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Localization of ecdysterone on polytene chromosomes of *Drosophila melanogaster*

(immunofluorescence/puff/photocrosslink/steroid hormones)

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ABSTRACT **Ecdysterone has been crosslinked *in situ* to polytene chromosomes of salivary glands of *Drosophila melanogaster* by photoactivation. The crosslinked hormone has been localized on the chromosomes by indirect immunofluorescence microscopy. At different developmental stages the hormone was detected at different chromosomal loci. These chromosomal sites correspond to ecdysterone-inducible puff sites. Thus, the hormone binds directly to chromosomal loci, whose transcription depends on the presence of the hormone. Ecdysterone was also crosslinked to one puff site that regresses during larval development. This indicates that (*i*) hormone binding to polytene chromosomes activates transcription at specific loci, and (*ii*) hormone-dependent regression of intermolt puff 68C is mediated by direct binding of ecdysterone. Ecdysterone was not detected on puff sites that are independent of hormone action or in chromosomal interbands. After heat shock, no ecdysterone became crosslinked to polytene chromosomes.**

The sequential changes that occur in the puffing activity of dipteran polytene chromosomes offer an excellent opportunity for the analysis of the control of gene activity during development. Puffs are reversible structural modifications which probably originate from one (or more) single band(s); the role of adjacent interbands is unclear (1, 2). Studies in several species have shown that, toward the end of larval development, dramatic changes occur in the pattern of puffed chromosome loci (2–4). It has been shown that these changes are initiated as a consequence of a hormonal stimulus. An increase in the titer of the steroid hormone ecdysterone switches the pattern of puffing activity from that characteristic of the intermolt to that characteristic of the molting or metamorphosing insect.

The temporal control of puffing of salivary gland chromosomes of *Drosophila melanogaster* by ecdysterone has been intensively investigated *in vivo* and *in vitro* (2, 4–7). The sequence of events has been divided into 21 "puff stages," each characterized by a unique set of puffed sites; 194 such sites have been described by Ashburner (4) and 341 by Zhimulev (6). According to Ashburner *et al.* (7), there are three different overall effects of ecdysterone with respect to puffing. The first one is that the hormone induces a regression of puff size in some sites, as exemplified by intermolt puff 68C.

The second and third effects are that the hormone induces puffing immediately at some sites (early puffs) and with a time lag of at least 3 hr at other sites (late puffs). Early puff sites are for example 23E, 63F, 74C, 74EF, and 75B. The majority of ecdysterone-induced puffs can be classified as late puffs. The best studied of those late puffs are sites 62E, 78D, 22D, 63E, and 82F.

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The hormonal regulation of puff induction could be correlated with specific molecular events at the transcriptional and translational level. Bonner and Pardue (8, 9) showed by *in situ* hybridization that the induction of RNA synthesis at puff sites 74EF/75B requires the presence of ecdysterone. Furthermore, proteins coded for by specific puffs have now been identified (10, 11).

The first step generating the complex series of cellular responses upon introduction of steroid hormones is generally believed to be the recognition of the hormone by its target cells and its binding to specific cytoplasmic receptor proteins. Up to now it is unclear in which way the hormone–receptor complex is able to generate the highly specific activation of genes in the nucleus which leads to the accumulation of a small number of mRNAs. For the action of estrogen it was suggested that the hormone–receptor complex is translocated into the nucleus and there recognized by chromosomal receptor protein(s), which then turn on and off the transcriptional machinery (for review, see ref. 12).

In *Drosophila*, attempts have been made to demonstrate directly the binding of ecdysone to chromosomes by autoradiography (13, 14). These experiments suffered from the low specific activity of the labeled ecdysone used and the results were not very conclusive.

We wanted to know whether ecdysterone binds directly to those chromosomal sites, which have been described as ecdysterone-inducible puff sites, and to study the correlation between hormone binding to chromosomes and gene activity. Besides autoradiography, indirect immunofluorescence microscopy has become a powerful technique for localizing molecules at individual sites of polytene chromosomes (15–17). Because ecdysone is washed out in squash preparations of polytene chromosomes (13), reversibly bound hormone was not detectable on polytene chromosomes by immunofluorescence microscopy. It was necessary to crosslink the hormone to polytene chromosomes *in situ* prior to squashing. In this paper, we report the photoinduced crosslinking of endogenous ecdysterone to chromosomes in salivary glands of *D. melanogaster* and the subsequent identification of chromosomal hormone binding sites and their correlation with puff sites.

MATERIALS AND METHODS

Preparation of Ecdysterone Antibodies. Antibodies directed against ecdysterone (Calbiochem) were prepared by coupling the hemisuccinate with thyroglobulin (18, 19). Antisera were raised in rabbits according to standard techniques. Antibody titers were measured by radioimmunoassay with [^{3}H]ecdysterone (1.1 Ci/mol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear). Sera with half-maximal antibody binding capacity of about 0.05 were used in immunofluorescence experiments.

Irradiation of Salivary Glands. Larvae from *D. melanogaster* Oregon R wild-type strain were dissected in Grace's medium (20) containing about 10 μM ecdysterone. For heat-shock experiments, larvae were exposed to 37°C for 45 min prior to dissection. Immediately afterward, 20–40 glands were quickly washed twice with irradiation buffer (2 mM KH_2PO_4/1 mM Na_2HPO_4/1.2 mM $MgSO_4$/1.2 mM $MgCl_2$/56 mM NaCl/40 mM KCl/80 mM sucrose, pH 6.6). Glands were then irradiated in 2 ml of buffer for 30 min in an air-cooled cylindrical cuvette with an HBO 500 W/2 high-pressure mercury lamp [Schoeffel (Trappenkamp, Germany); lamp housing: Oriel (Darmstadt, Germany)] through a 1-mm-thick WG 320 long-pass filter [80% transmission at 320 nm; Schott (Mainz, Germany)] at a distance of 5 cm. Irradiation was carried out under nitrogen atmosphere at room temperature. Temperature was controlled by an internal thermistor.

Immediately after irradiation, glands were transferred to a siliconized coverslip and fixed with ethanol/acetic acid, 3:1 (vol/vol), for 30 sec. After removal of the liquid, the glands were squashed in a solution of 45% acetic acid containing 3.7% formaldehyde and 10 mM $MgCl_2$. The slide was frozen in liquid nitrogen, and the coverslip was pried off with a razor blade. The slide itself was placed in 95% ethanol as postfixative solution for 30 min. Prior to treatment with antibodies, the slide was washed twice in phosphate-buffered saline (0.14 M NaCl/10 mM potassium phosphate, pH 7.5) for 30 and 10 min, respectively.

Chromosome preparations were treated for 30 min with anti-ecdysterone antiserum diluted 1:10 with phosphate-buffered saline and washed twice for 1 hr and for 10 min, respectively, in the buffered saline. Afterward, a 1:20 dilution of fluorescein isothiocyanate-coupled goat anti-rabbit immunoglobulins [Byk-Mallinckrodt (Dietzenbach, Germany)] was applied for 30 min and the slide washed again in buffered saline for at least 30 min.

For microscopy, preparations were mounted with a drop of 1 M Tris·HCl, pH 8.1/glycerol, 1:9 (vol/vol), a siliconized coverslip was applied, and the specimen was photographed by incident UV illumination in a Zeiss standard microscope with an incident UV-attachment and fluorescence optics. Phase-contrast photographs were taken after the preparations were stained with lacto-acetic acid/orcein (21).

RESULTS

Ecdysterone as a Δ^7-6-ketosteroid (22) contains an α,β-unsaturated ketone moiety which can be photoactivated by irradiation around 330 nm (23). Therefore, we attempted to crosslink endogenous ecdysterone to its chromosomal binding sites by irradiation of salivary glands of *D. melanogaster* third-instar larvae. During dissection of salivary glands, a 10 μM ecdysterone concentration was maintained in order not to lose some of the hormone-binding sites. Irradiation, however, was carried out in the absence of exogenous ecdysterone. Because the time interval between dissection and irradiation was in the order of 15 min, the puff stages seen are essentially *in vivo* puff stages.

The distribution of photocrosslinked ecdysterone on polytene chromosomes of *D. melanogaster* at different stages of development *in vivo* was investigated by indirect immunofluorescence microscopy. Results obtained after staining with fluorescent antibody were scored according to the guidelines discussed by Silver and Elgin (16). The major source of nonspecific fluorescent staining is the nonspecific binding of IgG molecules of the primary antiserum to salivary gland squashes. Only the chromosomal fluorescence that was of intensity equal to or higher than cytoplasmic fluorescence (due to adherence of IgG molecules to cytoplasmic debris) and that was reproducibly observed was interpreted to result from specific binding of ecdysterone-antibodies to chromosomal loci.

Fig. 1 shows fluorescence photographs of polytene chromosomes of *D. melanogaster* at three different stages of development after irradiation of salivary glands. In early third instar there were only a few fluorescent regions of different intensities; in late third-instar and prepupal glands there were many. Regions exhibiting details of special interest are presented at higher magnifications in Figs. 2–5.

Puffs at 75EF/75B had reached their maximal size at puff stages 3–7, whereas some intermolt puffs at other loci—e.g., 68C—had regressed (4–7). These particular puffs showed fluorescence after irradiation of appropriate salivary glands (Fig.

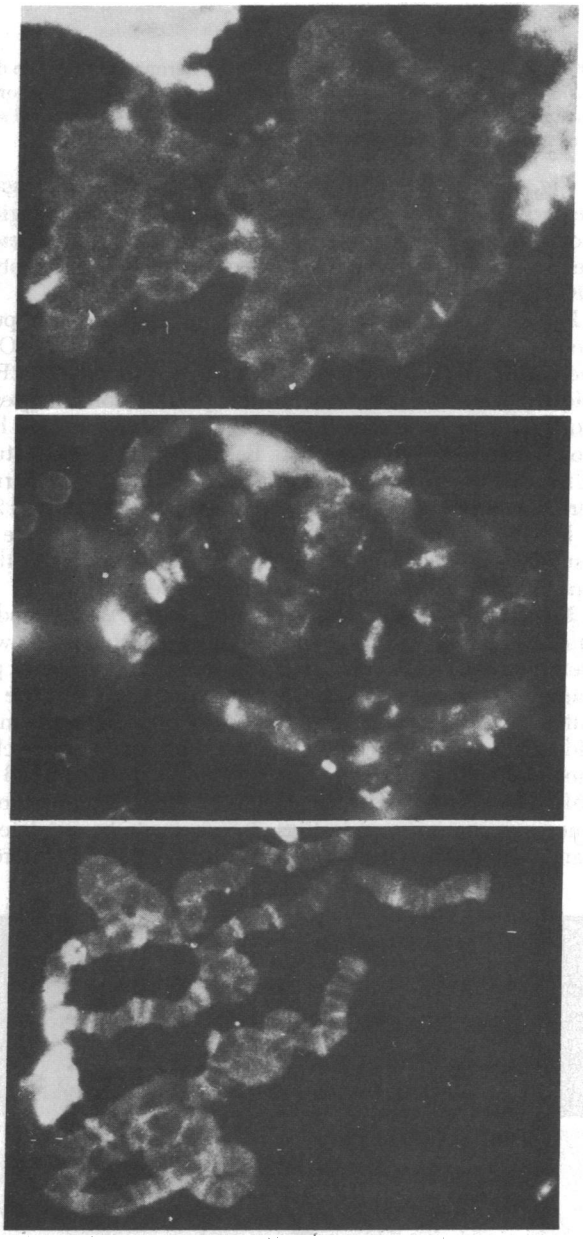

FIG. 1. Indirect immunofluorescence microscopy of chromosome squashes obtained from salivary glands of *D. melanogaster* after irradiation for 30 min. Glands were fixed in ethanol/acetic acid, 3:1 (vol/vol), squashed, and incubated with rabbit antisera directed against ecdysterone. Hormone–antibody complexes were visualized with fluorescent goat antibodies directed against rabbit IgG. (*Top*) Early third-instar larva. (*Middle*) Late third-instar larva. (*Bottom*) Prepupa. (×320).

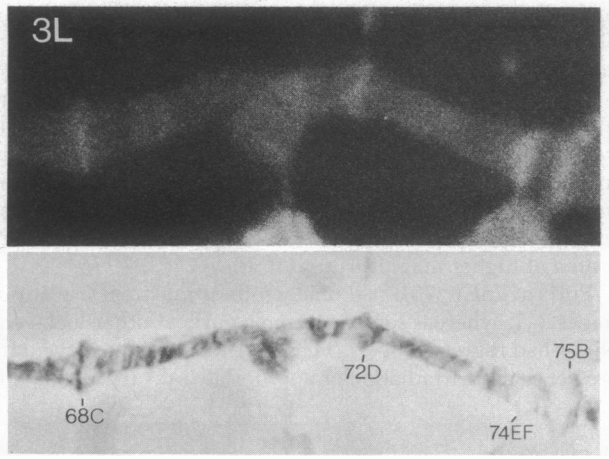

FIG. 2. (*Upper*) Photocrosslinked ecdysterone in the region 68C to 75B of chromosome 3L at puff stage 4 by indirect immunofluorescence microscopy. (*Lower*) Phase contrast pattern of the same segment after orcein staining. (×2000.)

2). Fig. 3 shows the distal part of chromosome 3L. Here again, the early puff 63F was brightly fluorescent, whereas regions known to respond later to the hormone (e.g., 63E) did not reveal the presence of any bound hormone in detectable amounts at this stage of development.

Fig. 4 shows the tip of the chromosome 2L. In this case, puffs occur in early puff stages at 21C, 22B, 23E, and 25AC (4). Only one region of chromosome 2L, which corresponds to 22B, is brightly fluorescent. The intermolt puff at 25AC regresses shortly after ecdysterone induction. Fig. 4 shows that no hormone was crosslinked to this puff. Because the cytologic features of irradiated chromosomes were inferior to those of normal chromosomes, the exact assignment of bands to 21C and 23E in Fig. 4 is ambiguous. However, this does not invalidate the result because the whole region of the chromosome, where these particular bands are located, did not fluoresce.

Fig. 5 shows the distal parts of chromosomes 2L, 3L, and 3R (puff stage 7–8); several brightly fluorescent sites, which were identified as 21F, 22C, 62E, 95F, and 98F, are apparent. At puff stage 7–8, the early puff at 63F has almost regressed; the late puff at 63E is not yet induced (4). Our crosslinking experiments did not reveal significant amounts of hormone at these chromosomal loci. Similarly, no hormone was detected at 22B and 96E which puff at earlier stages. In general, chromosomal fluorescence, as shown in Figs. 2–5, seems to be restricted to bands and puffs, whereas interband staining is not apparent.

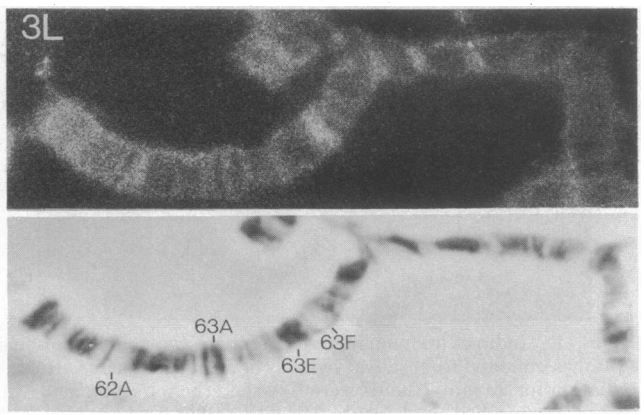

FIG. 3. (*Upper*) Photocrosslinked ecdysterone in the distal region of chromosome 3L at puff stage 4 by indirect immunofluorescence microscopy. (*Lower*) Phase-contrast pattern of the same segment. (×2000.)

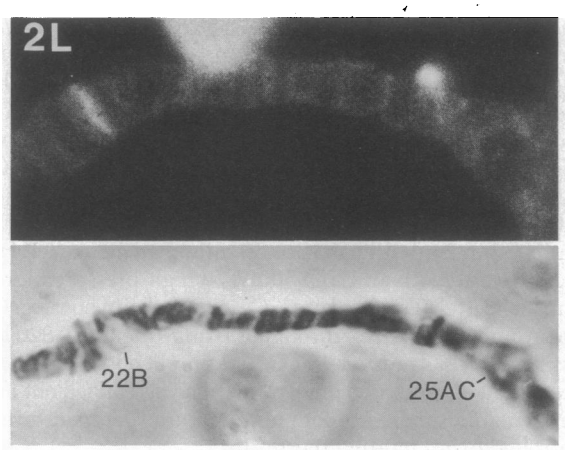

FIG. 4. Same as Figs. 2 and 3 except that the tip of chromosome 2L is shown. (×1300.)

Heat shock applied to *D. melanogaster* third-instar larvae produces a marked change in the puffing pattern of polytene chromosomes (2, 11). When *D. melanogaster* third-instar larvae or prepupae were exposed to 37°C for 45 min, dissected, and irradiated, no specific fluorescence was detected on the chromosomes, either on "heat shock puffs" or on any other chromosomal loci.

In our system we could also confirm the results of Emmerich (13), who was unable to detect [^{3}H]ecdysone on conventionally fixed chromsomes. Nonirradiated glands were fixed in etha-

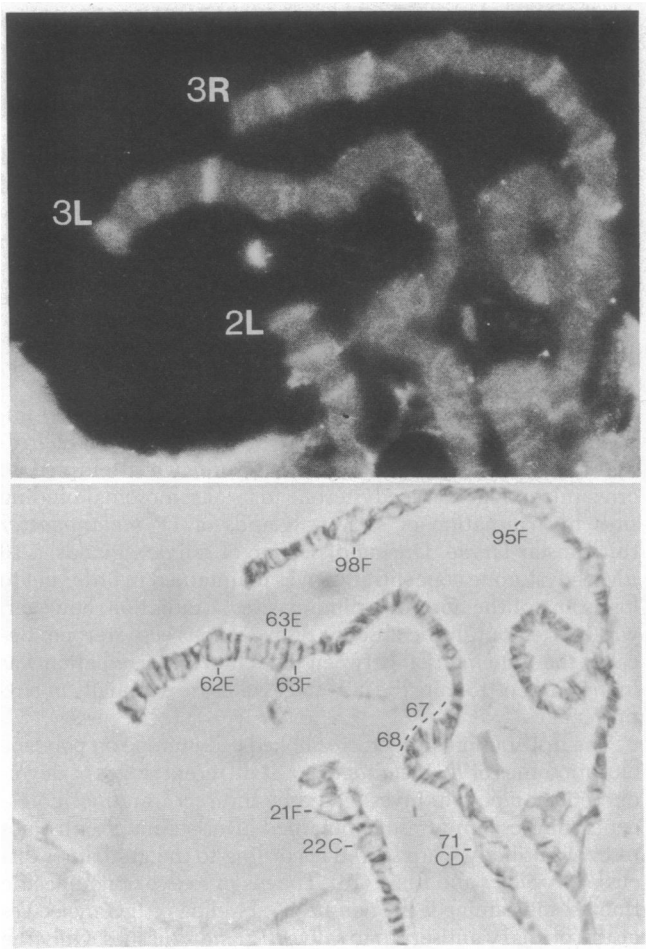

FIG. 5. Same as Figs. 2 and 3 except that the distal parts of chromosomes 2L, 3L, and 3R are shown at puff stage 7–8. (×1250.)

nol/acetic acid, formaldehyde, or glutaraldehyde. Squash preparations were incubated with antibodies for immunofluorescence microscopy under the conditions described in *Materials and Methods*. No specific fluorescence pattern could be detected. These experiments demonstrate that reversibly bound ecdysterone cannot be localized on polytene chromosomes by immunofluorescence techniques. It should also be pointed out that fluorescence was not detectable *in irradiated control* chromosomes that had been incubated either with preimmune serum or with nonimmune serum as primary antibody.

In view of these results, the following conclusions are drawn: (*i*) ecdysterone binds to chromosomal sites with high specificity; (*ii*) these sites are the same as the ones that respond to the hormone by puffing *in vivo;* (*iii*) the intermolt puff 68C, which regresses during development, is also a high-affinity binding site of the hormone, suggesting a negative control mechanism, and regression of intermolt puff 25AC is independent of ecdysterone; (*iv*) ecdysterone does not bind to interbands in detectable amounts; and (*v*) heat shock inhibits crosslinking of ecdysterone to its binding sites on polytene chromosomes.

DISCUSSION

Immunofluorescence studies on squashes of polytene chromosomes have already furnished considerable information on the distribution of chromosomal proteins (15–17). Small molecules like ecdysterone could not be localized by using this technique because they are reversibly bound and washed out during fixation. We therefore crosslinked the hormone to its binding sites prior to immunoreaction. The method of choice was irradiation of the salivary glands *in vitro*.

The results show that the photoinduced crosslinking reaction between endogenous ecdysterone and polytene chromosomes is specific. It only occurred at distinct chromosomal loci that are known to respond to the hormone (2). It did not occur at puff sites which do not respond to the hormone. The crosslinking reaction is completely abolished by heat shock. A model study of the crosslinking reaction between ecdysterone and bovine serum albumin demonstrated that yield of crosslinking depends on the lifetime of the steroid–protein complex (unpublished experiments). They, the chromosomal ecdysterone binding sites, detected by indirect immunofluorescence microscopy as shown in Figs. 2–5, must be high-affinity sites for the hormone.

The hormonal induction of puffing in *Drosophila* has many features in common with the induction of specific changes in RNA and protein synthesis occurring after stimulation of vertebrate tissues by appropriate steroid hormones (12). It is convenient to consider the response of any tissue as falling into three logical steps: (*i*) the recognition of the hormone by the target cell, (*ii*) the generation of intracellular signal(s), some of which may lead to (*iii*) the production of one or more specific RNAs. Studies on vertebrate steroids have emphasized those processes that fall into the first and third of these steps—namely, interactions of the hormone with specific cytoplasmic receptor proteins, and the production of specific messengers or their translation products.

There are certain similarities between ecdysterone and the vertebrate steroids, at least with respect to the accumulation of hormone by target cells and their nuclei and the identification of cytoplasmic protein fractions with hormone-binding activity (24, 25). But up to now, in Diptera, no conclusive experiments have been performed that describe the pathway from cytoplasmic binding to specific puff induction, although two classes of model have been put forward. Karlson (26, 27) suggested a direct hormonal control of gene activity at the genome level; Kroeger (28, 29) postulated the cell membrane as the primary site of hormone action. According to his hypothesis, which was recently critically reviewed by Ashburner and Cherbas (30), changes in the membrane permeability would give rise to an alteration of the intracellular ion balance with a consequent change in the activity of certain genes.

Our results strongly support the view of Karlson (26, 27). We have demonstrated that, in *Drosophila*, ecdysterone accumulated within the nucleus at those chromosomal loci known to respond to the hormone by puffing and by specific transcription.

A model for the temporal control of puffing activity in polytene chromosomes has been presented by Ashburner *et al.* (7). The first stage in this model is the reversible binding of ecdysterone by a receptor protein which induces the early puffs while repressing the late ones by acting directly on the genome. In the next step, the translation product(s) of the early puff(s) is thought to compete at the late puff sites for bound ecdysterone–receptor complex, displacing the complex and causing the expression of these puffs because the affinities of late puff sites for receptor and ecdysterone–receptor are assumed to be of the same order of magnitude. According to this model, we should have expected that, by the time late puffs are active, ecdysterone would have been displaced. However, this is not the case. On the other hand, the present data are not sufficient to warrant the formulation of an alternative model.

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