

The Metabolism of Chromosomal Ribonucleic Acid in *Drosophila* Salivary Glands and its Relation to Synthesis of Desoxyribonucleic Acid*

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ABSTRACT

Incorporation of adenine-8-C¹⁴ into chromosomal nucleic acids of *Drosophila* salivary glands has been observed with the autoradiographic technique. RNA-C¹⁴ and DNA-C¹⁴ were detected as the fractions extractable by ribonuclease digestion and resistant to ribonuclease, respectively. Extractions with desoxyribonuclease and acids were also used to identify the nucleic acids.

Time-course curves were determined from grain counts. RNA-C¹⁴ concentration reached a maximum in 2 hours, and decreased after the 4th hour. DNA-C¹⁴ concentration reached its maximum within 8 hours, and showed no decreases during a 48-hour experiment.

In the latter part of the period of observation, morphological differentiation of the gland occurred, the decrease in RNA-C¹⁴ became very rapid, and a large increase in DNA-C¹⁴ was observed. Marked decrease in RNA-C¹⁴ and increase in DNA-C¹⁴ were detectable in a few hours when isotope was administered shortly before visible differentiation of the gland.

Measurements of nuclear size indicated no significant decreases in RNA-C¹⁴ amount prior to the period of differentiation. During this later period, a large decrease in RNA-C¹⁴ amount occurs suddenly, and the same amount of C¹⁴ is added simultaneously to the DNA fraction, as expected if RNA-C¹⁴ is utilized in the synthesis of DNA.

In the many studies of nucleic acids localized in the various cellular structures, the chromosomal RNA has received little attention. Its study in cytological preparations has been hampered by the presence of DNA in the chromatin coupled with the lack of techniques which specifically reveal the ribose form of nucleic acid. Its study in isolated nuclear fractions has also been complicated, in this case by the close association of the nucleolus, and its RNA, with the chromatin. Although the fraction known as isolated chromosomes clearly contains RNA, in association with the "residual chromosomes" (11), observations of the preparative process have not clearly indicated a

chromosomal origin of the RNA (8, 17). The RNA of isolated nuclei has been studied extensively in more recent work, and usually designated simply as the nuclear RNA since more precise identification of the fraction is lacking.

Chromosomal RNA has been demonstrated in cytological preparations since nucleases which distinguish between RNA and DNA have become available (7). Rasch and Swift (14) have reported an ultraviolet absorption curve typical of nucleic acid for the substance removed from chromatin by ribonuclease. In further work with nucleases, RNA has been demonstrated by basic staining of the chromatin after removal of DNA (16, 22), and as labelled nucleic acid removed by ribonuclease after radioisotope incorporation (4, 10, 13).

The chromosomal RNA of *Drosophila* salivary glands is a large fraction compared with the nucleolar RNA, and the two fractions can be studied separately with relative ease in cyto-

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logical preparations because of the large size of the corresponding structures. Radioisotope incorporation into cytoplasmic, nucleolar, and chromosomal nucleic acid fractions can be compared by autoradiographic studies, and an earlier study with P^{32} (10) indicated that chromosomal RNA has a metabolism distinct from that of the other cellular fractions.

The characteristics of adenine-8- C^{14} incorporation into chromosomal RNA and DNA are reported here. In time-course studies we have observed a striking increase in the DNA- C^{14} at a particular stage of development; chromosomal RNA- C^{14} showed a large decrease at the same stage. A quantitative investigation indicated a relation between these two fractions which implies a conversion of RNA to DNA. The increase in the DNA fraction is simultaneous with a sudden decrease in the chromosomal RNA- C^{14} , and the changes in the two fractions involve equal amounts of C^{14} .

Materials

The conditions and foods used for growing *Drosophila repleta* have been described in previous papers (10, 19). Larvae used in the experiments described here were all obtained from the same culture. A fairly synchronous population resulted from eggs which were deposited during a 4 hour period. Isotope was administered to larvae of 2 different ages: in the first experiment to larvae in the 2nd day of 3rd instar (8 days after egg laying, 1 day after the larvae entered 3rd instar), and in the second experiment to larvae 19½ hours older.

On the 3rd day of 3rd instar, during the course of the experiments reported here, a striking differentiation of the cells of the salivary glands occurs (9), and particularly interesting features of the incorporation studies are seen during this period of development. The most outstanding change in the cells, and the easiest to detect, is the appearance, throughout the cytoplasm, of spherical granules which are stained by the Bauer reaction for polysaccharides. Neither granules nor polysaccharides are seen in younger glands. The earliest granules are very small and careful examination with the light microscope, after the Bauer stain, is necessary to detect them. After a few hours they are noticeably larger. After advanced granule formation the cytoplasm gives an intense Bauer reaction (all localized in the granules); the granules appear as empty spaces after basic staining, or in unstained preparations observed with phase optics. The cytoplasm in-

creases in size rapidly during granule formation, and its basophilia decreases. The chromosomes also become more distinct, and the nucleoli decrease in size.

In the 1st experiment small granules were present in all larvae of the 32 hour sample, large granules in the 48 hour sample, and none earlier. In the 2nd experiment small granules were apparent at the 9th and 12th hours, and large ones at 26 hours; 1 larva of the 3 hour sample contained very early granules.

The nuclear material of early 3rd instar larvae appears homogeneous in the unstained preparations (observed with phase) which were used for grain counts, and no precise correlation of grain localization with localization of chromosomes was made. No basophilic or Feulgen-positive material is seen in the nuclear sap with staining conditions which reveal intensely stained chromosomes in the material; therefore, non-nucleolar nucleic acid of the nucleus is considered to be chromosomal.

Methods

Isotope was administered in food which contained 5 μ c. of adenine-8- C^{14} (specific activity 3.7 c. per mole) per 100 mg. yeast, 15 mg. agar, and 1 cc. of mineral solution. Larvae were given this food for the first 2 hours of the experiments, and non-radioactive food for the rest of the time.

Material was fixed by freeze-substitution (3) followed by 2 hours in 80 per cent alcohol at 60°C. Cross-sections of the larvae were cut at 4 μ and mounted serially on different slides so that adjacent sections could be compared after various treatments. All slides were extracted with boiling alcohol-ether (3:1 for 5 minutes) and cold trichloroacetic acid (5 per cent for 5 minutes at 2-4°C.). One slide from each sample was subjected to ribonuclease digestion (0.2 mg. per cc., pH 6.0 to 6.5, for 2 hours at 37°C.). Desoxyribonuclease was used at a concentration of 0.01 per cent in 0.005 M $MgSO_4$ containing 0.1 per cent gelatin, at pH 6.0-6.5, at 37°C. for 2 hours. For acid hydrolyses 1 N HCl at 60°C. for 5 minutes, and boiling 5 per cent trichloroacetic acid for 30 minutes were used. The autoradiographic technique was followed as described previously (18).

Isotope concentration was estimated by counting all grains throughout the thickness of the film in an area of 160 μ^2 above sections of nuclei which did not include nucleoli. Counts were made at random positions above 5 to 10 nuclei for each larva of a sample. Studies were restricted to the most distal cell layers of the gland as previously described (10). The following number of animals was studied in each sample: in Experiment I, 3 animals at 1 hour, 2 at 2 hours, and 4 for each succeeding time point; in Experiment II, 7 at 3 hours, 6

at 6 hours, and 7 at each succeeding time point. Means and standard errors were calculated for the average animal, rather than treating all the cells from different animals as a single population; standard errors therefore indicate the variability among animals of each sample.

Nuclear volume was calculated from diameters measured with an eyepiece micrometer, using the formula for a sphere. For the 1st experiment nuclear volume was determined for each larva that was counted. In the 2nd experiment sample size ranged from 2 to 6 larvae.

RESULTS

After administration of adenine- C^{14} , nucleic acid- C^{14} appears throughout the salivary gland cell. The cytoplasmic and nucleolar activity is associated with RNA, as shown by its complete removal by digestion with ribonuclease. Part of the chromosomal activity is removed by RNase; the RNase-resistant fraction is evidently DNA- C^{14} , as seen below.

Identification of the RNase-Resistant Fraction:

The activities remaining after extractions with enzymes and acids are shown in Table I. Cytoplasmic nucleic acid- C^{14} was used as a control RNA for comparison. (Larvae from the 6, 9, and 12 hour samples of the 2nd experiment were used. During this period essentially constant total and RNase-resistant counts occurred. 70 per cent of the total nucleic acid- C^{14} remains after treatment with RNase, as shown in Table III, which lists counts for a large number of these larvae.)

The activity remaining after RNase is completely removed by further digestion with desoxyribonuclease, as seen in Table I. Although a non-specific extraction of cytoplasmic RNA- C^{14} by DNase was noted, the RNA was only partially removed; this is in contrast to the *complete* removal of chromosomal RNase-resistant C^{14} , which is expected only of DNA. The chromosomal activity found after DNase alone is consistent with the removal of all the RNase-resistant fraction, as expected of DNA, and of some of the RNA in addition. (Extraction of RNA by the gelatin solution in which DNase is dissolved has been observed previously (18)).

Short hydrolysis with HCl removed all cytoplasmic RNA- C^{14} , and would be expected to remove a large fraction of the DNA purines, as well as RNA, from the chromatin. Activity amounting to 20 per cent of the RNase-resistant C^{14} remained in the chromatin after HCl, which

can be explained by the release of all the RNA and all but 20 per cent of DNA purine- C^{14} . After hydrolysis with hot TCA, which removes both RNA and DNA, no activity was left.

It seems clear that the activity remaining after ribonuclease is associated with DNA. The fraction also shows the localization typical of DNA; it is not found in the cytoplasm or nucleolus, although high concentrations of nucleic acid- C^{14} have been observed in both structures.

Courses of Incorporation into DNA and Chromosomal RNA:

The incorporation of adenine into the RNA fraction is characterized by an early maximum activity, which does not persist, while the DNA reaches its maximum activity later, and shows no subsequent decrease in activity. The activities found after isotope was administered on the 2nd day of 3rd instar are shown in Fig. 1 and Table II. (Activities are expressed as grain counts per unit area of chromatin. Since all sections were of the same thickness, the counts are a measure of amounts of C^{14} associated with nucleic acid per unit *volume* of chromatin material, *i.e.*, the concentrations of nucleic acid- C^{14} in the chromatin.)

RNA- C^{14} concentration reaches a maximum by the 2nd hour, and decreases slowly after the 4th hour. Incorporation into DNA seems to begin later than into RNA. DNA- C^{14} shows a slower increase to its maximum concentration, found by the 8th hour, and this same concentration is seen through the 32nd hour.

Abrupt changes in the concentrations of both fractions occur in the later part of the experiment. The decrease in RNA- C^{14} concentration becomes

TABLE I
Chromosomal Nucleic Acid- C^{14} Remaining after Extraction with Enzymes and Acids
Average counts per larva, with standard errors. Forty-two day exposure.

Extraction	Grains per 160 μ^2	
	Chromatin	Cytoplasm
Ribonuclease	39.8 \pm 5.6	No activity
Desoxyribonuclease	12.0 \pm 2.8	40 per cent remaining
N HCl (5 min. 60°)	7.7 \pm 1.5	No activity
5 per cent TCA (30 min., boiling)	No activity	No activity
RNase followed by DNase	No activity	No activity

very rapid, and the activity of this fraction is very low by the end of the experiment. DNA-C¹⁴ also shows a marked change, but in the opposite direction: after the same concentration has persisted for many hours, a large increase in concentration suddenly occurs between the 32nd and 48th hours.

the younger larvae. About the same amount of incorporation was found in the two experiments. (Slides were exposed longer in the 2nd experiment, and therefore should be multiplied by 24/42 for comparison with the 1st experiment.)

The older and the younger larvae differ from each other in several respects, however. In the

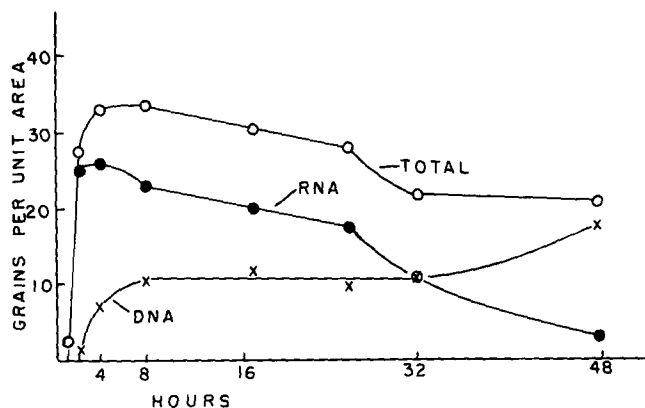


FIG. 1. Adenine-C¹⁴ incorporation into chromosomal nucleic acid fractions after administration to larvae in 2nd day of 3rd instar. Concentration of nucleic acid-C¹⁴ is expressed as grain counts per 160 μ^2 .

TABLE II
Concentrations of Chromosomal Nucleic Acid-C¹⁴ after Administration of Adenine-8-C¹⁴ on 2nd Day of 3rd Instar

Average counts per larva for RNase-extractable fraction (RNA), RNase-resistant fraction (DNA) and total nucleic acid, and also average nuclear volumes, with standard errors. Twenty-four day exposure.

Time hrs.	Grains per 160 μ^2			Nuclear volume 10 ² μ^3
	Total	RNase- resistant	RNase- extractable	
1¼	2.4 ± 3.1			21.6 ± 2.3
2¼	26.3 ± 3.5	1.5 ± 0.8	24.8 ± 2.5	22.2 ± 0
4¼	32.9 ± 4.1	7.0 ± 3.2	25.9 ± 5.2	31.5 ± 6.9
8	33.7 ± 6.3	10.4 ± 3.3	23.3 ± 7.1	26.0 ± 4.4
17½	30.4 ± 5.3	11.4 ± 2.3	19.0 ± 5.7	55.0 ± 9.6
26	27.9 ± 3.3	9.3 ± 1.0	18.6 ± 3.4	67.4 ± 9.4
32	21.6 ± 3.3	10.9 ± 1.6	10.6 ± 3.6	114.5 ± 12.0
47½	20.5 ± 3.0	17.5 ± 4.3	3.0 ± 5.2	121.0 ± 12.7

Adenine incorporation into older larvae is shown in Fig. 2. (In this experiment, isotope was administered 19½ hours later than in the preceding one.) RNA-C¹⁴ again reached an early maximum concentration, and then decreased in activity, while the DNA showed a later maximum and no decreases in activity, as found in

older larvae DNA-C¹⁴ amounts to about 70 per cent of the total nucleic acid-C¹⁴ by the 6th hour after administration, while in the younger larvae DNA-C¹⁴ is only about one-third of the total prior to the 32nd hour. The decrease in RNA-C¹⁴ in the older larvae is rapid and large; between the 3rd and 6th hours a 50 per cent decrease occurred. In the younger larvae, in contrast, decreases in RNA-C¹⁴ were gradual prior to the 26th hour, and the concentration was reduced by only about one-third after 20 hours of decreasing.

The early part of the curves in the 2nd experiment corresponds well with the later part of the curves of the 1st experiment, when the cells were in comparable stages of differentiation. Thus a large and rapid decrease in RNA-C¹⁴ concentration, and a high ratio of DNA-C¹⁴ to RNA-C¹⁴ occurs in both experiments. They are associated with a given stage of salivary development rather than occurring at a particular time after isotope administration. In the 1st experiment cytoplasmic granules were first apparent at the 32nd hour, and the rapid decrease in RNA-C¹⁴ concentration which is marked after the 26th hour occurs during this second period of development, which must have begun some time before

it was detectable morphologically. Larvae of the 2nd experiment were of comparable stage of development, since granules were visible very shortly after isotope administration.

Amounts of Nucleic Acid-C¹⁴ per Nucleus:

To determine if the observed decreases in RNA-C¹⁴ concentration represent losses of RNA-C¹⁴ from the chromatin, or simply dilution of

the amount of RNA-C¹⁴ per nucleus is observed through the 32nd hour. During this time all concentration decreases occur with nuclear enlargement. A large increase in amount occurred between the 8th and 17th hours, while nuclear size doubled. After this time the same amount occurred through the 32nd hour, notwithstanding the very large decrease in concentration between the 26th and 32nd hour. The further decrease in

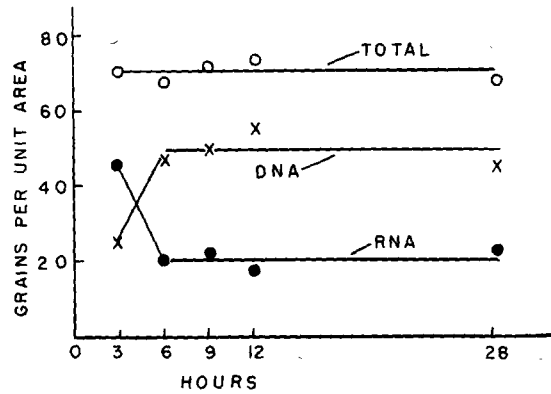


FIG. 2. Adenine-C¹⁴ incorporation into chromosomal nucleic acid fractions after administration to larvae in the 3rd day of 3rd instar. Concentration of nucleic acid-C¹⁴ in the nucleus is expressed as grain counts per 160 μ².

TABLE III

Concentrations of Chromosomal Nucleic Acid-C¹⁴ after Administration of Adenine-8-C¹⁴ on 3rd Day of 3rd Instar (19½ Hours Later than in 1st Experiment)

Average counts and nuclear volumes per larva with standard errors. Forty-two day exposure.

Time	Grains per 160 μ ²			Volume 10 ² μ ³
	Total	RNase-resistant	RNase-extractable	
hrs.				
3	70.0 ± 6.8	25.1 ± 3.2	44.9 ± 7.6	84.7 ± 1.8
6	67.1 ± 8.1	46.8 ± 4.2	20.3 ± 8.7	80.2 ± 52.8
9	71.4 ± 6.2	49.4 ± 5.0	22.0 ± 8.2	81.0 ± 3.0
12	72.7 ± 6.5	55.3 ± 6.2	17.4 ± 9.1	88.3 ± 22.8
28	67.3 ± 6.2	44.8 ± 5.2	22.5 ± 8.1	99.9 ± 5.9

the activity due to nuclear growth during the course of the experiment, nuclear volumes were measured, and are shown in Tables II and III. The amount of nucleic acid-C¹⁴ per nucleus was calculated as the product of grain count per unit area times nuclear volume. The amounts occurring in the 1st experiment are shown in Fig. 3.

Although the RNA-C¹⁴ concentration decreased continuously after the 4th hour, no decrease in

concentration seen at the 48th hour occurred without any change in nuclear volume, however, and during this time 2/3 of the RNA-C¹⁴ disappeared from the chromatin.

The amount of DNA-C¹⁴ per nucleus increased in proportion to the volume increases through the 32nd hour and increased further by the 48th hour, while nuclear volume was constant, as the concentration of DNA-C¹⁴ increased. This increase in concentration occurred at the same time that loss of RNA-C¹⁴ was observed.

A decrease in the amount of RNA-C¹⁴ per nucleus in this later stage of development is also indicated by the 2nd experiment. Since nuclear volume was essentially constant, as seen in Table III, changes in concentration therefore represent changes in amount. A large decrease in the amount of RNA-C¹⁴ and an equally large increase in DNA-C¹⁴ occurred between the 3rd and 6th hours, followed by no further change in either fraction.

Loss of C¹⁴ from the chromosomal RNA is clear only in older larvae. However, any loss would be difficult to detect, if it should occur, in younger larvae, since RNA-C¹⁴ is accumulating rapidly. There is an indication of a small decrease in RNA-C¹⁴ amount, with equal increase

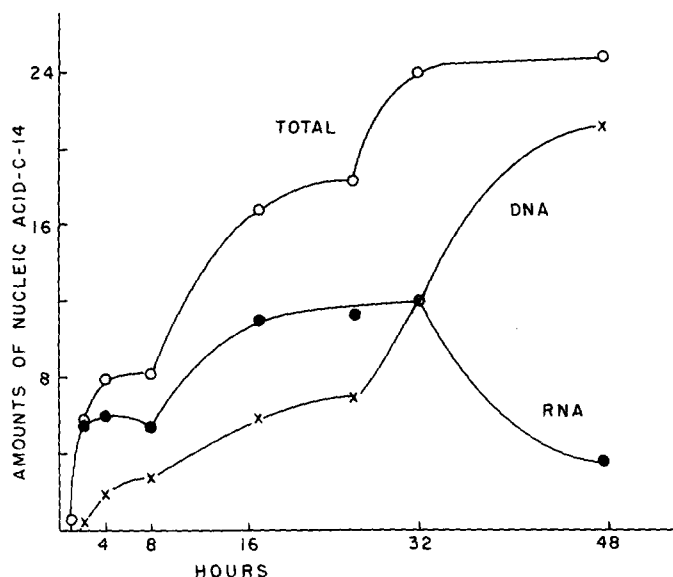


FIG. 3. Amounts of chromosomal nucleic acid- C^{14} per nucleus, expressed as the product of grain count per $160 \mu^2$ times volume. (1 larva of the 4 hour sample had an exceptionally large nucleus ($5 \times 10^2 \mu^2$), and for estimating amount average nuclear volume was recalculated without this value.)

in the DNA fraction, between the 4th and 8th hours in the young larvae. The data are not precise enough to determine whether such a decrease actually occurred, however.

Neither nucleolar nor cytoplasmic RNA- C^{14} show decreases in amount specifically associated with the later period of salivary development, as the chromosomal RNA does. (Although there does appear to be a general cessation of increases in RNA amount in all structures during this period.) RNA- C^{14} amounts were calculated for all fractions of the 1st experiment (not shown here), and indicated no decreases in cytoplasmic RNA- C^{14} throughout the 48 hour period, while the nucleolar amount decreased continuously after the 2nd hour until it reached a plateau value at about the 26th hour.

DISCUSSION

After the absence of any decrease in RNA- C^{14} amount during a 32 hour period, the disappearance of $\frac{2}{3}$ of this fraction between the 32nd and 48th hours is striking. In connection with the problem of what happens to the RNA- C^{14} , and what cellular fraction receives it, the DNA- C^{14} immediately attracts attention. It shows a sudden increase in concentration at this time after a long period of constant concentration. Further,

its increase in concentration was equal to the simultaneous decrease in the RNA fraction; no change in the total nucleic acid- C^{14} concentration occurred, as seen in Fig. 1. Since nuclear volume was the same at 32 and 48 hours, the increase in amount of the DNA fraction is equal to the decrease in amount seen in the RNA fraction. A similar increase in DNA- C^{14} , equal to and simultaneous with the decrease in RNA- C^{14} , is seen in the 2nd experiment. The DNA fraction thus shows just the behavior expected if it is receiving the RNA- C^{14} which disappears.

Brachet's proposal that RNA is utilized materially in the synthesis of DNA (2) was one of the earliest hypotheses arising from studies of nucleic acids. The correspondence between changes in RNA and DNA in *Drosophila* salivaries clearly supports the hypothesis. Such a relation between the total RNA and the DNA was reported by Brachet from early studies of sea urchin development, but was not confirmed by later measurements (15). Volkin and Astrachan (21) recently noted an RNA from *E. coli* which has incorporation characteristics suitable for a precursor of phage DNA, and apparently has a base ratio suitable for direct conversion to phage DNA.

The opposite changes in chromosomal RNA- C^{14} and DNA- C^{14} which occur in salivaries may be explained most simply by a direct transfer of RNA purines to DNA, without a preceding breakdown of the RNA and mixing of the purines in a general precursor pool. Whether the whole RNA molecule, or only the purines, are transferred is not indicated, since only the purines are labeled by adenine. For either of these processes to occur, the presence of DNA and its precursor RNA in the same structure, as indicated, would be expected.

More complicated explanations cannot be ruled out, however. Synthesis of DNA from an acid soluble precursor which has the same specific activity as the RNA, would result in the same data if the amount of DNA synthesized is equal to the amount of RNA lost from the chromatin. Equal changes in amounts of the two nucleic acids, in this case, would imply an indirect relation between RNA and DNA synthesis, such as competition for the same sites in the chromatin.

The retention of radioisotopes by the RNA of isolated nuclei has been studied extensively in mammalian tissues (5, 6, 20), but it is difficult to relate the findings to the behavior of chromosomal RNA since definite identification of the RNA has yet to be made (1). In salivaries it is the nucleolar, rather than the chromosomal, RNA which shows the metabolic characteristics typical of the RNA of isolated nuclei (10). Incorporation of isotopes into chromosomal RNA has been observed in other autoradiographic studies (4, 13), but time-course curves have not been reported. Pelc (12) has suggested, from a study of adenine incorporation, that DNA of mouse spermatogenous cells is derived from RNA, but chromosomal RNA was not distinguished from the other RNA fractions of the cell, nor evidence of the kind found in *Drosophila* reported.

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