

DEMONSTRATION OF BALBIANI RING RNA SEQUENCES IN POLYSOMES

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On the basis of morphological observations, it was originally proposed that puffs in polytene chromosomes of Diptera represent active genes (1). This concept received support from cytogenetic investigations which suggested that particular puffs of giant size, the Balbiani rings, in *Chironomus salivary glands* are correlated with the synthesis of salivary polypeptides (2, 3, 4). Biochemical analyses of the Balbiani ring products in *Chironomus tentans* are furthermore compatible with the idea that puffs generate messenger RNA molecules. Both Balbiani rings 1 and 2 (BR 1 and BR 2) produce primary transcripts of very large size (75S RNA) (5, 6). These RNA molecules are transported via nuclear sap into the cytoplasm, without a measurable size reduction (7, 8). Recently, polysomes from the salivary gland cells were found to contain large RNA molecules including 75S RNA (9). In this paper we present results of *in situ* hybridization experiments, showing that RNA extracted from these polysomes contains sequences complementary to the DNA of BR 1 and BR 2.

MATERIALS AND METHODS

Fourth instar larvae of *Chironomus tentans* were incubated at 18°C for 3 days in 20 ml of ordinary culture medium supplied with 400 μ Ci of tritiated cytidine (25.6 Ci/mmol) and 400 μ Ci of tritiated uridine (49 Ci/mmol). Eight salivary glands were removed from the larvae and transferred to a polysome extraction me-

dium consisting of 0.5% Tween 80, 0.5% sodium deoxycholate, 0.1% β -mercaptoethanol in TEAKM (0.1 M KCl, 0.003 M $MgCl_2$, 0.02 M ethanolamine-HCl, pH 7.6) kept at 2–4°C. Extraction was performed for 10–15 min as reported elsewhere (9). The polysome extract was divided into two halves, one of which was treated with 0.02 M EDTA.

The two samples were then layered on top of 15–60% (wt/wt) sucrose gradients. Centrifugation was performed at 4°C for 30 min at 40,000 rpm in a SW65 rotor (Beckman Instruments, Spinco Div., Palo Alto, Calif.). The gradients were fractionated and the radioactivity in each, or in the polysome region in each second fraction, was determined as described in the accompanying paper (9). The remaining fractions from the heavy polysome (HP) region and those from the light polysome (LP) region were pooled separately for each gradient (see Fig. 1).

RNA was released from each of the four samples by adding an equal volume of TEAKM containing 25 μ g of *Escherichia coli* RNA and 0.1 volume of a preincubated Sarkosyl-pronase solution (5% Sarkosyl and 10 mg/ml pronase). The samples were dialysed against $2 \times$ SSC (SSC is 0.15 M sodium-chloride and 0.015 M sodium citrate) in the cold for 3 hours to remove Sarkosyl. The RNA was precipitated by addition of 2 vol of cold ethanol and stored at $-20^\circ C$ overnight. The precipitate was collected by centrifugation and dissolved in 30 μ l of $2 \times$ SSC.

For *in situ* hybridization, each sample was split into two 15- μ l portions and added to salivary gland squash preparations which previously had been denatured in 90% formamid in $0.1 \times$ SSC at 62°C for 2 h. The hybridization reaction was carried out at 62°C for 4 h.

The preparations were RNase treated, washed extensively in $2 \times$ SSC, and dehydrated. Further details were carried out as described earlier (12). Autoradiography was performed with Kodak AR 10 stripping film, and the slides were stained with Giemsa. The exposure time was 17 wk.

RESULTS

After sedimentation in a 15–60% sucrose gradient, the salivary gland polysomes were distributed in a broad range corresponding to 200–2,000S with an average peak value of 700S (Fig. 1). This result agrees with a recent study of the biochemical and electron microscopic properties of the salivary gland polysomes (9). When the polysomes were dissociated with EDTA, most of the rapidly sedimenting material shifted towards the top of the gradient (Fig. 1).

Polysome-containing fractions were pooled into two samples, one containing material with S higher than 1,000S (HP or heavy polysomes) and one containing material with S between 200 and 1,000S (LP or light polysomes) (Fig. 1). From each of these polysome preparations, RNA was released and analysed by *in situ* hybridization. The material remaining in the corresponding gradient fractions upon EDTA treatment were pooled in the same way and the RNA was hybridized *in situ*.

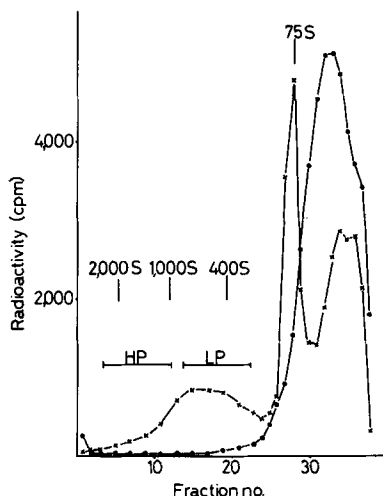


FIGURE 1 Sedimentation analysis of a polysome extract from *Chironomus tentans* salivary glands with and without EDTA. In each gradient, fractions from the heavy polysome (HP) region (4,6 ... 12) and fractions from the light polysome (LP) region (14,16 ... 22) were pooled separately and saved for *in situ* hybridization. Non-EDTA-treated extracts: X—X; EDTA-treated extracts: ●—●.

The results of the *in situ* hybridizations are shown in Fig. 2. RNA from heavy polysomes produced a large number of grains over BR 2 and a somewhat less but still significant number of grains also over BR 1 (Fig. 2A). Few grains were found over BR 2 as well as over BR 1 after EDTA treatment of the polysome samples (Fig. 2C): on average, the number of grains were reduced by about 80% for both BR 1 and BR 2. When RNA from light polysomes was hybridized to squash preparations, grains were also recorded over BR 1 and BR 2 (Fig. 2B). Again, the number of grains was considerably reduced after EDTA treatment of the polysome samples (Fig. 2D). It can therefore be concluded that RNA from BR 1 and RNA from BR 2 are present in rapidly as well as in slowly sedimenting polysomes.

It can also be noted (see Fig. 2) that the relative grain density of BR 1 to BR 2 is higher after hybridization with the LP than with the HP fraction. This result suggests that the BR 1 and BR 2 sequences are not distributed in the same manner along the gradient, the BR 1 sequences being relatively more abundant in LP than in HP when compared to BR 2 sequences. However, because of the difficulties in assessing the hybridization conditions during the *in situ* reaction, the interpretation can only be tentative.

During the present hybridization conditions, polysomal RNA produces very few grains over the chromosomes except those over the BRs. Additional significant and reproducible label was seen only over region 2A of chromosome II, the site of the 5 S RNA genes (10), and over the nucleolar organizers. Hybridization with EDTA-treated HP and LP fractions showed a reduction in the number of grains over the nucleolar organizers similar to that found over BR 1 and BR 2.

DISCUSSION

The present work demonstrates that RNA sequences complementary to BR 1 and BR 2 DNA are located in polysomes, but the size of the polysomal RNA responsible for the *in situ* hybridization has not been directly determined. RNA extracted from the HP as well as from the LP fraction evidently hybridizes efficiently with BR 1 and BR 2 (Fig. 2). In both these fractions, the RNA molecules have S-values ranging from 10S to 75S, with the highest molecular weight RNA (50–75S) being relatively enriched in the HP fraction (9). It was previously shown that cytoplasmic 75S RNA hybridizes specifically with BR 1 and BR 2, while

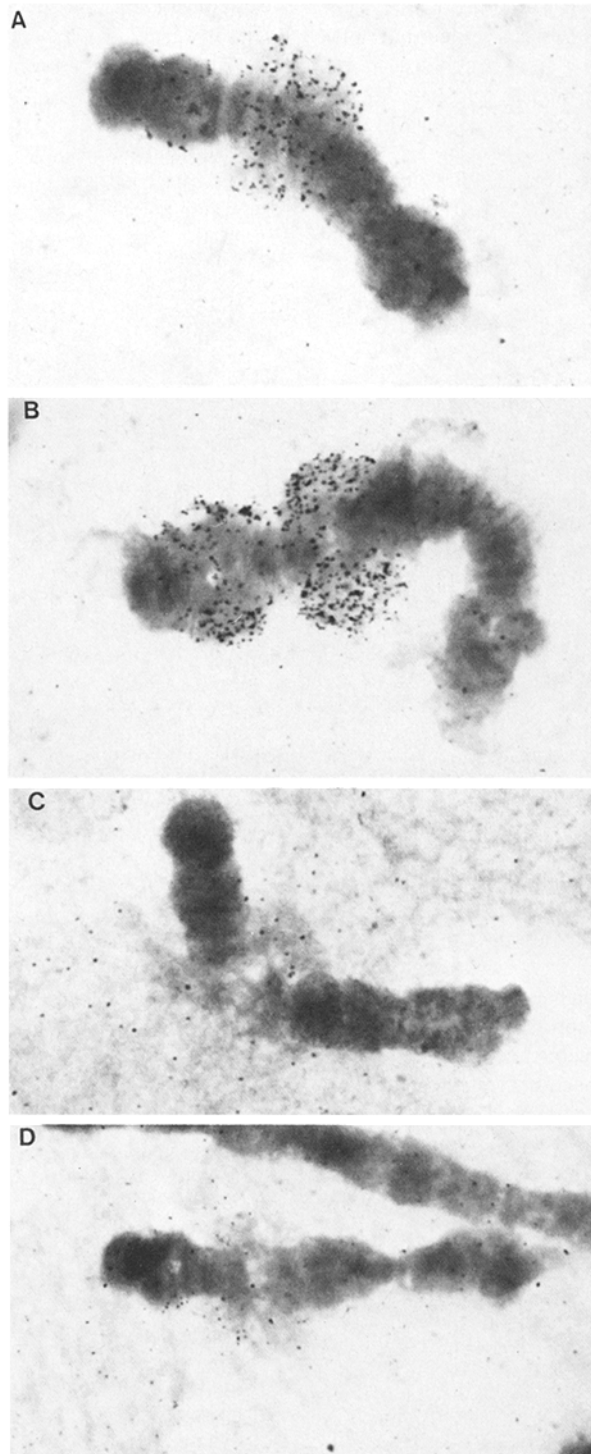


FIGURE 2 *In situ* hybridization of labeled RNA from the rapidly (*HP*) and slowly (*LP*) sedimenting samples obtained as described in Fig. 1. 2 A, RNA from the HP region of the non-EDTA-treated extract; 1,050 cpm. 2 B, RNA from the LP region of the non-EDTA-treated extract; 13,000 cpm. 2 C, RNA from the HP region of the EDTA-treated extract; 50 cpm. 2 D, RNA from the LP region of the EDTA-treated extract; 200 cpm. The length of chromosome IV is about 80 μm .

negative results were obtained with high molecular weight cytoplasmic RNA smaller than 75S RNA (8). It can therefore be concluded that polysomal 75S RNA is likely to be at least partly responsible for the hybridization *in situ* to the BRs. Since 75S RNA is partially degraded during polysome extraction (9), it is also possible that degraded polysomes containing 75S RNA sequences are recorded mainly in the LP fraction. This could contribute to the hybridization obtained with RNA from the LP fraction. However, it can not be excluded that native polysomal RNA smaller than 75S RNA hybridizes *in situ* to BR 1 and BR 2. In summary, the *in situ* hybridization results do not permit a direct conclusion as to the size of the BR-specific sequences contained in polysomes. It should, however, be pointed out that all results are compatible with the idea that BR-generated 75S RNA is present in polysomes (9).

In agreement with earlier studies (for review, see reference 11), the demonstration of BR sequences in polysomes strongly indicates a messenger function for BR RNA. This is important since it provides a potential functional meaning to the BR RNA sequences which earlier have been recorded outside the BRs in the nuclear sap (12) and in the cytoplasm (13). Our results also agree with *in situ* hybridization studies of polysomal RNA obtained from cultured cells of *Drosophila* embryos (14, 15). These studies suggest that certain heat-induced loci, which correspond to puffs in polytene tissues, synthesize RNA that also appears in polysomes. The present information on the fate of puff products in *Drosophila* and *Chironomus* therefore strongly supports the puffing concept, i.e. that puffs represent active genes producing messenger RNA sequences to be utilized in the protein-producing machinery of the cell. It is, however, evident that the nature of the amino acid coding sequences in puff RNA has to be further analysed in a suitable translational system.

SUMMARY

A polysome extract from salivary glands of *C. tentans* was sedimented in a 15–60% sucrose gradient. Fractions from the heavy polysome region (1,000–2,000S) and fractions from the light polysome region (200–1,000S) were pooled separately, and the long-term labeled RNA was released by Sarkosyl/pronase and analysed by *in situ* hybridization. The results showed that BR 1 and BR 2 sequences were present in the heavy and the

light polysome regions of the sucrose gradient. From control experiments with EDTA-treated extracts, it was concluded that most of the recorded BR 1 and BR 2 sequences were in fact located in polysomes. The finding that BR products enter polysomes suggests that they act as messenger RNA molecules. This study therefore strongly supports the concept that chromosome puffs represent active genes.

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