STRUCTURAL AND FUNCTIONAL PROPERTIES OF POLYTENE NUCLEI ISOLATED FROM SALIVARY GLANDS OF *DROSOPHILA HYDEI*

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ABSTRACT

Salivary gland nuclei of *Drosophila hydei*, isolated by a modification of the procedure described by Boyd et al. (9), retain their normal morphology during the isolation and subsequent incubation procedure. RNA synthesis was studied in isolated nuclei by biochemical and cytological techniques. In radioautographs 70% of the nuclei displayed a distribution of labeled RNA over the nuclear constituents similar to the distribution obtained after in vivo incorporation of radioactive precursor. Chromosome puffs and the nucleoli were specifically labeled. The remaining 30% of the nuclei showed a weak to very weak incorporation of radioactive precursor. In these nuclei most of the radioautographic grains were concentrated over the nucleolus, and a few grains were randomly distributed over the chromosomes. Actinomycin D and the absence of ATP, GTP, and CTP in the medium inhibited incorporation of radioactive precursor. The radioactive product was sensitive to combined pronase and RNase digestion. Addition of *E. coli* RNA polymerase to the incubation medium enhanced the specific labeling over the puffed regions. The sedimentation behavior of the RNA synthesized in isolated nuclei was different from that of RNA synthesized during a 20 min pulse of radioactive precursor administered to whole glands in vivo and in vitro. Neither the steroid ecdysterone nor a temperature treatment was effective in inducing new puffs in isolated nuclei.

INTRODUCTION

Microscopic and chemical studies of polytene chromosomes have provided some insight into the process of gene activity in cells of higher organisms (4; Pelling, C. Data in preparation). There is good evidence that Balbiani rings and puffs represent morphological manifestations of gene activity (3). Experimental activation and/or inactivation of particular chromosomal loci has revealed the interrelationships of some of the factors involved in the complex processes of puff formation, puff maintenance, and puff regression (8).

So far, however, it has been difficult to investigate the physiology of polytene nuclei by conventional biochemical techniques because insufficient material could be obtained. A recently developed method for the mass isolation of polytene nuclei from salivary glands of *Drosophila hydei* (9) has provided the basis for further analysis of nuclear ac-

tivity by conventional biochemical techniques. The effect of the absence of cytoplasm on the response of the genome to experimental activation of certain groups of chromosomal loci can now be investigated in more detail. The aim of the present study is the characterization of the structural and of some of the functional properties of isolated polytene nuclei from salivary glands of *Drosophila hydei*. The results of experiments designed to induce specific puffs in isolated nuclei will be discussed.

MATERIAL AND METHODS

A wild type stock of *Drosophila hydei* was used. The culture conditions, the isolation of the salivary glands, and the isolation of the salivary gland nuclei were performed according to the method of Boyd et al. (9). Two modifications of the method were employed. (*a*) Instead of centrifuging the isolated tissues in a discontinuous Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) gradient in order to separate the salivary glands from the other tissues, the contaminating tissues were removed manually with a Pasteur pipette. The resulting preparation contained about 95% salivary glands. (*b*) For isolation of the nuclei, 0.4 ml of 5% NPT 12 Melle Bezons, France) was added to 5 ml of the isolation buffer instead of the previously used Triton X 100. The percentage of nuclei with aberrant chromosome morphology was greatly reduced after use of NPT 12.

Incubation Medium and Assay for RNA Synthesis

After isolation the nuclei were resuspended in 0.5 ml of the following incubation buffer which included 0.1 ml of the nuclear isolation buffer (9): 0.01 M Tris-HCl (pH 8.2), 4 mM $MgCl_2$, 0.4 mM $(NH_4)_2SO_4$, 0.2 mM mercaptoethanol, 0.6 mM $MnCl_2$, and 0.06 mg of each of the nucleoside triphosphates ATP (adenosine triphosphate), GTP (guanosine triphosphate), and CTP (cytidine triphosphate) (Zellstofffabrik: Waldhof, Mannheim, Germany). Per aliquot (0.5 ml), 5 μc of uridine-^{3}H-5′-triphosphate (UTP-^{3}H) (New England Nuclear Corp., Boston, Mass. Specific activity, 1.37 c/mmole) was added. In one series of experiments the medium was deprived of cold ATP and supplemented with cold UTP and 1 μc of adenosine-5′-triphosphate-8-^{14}C (The Radiochemical Centre, Amersham, U. K. Specific activity 25 μc/mmole) instead of UTP-^{3}H. Incubation was carried out at 25°C. The reaction was stopped by chilling and immediately adding 5 ml of 4% TCA (trichloroacetic acid) and 0.25 ml of saturated Na-pyrophosphate. The acid-insoluble material was pelleted by low-speed centrifugation (3000 rpm), washed three times with 5 ml of 4% TCA, collected on Millipore filters (HA 0.45 μ), and dried at 60°C. The radioactivity was measured in a PPO-POPOP-toluene scintillator with a Packard Tricarb liquid scintillation spectrometer.

RNase sensitivity of the acid-insoluble material was established with a combined pronase-RNase digestion. The nuclear suspension was supplemented with pronase (Calbiochem, Los Angeles, Cal. B-grade) to a concentration of 25 μg/ml and incubated for 30 min at 25°C. The preparation was subsequently heated at 100°C for 30 min, followed by an incubation with 25 μg/ml RNase (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. The pronase treatment prior to the RNase treatment was applied to break down eventual protein-RNA complexes in which the RNA is insusceptible to RNase.

The sedimentation pattern of RNA synthesized by whole salivary glands and isolated nuclei was studied with the method of C. Pelling (Data in preparation) with a linear sucrose gradient (25–5%).

The *E. coli* RNA polymerase used in some of the experiments was kindly supplied by Dr. P. Hausen (gift from Dr. W. Zillig, Munich). The solution contained 6000 u/ml (13).

Cytology

The presence and location of acidic protein in the isolated nuclei was studied after fixation of the nuclei with 5% neutral formaldehyde followed by staining with acetocarmine and subsequently with 0.1% fast green at pH 2.4.

For electron-microscopic studies, the nuclei were pelleted in a conical plastic tube, fixed for 10 min at 0°C in 0.5 ml of 3.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.0), and postfixed in 1% OsO_4 in the same buffer. For dehydration the pellet was passed through an ethanol series (10 min each step). The 70 and 95% ethanol solutions were saturated with uranyl acetate. The pellet was embedded in Epon.

For radioautographic analysis a drop of the nuclear suspension was placed on a gelatinized slide. The incubation medium was replaced by a drop of acetocarmine, and the nuclei were stained for 5 min. After staining, the nuclei were slightly squashed. Radioautographs were made as described by Berendes (6) and were exposed for 14 days.

RESULTS

Electron Microscopic Observations

Electron micrographs of nuclei pelleted by low-speed centrifugation reveal an apparently normal morphology of the nuclear organelles as compared with nuclei of salivary glands fixed *in toto* immediately after dissection. The polytene chromosomes

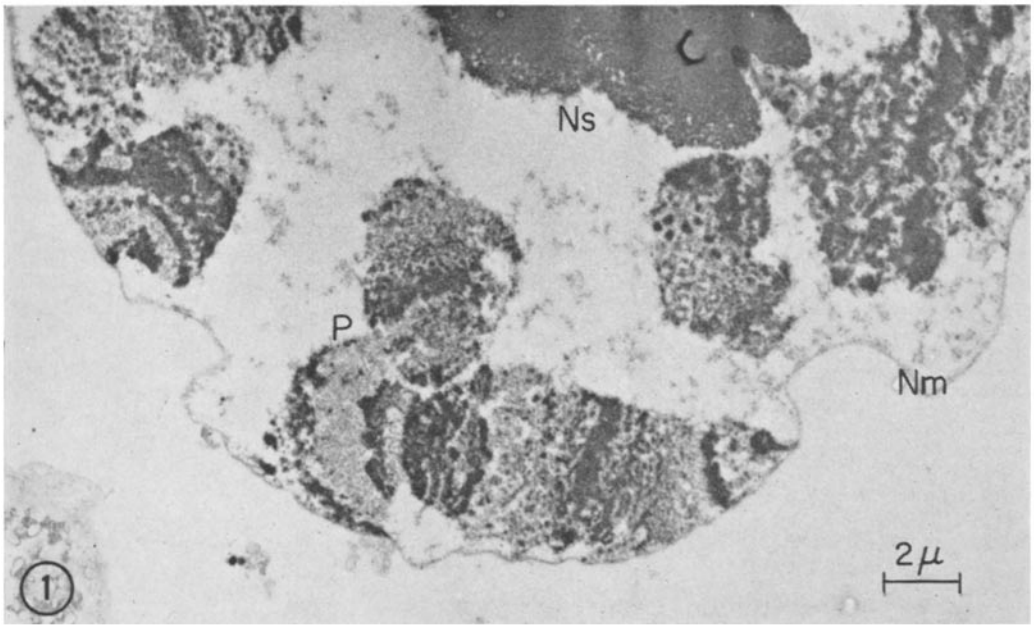

FIGURE 1 Low-power electron micrograph of an isolated salivary gland nucleus revealing a normal arrangement of the nuclear constituents. Nucleolus (*Ns*), a chromosome puff (*P*), and the nuclear membrane (*Nm*) are indicated. × 5000.

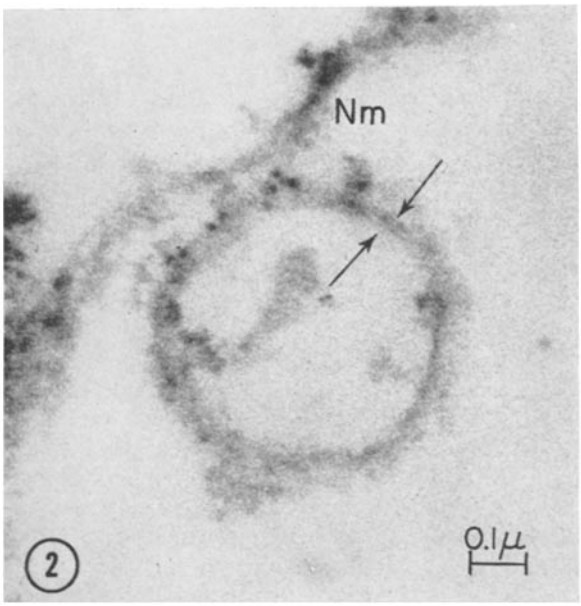

FIGURE 2 Detail of part of the nuclear membrane (*Nm*) showing a nuclear outpocketing. 200-A granules are attached to the outer membrane. The two units of the membrane are indicated (arrows). × 70,000.

retain their characteristic banding pattern (Fig. 1). In the nucleolus two areas can be recognized, a central region composed of tightly packed 100–150-A granules which is surrounded by an area containing similar size granules with a less compact organization. Most of the nuclei displayed a nuclear membrane free of cytoplasmic contamination. In a few cases, 200-A granules attached to

TABLE I

In Vitro RNA Synthesis in Isolated Polytene Nuclei at 25°C

Medium	DNA	Incubation period	Posttreatment	
	μg	*min*		*cpm**
A. Incorporation of adenosine-5′-triphosphate-8-^{14}C				
a. Complete medium	80	20	—	1938
b. Complete medium + 0.05 M sucrose	80	20	—	1138
c. Medium without UTP, GTP, and CTP	80	20	—	190
d. Complete medium	80	20	30 min pronase (25°) 30 min at 100°C 30 min RNase (37°)	125
B. Incorporation of uridine-^{3}H-5′-triphosphate				
a. Complete medium	150	20	—	3886
b. Complete medium	150	20	30 min pronase (25°) 30 min at 100°C 30 min RNase (37°)	177
C. Incorporation of uridine-^{3}H-5′-triphosphate				
a. Complete medium	85	25	—	1572
b. Complete medium + 3.2 μg/ml Actinomycin D	85	25	—	242

* 1000 cpm represents 0.05 μμM uridine-^{3}H.

the outer membrane were observed (see Fig. 2). The membrane has a cross-section varying from 180 to 280 A. Two units of the membrane can be recognized at those locations with cross-section of 250–280 A. In contrast to the membrane structure in freshly fixed glands, pores could not be detected in the membrane of isolated nuclei. The typical blebs, outpocketings of the nuclear membrane into the cytoplasm, remain attached to the nuclear membrane during the isolation procedure (Fig. 2). In the chromosomal puffs the characteristic puff particles of 280–350 A in diameter are abundantly present. Particles with similar diameters occur in the nuclear sap and are attached to the nuclear membrane.

RNA Synthesis

The medium of Pogo et al. (11) was modified in order to permit *de novo* RNA synthesis in isolated polytene nuclei while maintaining their normal morphology. The locations of chromosome regions active in RNA synthesis could thus be compared with the locations of those regions that are active in vivo. In order to retain the normal polytene chromosome structure the $(NH_4)_2SO_4$ concentration used in the medium of Pogo et al. had to be reduced to 1/100. The presence of sucrose in the incubation medium produced alterations in chromosome structure, even at very low concentrations (0.05 M), and also reduced the rate of RNA synthesis (Table I). Sucrose was therefore omitted from the medium.

In order to ascertain whether the incorporation of radioactive precursor into acid-insoluble material is a reflection of *de novo* RNA synthesis, the following controls were used: *a*, incubation with actinomycin D; *b*, incubation in a medium deprived of the three unlabeled nucleoside triphosphates; and *c*, a combined pronase-RNase digestion after incubation. Actinomycin D was added to the nuclear suspension at a concentration of 3.2 μg/ml. The incorporation of the radioactive precursor after 25 min of incubation at 25°C was measured in the nuclear suspension containing actinomycin D and also in a parallel sample lacking the antibiotic, and the values were compared. The incorporation of UTP-^{3}H in the presence of the actinomycin D was only 15% of that obtained with the complete medium lacking the antibiotic (Table I, *C*; Fig. 3). A comparison of the incorpo-

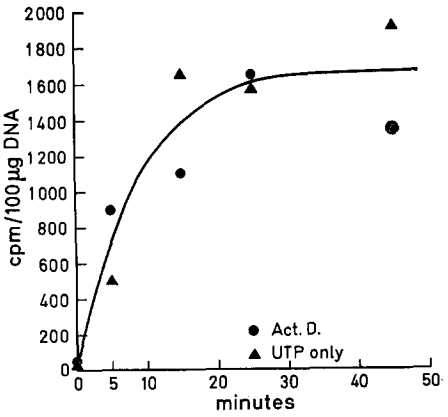

FIGURE 3 Kinetics of the in vitro incorporation of UTP-^{3}H into nuclear RNA at 25°C. The experiments were started by addition of the radioactive precursor to a series of identical nuclear suspensions, followed by immediate transfer of the nuclear suspensions from 0° to 25°C. The radioactivity in the acid-insoluble material was established after 5, 15, 25, and 45 min of incubation. In one series of experiments (circles), actinomycin D (3.2 µg/ml) was added to one of the samples, and that sample was incubated for 25 min. In the other series (triangles), one of the samples (UTP only) was supplied with UTP-^{3}H and deprived of the unlabeled nucleoside triphosphates ATP, GTP, and CTP and was incubated for 25 min. 1000 cpm represents 0.05 µµM.

ration in an incubation medium lacking the three unlabeled triphosphates and the incorporation obtained in complete medium revealed a very weak (10%) incorporation in the absence of the complete group of nucleoside triphosphates (Table I, *A*; Fig. 3). At least 90% of the radioactivity incorporated during 20 min of incubation was removed by a combined pronase-RNase treatment (Table I, *A* and *B*). A similar control was performed with preparations of isolated nuclei used for radioautographic analysis of RNA synthesis. Of a group of 10 slides containing nuclei from the same sample, 5 slides were treated with 25 µg/ml pronase (30 min at 25°C) followed by thorough rinsing in distilled water and incubation in 25 µg/ml RNase (30 min at 37°C). This treatment removed most of the labeled material from the nuclei (Fig 4). These experiments strongly indicate that at least 90% of the acid-insoluble material represents newly synthesized RNA.

The time course of RNA synthesis in isolated nuclei was established by incubating identical aliquots of nuclear suspensions for various times at

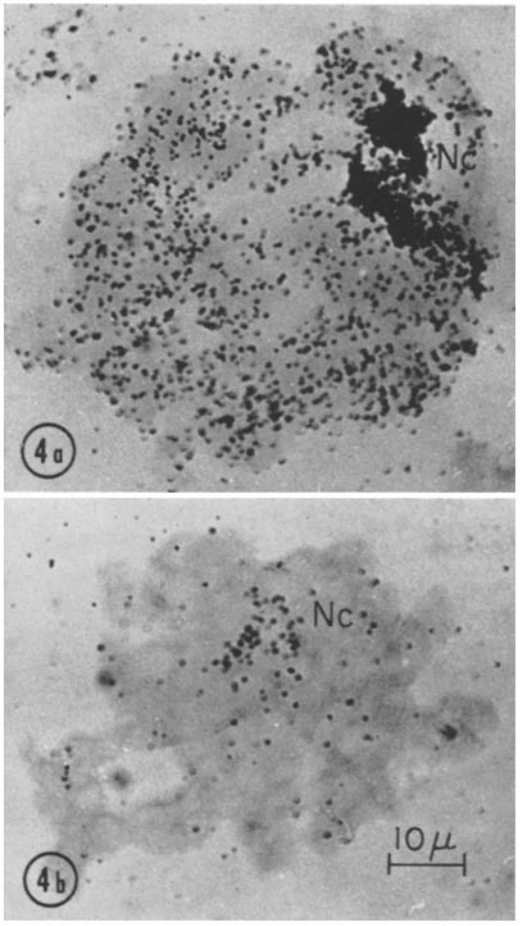

FIGURE 4 Radioautographs of nuclei incubated for 20 min at 25°C. *a*, Nucleus displaying a normal incorporation pattern. The nucleolus is heavily labeled (*Nc*). Because the chromosomes of this nucleus are little spread, it is difficult to identify the correspondence between puffs and distribution of label as indicated in the chromosome region aside the nucleolus. *b*, Nucleus of the same sample after combined pronase-RNase digestion. × 1000.

25°C. As controls in these series, a nuclear suspension containing 3.2 µg/ml actinomycin D or a suspension deprived of unlabeled nucleoside triphophates was used. Fig. 3 shows that *de novo* RNA synthesis occurs during the first 20 min of incubation. The question of whether the decrease in the rate of synthesis after 20 min is due to exhaustion of the precursors was studied with a preincubation experiment. A nuclear suspension was incubated for 20 min in the presence of all four unlabeled nucleoside triphosphates (12 µg UTP, and 60 µg

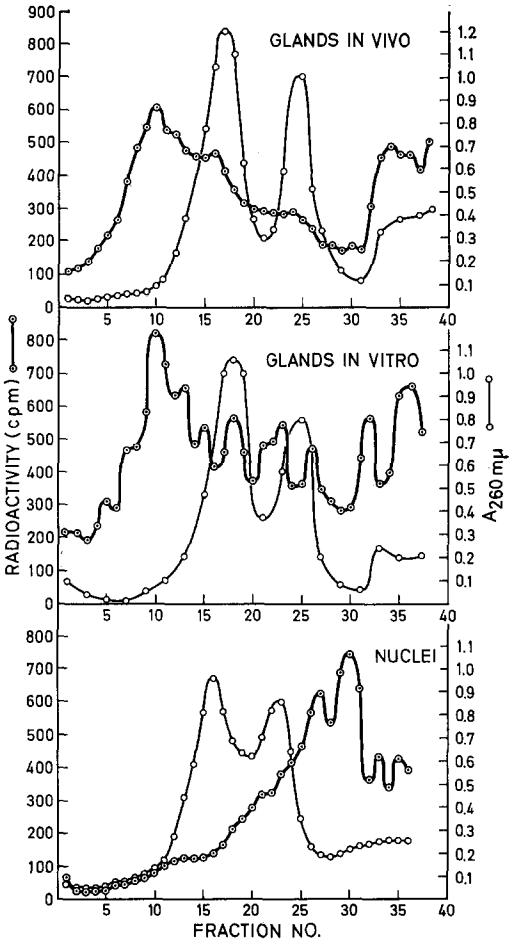

FIGURE 5 Sedimentation patterns of labeled RNA in a linear sucrose gradient. Upper graph: RNA extracted from intact glands (286) labeled with uridine-^{3}H (20 min) in vivo. Middle graph: RNA extracted from intact glands (200) labeled with uridine-^{3}H (20 min) in vitro. Lower graph: RNA extracted from isolated nuclei labeled with UTP-^{3}H in vitro. Each of the graphs is representative for the results obtained from three experiments. Nonradioactive RNA extracted from imagines was used as carrier. 1000 cpm represents 0.05 $\mu\mu$M.

of each of ATP, GTP, and CTP); subsequently, another 60 μg of each of the unlabeled triphosphates ATP, GTP, and CTP, and 5 μc of UTP-^{3}H were added to the medium. The radioactivity was measured after 20 min. Under these conditions very little incorporation occurred (80 cpm/165 μg DNA).

The sedimentation behavior of the RNA synthesized in isolated nuclei was analyzed by sucrose

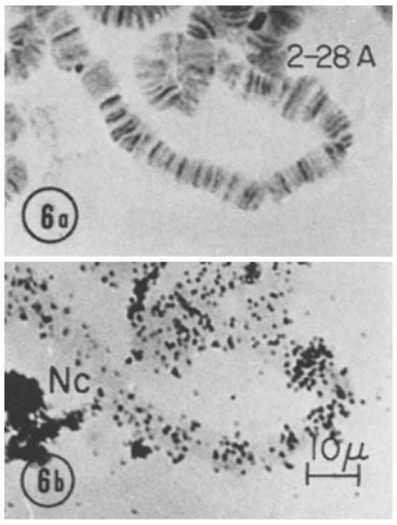

FIGURE 6 Pattern of incorporation of UTP-^{3}H, after 20 min of incubation at 25°C, into part of chromosome 2. The label distribution follows the pattern of puffs (*2-28A*). *Nc*, nucleolus. × 650.

gradient centrifugation and compared with that of RNA synthesized during a 20 min pulse of uridine-^{3}H in intact glands in vivo and in vitro. The data in Fig. 5 indicate that the RNA extracted from isolated nuclei has a sedimentation behavior different from that of RNA extracted from intact glands in vivo and in vitro.

Radioautographic analyses of UTP-^{3}H incorporation into isolated polytene nuclei revealed that the label in most of the nuclei is distributed over the nuclear constituents as it is in nuclei after in vivo incubation of glands with uridine-^{3}H. The nucleolus and the active sites on the polytene chromosomes (puffs) show clear concentrations of labeled material (Figs. 4 *a* and 6). A detailed comparison of puffing pattern and UTP-^{3}H incorporation in at least 30 well-stretched chromosome arms revealed an obvious correspondence between puffs and distribution of label. The specific incorporation of the precursor into puffed regions was enhanced by the addition of 3 units of *E coli* RNA polymerase. This enhancement was particularly well demonstrated in puffs which were induced by a temperature treatment (transfer of the larvae from 25° to 37°C for 20 min) prior to the isolation of the nuclei (Fig. 7). The excessive incorporation of precursor into the newly activated puffs is similar to the incorporation into these puffs observed after in vivo incorporation of uridine-^{3}H.

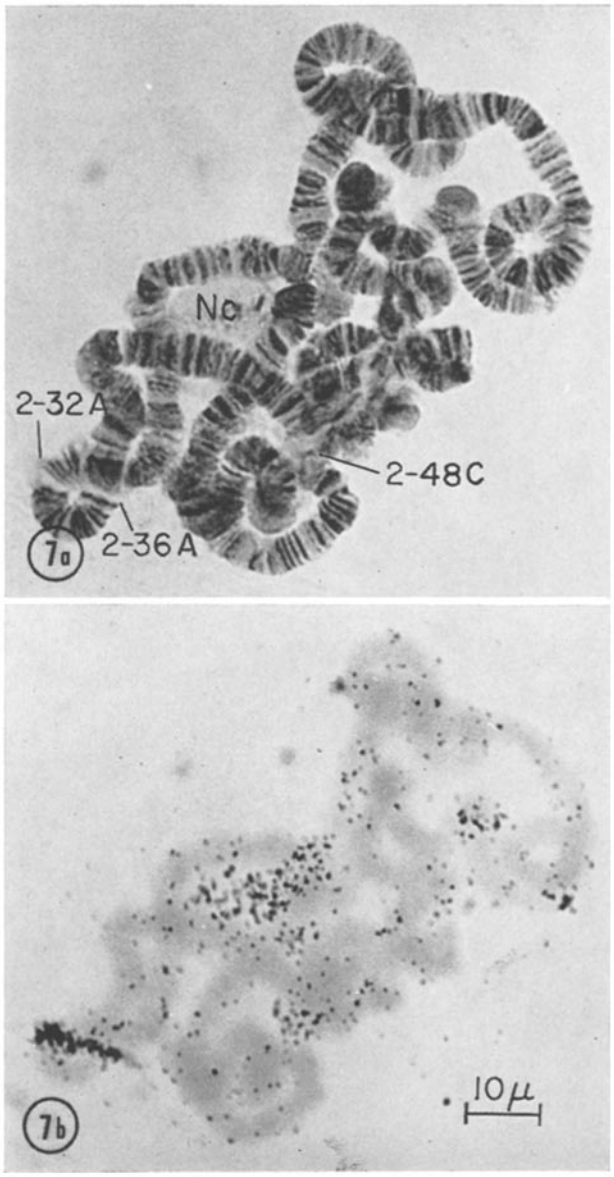

FIGURE 7 Enhanced incorporation of UTP-^{3}H into the puffs *2-32A*, *2-36A*, and *2-48C*. These puffs were activated by a temperature treatment (see text) prior to the isolation of the nuclei. The incubation medium contained 3 units of *E. coli* polymerase/125 μg DNA. Incubation for 20 min at 25°C. *Nc*, nucleolus. × 1000.

Protein Staining Characteristics

Staining of formaldehyde-fixed, isolated nuclei with fast green at pH 2.4 revealed that the distribution of acidic protein in isolated nuclei is identical with that of acidic protein in nuclei of salivary glands fixed *in toto* immediately after dissection. Acidic proteins were demonstrated in the nuclear sap, in the nucleolus, and at various locations along the chromosomes. The localized staining reaction in the chromosomes is generally correlated with a puffed appearance of the region. In puffs induced by a temperature treatment prior to the isolation of the nuclei, no detectable loss of accumulated acidic protein could be established (Fig. 8).

Puff Formation and Puff Regression

So far, the administration of ecdysterone, a steroid which induces the formation of specific puffs both in vivo and in vitro (7), has no effect on

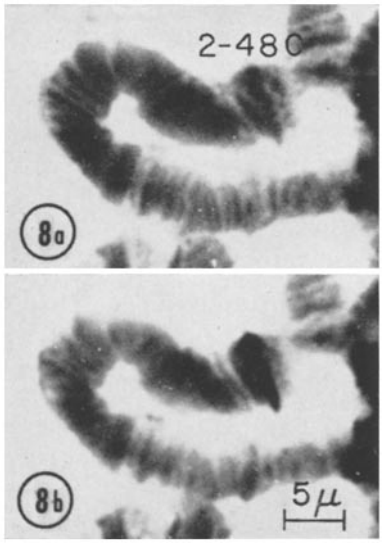

FIGURE 8 Demonstration of acidic protein with fast-green staining at pH 2.4 in puff *2-48C*. This puff was induced by a temperature treatment prior to the isolation of the nuclei. *a*, green filter (635 nm); *b*, red filter (500 nm). It can be concluded that acidic proteins accumulated in vivo upon experimental activation of region 48C are retained during the nuclear isolation procedure. × 1500.

the puffing pattern in isolated nuclei. With various concentrations of the steroid ranging from 10^{-2} to 10^{-6} mg/ml, it has not been possible to demonstrate a localized accumulation of acidic protein, localized RNA synthesis, or a change in chromosomal diameter of the normally responding loci. Temperature treatments performed by transfer of the nuclear suspensions from 25° to 37°C also failed to produce any sign of activation of those loci which respond to a similar treatment in vivo and in vitro.

Regression of those puffs which are induced by a temperature treatment in vivo prior to the isolation of nuclei did not occur, although the nuclear suspension was kept for 30–40 min at 25°C. The same puffs if induced in vivo or in vitro regress completely upon transfer from 37° to 25°C within 30 min.

DISCUSSION

It is well established that polytene nuclei of dipteran salivary glands provide a unique system for the analysis of local chromosomal activity under in vivo and in vitro conditions (1–3, 5, 10). The present experiments indicate that isolated polytene nuclei may also provide a useful tool for studies on the regulation of gene activity in the absence of cytoplasm. We have shown that chromosome morphology, the typical distribution of acidic protein in the nucleus, and the capacity of the nuclei for RNA synthesis are retained during the isolation procedure. Radioautographic analysis revealed that chromosome puffs and the nucleolus become specifically labeled. The specific incorporation of precursor into puffed regions was enhanced by addition of *E. coli* RNA polymerase to the incubation medium. This observation suggests that the *E. coli* polymerase is active at the chromosomal level in insect nuclei and that it either supplemented or replaced the endogenous RNA polymerase.

Although the pattern of incorporation of UTP-^{3}H into isolated nuclei is apparently similar to the pattern of uridine-^{3}H incorporation under in vivo conditions, the sedimentation behavior of the radioactively labeled RNA is strikingly different in the two situations. The low molecular weight RNA obtained from isolated nuclei is most likely due to RNase activity in our system, which may be enhanced by the isolation procedure. Similar low molecular weight RNA fractions were recovered from isolated polytene nuclei of *Chironomus tentans* (12). More detailed studies with nuclei isolated from imaginal discs of *Drosophila* indicate that the recovery of low molecular weight RNA from isolated polytene nuclei may well be an artifact of the RNA isolation procedure.[1]

So far, treatments with the steroid ecdysterone at concentrations which activate a particular group of chromosome loci in vivo and in vitro have failed to produce any response of these loci in isolated nuclei. This failure may be due to changes in nuclear membrane structure which prevent the steroid molecules from entering the nucleus. However, the nucleoside triphosphates, which have molecular weights of the same order of magnitude as the ecdysterone molecule, do enter the nucleus. Apart from the question of whether the ecdysterone molecules can enter the nucleus, it cannot be excluded that during the isolation procedure the molecular composition of the genome underwent changes which are not expressed in the morphological appearance of the genome. Such changes could be responsible for the failure to respond to experimental activation. This could also be the explanation for the failure of puff regression of those

[1] J. W. Fristrom. Personal communication.

puffs which were induced prior to the isolation of the nuclei. However, it may also be possible that the failure of response of the genome is a consequence of the absence of the cytoplasm.

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