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ON THE ACTION OF RIBONUCLEASE IN SALIVARY GLAND CELLS OF DROSOPHILA*

By

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With 4 Figures in the Text

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Introduction

It has been found that ribonuclease can induce many puffs in the salivary gland chromosomes of *Drosophila buskii* (RITOSSA and VON BORSTEL, 1964). On the other hand, it is known that RNase can work *in vivo* in this material (RITOSSA, 1962) and that puffs are regions of high RNA content (PELLING, 1964; SWIFT, 1962; RITOSSA, in press). To understand this apparent paradox, we undertook this series of investigations to determine the effects of ribonuclease on pre-existing and newly synthesized RNA.

Materials and Methods

Third instar larvae of *Drosophila buskii* have been used throughout this work. The larvae were grown on a normal medium enriched 20% by fresh yeast. The temperature was kept at 25°C. In order to induce puffs by anaerobiosis the larvae were left under a flow of nitrogen for 10 minutes, then left in the nitrogen atmosphere for 35 minutes further. Air was then given and the salivary glands were excised and maintained in Ephrussi-Beadle solution (EPHRUSSI and BEADLE, 1936). Alternatively the larvae were kept under mineral oil for 4 hours and then the salivary glands were excised in Ephrussi-Beadle solution. About 10 minutes after readministration of air, three puffs arise.

In order to induce puffs with ribonuclease, the excised glands were incubated in Ephrussi-Beadle solution containing 5 mg/ml ribonuclease (Light and Co. or Worthington) for 5 hours. Control glands were incubated in plain Ephrussi-Beadle solution. In some experiments 5 mg/ml of histone or protamine were added.

The cells were fixed in 60% acetic acid and squashed after brief staining with aceto lactic orcein. The siliconized coverslips were removed after immersion in liquid nitrogen. Nucleotides were removed by extraction in 5% cold TCA.

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For colorimetric detection of RNA in the puffs, fixed and squashed preparations were stained for one hour at 60°C in a 0.25% solution of Azure B (National Aniline Division) in phosphate buffer at pH 4. The slides were rinsed in water, passed through three changes of t-butyl alcohol, cleared in xylol, and mounted in clarite.

Ribonuclease digestion of the fixed cells was performed at 30°C for $2^1/_2$ hours. The concentration of the enzyme was 0.5 mg/ml and the pH was adjusted to 7.

NTB-3 nuclear emulsion was used for autoradiography. All the labeled compounds used were given in a solution containing 0.1 mC/ml. Their specific activities were as follows: uridine 4 C/mM; thymidine 6 C/mM; L-histidine 1.1 C/mM; L-lysine 0.4 C/mM; L-isoleucine 0.5 C/mM; L-phenylalanine 1.65 C/mM; L-leucine 0.5 C/mM; D-L tryptophan 0.6 C/mM. Uridine and tryptophan were from New England Nuclear Corporation; all the others were from Schwartz Bioresearch Corporation.

Results

Effect of ribonuclease on RNA and RNA synthesis in living cells. In order to test the effects of ribonuclease on RNA synthesis, puffs induced either by anaerobiosis or by ribonuclease were used. We shall first consider the anaerobiosis-induced puffs. For the control, the larvae were placed in a nitrogen atmosphere for 45 minutes (or under mineral oil for four hours); salivary glands were dissected from the larvae and incubated under a shallow oil drop in Ephrussi-Beadle solution containing H^3-uridine for two hours. This anaerobic treatment induces three puffs which are heavily labeled (Fig. 1a). The nucleolus is also heavily labeled and some radioactivity is found in the cytoplasm (Fig. 2). When the Ephrussi-Beadle solution contains 5 mg/ml ribonuclease as well as the H^3-uridine, only the nucleus is labeled; the label is restricted to the puffs and the nucleolus organizer (Fig. 1b and 1c). The nucleolar profile disappears. That the disappearance is real is confirmed by the examination of the ribonuclease-treated cells under phase optics or just after fixation in 60% acetic acid.

The course of RNA synthesis follows a similar pattern in puffs induced by ribonuclease itself. As a control the salivary glands are dissected away from the larvae and incubated for five hours in Ephrussi-Beadle solution containing H^3-uridine (Fig. 3a). The nucleolus and various chromosomal regions are labeled in the nucleus. When 5 mg/ml ribonuclease is incubated with the same mixture for five hours, the only regions in which label can be seen are newly arisen, ribonuclease-induced puffs and the nucleolus organizer (Fig. 3b). The amount of radioactivity is considerably less than that seen in the puffs induced by anaerobiosis after only two hours of H^3-uridine treatment.

In order to determine whether the puffs were still functioning, the cells were first incubated in ribonuclease for five hours and then given a 30-minute pulse of H^3-uridine. The same results were obtained: the puffed regions and the nucleolus organizer were lightly labeled, indicating that RNA was still being synthesized in these regions.

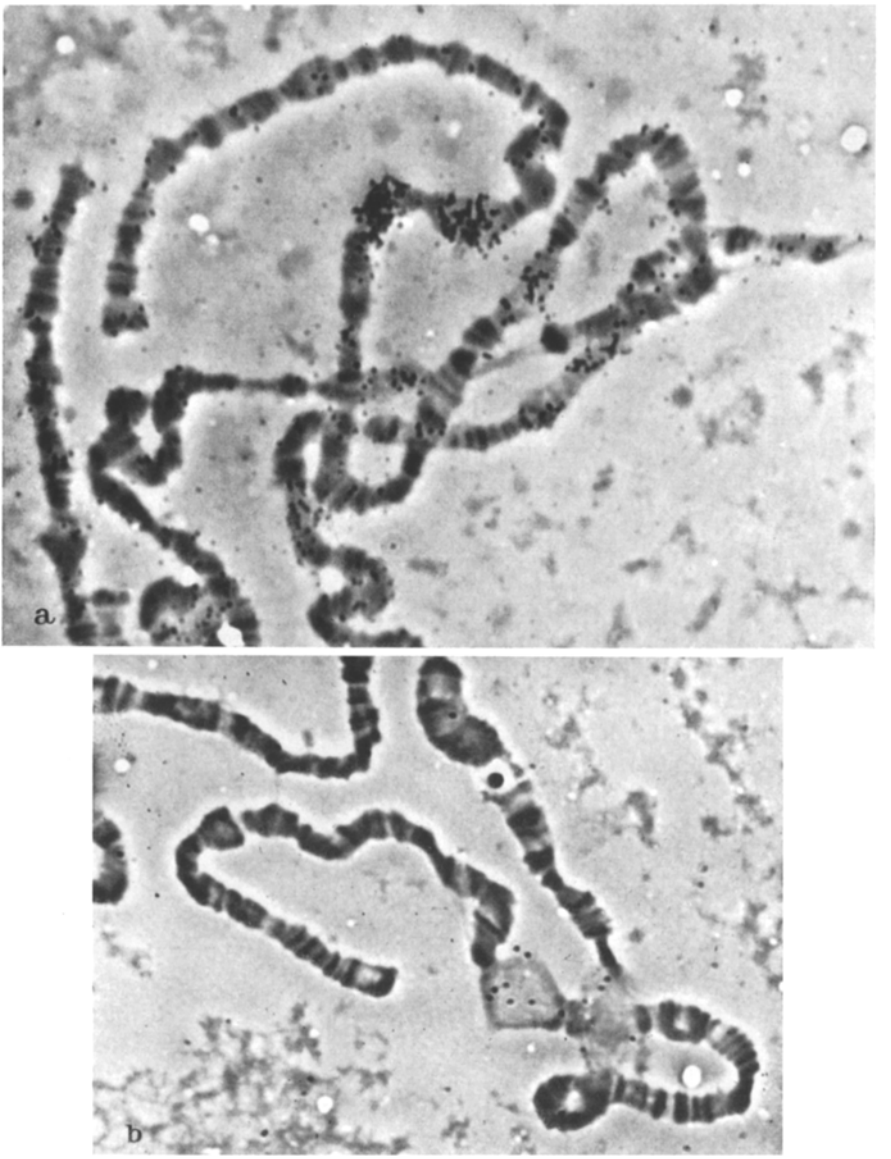

Fig. 1a. Squashed preparations of salivary gland chromosomes of third instar larvae of *D. busckii*. The larvae were kept under mineral oil for four hours, then incubated for two hours in Ephrussi-Beadle solution containing H^3-uridine in order to induce the puffs in regions 30 B, 31 B and 38 A of the 2 L chromosome. These puffs appear to be heavily labeled Fig. 1b. The cells have been treated as in Fig. 1a but 5 mg/ml ribonuclease was added to the Ephrussi-Beadle solution. Some radioactivity is still found in the puffed regions. Fig. 1c. The cells were subjected to exactly the same treatment as in Fig. 1b. This photograph shows the labeled nucleolus organizer and the puffs

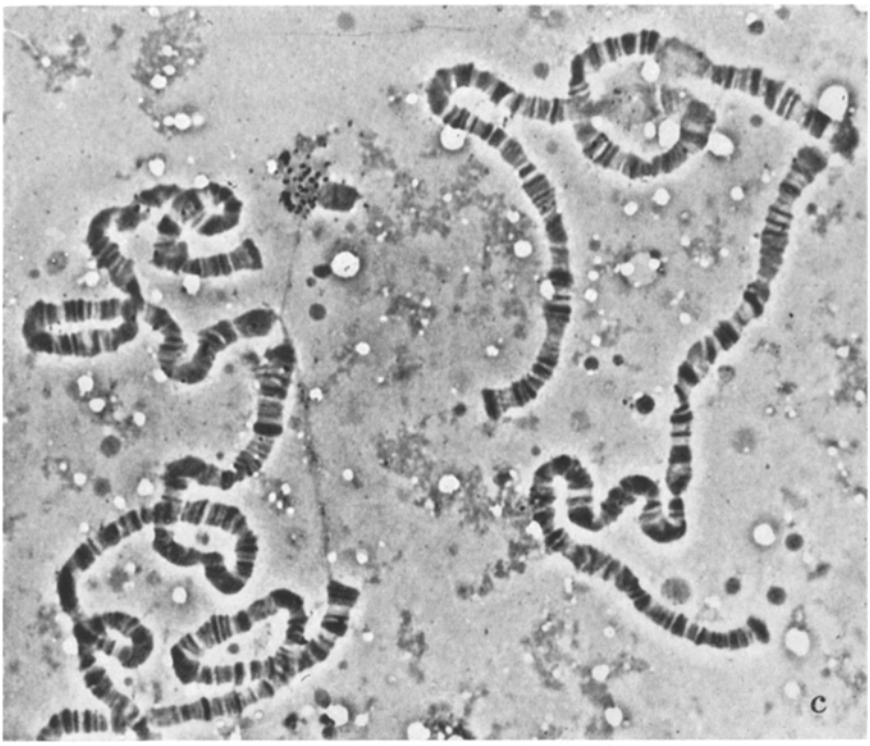

Fig. 1 c

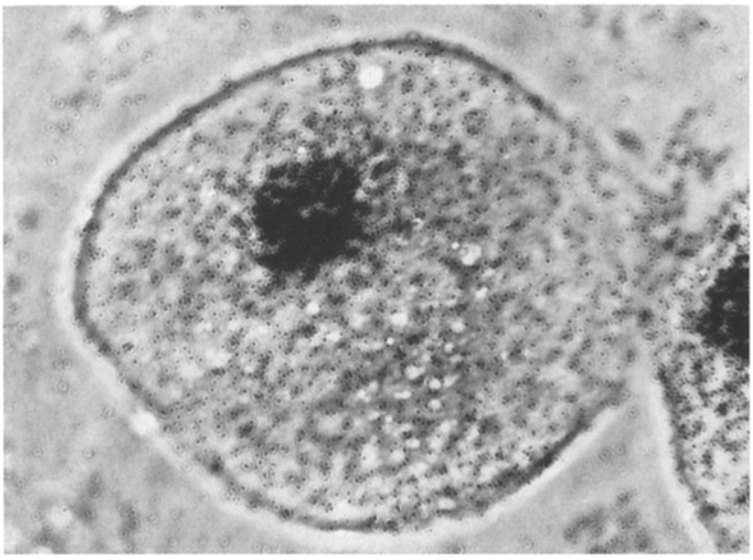

Fig. 2. The same treatment has been applied to the salivary gland cells as in Fig. 1 a. Liquid emulsion was applied over the entire cell after fixation. The nucleolus and the cytoplasm are particularly heavily labeled

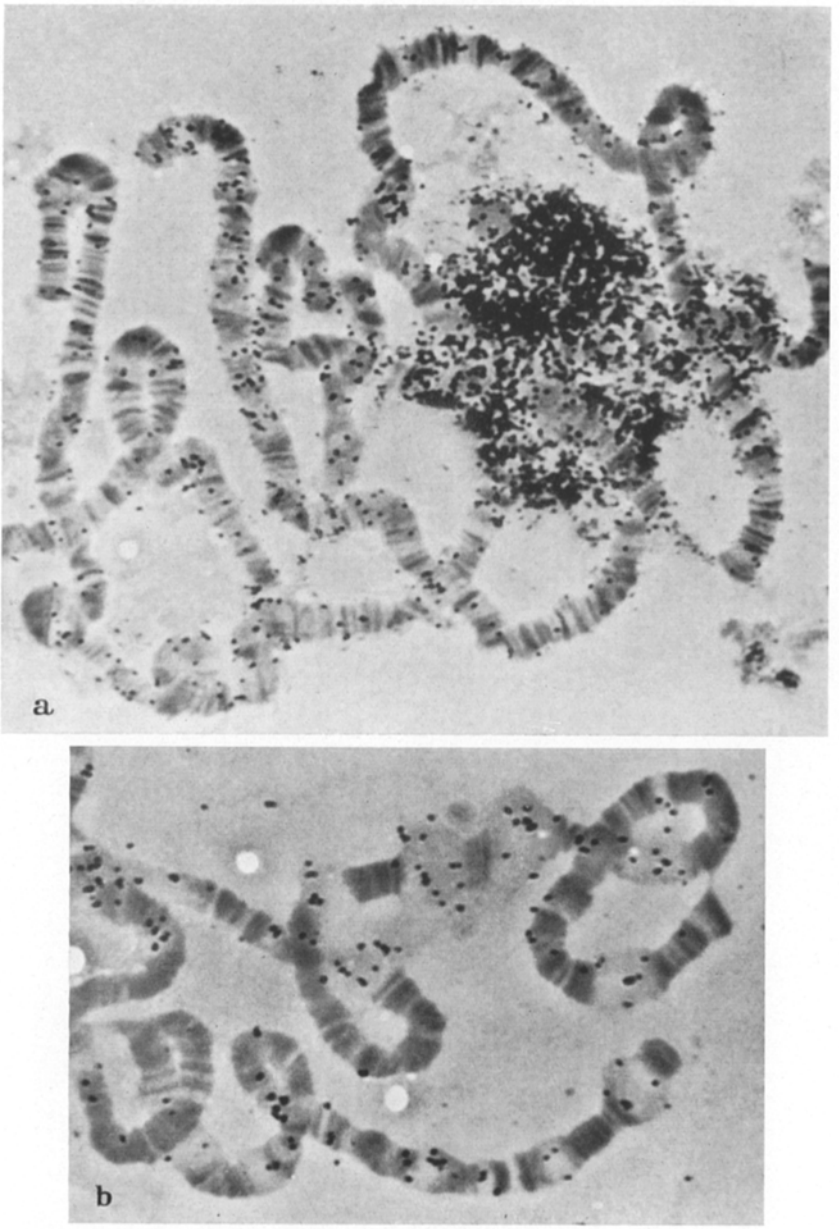

Fig. 3a. Salivary glands of *D. busckii* were incubated for five hours in Ephrussi-Beadle solution containing H^3-uridine. The nuclear emulsion was applied over the squashed cells Fig. 3b. The salivary glands were treated as in Fig. 3a but 5 mg/ml ribonuclease was added to the Ephrussi-Beadle solution. The ribonuclease-induced puffs are palely labeled

Ribonuclease removal of RNA from fixed cells. When cells are fixed in 60% acetic acid, incorporated H^3-uridine is completely removed by the ribonuclease digestion as indicated in the methods; H^3-uridine is not removed in a water control.

RNA content of ribonuclease-induced puffs. When ribonuclease-induced puffs are stained with azure B, the puffs are intensely colored.

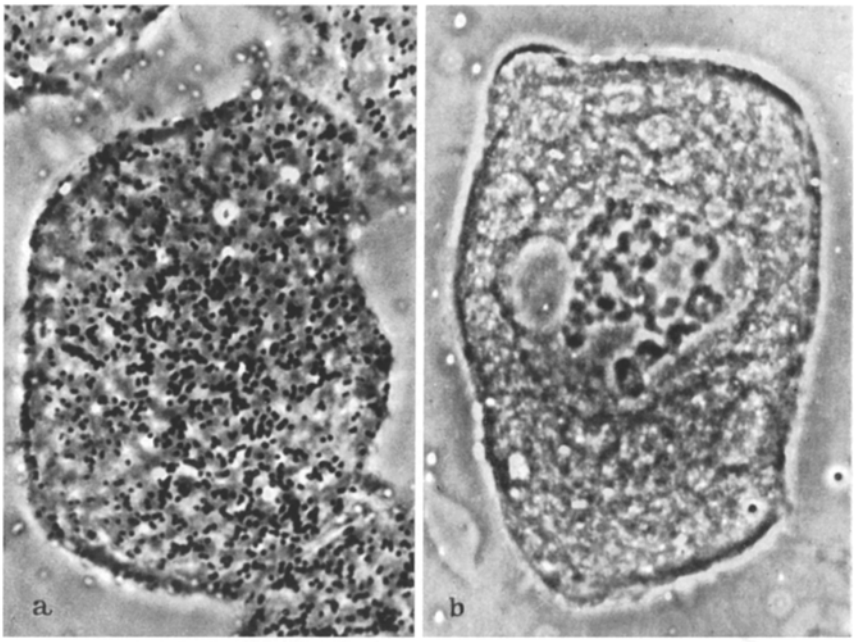

Fig. 4a. The salivary glands of *D. busckii* were incubated for two hours in Ephrussi-Beadle solution, then a 30-minute pulse of H^3-leucine was given
Fig. 4b. The salivary glands were incubated for two hours in Ephrussi-Beadle solution containing 5 mg/ml ribonuclease, then a 30-minute pulse of H^3-leucine was given. No radioactivity is found in the cell

The amount of staining is much heavier in the ribonuclease-induced puffs than in the normally occurring puffs or in the anaerobiosis-induced puffs, even those that involve the same chromosomal regions.

Effects of ribonuclease on amino acid incorporation. When a salivary gland is incubated in Ephrussi-Beadle solution for two hours and then a H^3-labeled amino acid is added to the incubation mixture for 30 minutes, the cells become strongly labeled (Fig. 4a). When 5 mg/ml ribonuclease is added to the incubation mixture for the first two hours of incubation, no labeling takes place (Fig. 4b). Tritium-labeled histidine, lysine, leucine, isoleucine, phenylalanine, and tryptophan were used separately.

Effects of ribonuclease on DNA synthesis. When H^3-thymidine is added to the incubation mixture after five hours of ribonuclease treatment, the H^3-thymidine incorporation is apparently normal. It has been found previously that puffs are never labeled with H^3-thymidine when the chromosomes are labeled discontinuously along their length, but the puffs are, of course, labeled in the continuously labeled chromosomes (Ritossa, in press). It is of interest to note that when approximately 40 puffs are induced by ribonuclease, puffs remain unlabeled in the discontinuously labeled chromosomes.

Discussion

Since only remnants of the nucleolus are left after incubation with ribonuclease, and RNA and DNA syntheses are not blocked, the evidence appears to be incontrovertible that ribonuclease can break down RNA in cells that are actively metabolizing. Alfert and Das (1962) have suggested that removal of RNA from ribonuclease-treated cells of root tips does not occur in the living condition but only after fixation. Our observations cannot be explained in this way because loss of nucleolar material can be observed in the living cell.

The decreased labeling of the puffs with H^3-uridine in the ribonuclease-induced puffs appears to be caused by isotope dilution rather than by ribonuclease interference with RNA synthesis or by digestion of RNA in these sites. There is more RNA in the ribonuclease-induced puffs than in other puffs, and these puffs are still synthesizing RNA; it seems likely therefore that the contribution from broken cytoplasmic and nucleolar RNA to the nucleotide pool is such to dilute the added tritium-labeled uridine. It thus appears that RNA at the sites of synthesis is not susceptible to degradation by ribonuclease.

The induction of many puffs by ribonuclease is an interesting phenomenon. There are undoubtedly many possible explanations for this: one of the more plausible is that with inhibition of protein synthesis there is a feedback to the genes which call for more gene product to be formed. If this were true, then specific antimetabolites should be able to induce specific puffs, while puromycin should be able to induce a number of puffs. Up to now, attempts to induce puffs with puromycin and other antimetabolites have been unsuccessful. The general model that can be formulated from our experiments is that the appearance of the puffs induced by ribonuclease is caused as a consequence of the breakdown of RNA, either by feedback of RNA breakdown products, or by release of possible RNA repressors.

When puffs are formed there is an increase of protein as well as RNA in the puffs (Beermann, 1959; Swift, 1962). After two hours of incubation in ribonuclease, amino acid incorporation into protein has ceased,

yet puffs arise after five hours of incubation while protein synthesis is not resumed. The evidence appears to be conclusive that the protein that accumulates in puffs during puff production must pre-exist.

Summary

It was shown that ribonuclease degrades the nucleolus in actively metabolizing cells. It does this without inhibiting RNA synthesis in the puffs and the nucleolus organizer. DNA synthesis still continues before or after puff formation, while amino acid incorporation is inhibited before the puffs are formed, indicating pre-existence of proteins involved in the process of puff formation.

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