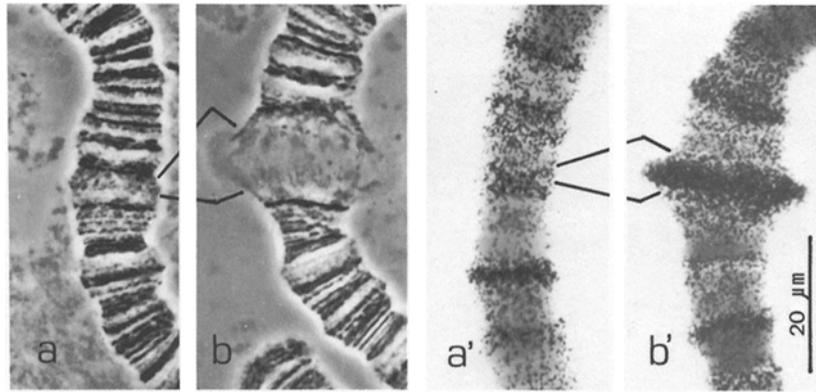


FIGURE 4 Induction of puff X15E by diethyl ether anesthesia. Left: Chromosome from the salivary gland of a control larva (a) and from a larva sacrificed 100 min after treatment (b). Right: $[^3\text{H}]$ uridine incorporation into a chromosome X from the salivary gland of a control larva (a') and of a larva sacrificed 100 min after treatment (b'). $\times 900$.

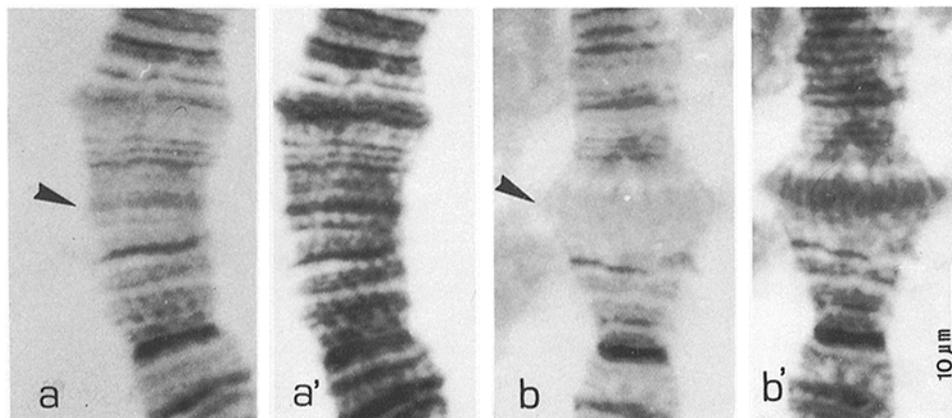


FIGURE 5 Chromosome X from a control larva (left) and from a treated larva (right) sacrificed 80 min after the end of the diethyl ether treatment. The preparations were double-stained with orcein and acidic fast green and photographed with a green filter (a and b) and with a red filter (a' and b'). $\times 1,800$.

ized gene activity. However, an increase of labeling in a specific chromosome region could also be interpreted as a decrease in the rate of release of the newly formed RNA. The analysis of autoradiographs of *T. pubescens* salivary glands, pulse-labeled in vivo with $[^3\text{H}]$ uridine, revealed that, in pulses up to 20 min, labeling is restricted to the chromosomes; no detectable labeling is seen either in the cytoplasm or in the nuclear sap. These findings suggest that the newly synthesized RNA of the salivary glands of *T. pubescens* is temporarily retained within the chromosomes over a period of at least 20 min. Storage of newly synthesized RNA has been considered to be one of the characteristics of the puff (4). The most plausible hypothesis, taking into account these

findings and those obtained in other species (6), is that the increase of incorporation of $[^3\text{H}]$ uridine observed in section X15E of the salivary gland chromosomes of *T. pubescens*, after diethyl ether anesthesia, is due to an increase in gene transcription at this region. Thus, this treatment brings about the induction or increase of gene transcription at specific chromosomal loci.

The analysis of the autoradiographs of L_1 and L_{5-6} control larvae revealed a low incorporation of RNA precursor into section 15E of chromosome X of the salivary gland cells. In these larvae, a sharp fast green-stained band associated with band 3 of region X15E of the salivary chromosomes can be seen. These findings suggest that a small puff, reflecting a low activity of gene transcription,

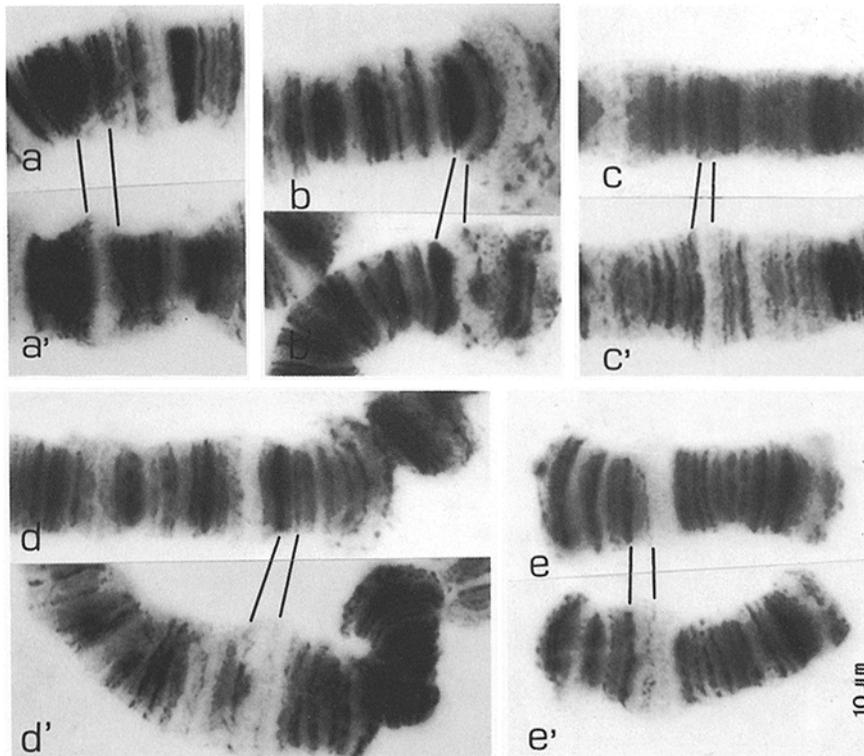


FIGURE 6 Other puffs in the salivary gland chromosomes induced by diethyl ether or chloroform treatment. Sections X18D, X11D, C6C, B10G-H, and B4E are indicated, respectively, by letters *a*, *b*, *c*, *d*, and *e*. Unprimed letters indicate chromosomes from control larvae and primed letters, chromosomes from larvae sacrificed 90 min after diethyl ether treatment. $\times 1,200$.

is present in section X15E of the salivary gland chromosomes up to the end of the fourth larval instar. Thus, it is possible that diethyl ether or chloroform treatment just enhances the activity of certain previously active genes. However, when one examines other loci such as C6C, B4E, and B10G-H, no detectable gene activity is observed before the treatment; gene activity at these loci seems to be induced by the treatment. It could be thought also that puff X15E is induced by the treatment and that the low gene activity observed in the untreated larvae is due to a small puff in the vicinity of the band that is the origin of the anesthesia-induced puff.

The induction of these puffs could be mediated by proteins mobilized from the cytoplasm to the nucleus upon stimulation, as seems to occur in other cases (7, 21). Part of the protein which accumulates at puff X15E during its expansion could be involved in the activation of this locus. A plausible hypothesis to explain the capability of a

chromosomal locus to form a puff in one type of cell and not in another could be that only the type of cell in which the puff is formed has such cytoplasmic inductor factors available.

Another problem of special interest is how diethyl ether or chloroform induces gene transcription at specific chromosomal loci. The first point to be investigated is whether salivary glands cultured *in vitro* do respond to the treatment. Preliminary results demonstrated that when salivary glands from midfourth instar larvae are cultured in Cannon's medium, a small puff is induced in region X15E even in the absence of the treatment with diethyl ether or chloroform. It seems that the induction of such a puff is brought about when salivary gland cells are submitted to conditions of stress. As the effective inducers—diethyl ether and chloroform—are lipid solvents, it is possible that specific alterations in the permeability of the plasma membrane may be involved in the induction of those puffs. Experiments are

being carried out to investigate the mechanisms of induction of those puffs as well as their end products.

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