INTRACELLULAR DISTRIBUTION OF LOW MOLECULAR WEIGHT RNA IN *CHIRONOMUS TENTANS*

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ABSTRACT

Low molecular weight RNA species are described in isolated nuclear components and cytoplasm of salivary gland cells of *Chironomus tentans*. In addition to 4S and 5S RNA and RNA in the 4–5S range previously described, at least three other components in the range below 16S are present. RNA, the molecular weight of which was estimated to 2.3×10^5 and designed 10S RNA, can be observed only in nucleoli; other RNA, the molecular weight of which was estimated to 1.3×10^5 and designed 8S RNA, was detected in the chromosomes, the nuclear sap, and the cytoplasm but not in the nucleoli; and a third type of RNA, the molecular weight of which was estimated to 8.5×10^4 and designed 7S RNA, was present in nucleoli, chromosomes, nuclear sap, and cytoplasm. The substituted benzimidazole, 5,6-dichloro-1(β-D-ribofuranosyl)benzimidazole (DRB), which gives a differential inhibition of the labeling of heterodisperse, mainly high molecular weight RNA in the chromosomes, does not inhibit the labeling of 8S RNA. The relative amounts of label in 8S RNA and 4–5S RNA (including 4S RNA and 5S RNA) in different isolated chromosomes, are distributed in proportion to the chromosomal DNA contents. The 8S RNA as well as the 7S RNA show a relative accumulation in chromosomes and nuclear sap with prolonged incubation time and are in this respect similar to intranuclear low molecular weight RNA species described by previous workers. Our data suggest, however, that these two types of RNA may differ in an important aspect from the previously described types since they are also present in the cytoplasm.

INTRODUCTION

New analytical techniques have permitted the detection of new classes of RNA species in the 4–7S range associated with the nuclei of various kinds of cells. The existence of low molecular weight RNA species in addition to 4 and 5S RNA was first indicated by the work of Knight and Darnell (1). Such low molecular weight RNA's have been found in HeLa cells (2), chicken embryo brain (2), rat liver (3, 4), Novikoff hepatoma cells (5), salivary gland cells of *Chironomus tentans* (6), 3T3 and WI 38 cells (7), and Chinese hamster ovary cells (8). The presence of a 7S RNA as a ribosomal component of mammalian cells was demonstrated by Pene, Knight, and Darnell (9). Except for this species, information about the functional involvement of the newly described low molecular weight RNA species is lacking.

The intracellular distribution of the low molecular weight RNA fractions with an unknown function was investigated by Weinberg and Penman (2). They found them to be restricted to the nucleus. All of them may be present in the nucleolus, and some also in the nucleoplasm. It is possible, therefore, that they all have a nu-

cleolar origin. In this paper, analyses of low molecular weight RNA were performed in microisolated nucleoli, chromosomes, nuclear sap, and cytoplasma of dipteran salivary gland cells with polytene chromosomes. Three new low molecular weight RNA types, each with a characteristic intracellular distribution, will be described.

MATERIALS AND METHODS

Salivary glands from late fourth instar larvae of *Chironomus tentans* were incubated in Cannon's modified insect medium (10, 11) containing tritiated nucleosides (The Radiochemical Centre, Amersham, Buckinghamshire, England) for 45 min, 180 min, and 16 hr. When labeling with tritiated nucleosides was performed in the presence of 5,6-dichloro-1(β-D-ribofuranosyl)benzimidazole (DRB) (Merck, Sharp and Dohme, Haarlem, Holland) the glands were preincubated with the drug for 60 min in the absence of isotopes. After incubation the glands were fixed in a medium containing absolute ethanol, formaldehyde, and glacial acetic acid (12). Chromosomes, nucleoli, nuclear sap, and cytoplasm were isolated by micromanipulation (13) and then digested for 3 hr with a buffered pronase–sodium dodecyl sulphate solution (12). The digest was then taken up in a small piece of filter paper, transferred to an ethanol-potassium acetate solution, and dried in air before being placed in 10 μl E buffer (0.02 M Tris-HCl buffer (pH 8.0), 0.02 M NaCl, and 0.002 M EDTA) as described previously (12). *Escherichia coli* RNA used as marker was obtained by sodium dodecyl sulphate–phenol extraction from frozen bacteria. The RNA samples were subjected to electrophoresis in slabs of 7.5% polyacrylamide gel (12). In some cases a part of the gel containing 4–5S RNA was removed and the material was used for special purposes. The marker RNA bands were localized by staining with toluidine blue. The gel was sliced and the slices were transferred to disposable plastic scintillation vials (Packard Instrument Co., Downers Grove, Ill.) containing 10 ml of toluene which, per liter, contains 5 g PPO, 0.5 g dimethyl-POPOP, and 30 ml *N*-chlorosuccinimide (Nuclear Chicago Corp., Des Plaines, Ill.), incubated at 37°C overnight, and counted in a Packard liquid scintillation spectrometer (Packard Instrument Co.), at an efficiency of 33% at a background of 15 cpm. Salivary glands were also analyzed after the animals had been allowed to incorporate tritiated cytidine in vivo after they had been injected with precursor as described in the text to Fig. 4.

RESULTS

It was demonstrated in a previous paper (14) that the substituted benzimidazole DRB has a differential inhibitory action on labeling of chromosomal and nuclear sap RNA. After administration of DRB labeling of chromosomal and nuclear sap, heterodisperse RNA is almost completely inhibited. Analyses of chromosomal, nuclear sap, and nucleolar RNA in 7.5% polyacrylamide gel after 60 min preincubation in DRB followed by incubation for 180 min with labeled nucleosides are shown in Fig. 1 *a*. As can be seen, the chromosomes as well as the nuclear sap contain, in addition to the 4–5S RNA complex which was characterized in a previous report (12), a radioactivity peak in the 8S region. This RNA cannot be observed in the nucleoli which, on the other hand, contain RNA at 10S, i.e. migrating slower than the 8S RNA fraction. The controls to the DRB-treated glands (Fig. 1 *b*) produce a gel electrophoretic curve which suggests that the 8S RNA is not an artifact due to DRB treatment but is also present in uninhibited glands. This was confirmed in other analyses, particularly in which RNA was used from glands incubated for extended time periods (*vide infra*). The nucleolar RNA in the 10S region is not very distinct in the controls and one cannot exclude that its relative labeling is somehow promoted by DRB. Similar RNA is, however, present also in uninhibited glands as could be shown with glands incubated for long time periods. We do not know whether the variation in amounts of this kind of RNA has biological or experimental causes. 8S RNA is present in the cytoplasm of both DRB-treated glands and controls (Fig. 1 *c*), but no peak can be observed corresponding to 10S RNA. The treatment of labeled chromosomal + nuclear sap RNA with pancreatic ribonuclease (10 μg/ml) for 60 min at 37°C resulted in a removal of all radioactivity from the electrophoretic spectrum.

The labeling of 8S RNA was further studied in the absence of DRB. For this purpose a relatively short period of 45 min and a long one of 16 hr were chosen for incubation of glands, and RNA species migrating between 5 and 16S were analyzed. The appearance of 8S RNA in the chromosomes, nuclear sap, and cytoplasm could be demonstrated in these analyses already after 45 min of labeling (Fig. 2 *a*, *b*). Nucleolar RNA contained no labeled peak in the 8S region. After incorporation for 16 hr the relative amount of radioactivity in 8S RNA increased in the chromosomes and nuclear sap and could be clearly demonstrated in spite of high background level of heterodisperse material (Fig. 3 *a*). The cytoplasm

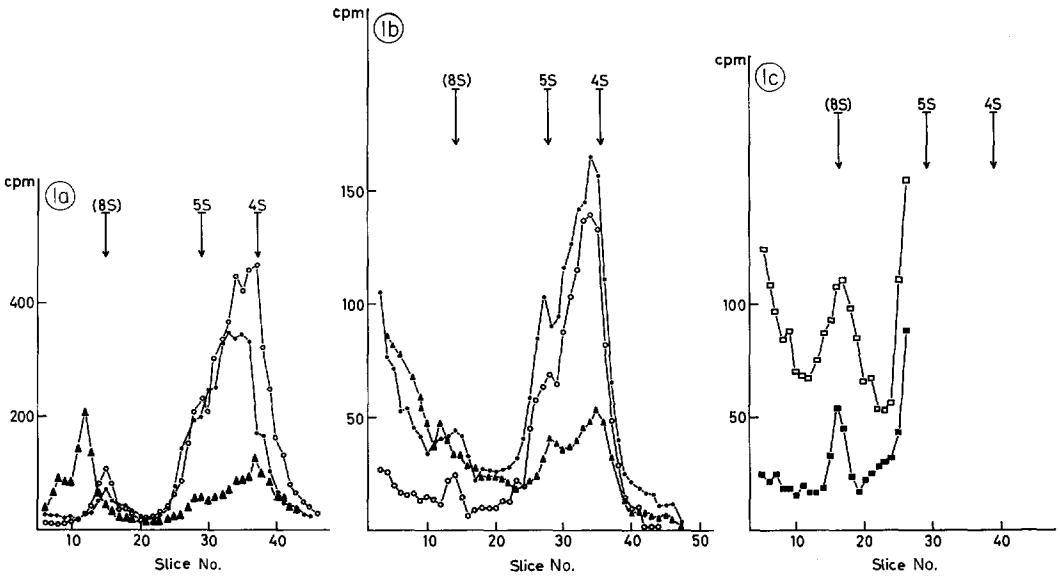

FIGURE 1 180 min labeling of low molecular weight RNA in the presence and absence of DRB. The glands were preincubated at 18°C for 60 min in 25 μl of Cannon's modified insect medium containing 20 μg DRB per ml. They were then transferred to another 25 μl of the same medium containing 100 μCi cytidine (sp. act. 27 Ci/mmole) and 100 μCi uridine (sp. act. 24 Ci/mmole) and incubated for 180 min at 18°C. Labeling with tritiated nucleosides in the absence of DRB was preceded by preincubation for 60 min in the same medium but without the isotope. Chromosomes, nucleoli, nuclear sap, and cytoplasm were isolated from: (*a*) 70 DRB-treated cells (2 glands), (*b*) 30 untreated cells (1 gland), and (*c*) 30 DRB-treated and 30 untreated cells (1 gland in both cases). The electrophoresis was carried out in 7.5% polyacrylamide. The positions of 4S, 5S, and 8S are indicated by arrows. The position of 8S RNA was determined in 0.5% agarose–2.25% acrylamide composite gel. For other details, see Materials and Methods. Cpm values are not shown for the first slices. The sum of these values for the components analyzed in Fig. 1 *a* were, for chromosomes, 1042 cpm; nucleoli, 13,300; nuclear sap, 217; in Fig. 1 *b*, for chromosomes, data missing; nucleoli, 6100; nuclear sap, 1254; in Fig. 1 *c*, for DRB-treated cytoplasm, 1267; and for control cytoplasm, 9500 cpm. Chromosomes (closed circles); nucleoli (closed triangles); nuclear sap (open circles); cytoplasm in presence of DRB (closed squares); cytoplasm in absence of DRB (open squares).

also contains a distinct 8S RNA peak (Fig. 3*b*). In the chromosomes, nuclear sap, and cytoplasm there are small amounts of label in peaks at 7S undetectable after short periods of labeling. Similar RNA as well as the previously mentioned 10S RNA are clearly distinguished in the nucleolar analyses (Fig. 3 *b*).

When the time of incubation with tritiated nucleosides was increased from 45 min to 16 hr the relative amount of 8S RNA (approximate determination) increased from 0.5 to 1.2% (of the total RNA label) in the chromosomes and from 1.6 to 3.2% in the nuclear sap, whereas the cytoplasmic ratio remained unchanged at around 0.7%. In comparison, the relative amount of radioactivity in the 4S region decreased from 11 to 5% in the chromosomes and from 15 to 10% in the nuclear sap but increased from 15 to 46% in the cytoplasm under identical conditions.

With glands from animals allowed to incorporate tritiated cytidine for 16 hr in vivo it could be shown that at least the 8S RNA component is formed in vivo and therefore is no artifact due to in vitro incubations (Fig. 4).

Evidence was obtained in previous work (12) that the RNA's located between the 4S and 5S marker constitute precursors to RNA migrating as 4S RNA. After 180 min of labeling with tritiated nucleosides there is radioactivity in the 4S position in the chromosomes and nuclear sap probably because there has been enough time for some precursor material to mature. A comparison between DRB-treated glands (Fig. 1 *a*) and their controls (Fig. 1 *b*) shows similar electrophoretic

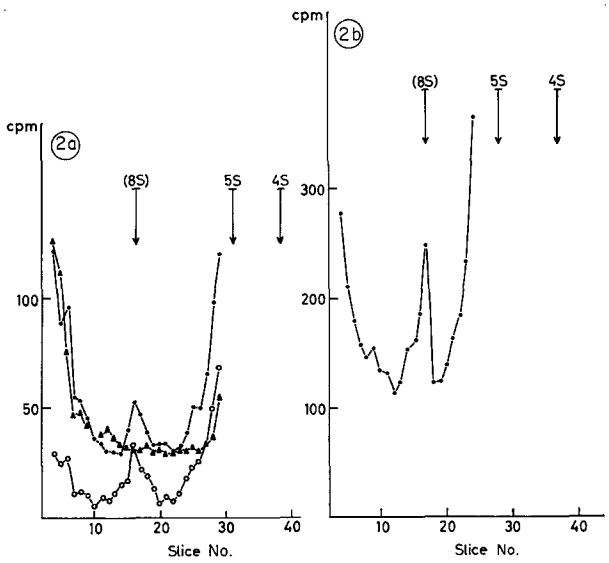

FIGURE 2 45 min labeling of low molecular weight RNA. The glands were incubated with 100 μCi cytidine (sp. act. 27 Ci/mmole) and 100 μCi uridine (sp. act. 25 Ci/mmole) in 25 μl Cannon's modified insect medium at 18°C for 45 min. Chromosomes, nucleoli and nuclear sap (*a*), and cytoplasm (*b*) were collected from 90 cells (3 glands). The gel in the 4–5S range was extracted for other purposes, but the position of the markers is indicated. Cpm values are not shown for the first slices. The sum of these values for the components analyzed in Fig. 2 *a* were, for chromosomes, 8900 cpm; nucleoli, 28,500 cpm; nuclear sap, 2400 cpm; in Fig. 2 *b*, for cytoplasm, 8728 cpm. For other details, see Materials and Methods. Chromosomes (closed circles); nucleoli (closed triangles); nuclear sap (open circle).

patterns, indicating that DRB is not interfering with the postulated maturation process of 4S RNA. With regard to the action of DRB on labeling of nucleolar low molecular weight RNA, we have no evidence that the pattern of labeling of these RNA species is changed.

The electrophoretic pattern of labeled low molecular weight RNA after incubation with tritiated nucleosides for 180 min in the presence of DRB from the three chromosomal fractions constituted by chromosome I, chromosomes II + III, and chromosome IV, respectively, revealed that there is a radioactivity peak at a position corresponding to 8S RNA for each chromosome fraction analyzed. It was furthermore found that the relative amount of radioactivity in the 8S region approximately correlated with the number of bands or DNA content of the different chromosome fractions (Table I), as is also the case for the 4–5S RNA complex (reference 13 and Table I).

In separations of RNA from DRB-treated glands in low per cent polyacrylamide (2.25%)–agarose (0.5%) composite gels, the migration rate of 8S RNA was determined in relation to the 16S and 23S ribosomal RNA components and 4S RNA from *E. coli*. It was shown by Peacock and Dingman (15) for this type of gel that there exists an inverse relationship between the logarithm of the molecular weight and the migration rate also for the range down to 4S. In agreement with these observations, we found that the three markers 23S, 16S, and 4S RNA formed a linear relation in a plot. It must be pointed out, however, that determinations of molecular weight based only on electrophoretic mobility are beset with uncertainties because base composition and secondary structure may influence the migration rate (16). The value obtained for 8S RNA was 1.3×10^5 daltons, corresponding to 400 nucleotides.

Using data for 8S RNA and added 5S RNA, we calculated the molecular weight for the nucleolar 10S RNA fraction and for a type of RNA migrating as 7S RNA. The values for these molecular weights were, respectively, 2.3×10^5 and 8.5×10^4.

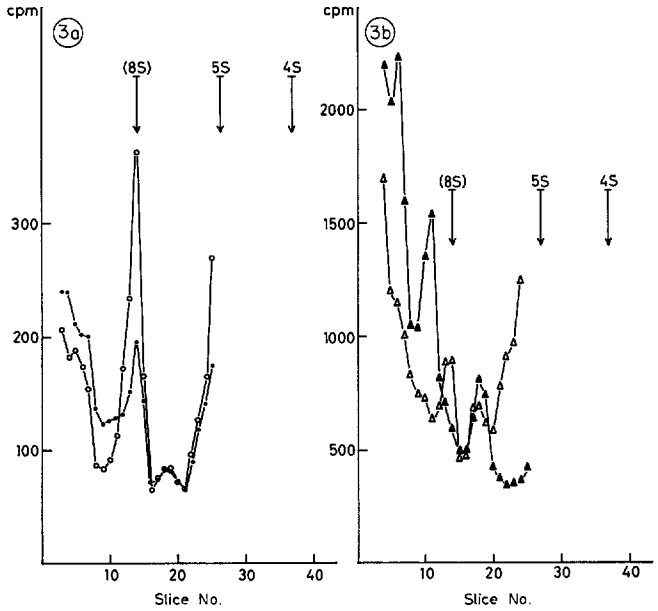

FIGURE 3 16 hr labeling of low molecular weight RNA. The glands were incubated with 100 μCi cytidine (sp. act. 27 Ci/mmole) and 100 μCi uridine (sp. act. 25 Ci/mmole) in 25 μl Cannon's modified insect medium of 18°C for 16 hr. Chromosomes, nuclear sap (*a*), nucleoli, and cytoplasm (*b*) were isolated from 80 cells (3 glands). Other details as for Fig. 2. Cpm values are not shown for the first slices. The sum of these values for the components analyzed in Fig. 3 *a* were, for chromosomes, 17,350 cpm; nuclear sap, 13,950 cpm; in Fig. 3 *b*, for nucleoli, 53,300 cpm; cytoplasm, 16,800 cpm. Chromosomes (closed circles); nuclear sap (open circles); nucleoli (closed triangles); cytoplasm (open triangles).

DISCUSSION

Recent findings suggest the existence of nuclear low molecular weight RNA species other than 4 and 5S RNA in many cell types. These RNA species in the 4–7S region found in the nuclei of different cell systems were not detected in the cytoplasm and may have a nucleolar origin (2).

The 8S RNA described here has a distribution which is different from that of the previously described low molecular weight RNA's since it is present in chromosomes, nuclear sap, and cytoplasm but cannot be detected in nucleoli. In this respect it resembles the heterodisperse chromosomal RNA (18). It differs, however, from such RNA in showing a low sensitivity to DRB-induced inhibition of nucleoside labeling, which was in fact the reason why its presence was originally noticed. The fact that it is present in the cytoplasm is of special interest since this should offer hopes of a functional characterization.

It is of course not excluded that the cytoplasmic 8S RNA is a different RNA species that the nuclear fraction, although the agreement in electrophoretic mobilities and the similar lack of sensitivity towards DRB seem to make such an assumption less likely.

A second low molecular weight RNA type, 7S RNA, was also observed, although, because of the low amount of label taken up in this region, it could not be demonstrated with the same distinctness as 8S RNA in chromosomes and nuclear sap. This RNA is distributed in a fashion which is different from that of 8S RNA since it seems to be present also in the nucleoli. It should again be stressed that agreement in migration rates does not necessarily imply identity although such an interpretation appears likely.

Thus both types of RNA described here are different from those previously described by others in a number of papers insofar as, in both cases, RNA of the same migration properties is present in the cytoplasm while, furthermore, there is no evidence, in one case, for 8S RNA, that it is present in the nucleolus.

Both types of RNA are similar to the previously described species in one respect; however, they

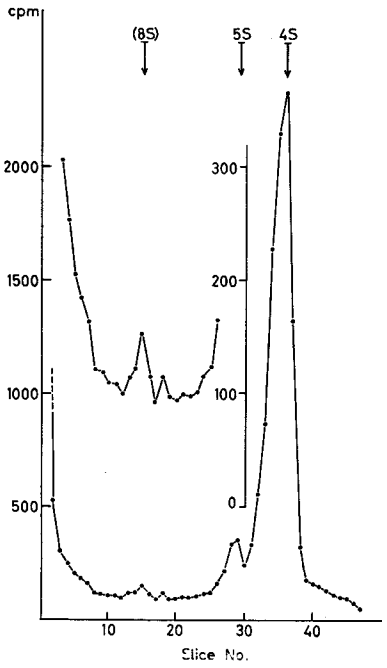

FIGURE 4 Separation of in vivo labeled low molecular weight RNA from whole salivary glands. Each of eight larvae was injected with 10 μCi tritiated cytidine (23.8 Ci/mmole) in 1 μl isotonic salt solution. The injection was introduced into the body cavity with a micropipette about 10 μ in diameter which was manoeuvered with a de Fonbrune micromanipulator under control in a stereo-microscope. The injections were done at the height of the third or fourth segment. About 16 hr after the injections the salivary glands were excised from the animals as well as from 30 other uninjected animals and homogenized in the cold in 200 μl of 0.02 M Tris-HCl, pH 7.4, 0.5% sodium dodecyl sulphate, 0.1 M NaCl. An equal volume of water-saturated phenol was added and the mixture was shaken for 45 min at 4°C. The aqueous phase was then removed and the phenol treatment was repeated once. The RNA was precipitated with two volumes of ethanol at −20°C overnight. Electrophoretic separation was afterwards carried out as described under Materials and Methods. The radioactivity value for the first slice (the trough), which is not indicated in the graphs is 10,396 cpm. Insert shows the 8S region with extended radioactivity scale.

show signs of relative accumulation in the nucleus with prolonged incubation times. At least 8S RNA, on the other hand, does not show a differential accumulation in the cytoplasm.

In the untreated cells, but not in DRB-treated material, the monodisperse 7S and 8S RNA peaks observed in chromosomes, nuclear sap, and cytoplasm were superimposed upon a heterogeneous background activity. This activity is part of broader profile of heterogeneous RNA which shows a maximum of radioactivity in the 35S–40S region (18) and it constitutes, in terms of radioactivity, a relatively small part of this population.

The nucleolar 10S RNA shows a relatively irregular behavior in appearing in widely differing amounts in different analyses. It is possible that the high amounts of preribosomal RNA from the nucleolus interfere with the migration of this material; the position is also close to the start in the gels, which may be unfavorable. Therefore, conclusions cannot be drawn with respect to effects of DRB on its degree of labeling or whether it shows any signs of accumulation with time. We could show, however, that there is no evidence for its presence in the nucleus outside of the nucleolus or in the cytoplasm.

It was suggested by Weinberg and Penman (19) that low molecular weight monodisperse nuclear RNA species from HeLa cells originate from a different synthetic system than the transfer RNA or nucleoplasmic heterogeneous RNA because of the character of the sensitivities towards different drugs (acti-dione, low actinomycin, and cordycepin). The 8S RNA fraction described

TABLE I

Comparison between DNA Content and Amount of Labeled 4–5S and 8S RNA in Individual Polytene Chromosomes of Chironomus tentans

Three glands were preincubated at 18°C for 60 min in 25 μl of Cannon's modified medium containing 20 μg DRB per ml. They were then transferred to another 25 μl of the same medium containing 100 μCi cytidine (sp. act. 27 Ci/mmole) and 100 μCi uridine (sp. act. 24 Ci/mmole) and incubated for 180 min at 18°C. Chromosomes I and IV were isolated from 90 cells, chromosomes II + III from 70 cells. Other data, see Materials and Methods.

Chromosome No.	*DNA content in $\mu\mu$g per chromosome	Radioactivity in cpm per chromosome		cpm in 4–5S per $\mu\mu$g DNA × 10^{-3}	cpm in 8S per $\mu\mu$g DNA × 10^{-3}
		4–5S	8S		
I	1044	15.3	1.4	147	13.4
II + III	1935	27.0	2.8	140	14.5
IV	361	5.3	0.5	147	13.9

* Values taken from Daneholt and Edström (17).

here shows a similar lack of sensitivity towards DRB as 4–5S RNA, in contrast to heterodisperse RNA the labeling of which is almost completely inhibited. Thus, in this respect there is no evidence to indicate whether 8S RNA is different in drug sensitivity from other low molecular weight RNA's.

It has been found that 4–5S RNA shows one type of distribution in the chromosomes and that the heterodisperse, high molecular weight RNA shows another one. The 4–5S RNA label is proportional to the DNA content of the chromosomes, which is in turn proportional to the number of chromosome bands or puffs (reference 13, and present data). Thus the fourth chromosome with 10% of the number of puffs (20) contains about 10% of the 4–5S RNA also after 45 min incorporation (unpublished data). The heterodisperse RNA, on the other hand, is present in larger quantities in large puffs than in small puffs, and after 45 min labeling, 25% of the chromosomal content of heterodisperse RNA is located on the fourth chromosome which contains three giant puffs, Balbiani rings (18). The 8S RNA has a distribution which parallels that of 4–5S RNA (Table I). The present investigations were carried out with glands incubated for altogether 4 hr when the Balbiani rings have largely regressed. It can therefore not be decided whether the labeling of 8S RNA is correlated with puff size. The fact that the amounts are correlated with the chromosome size could imply either that the 8S RNA, is unspecifically adsorbed from the sap or that its widespread occurrence reflects a biological situation.

Unspecific adsorption of 4–5S RNA to the chromosomes during our preparative procedures cannot be excluded but it is unlikely because the amounts of 4–5S RNA in different chromosome segments show characteristic differences not related to the size of these segments (21). The results of others working with RNA fractions obtained after isolation of nuclei in bulk in aqueous media show that 4S RNA and its precursor stages are present in low or nondetectable amounts in such nuclei (2, 22). Considering our positive results, it may be justifiable to discuss whether the nuclear localization of low molecular weight RNA could be the result of a contamination of the nuclei by cytoplasmic fractions. During early labeling times the amount of label in the nuclear fractions is of the same order for 4–5S RNA as for the cytoplasm. The cytoplasm represents, however, a volume one to two orders of magnitude larger than that of the nucleus, and the concentration of label in 4–5S RNA in the cytoplasm is consequently many times lower than that of the nucleus. It is therefore not possible to explain the nuclear content as due to cytoplasmic contamination. It should also be pointed out that the specific spatial and temporal patterns of distribution for low molecular weight RNA types argue against major contamination artifacts.

It is unlikely, finally, that the low molecular weight components described here are the result of degradation of RNA fractions of higher molecular weights. This can at least be shown for 8S RNA and 4–5S RNA in the chromosomes and the nuclear sap because they appear also when the labeling of high molecular weight RNA is suppressed by DRB treatment. Nor are these fractions artifacts due to the in vitro incubation conditions, since they appear in the same relative proportions after in vivo incubation.

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