

Evidence for Transport of 75S RNA from a Discrete Chromosome Region via Nuclear Sap to Cytoplasm in *Chironomus tentans*

(Balbiani ring/dipteran/high molecular weight RNA/midge/salivary glands)

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ABSTRACT Salivary glands of the dipteran *Chironomus tentans* were exposed to tritiated pyrimidines for different time periods either *in vitro* or *in vivo*. Nonribosomal, high molecular weight RNA molecules of very different sizes (15-100 S) were labeled on chromosomes I-III, while only one main species, 75S RNA, was recorded in Balbiani ring 2, a giant chromosome puff on chromosome IV. 75S RNA was present as a prominent fraction in nuclear sap, but not in cytoplasm, after 90 min of incubation *in vitro*. During the ensuing 90 min, 75S RNA also appeared in cytoplasm. After 1 week of labeling *in vivo*, radioactive 75S RNA accumulated heavily in cytoplasm. Absorbance measurements showed that 75S RNA constituted as much as 1.5% of the total salivary-gland RNA. The half-life of 75S RNA was estimated to exceed 35 hr.

Available data on the intracellular distribution and metabolic stability of nonribosomal, high molecular weight RNA from Balbiani ring 2 and chromosomes I-III indicate that most of the cytoplasmic 75S RNA is transcribed in the Balbiani ring 2 region of chromosome IV. 75S RNA molecules are, therefore, likely to be transferred from Balbiani ring 2, via nuclear sap, to cytoplasm without being measurably reduced in molecular size.

One important step in gene expression of eukaryotic cells is the transfer of genetic information from nuclear DNA to polysomes in cytoplasm. Available data strongly indicate that this information transfer is accomplished by RNA, but the details of the process are far from clear (1). Although different defined messenger RNA species have been demonstrated in cytoplasm (2-5), it has been more difficult to identify the individual corresponding precursors in the nucleus. The main reason for this failure is that the nonribosomal, high molecular weight RNA of the nucleus is a complex mixture of molecules, with concomitant identification problems. In transformed cells, it is feasible to select for one type of RNA, the virus-specific RNA, and thus simplify the analysis (6-8). Another approach is to extensively fractionate the nucleus in order to obtain defined, nonribosomal, high molecular weight RNA species. When such a procedure was applied recently to salivary gland cells of the midge *Chironomus tentans*, a defined RNA species (75S RNA) was recorded in a restricted chromosome region (9). The present investigation shows that it is possible to follow the migration of that particular RNA species from its site of synthesis via nuclear sap to cytoplasm. This transfer of 75S RNA occurs without any major reduction in molecular size.

Abbreviation: SDS, sodium dodecyl sulfate.

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MATERIAL AND METHODS

Materials. The dipteran *Chironomus tentans* was cultured according to Beermann (10). The paired salivary glands of rapidly growing fourth-instar larvae were used. Each salivary gland cell harbors four giant chromosomes, two of which carry nucleoli. Chromosome regions active in RNA synthesis are somewhat swollen, and are called puffs. On the small chromosome IV there are three giant puffs, the Balbiani rings [1, 2, and 3].

Labeling Conditions. *In vitro*: Four salivary glands were explanted into 25 μ l of modified Cannon's medium (11), supplied with 100 μ Ci of [3 H]uridine and 100 μ Ci of [3 H]-cytidine (29 Ci/mmol and 27 Ci/mmol, respectively), and incubated for 90 or 180 min at 18°. *In vivo*: 10 Fourth-instar larvae were bathed for a week at 18° in 20 ml of medium and provided with 400 μ Ci of [3 H]uridine and 400 μ Ci of [3 H]-cytidine.

Isolation of Cellular Components. The salivary glands were fixed in ethanol-acetic acid 3:1 at 4° for 30 min, rinsed in 70% ethanol three times for 10 min (each time) at 4°, and finally transferred to an inert mixture of ethanol-glycerol 1:1 for 60 min at 4°. The glands were then microdissected under paraffin oil as described in detail elsewhere (12). Each nucleus was divided into the following components: nucleoli, chromosomes I-III, Balbiani ring 2 (the remainder of chromosome IV was discarded), and nuclear sap. In the dissected cells, Balbiani rings 2 were always large, while Balbiani rings 1 and 3 were small or absent. When cytoplasm was also collected, the peripheral part of cytoplasm (about half of the total cytoplasm) was first isolated, then the remainder was used as a source of the nucleus. This procedure guaranteed pure nuclear and cytoplasm fractions. The number of particular components analyzed in each experiment is given in the figure legends.

Extraction of RNA. Microisolated Components: Each sample was dissolved for 3 min at 25° in a preincubated, sodium dodecyl sulphate (SDS)-Pronase solution [5 mg/ml of SDS, 1 mg/ml of nuclease-free Pronase (Calbiochem, Los Angeles) in 0.02 M Tris-HCl (pH 7.4)] with carrier RNA (25 μ g of *Escherichia coli* RNA) added. If electrophoresis was to be performed immediately (convenient for nuclear components), the labeled RNA was released in only 15 μ l of SDS-Pronase solution. If the sample was to be precipitated with ethanol before electrophoresis (necessary for cytoplasmic samples

containing labeled, ethanol-soluble substances that would disturb the radioactivity pattern unless extracted), each sample was treated with 100 μ l of SDS-Pronase solution. RNA was then precipitated overnight at -20° after 10 μ l of 1 M NaCl and 250 μ l of 100% ethanol had been added. The precipitated RNA was pelleted centrifugally and redissolved in 15 μ l of buffered SDS solution [5 mg/ml of SDS in 0.02 M Tris·HCl (pH 7.4)].

Whole Glands. About 35 salivary glands, fixed as above, were transferred to 400 μ l of SDS-Pronase solution and homogenized at 25° in a loose Dounce homogenizer with 10 strokes. When the glands were dissolved (after about 5 min), 50 μ l of NaCl and 1 ml of ethanol were added, the sample was stored at -20° overnight, and the precipitated RNA was collected by centrifugation. Usually two such precipitates were dissolved in the same 15 μ l of buffered SDS solution. About 30 μ g of RNA was obtained from 70 glands.

Electrophoresis. The technique with agarose gel slabs has been described in detail (13). 1% Agarose gels were prepared in 0.02 M Tris·HCl (pH 8.0)–2 mg/ml of SDS–0.02 M NaCl–2 mM EDTA. After completion of electrophoresis, the gel was cut into slices. These were put into Packard scintillation vials, each containing 10 ml of toluene scintillator [30 ml Soluene (Packard), 20 ml methoxyethanol, and 5.5 g of Permablend III (Packard) per 1000 ml of toluene] and incubated overnight at 37° . The samples were then counted in a Packard Tricarb liquid scintillation spectrometer, model 3380.

When needed, the absorbance of the electrophoretic separation was recorded at 254 nm with a Vitatron densitometer (UFD 500) and recorder (UR 100), and the relative amounts of the different RNA fractions were determined. When the fractions were to be tested for RNase sensitivity, the scanned migration path was cut longitudinally into halves, one of which was kept for 3 hr at room temperature in 0.02 M Tris·HCl (pH 7.4) containing 100 μ g of RNase per ml, and the other in the same buffer without added RNase. After the treatments the two gel sections were again scanned for absorbance at 254 nm. They were then kept overnight in RNase for extensive treatment and afterwards scanned for background.

RESULTS

Intracellular transport of 75S RNA

The synthesis and transport of RNA in salivary gland cells of *Chironomus tentans* were studied in explanted glands, incubated for 90 and 180 min in modified Cannon's medium supplied with [3 H]uridine and [3 H]cytidine. Different cellular compartments were then isolated and analyzed separately. In one series of experiments (Fig. 1) the purpose was to investigate different nuclear components (nucleoli, chromosomes I-III, Balbiani ring 2, and nuclear sap); in another series (Fig. 2) the two main cellular compartments (nucleus and cytoplasm) were studied. These studies supplement each other and make it possible to follow the migration of defined RNA species from the chromosomes via nuclear sap into cytoplasm. Although the object of interest is 75S RNA, it is useful first to outline the synthesis, processing, and transport of (pre)-ribosomal RNA and low molecular weight RNA as they occur in the present analyses.

Nucleolar and Ribosomal RNA. The ribosomal RNA precursor, 38S RNA, is synthesized in the nucleoli, and is also

converted there to 30S and 23S RNA (14). During 90 min of incubation, some 38S RNA had already been cleaved to 30S and 23S RNA (Figs. 1A and 2A). The split products accumulated during 90 min of additional incubation (Figs. 1B and 2B). Particularly during *in vivo* conditions, (pre)ribosomal RNA can also be recorded on the chromosomes (11). In the present *in vitro* experiments (Fig. 1) substantial amounts were, however, not detected on the chromosomes, possibly suggesting an impaired migration of (pre)ribosomal RNA to the chromosomes, not necessarily related to the transport of ribosomal RNA to cytoplasm.

The appearance in cytoplasm of the finished ribosomal components, 28S and 18S RNA, has been established *in vitro* (11, 15). The small ribosomal RNA component emerged in cytoplasm after about 90 min of incubation. In two analyses 18S RNA had not entered cytoplasm after 90 min (Fig. 2A), while in two other cases it was present in small amounts (not shown). The large ribosomal RNA component, 28S RNA, could not be detected in cytoplasm after 90 min of incubation (Fig. 2A). After 180 min, 18S RNA, as well as 28S RNA, were recorded in cytoplasm (Fig. 2B).

Low Molecular Weight RNA (4–5S RNA). The low molecular weight RNA is synthesized in the nucleus and is rapidly transported to cytoplasm (16). After 90 min, considerable amounts of low molecular weight RNA had reached the cytoplasm (Fig. 2A); still more appeared after another 90 min (Fig. 2B).

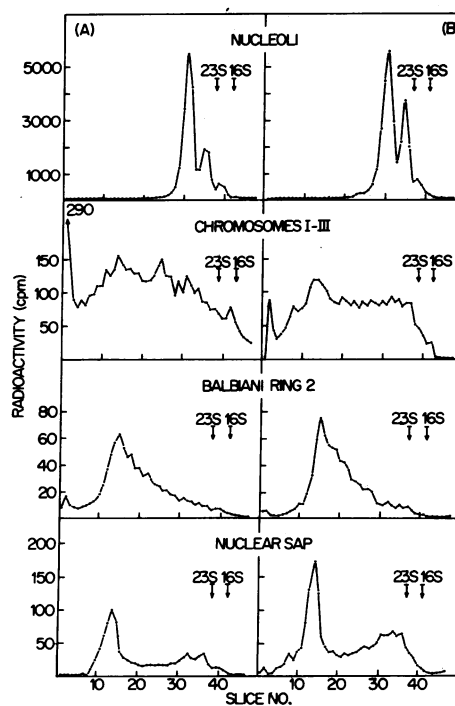


FIG. 1. Electrophoresis in 1% agarose gels of labeled RNA from different nuclear components after 90 min (A) and 180 min (B) of incubation of explanted salivary glands at 18° . Nuclear components were collected from 30 fixed cells. Each sample was dissolved in 15 μ l of SDS-Pronase solution for 3 min at 25° , and analyzed immediately by electrophoresis. For reference, the positions of the carrier RNA components (*E. coli* 23S, 16S, and 4S RNA) have been indicated.

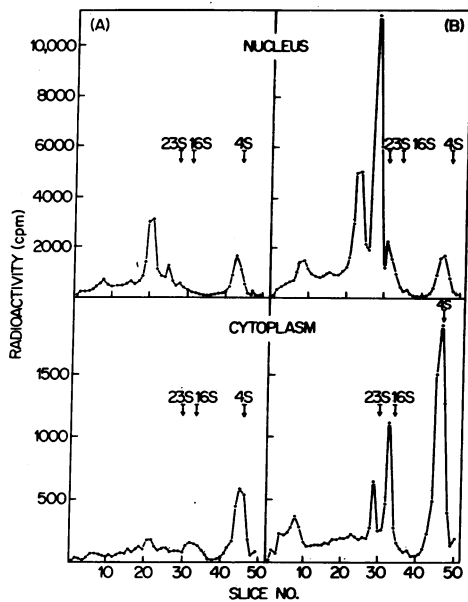


FIG. 2. Electrophoresis in 1% agarose gels of labeled RNA from nucleus and cytoplasm after 90 min (A) and 180 min (B) of incubation of explanted salivary glands at 18°. Nucleus and cytoplasm were dissected from 15 fixed cells. Each sample was dissolved in 100 μ l of SDS-Pronase solution for 3 min at 25°, precipitated in ethanol overnight, redissolved, and analyzed by electrophoresis.

75S RNA and Other Nonribosomal, High Molecular Weight RNAs. Many nonribosomal RNA species are synthesized on the four giant chromosomes (15, 17). These RNA fractions represent unbroken, single-stranded RNA molecules, partly of very high molecular weights (13). On chromosomes I-III they ranged in size from 15 S to about 100 S (Fig. 1). In Balbiani ring 2, however, there was only one main RNA species (Fig. 1), earlier determined to have a sedimentation value of 75 S (9). For chromosomes I-III, as well as for Balbiani ring 2, no change in the activity distribution, or in the total activity was observed between 90 (A) and 180 min (B) of incubation. This result is in agreement with earlier

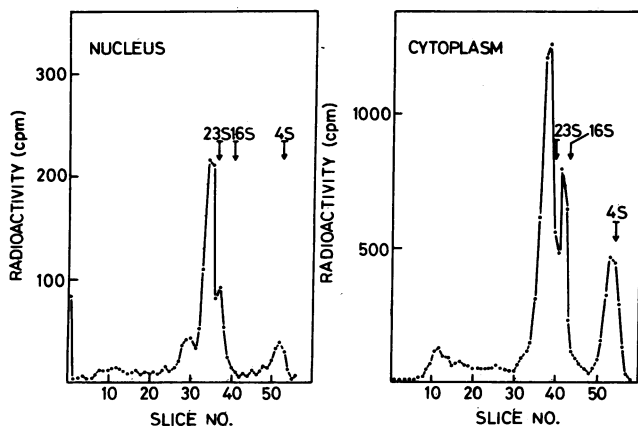


FIG. 3. Electrophoresis in 1% agarose gels of nuclear and cytoplasmic RNA from salivary glands after 1 week of labeling *in vivo* at 18°. Nucleus and cytoplasm were dissected from 50 fixed cells. Each sample was dissolved in 100 μ l of SDS-Pronase solution for 3 min at 25°, precipitated in ethanol overnight, redissolved, and analyzed by electrophoresis.

data (15) indicating a life time on the chromosomes of not more than 45 min for these RNA species.

Molecules corresponding to the whole size range of total chromosomal RNA were present after 90 and 180 min in nuclear sap (Fig. 1A and B). One fraction was, however, by far the most prominent. It had the same migration properties as 75S RNA in Balbiani ring 2 and is, therefore, conveniently designated 75S RNA in this presentation. The nuclear sap profile did not change shape between 90 and 180 min of incubation (Fig. 1A and B). As nuclear sap was not collected quantitatively, we cannot decide whether there was an accumulation of labeled RNA in nuclear sap between 90 and 180 min.

Finally, in cytoplasm a heterogeneous distribution of molecules similar in size to total chromosomal RNA could be recorded after 90 and 180 min of incubation (Fig. 2A and

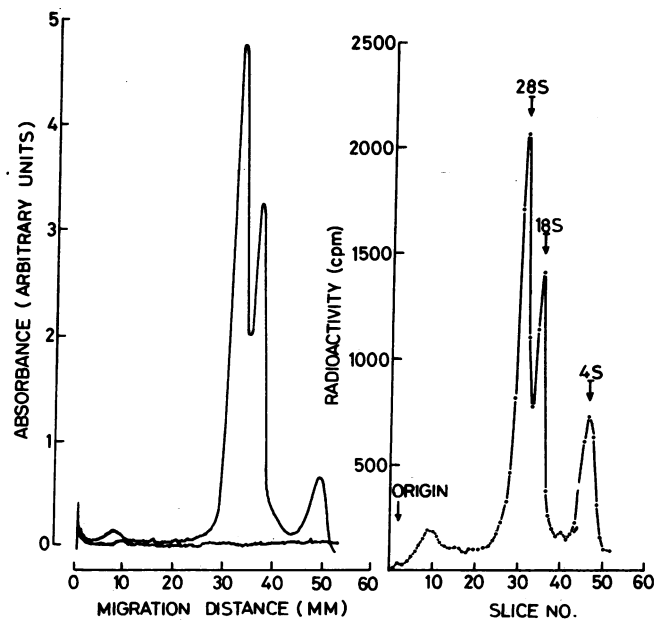


FIG. 4. Electrophoresis in 1% agarose gels of total salivary gland RNA (left, absorbance) containing as a control gland RNA labeled *in vivo* for 1 week at 18° (right, radioactivity). 10 *Chironomus tentans* larvae were kept for 7 days at 18° in a rearing medium provided with [3 H]uridine and [3 H]cytidine. Two gland extracts were then prepared. For each, three salivary glands were isolated from the radioactive larvae and combined with about 30 unlabeled glands. After fixation, total salivary gland RNA was extracted in 400 μ l of SDS-Pronase solution for about 5 min at 25° and precipitated in ethanol. The two RNA precipitates were collected and dissolved in the same 15 μ l of buffered SDS solution and run at a 1% agarose gel. The electrophoretic separation was scanned at 254 nm (left, upper tracing). The gel was then cut longitudinally along the migration path into two equal portions. For extensive RNase treatment, one portion was incubated overnight at 25° in an RNase solution [100 μ g/ml of RNase in 0.02 M Tris-HCl (pH 7.4)] and afterwards scanned (left, lower tracing). A small RNase-insensitive peak, migrating slightly ahead of the 75S RNA, was frequently observed. It was sensitive to DNase. The absorbance peak, recorded just ahead of the origin in both tracings, is due to an optical artifact at the trough, and not to substances absorbing ultraviolet light. The remaining gel portion was sliced as usual and analyzed for radioactivity distribution (right). The positions of the main RNA species in *Chironomus tentans* (28S, 18S, and 4S) have been indicated for reference.

B). No distinct 75S RNA fraction was observed in cytoplasm, after 90 min of labeling (A). 75S RNA was easily recognized after 90 min of additional incubation (B). Thus, 75S RNA also appeared in cytoplasm, but only after a lag period.

Thus, 75S RNA was synthesized on the chromosomes and was recovered in nuclear sap. After a delay, 75S RNA also appeared in cytoplasm. These data indicate that 75S RNA molecules migrate from the nucleus to the cytoplasm without being reduced in molecular size. Whether this property is also true for other nonribosomal, high molecular weight RNA is not known.

Accumulation of 75S RNA in cytoplasm

Long-term labeling of salivary glands was performed *in vivo* in order to determine whether or not 75S RNA accumulated. Larvae were kept for 7 days in rearing medium containing tritiated pyrimidines. The labeling conditions were tested with control larvae, some of which were kept in the medium during the first day (day-one larvae) and some during the day after the 1-week experiment was completed (day-eight larvae). Four salivary glands were isolated from each of the two groups of control larvae and 15 cells were dissected from each gland; the acid-precipitable, RNase-sensitive activity was determined. The average activity of 15 cells from day-eight larvae (475 cpm) amounted to about 40% of that from day-one larvae (1250 cpm). This result indicated that the rearing medium contained substantial amounts of radioactive RNA precursors all through the 1-week experiment. Thus, the larvae kept in the radioactive medium for 7 days should be suitable for demonstration of an accumulation of 75S RNA. The electrophoretic profiles of nuclear and cytoplasmic RNA from the 1-week larvae are presented in Fig. 3. The nuclear sample displayed the preribosomal RNA species (38S, 30S, and 23S), as well as 4-5S RNA and nonribosomal, high molecular weight RNA. No substantial nuclear accumulation of 75S RNA was indicated. The cytoplasm contained finished ribosomal RNA (28S and 18S), 4-5S RNA, 75S RNA, and other nonribosomal, high molecular weight RNA. The 75S RNA peak was completely abolished if RNase treatment (100 μ g/ml of RNase for 15 min at 25°) preceded electrophoresis. The long-term labeling experiment shows that 75S RNA was exported to cytoplasm *in vivo* and accumulated there. It can be calculated from Fig. 3 that the cytoplasm contained more than 95% of the total labeled 75S RNA after 1 week of labeling *in vivo*.

Total amount of 75S RNA

Sufficient quantities of 75S RNA accumulate in salivary gland cells to be measured by absorbance scanning of electrophoretic separations of total gland RNA. A modified extraction procedure was applied to several salivary glands. The RNA species were separated by electrophoresis (Fig. 4). Apart from the ribosomal and 4S RNA fractions, a peak in the 75S RNA region sensitive to RNase was obtained. The total gland RNA extract was of about the same quality as that obtained from microisolated components, as demonstrated by addition of some labeled glands to the unlabeled ones before extraction. The electrophoretic analysis of the radioactive RNA from whole glands (Fig. 4, *right*) was compared with that from microdissected cytoplasm (Fig. 3, *right*). The profiles were similar, indicating that the two extraction techniques were equivalent. It could then be estimated from four absorbance

analyses that 75S RNA constitutes 1.5% (1.3-1.8%) of total RNA. As the RNA content of a large salivary gland cell is 70,000 pg (18), the 75S RNA in such a cell amounts to about 1000 pg.

DISCUSSION

The Transcription Site of Transported 75S RNA. Non-ribosomal, high molecular weight RNA is synthesized on the four giant chromosomes in the salivary glands of *Chironomus tentans*. Many different molecules (15-100 S) are recorded on chromosomes I-III, while only one main species, 75S RNA, is recovered in the short chromosome IV region that carries Balbiani ring 2. It is known that 75S RNA in Balbiani ring 2 is actually transcribed in the ring, as its complementary sequences are localized there (19). The distribution of the rapidly labeled RNA in the nuclear sap is characterized by a prominent 75S RNA fraction. After a lag period, labeled 75S RNA also appears in cytoplasm; after long labeling times, it constitutes the main nonribosomal, high molecular weight RNA in cytoplasm. The best explanation for the present results is that Balbiani ring 2 RNA is released into nuclear sap and, after some delay, released into cytoplasm, without being measurably reduced in molecular size. Such an interpretation is supported from earlier studies (19, 20) that demonstrate that Balbiani ring 2 RNA is preserved (at least to a very large extent), while most RNA from chromosomes I-III is rapidly degraded to acid-soluble products. Furthermore, it has recently been shown by *in situ* hybridization (B. Lambert, Nature, submitted) that Balbiani ring 2 RNA is readily recorded in nuclear sap as well as in cytoplasm. The transcription site of cytoplasmic 75S RNA has not, however, been directly demonstrated.

It is interesting to note that cytoplasmic 75S RNA displays certain characteristics that can be predicted for an RNA species synthesized in a Balbiani ring. It is likely from cytogenetic data that the Balbiani rings in salivary gland cells are responsible for generation of messenger RNAs for salivary proteins (21, 22). As the salivary protein synthesis in these cells comprise more than 80% of total protein synthesis (23), it can be anticipated that the corresponding messenger RNAs are major nonribosomal, high molecular weight RNA species. Furthermore, since not even a 24-hr inhibition of RNA synthesis substantially influences salivary protein synthesis (24-26), the messengers are probably also stable or turning over very slowly. In the present investigation, absorbance measurements showed that 75S RNA constitutes as much as 1.5% of total RNA. This figure can be compared with the proportion of fibroin messenger of total RNA in the silkworm *Bombyx mori* (0.8-1.4%) (27). 75S RNA also displays a high metabolic stability (see below). Thus, cytoplasmic 75S RNA has certain predicted properties of a salivary protein messenger, likely to be transcribed in a Balbiani ring.

The Transport of 75S RNA. The most interesting feature of the 75S RNA migration is that the molecule does not seem to be reduced in size when it is transferred from nucleus to cytoplasm. It cannot, of course, be ruled out that a very minor segment is split off. Evidence for giant RNA in cytoplasm of the salivary gland cells in *Chironomus tentans* has been presented (15). This property does not seem to be a unique feature of these cells. Working with silk worms, Suzuki and Brown (27) found that the messenger RNA for silk fibroin

sedimented at 45–65 S. Recently, Giudice *et al.* (28) detected giant RNA in the cytoplasm of sea urchin embryos. There is now also good evidence in mammalian polysomes for non-ribosomal RNA that is considerably larger than earlier anticipated. Sedimentation in the presence of dimethyl sulfoxide showed that 50% of the rapidly labeled, polysomal RNA in KB cells sedimented faster than the 28S ribosomal RNA (29).

The Stability of 75S RNA. In the present study, different lines of evidence suggest that cytoplasmic 75S RNA has a high metabolic stability. After a 1-week labeling period, more than 95% of the labeled 75S RNA is recovered in cytoplasm.

The half-life of 75S RNA can be estimated from the information about the total amount of 75S RNA per salivary gland cell (1000 pg) and the production rate of 75S RNA in Balbiani ring 2. The rate has been assessed *in vitro* as 20 pg per cell per hr (15). Provided that the cell is in a steady state, it can then be assumed that about this amount of 75S RNA is also degraded per hour. If 75S RNA decays at a logarithmic rate, the half-life can be calculated as 35 hr. It should be kept in mind, however, that this is probably a minimum estimate. Particularly important is the observation that the salivary gland cells of rapidly growing larvae increase in volume more than 10-fold during the fourth-larval instar (lasting 2–4 weeks) (10). It seems plausible that this considerable expansion is accompanied by a continuous increase in messenger content; thus, the salivary gland cells should be looked upon as systems in expansion rather than in steady state. The half-life of 75S RNA is accordingly likely to exceed 35 hr.

The Genetic Information in 75S RNA. The giant size of some cytoplasmic RNA, presumably acting as messenger, suggests that such molecules might contain a considerable amount of genetic information. The very fact that Suzuki and Brown (27) obtained good agreement between the results from partial sequence analysis of the rapidly sedimenting RNA and the predicted sequence of fibroin messenger strongly indicated that most, if not all, of the RNA carried the genetic message for fibroin. If the *Chironomus tentans* 75S RNA [15–35 × 10⁶ daltons (9)] contains genetic information for salivary polypeptides all along the molecule, then each molecule can accommodate more than one salivary polypeptide cistron. This conclusion is true even for the largest salivary polypeptide, which is of exceptional size in *Chironomus tentans* [about 500,000 daltons (30)]. Although the question of whether 75S RNA is poly- or mono-cistronic cannot yet be answered, some information is available on the structure of 75S RNA. First, different segments of the molecule probably contain the same base composition, specific for Balbiani ring 2, indicating a similar structure along most of the molecule (31). Furthermore, using biochemical as well as *in situ* hybridization techniques, Lambert (32) has directly demonstrated that 75S RNA contains repeated sequences. These data may imply that the 75S RNA molecule contains more than one copy of the same cistron or, alternatively, two or more structurally related cistrons. When the repeated structure of 75S RNA is considered, it is also important to recall that there can be a substantial number of repeated amino acid sequences within a structural polypeptide. This effect has been nicely demonstrated for silk

fibroin (33), which forms the cocoon of silk worms. It is interesting that the *Chironomus tentans* larvae use the salivary proteins to surround themselves by a special tube, i.e., a structure analogous to that of the cocoon of *Bombyx mori*.

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