*CONTROL OF GENE ACTIVITIES IN THE POLYTENE CHROMOSOMES OF CHIRONOMUS TENTANS BY ECDYSONE AND JUVENILE HORMONE**

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Abstract.—The injection of ecdysone (molting hormone) into chironomid larvae is known to result in the stimulation of specific gene activities (increased RNA synthesis). The morphological manifestation of this gene activation is the formation of a puff. Cytological and autoradiographic analysis of several regions of the salivary gland chromosomes of *Chironomus tentans* revealed that ecdysone also stimulates Balbiani ring 1. In contradistinction to the puffs, the Balbiani rings are tissue specific and account for a significant percentage of the cell's non-nucleolar RNA.

The application of juvenile hormone results in a decreased activity of Balbiani ring 1, suggesting that the two hormones may act antagonistically. In addition, juvenile hormone induces a puff at chromosome region I-19-A and this puff becomes less active after a short-term treatment with ecdysone. These data demonstrate an effect of juvenile hormone on puffing and are the first to demonstrate activation of a Balbiani ring by ecdysone.

The original and important observation that the insect molting hormone, ecdysone, can influence the activity of two specific loci on the polytene, salivary gland chromosomes of larval *Chironomus tentans*, was made a decade ago by Clever and Karlson.[1] In that case, injection of ecdysone stimulated the formation of puff I-18-C and caused the regression of puff I-19-A. This finding, as well as subsequent work on the polytene chromosomes of Diptera, led to the conclusion that ecdysone acts either directly upon the chromosome or indirectly via changes in the ionic environment.[2]

In regard to salivary gland function, however, the activation or deactivation of a puff-forming region can be considered a minor effect since a puff only constitutes between 0.5 to 3.0% of the total non-nucleolar RNA in the salivary gland nucleus.[3, 4] In addition, the puffs alluded to above[1] are not specific to the salivary gland chromosomes.[5] The Balbiani rings (BR1, BR2, BR3), on the other hand, are considered to be sites of gene activity specific to the salivary gland chromosomes[6] and produce 10–60 times more RNA than a puff.[3, 4] The present paper describes the effect of ecdysone on Balbiani ring 1.

Although ecdysone induces molting in insects, it is the juvenile hormone titer that determines the nature of the molt. Attempts to produce significant effects on chromosome puffing with juvenile hormone have failed so far.[7, 8] This lack of success may have been due to both the use of crude extracts since pure juvenile hormone has only been recently available,[9] and to the lipoidal characteristics of juvenile hormone that make it difficult to administer to aquatic forms, such as

Chironomus larvae. The present paper describes the effect of juvenile hormone on chromosome puffing.

Materials and Methods.—Animals: Fourth instar larvae of *Chironomus tentans* were staged according to the method of Kroeger.[8] Young larvae are no older than stage I and presumably have little or no endogenous ecdysone, while old prepupae correspond to animals of stage V and are believed to possess a critical hemolymph ecdysone titer.

Ecdysone treatment: Synthetic ecdysone (courtesy of Dr. P. Hocks, Schering AG, and Hoffmann-La Roche) was dissolved in physiological saline (0.65% NaCl) to yield a concentration of 0.05 mg/ml. Young larvae were injected with 0.5 to 0.75 μl of solution (0.025 to 0.0375 μg of ecdysone) by methods described previously.[10] Control animals received only saline solution.

Juvenile hormone treatment: The animals were treated with the following substances possessing juvenile hormone activity in Diptera.[11] DL-juvenile hormone (DL-methyl *trans, trans, cis*-10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate; courtesy of Dr. H. Röller, Texas A & M University); C_{17} methyl ester (methyl *trans, trans, cis*-7-ethyl-3,11-dimethyl-2,6,10-tridecatrienoate; courtesy of Dr. H. Röller); synthetic mixture.[12] They were either diluted in olive oil and injected into old prepupae or dissolved in acetone and applied externally. For the latter treatment, 1 μl of hormone in acetone was placed on the posterior region of the anesthetized animal. After 1 min, 1 μl of an olive oil:acetone solution (1:10 v/v) was applied to the same region to seal the cuticle. Control animals were treated identically except for the omission of the hormonally active substance.

Cytological techniques: The animals were sacrificed 2 or 4 hr after treatment and the salivary glands fixed, stained with acetic orcein, and squashed. These preparations were scored within 2 days and the frequency (expressed as percentages) of a given puff or Balbiani ring of a specific stage, was determined. The frequencies of the treated and control animals were averaged and compared by utilizing Fischer's t-test for the determination of significant differences. For conciseness we have classified the cytological state of the Balbiani rings into three stages and they will be referred to by this terminology throughout the paper.

Stage I—condensed interiorly with few exterior fibers (Fig. 1*a*).

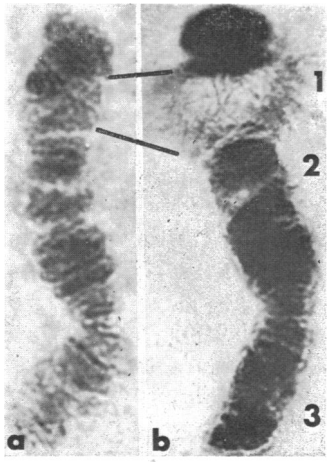

FIG. 1.—Chromosome IV from young 4th instar larvae. *a*, control; *b*, animal injected with ecdysone. Balbiani rings 1, 2, and 3 are indicated.

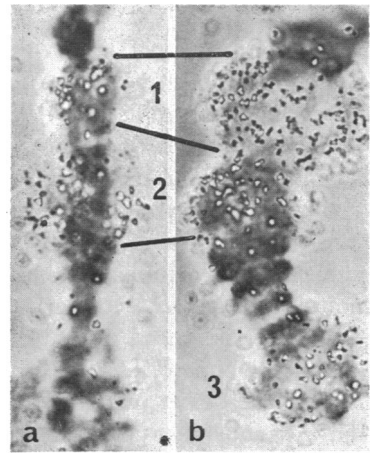

FIG. 2.—Autoradiograms of chromosome IV from young 4th instar larvae demonstrating ^{3}H-uridine incorporation into Balbiani rings 1, 2, and 3. *a*, control; *b*, animal injected with ecdysone. Phase contrast (note: silver grains appear black over the bright areas and white over the dark areas).

Stage II—intermediate stage between I and III; interior slightly unraveled.

Stage III—extremely large and completely unraveled both interiorly and exteriorly (Fig. 1*b*).

Autoradiographic techniques: To determine uridine incorporation, the ecdysone solution or saline alone was supplemented with 1 mc/ml H^3-uridine (20 c/mM) and injected into young larvae. After 4 hr, the salivary glands were removed and autoradiographs prepared.[13] After 2 weeks of exposure, the autoradiographs were examined for ^3H-uridine incorporation. The degree of labeling in each Balbiani ring of chromosome IV was determined either by grain counts or by measuring the blackened areas in those chromosomes that were densely labeled. The ratios between the three Balbiani rings were calculated by giving BR2 a unit value since it is the most stable of the Balbiani rings. The ratios were then averaged.

Results.—Effect of ecdysone: Table 1 summarizes the data on the puffing behavior of four selected chromosome regions in response to the injection of ecdysone into young larvae. It reveals that there is a definite increase in puff frequency at regions I-18-C and IV-2-B and a decrease at region I-19-A. These results confirm previously reported data.[1, 14] However, the observation that ecdysone also affects the Balbiani rings by increasing the frequency of occurrence

TABLE 1. *Effects of ecdysone injection on chromosomal morphology.*

Treatment	Average puff frequency (%) of regions: I-18-C	IV-2-B	I-19-A	Average frequency (%) of stage III BR1
A. Saline injected	38	35	60	4
B. Ecdysone injected	80	90	28	38
B-A	+42*	+55*	−32†	+34*

Young fourth instar larvae were utilized. Twelve were injected with saline and 12 with ecdysone. If B-A is positive, ecdysone stimulated puffing or Balbiani ring activation. If B-A is negative, an existing puff regressed after treatment. See *Materials and Methods* for other details.

* = $P < 0.01$.

† = $P < 0.02$.

of stage II and stage III BR1 has not been reported previously. Stage III is probably the most active phase of BR1 in terms of ^3H-uridine incorporation into acid insoluble material (Fig. 2). On the basis of these observations we conclude that ecdysone activates BR1 in young larvae. This supposition is supported by the labeling ratios of BR1:BR2 where the controls are as low as 0.25 and the ecdysone treated animals as high as 1.4 (average values in Table 2). This enhancement of BR1 activity by ecdysone may result in a significant change in the nuclear RNA complement since it may be responsible for 10–20 per cent of the non-nucleolar RNA in the salivary gland nucleus. Although the activity of BR3 is also enhanced by ecdysone, the absolute increase is much less (3–6% of the non-nucleolar RNA) than that observed with BR1 (Table 2).

Effect of substances with juvenile hormone activity: When old prepupae are treated with substances possessing potent juvenile hormone activity the following is observed. Within 2–4 hours, a puff is formed at region I-19-A (Fig. 3) while puff I-18-C and BR1 are reduced (Tables 3 and 4). This suggests that juvenile hormone and ecdysone may act antagonistically at the chromosome level although in many nuclei both the juvenile hormone-specific and ecdysone-specific puffs are observed together (Fig. 3*b*). (The assay animals already have a critical level of molting hormone when injected.) This occurrence of both ecdysone-

TABLE 2. *Effects of ecdysone on ^3H-uridine incorporation into the Balbiani rings.*

Treatment	Label ratio (average) BR1/BR2	BR3/BR2
A. Saline injected	0.52	0.13
B. Ecdysone injected	1.25	0.35
B-A	+0.73*	+0.22

Four young fourth instar larvae were injected as described in *Materials and Methods* and 34 chromosomes were evaluated.
* = $P \ll 0.01$.

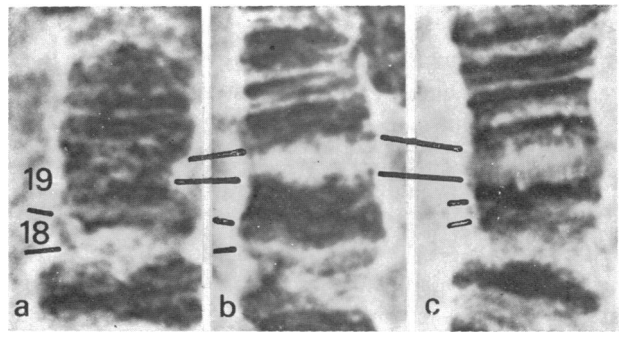

FIG. 3.—Chromosome regions I-18-C and I-19-A from old prepupae. *a*, control; *b*, animal injected with C_{17} methyl ester; *c*, animal injected with synthetic mixture. (18 = region I-18-C; 19 = region I-19-A).

specific and juvenile hormone-specific puffs was especially common when 2500–5000 Tenebrio units of DL-juvenile hormone or C_{17} methyl ester were injected. Both of these substances always acted identically on the puffing pattern. When synthetic mixture was injected, the formation of puff I-19-A and regression of puff I-18-C was more frequent and more complete (Fig. 3*c* and Table 3). Although injection usually results in greater effects than external application, the results are more variable among the cells of a single salivary gland after injection. The more uniform response resulting from external application may be the result of a more homogeneous distribution of the active principle within the animal.

During normal development, the activity of BR1 (frequency of stage II or III) is low at the beginning of the fourth larval instar, high in the middle of the instar and in the prepupa (Table 4), but decreases immediately preceding pupation. This sequence of low activity → high activity → low activity is an unexpected

TABLE 3. *Effects of substances with juvenile hormone activity on chromosome morphology.*

Treatment	Region I-19-A average puff frequency (%)	B-A	Region I-18-C average puff frequency (%)	B-A
A. Olive oil injected (13)	31		71	
B. DL-juvenile hormone or C_{17} methyl ester injected (13)	67	+36*	60	−11
A. Olive oil injected (5)	26		88	
B. Synthetic mixture injected (5)	79	+53*	42	−46*
A. Acetone applied externally (19)	49		77	
B. Synthetic mixture applied externally (19)	76	+27	68	−9

Numbers in parentheses denote number of animals utilized. A total of 1 μl was injected or applied to old prepupae. The quantity injected contained 2500–5000 Tenebrio units of DL-juvenile hormone or C_{17} methyl ester or 0.1 μl of synthetic mixture. For external application, 0.1 μl of synthetic mixture was dissolved in 1 μl of acetone and applied.
* = $P < 0.05$.

TABLE 4. *Effects of synthetic mixture on Balbiani ring 1 morphology.*

Treatment	Average Frequency (%) Old Prepupae Stage II	Old Prepupae Stage III	Young Larvae Stage II	Young Larvae Stage III
A. Acetone applied externally	74	11	34	1
B. Synthetic mixture applied externally	38	2	5	0
B-A	−36*	−9	−29	−1

Twenty old prepupae each received 1 μl acetone and 20 each received 1 μl of a solution containing synthetic mixture diluted 1:10 in acetone. Six young larvae each received 0.5 μl acetone and 6 each received 0.5 μl of a solution containing synthetic mixture diluted 1:10 in acetone.
* = $P < 0.01$.

phenomenon but is exhibited by region IV-2-B which is also an ecdysone-specific region and has been extensively studied.[15] Region I-19-A is also repressed prior to pupation but is highly active in the young larva, a time when BR1 and region IV-2-B are either slightly active or completely inactive (Tables 1 and 2; see also ref. 16). Treatment of young larvae with synthetic mixture further decreases the activity of BR1 (Table 4) while increasing the activity of region I-19-A.

Discussion.—It is the Balbiani rings that are most likely involved in the control of salivary gland secretion which is the principal physiological function of this structure. By extrapolating the data on *Chironomus thummi*, we can assume that the salivary gland secretion of our experimental insect changes in composition during the final larval instar (for literature, see ref. 2). Our data reveal that the activity of a specific Balbiani ring (BR1) is low at the beginning of the final larval instar when the juvenile hormone titer is presumably high and the ecdysone titer is low, but very high in the prepupa when the juvenile hormone titer is presumably low and the ecdysone titer is high. (See refs. 17 and 18 for studies on hormone titer.) This apparent relationship between hormone titer and the activity of BR1 was experimentally produced by treating last instar larvae with ecdysone or substances possessing juvenile hormone activity.

Clever[14] was unable to relate the activity of BR1 to the concentration of ecdysone in the hemolymph either during normal development or after injection of ecdysone. This may be due to the difficulty involved in determining Balbiani ring activity solely on the basis of morphological observations. Our autoradiographic studies demonstrated that Balbiani ring 1 incorporates the greatest amount of ^{3}H-uridine when it is maximally expanded (stage III) and we therefore consider this to be the most active stage.

A short-term juvenile hormone treatment of young larvae causes induction of puff I-19-A and this puff is reduced with a short-term treatment of ecdysone. The normal behavior of this chromosomal region is what one would predict for a region exhibiting a juvenile hormone-specific puff. Region I-19-A is active (puffs) during the third larval instar, during the molt to the fourth instar (simultaneously with the ecdysone-specific puff at region I-18-C), and at the beginning of the fourth larval instar.[7] However, in contrast to I-18-C, I-19-A is not active during the pupal molt[16] when the juvenile hormone titer decreases. Clever classified the puff at I-19-A as ecdysone-specific because it reappears after *prolonged* ecdysone treatment even though it is normally absent at the pupal molt. On the basis of this delayed induction of a puff (response after a single injection of ecdysone takes a minimum of 12 hr), Clever[14] postulated the concept of sequen-

tial gene activation. Since region I-19-A is activated in only 2–4 hours after application of juvenile hormone, we feel that it is primarily a juvenile hormone-specific puff and that Clever's concept must be reexamined. It may be that the induction of puff I-19-A after prolonged ecdysone treatment is not a physiological response.

The exact mechanisms by which ecdysone and juvenile hormone activate the specific chromosome regions described previously are not known with certainty. However, ecdysone appears to increase the K^+/Na^+ ratio in intact salivary glands,[19] whereas juvenile hormone appears to decrease this ratio.[20] Puff formation or Balbiani ring enlargement at the ecdysone-specific loci I-18-C, IV-2-B, and BR1 can be induced by incubating isolated nuclei in a medium rich in K^+, whereas the juvenile hormone-specific puff can be elicited by incubating isolated nuclei in a Na^+ rich medium.[13, 21] Recent experiments with both isolated chromosomes and chromatin suggest that K^+ and Na^+ interact with the histone-DNA bonds and that their specificity depends on the presence of bivalent cations.[21]

Note added in proof: It has recently been reported that when *Chironomus thummi* larvae are grown in a medium containing juvenile hormone, their development is arrested and the pattern of chromosome puffing is effected [Laufer, H. and H. Greenwood, *Am. Zoologist*, **9**, 603 (1969)].

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