The complexity of 75S premessenger RNA in Balbiani ring granules studied by a new RNA band retardation assay

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ABSTRACT

Under normal growth conditions, Balbiani ring granules constitute premessenger ribonucleoprotein (RNP) particles synthesized in two chromosomal puffs, Balbiani ring (BR) 1 and 2, in the larval salivary glands of Chironomus tentans. At least three genes encoding 75S RNA are present in these two BRs: one in BR1 and two in BR2 (BR2.1 and BR2.2). The complexity of BR granule 75S RNA was studied by agarose gel electrophoresis under non-denaturing conditions. We recorded three main bands, designated I, II and III. Experiments with denaturing gels demonstrated that the differences in migration reflected mainly, but not exclusively, conformational differences. Northern blotting experiments showed that band I contained BR1 sequences, band II contained BR2.1 sequences, and band III contained BR2.2 sequences. To study whether additional genes contributed to the BR granule 75S RNA, an RNA band shift assay was developed. When an oligodeoxyribonucleotide complementary to repetitive BR1 and BR2.2 sequences was hybridized to 75S RNA prior to electrophoresis, bands I and III were retarded but not band II. An oligonucleotide complementary to a repetitive BR2.1 sequence only shifted band II. Since no detectable 75S RNA remained uncharged in these experiments, and all bands were identified by Northern blotting, all the BR granules are likely to originate from the BR1, BR2.1 and BR2.2 genes; no additional genes have to be invoked. Possible applications of the new RNA band shift assay are discussed.

INTRODUCTION

Specific premessenger RNP particles, the Balbiani ring granules of Chironomus tentans salivary glands, have served as a model to study the information flow from the genes to the protein synthesis machinery (1). The RNA molecules are synthesized and the RNP particles assembled in specific chromosomal regions of the polytene chromosomes, the Balbiani rings (BR) (2, 3). The particles have been observed in the nucleoplasm and during the transport through the nuclear pores (4–6). When they enter cytoplasm, they disassemble, and the RNA is incorporated into polyribosomes (7, 8).

Due to the intense transcriptional activity of the BR genes, the BR granules are abundant in the cell nucleus (1). Furthermore, they are exceptionally large, 500 Å in diameter, and can morphologically be readily identified. Each granule contains a 37 kb RNA molecule (75S RNA) (9) encoding a large-sized secretory protein (10–12). Electron microscope tomography has revealed that the BR particles consist of an RNP ribbon bent into a ring-like conformation (13).

The biochemical analysis of the total population of premessenger RNP particles, often referred to as heterogeneous nuclear RNP (hnRNP), has been severely hampered by the heterogeneity of hnRNP in most cells and by the low frequency of individual hnRNP species (14, 15). In the BR system, a considerable simplification of the analysis can be obtained. We have recently developed a method to isolate BR granules in their native state and have found that they form a distinct 300 S peak in sucrose gradients (16). The BR granule population is, however, not homogeneous, since the BR peak is slightly asymmetric in both sucrose gradients and CsCl buoyant density gradients. Moreover, in gel electrophoresis, we have observed several 75S RNA bands, presumably corresponding to RNP particles with slightly different sedimentation rates (16).

In order to better characterize the BR granule population we have now investigated the complexity of the 75S RNA in the granules with respect to their genetic origin. There are four known BR genes encoding 75S RNA: one gene in BR1, two genes in BR2 (BR2.1 and BR2.2) and one gene in BR6 (17, 18); the latter is only expressed under special culturing conditions (19). In the present study we describe that BR granule RNA comprises three main 75S RNA species (I–III), which contain transcripts from the BR1, BR2.1 and BR2.2 genes, respectively. To investigate whether additional gene products are present, we developed an RNA band retardation assay. Our results showed that all the observed RNA species can be shifted with oligonucleotides complementary to known BR genes, indicating...
that the BR1, BR2.1 and BR2.2 genes generate all the detectable 75S RNA found in BR granules under normal conditions of larval growth.

MATERIAL AND METHODS

Animals

Chironomus tentans larvae were cultured as described (20). Rapidly growing, four week old, fourth instar larvae were used. In some experiments, seven week old late fourth instar or prepupae were chosen.

Labelling of RNA in salivary glands

The animals were decapitated at 0°C. Four to five glands were transferred to 50 µl of Cannon's medium (21) containing 40 µCi of [α-32P] ATP (NEN; 800 Ci/mmol), and the sister glands to 50 µl of Cannon's medium containing 100 µCi of [5,6-3H] uridine (NEN; 47 Ci/mmol). All glands were incubated for 90 min at 18°C to label nuclear RNA.

Isolation of BR granules

The isolation procedure was performed as described (16). After the incubation the glands were transferred to 250 µl of TKE (0.1 M KCl, 10 mM EDTA, 10 mM triethanolamine-HCl, pH 7.0) plus 0.3% Nonidet P40 and 0.5 mg/ml of tRNA. They were homogenized on ice by 10 strokes in a Dounce homogenizer. The total homogenate was immediately loaded onto a 15–40% linear sucrose gradient made in TKE and centrifuged at 0°C for 1 h at 40,000 rpm in a Beckman SW 50.1 rotor. The radioactivity in the fractions was determined by Cerenkov counting (32P) or by liquid scintillation counting of a small aliquot (3H). BR granules sedimenting at 300S were precipitated at −20°C after the addition 2.5 volumes of ethanol and 25 µg/ml of tRNA.

Agarose gel electrophoresis

The precipitated BR granules were dissolved in 10 µl of 10 mM sodium phosphate buffer (pH 7.0), containing 1 mM EDTA, 0.5% SDS and 250 µg/ml of proteinase K (Boehringer), and incubated for 1 hour at 25°C. The samples were either loaded directly, or after denaturation, onto a 1.5 mm thick, 0.7% agarose slab gel. For denaturation the RNA was precipitated in ethanol, dissolved in 10 mM sodium phosphate buffer (pH 7.0) containing 1M glyoxal and 50% dimethyl sulfoxide, and incubated for 1 h at 50°C (22). In both cases the running buffer consisted of 30 mM sodium phosphate buffer (pH 7.0), provided with 3 mM EDTA and 0.5% SDS. The electrophoresis continued at room temperature for about 5 h at 5 V/cm. The slab gels were either dried for autoradiography of 32P-labelled RNA or used for Northern blot analysis.

Hybridization on filters

After electrophoresis the gels were kept in water for 1 h in order to remove SDS, which interfered with the transfer of the RNA. The gels were then soaked sequentially in 200 ml of 50mM NaOH and 0.02 M NaCl, in 400 ml of 0.2 M Tris-HCl (pH 7.0), and finally in 200 ml of 20×SSC; each incubation was performed for 40 min at room temperature, with one change of liquid. The RNA was blotted to Hybond N (Amersham) membrane filters in 20×SSC and fixed by UV irradiation. The filters were incubated for 2 h at 42°C in 50% formamide, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 10mM EDTA and 50mM sodium phosphate buffer (pH 7.0) provided with 250 µg/ml denatured salmon sperm DNA and 125 µg/ml E.coli tRNA. For hybridization, the solution was replaced by the same mixture with the addition of 10⁶ cpm/ml of a complementary RNA probe, prepared by in vitro transcription of various cloned BR repeats as described (23). The hybridization was allowed for 15 h at 42°C. The filters were washed once for 15 min in 2×SSC at room temperature and then at 65°C for 1 h in 2×SSC and 1% SDS, then 1 h in 2×SSC and 0.1% SDS and finally for 1 h in 0.1×SSC and 0.1% SDS. The radioactivity was revealed by autoradiography.

RNA band retardation assay

Three 18mers were synthesized in a Pharmacia Gene Assembler: 1) an oligonucleotide complementary to the coding strand of BR1γ: 5'-TGGTTTTCCTGTTCTAGG-3'; 2) an oligonucleotide complementary to the coding strand of BR2α: 5'-GCTTGGTTTGCTGTGTTTT-3'; and 3) an oligonucleotide identical with the coding strand of BR2α: 5'-AAACACAGCAAAACCAACG-3'. After synthesis the oligonucleotides were purified over Sephadex G50. They were eluted with water in the void volume and were concentrated to 0.6 mg/ml by freeze-drying. The oligonucleotides were labelled with T4 polynucleotide kinase and [γ-32P] ATP and electrophoresed on a sequencing gel. More than 90% of the synthesized material was 18 nucleotides long, and the oligonucleotides were used for hybridization without further purification.

BR granules used for the RNA band shift assay were prepared as described above, but the sucrose gradients were supplemented with 0.2 M NaCl to optimize the ensuing hybridization. Oligonucleotides were added to 4 µg/ml directly to the sucrose gradient fractions containing BR granules. The concentration of BR granule RNA was around 5 ng/ml, according to the number of granules seen in the EM. tRNA was added to 100 µg/ml, and hybridization was allowed for 5 min at 37°C. Then the mixture was put on ice and precipitated with 2.5 volumes of ethanol. RNA was released and analysed as described above. In control experiments, the BR granule preparation was replaced by RNA isolated from BR granules and cytoplasm.

RESULTS AND DISCUSSION

The electrophoretic complexity of 75S RNA in BR granules

The BR granules were isolated from a Chironomus salivary gland homogenate by sucrose gradient centrifugation. To trace nuclear RNP, the glands had been incubated in the presence of radioactive RNA precursors. A 90 min incubation time was chosen to assure that essentially only nuclear RNA was labelled (24). The BR granules sedimented at 300S and gave rise to a peak in the radioactivity profile (Fig. 1). When RNA, isolated from the 300S peak, was electrophoresed under non-denaturing conditions, three main 75S RNA bands were resolved (I−III) (Fig. 2). Bands I and II were regularly present in both fourth instar larvae and prepupae, while band III appeared preferentially in the late fourth instar and in prepupae (not shown). As a rule band II was by far the predominant one (Fig. 2). Sometimes one or more of the bands appeared as doublets (see e.g. band III in Fig. 5 and band I in Fig. 6). It should finally be noted that band I RNA was enriched in the more rapidly sedimenting 300S particles and band III RNA in the more slowly sedimenting ones (lanes A, B vs lanes E,F in Fig. 2).
Identification of the individual 75S RNA species

Glands from several larvae were incubated with [5,6-3H] uridine and their sister glands with [α-32P]ATP. The 3H- and the 32P-labelled BR granules were isolated separately, and their RNA was electrophoresed in parallel lanes in a non-denaturing gel and transferred to a membrane filter (Fig. 3). Lanes of the filter containing the 3H-labelled RNA were hybridized with 32P-labelled cRNA probes transcribed from cloned BR repeats (BR1γ, BR1β, BR2α or BR2β); lanes with the 32P-labelled 75S RNA served as a control showing the position of bands II and III and after long exposure also band I. By comparing the hybridized and the incorporated radioactivity patterns, we could assign BR sequences to specific 75S RNA bands.

To locate the transcripts of the BR1 gene we used two probes, a BR1γ and a BR1β sequence. These two probes correspond to large blocks of tandemly repetitive sequences within 75S RNA (25, 26). Direct sequence analysis of cloned genomic DNA fragments indicated that these two blocks reside within the same BR1 gene (G. Paulsson, C. Hög, K. Bernholm and L. Wieslander, manuscript in preparation). As shown in Fig. 3 B and D, the BR1γ and BR1β sequences both hybridized to band I RNA.

The BR2.1 gene transcripts were identified by a BR2α probe and the BR2.2 transcripts by a BR2β probe. Again these cRNA probes correspond to large blocks of repetitive sequences within the respective transcripts (27–29). The BR2α probe hybridized to band II showing that this band contains transcripts from the BR2.1 gene (Fig. 3F). The BR2β probe hybridized to band III (Fig. 3 H), indicating that the BR2.2 transcripts are present in this band.

The migration differences between the 75S RNA species observed under non-denaturing conditions may only to a minor extent be due to differences in molecular size. This was demonstrated by electrophoretic analyses of 75S RNA under denaturing conditions (Fig. 4). While the BR1 and BR2 transcripts were clearly separated without denaturation (Fig. 4A), they migrated so closely after denaturation (Fig. 4B–D) that only a slight asymmetry of the single band in Fig. 4D could be observed. Similarly, the BR2.1 and BR2.2 transcripts differed only slightly in size and could be separated under denaturing conditions only after prolonged electrophoretic runs (30). The remarkable separation of the various 75S RNA species under non-denaturing conditions is therefore likely to be largely due to differences in secondary structure.

The transcripts from the BR1, BR2.1 and BR2.2 genes may account for the complex pattern of 75S RNA observed in non-denaturing gels; we did not detect bands that did not hybridize to the BR probes used. It is, however, not possible in this type of experiment to exclude that there are additional comigrating RNA species with a different genetic origin. To this aim we worked out an RNA band shift technique.

The RNA band retardation assay

We found that it is possible to shift the position of specific BR RNA transcripts by prehybridization with complementary oligonucleotides. In our experiments we used three 18-mers. The first one was complementary to the BR2α RNA. This sequence was chosen so that it had the least possible complementarity to the BR1γ RNA sequence. The second oligonucleotide was complementary to the BR1γ transcript and had minimum complementarity to the BR2α transcript. This oligonucleotide is also complementary to 16 out of 18 nucleotides in the BR2β RNA sequence. The third oligonucleotide had the same sequence as the RNA strand of the BR2α sequence, i.e. it should not hybridize with any known BR RNA sequence.

In separate experiments the three oligonucleotides were hybridized to isolated BR granules, and RNA was extracted and electrophoresed without denaturation. The nucleic acids were transferred to membranes, and each sample was probed with four 32P-labelled BR cRNAs probes (BR1γ, BR1β, BR2α or BR2β). Separate samples with no oligonucleotides added were treated in parallel. Non-hybridized, 32P-labelled 75S RNA served as a marker to identify the positions of the various RNA species. The results are presented in Fig. 5. The added oligonucleotides are indicated above (C represents the marker), and the BR cRNA hybridization probes below the autoradiograms. Identical results were obtained when the BR probes were hybridized to isolated BR RNA instead of to BR granules (data not shown).

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The BR2a oligonucleotide complementary to the BR2.1 transcript (2α in Fig. 5) retarded the migration of BR2a RNA: the BR2a band was replaced by two, more slowly migrating bands (panel 3: lane 1 vs lane 4); the BR1γ and BR1β bands were not changed (panel 1 and 2: lane 1 vs lane 4 in both panels), nor was the BR2β band (panel 4; lane 1 vs lane 4). Thus, the BR2a oligonucleotide can selectively shift the BR2.1 transcripts. The two retarded bands probably represent two different conformational states of the hybrid between the oligonucleotide and the BR2α transcript. It is less likely that they indicate different transcripts containing the BR2α repeat, since the relative abundance of the bands depended on the hybridization temperature (data not shown).

The BR2α oligonucleotide with the same sequence as in the BR2.1 transcript (2α in Fig. 5) did not alter the position of any of the 75S RNA species (lane 2 vs lane 4 in all the four panels).

The BR1γ oligonucleotide retarded 75S RNA containing both BR1γ and BR1β sequences as well as the BR2β sequence (lane 3 vs lane 4 in panel 1, 2 and 4, respectively). As mentioned above, the BR1γ oligonucleotide is to a large extent complementary to the BR2β RNA sequence. The BR2α RNA was not appreciably retarded in the experiment with the BR1γ oligonucleotide (panel 3; lane 3 vs 4).

We conclude that the short, synthetic oligonucleotides can be used to shift the position of complementary transcripts. The BR2α oligonucleotide turned out to be specific for the BR2.1 transcripts, while the BR1γ oligonucleotide retarded both the BR1 and BR2.2 transcripts. The BR2α oligonucleotide identical to the RNA sequence did not change the migration rate of any 75S RNA species. These experiments therefore demonstrate that the two complementary oligonucleotides available, the BR1γ and the BR2α, can be used to shift the position of the BR1, BR2.1 and BR2.2 transcripts in the gel.

Application of the RNA retardation assay

If we hybridize 32P-labelled 75S RNA to non-radioactive oligonucleotides, we should be able to decide whether or not all the 75S RNA in a given band is displaced. Under conditions of excess oligonucleotides, RNA that did not shift should lack the BR sequences within the oligonucleotide. In such an experiment the BR2α complementary oligonucleotide (2α), but not the BR2α non-complementary oligonucleotide (2α), shifted the position of the BR2.1 band (Fig. 6: lanes 1 and 2 vs lane 4). As in the earlier experiments (Fig. 5) two major, more slowly migrating bands appeared. No radioactivity remained at the former position of the BR2.1 band. Furthermore, as a consequence of the shift of the BR2.1 band, a putative second BR1 band as well as the BR2.2 band can be clearly seen in lane 1 (the two BR1 bands have been indicated with bars and the BR2.2 band with a filled circle). The BR1γ oligonucleotide changed the position of the two BR1 bands and the BR2.2 band; two new bands appeared as well as additional material on the slowly migrating side of the BR2 band (lane 3 in Fig. 6). On the basis of the extent of the shifts of the BR1 and the BR2.2 bands in Fig. 5 and the relative intensities of the bands in Fig. 6, we suggest that the two new bands correspond to the two BR1 bands (bars), and that the additional activity corresponds to the BR2.2 band (filled circle). If this interpretation is correct, again no radioactivity was left at the former positions of the BR1 and BR2.2 transcripts. Thus, the two complementary oligonucleotides retarded all the transcripts in bands I, II and III. We conclude that all the 75S RNA species can be accounted for as transcripts from the BR1, BR2.1 or BR2.2 genes.

Our results support the view that only a very limited number of BR genes are active in the salivary glands. We base this conclusion on two findings: First, all the non-shifted and all the shifted bands were identified by hybridization with cRNA (Figs. 2, 3, 5, 6); this assay is gene specific. Second, all the RNA bands can be displaced by complementary oligos (Fig. 6). There is still the formal possibility that we overlook transcripts which meet three criteria: I) they comigrate with known transcripts, II) they hybridize to the same oligo and III) they are retarded and comigrate again with one of the known bands. We regard this possibility as unlikely, but not excluded. Moreover, our conclusion is in good agreement with a number of independent findings. In a hybridization saturation study Wieslander could show that there are probably not more than 4–5 transcriptionally active BR genes (31). It has been demonstrated that there are two genes in BR2 (BR2.1 and BR2.2) (30), while there is no evidence for more than one 75S RNA gene in BR1 (G. Paulsson, C. Höög, K. Bernholm and L. Wieslander, manuscript in preparation). The analysis of the expression pattern of the SP-1 secretory proteins, i.e. the proteins encoded in 75S RNA, lead to a similar result (32). Thus, we conclude that under normal growth conditions only three BR genes generate 75S RNA, i.e. the BR1, BR2.1 and BR2.2 genes.
We noted that one or more of the three main 75S RNA bands can sometimes occur as double bands. In our Northern blotting experiments with BR cRNA probes the double bands migrated as size variants (e.g. Fig. 5). Such a heterogeneity among the RNA transcripts is in good agreement with the heterogeneity reported for the corresponding giant-sized secretory polypeptides (33, 34).

In the present study we used the RNA band shift assay to establish whether a specific RNA band in a gel comprises one or more RNA species. This was feasible because the RNA which hybridized to the oligonucleotide was shifted from comigrating RNA molecules. This method might also be used for preparative purposes. Another application would be to determine whether two known, comigrating sequences reside in the same transcript. To this end the RNA molecule is shifted in the gel with an oligonucleotide complementary to one of the sequences. Subsequently, hybridization with the second sequence will establish whether or not this sequence has also moved to the new position. The feasibility of such an application was demonstrated in this study: the RNA species shifted with the BR1γ oligonucleotide also hybridized to the BR1β sequence (Fig. 5).

The precise reason for the retardation is not clear. As the shifted bands migrate between native RNA and fully denatured RNA, we assume that the shift is due to a change of the secondary structure, i.e. a partial unfolding of the RNA molecules. The shift depends on the presence of complementary oligonucleotides and, therefore, the unfolding should be brought about by molecular hybridization. The oligonucleotides could compete out intramolecular RNA double strands or, alternatively, the oligonucleotide-RNA double-stranded region could become more rigid than single-stranded RNA and in this way interfere with the folding of RNA. The striking finding was that the bands moved from one discrete position to a new discrete position and that there were no intermediate states which would give rise to a smear in the gel. Since in our experiments the oligonucleotide was present in about a thousand fold excess, it seems likely that all binding sites have been saturated.

In the RNA band shift assay we used oligonucleotides complementary to parts of tandemly repetitive elements present in well above one hundred copies along each 75S RNA molecule (17, 18). To test whether the retardation assay is applicable not only to repetitive sequences, we subjected a short in vitro transcript from the 5' end of the E. coli ompA RNA to the same experimental system. This RNA contains a well-established stem-loop structure (35), and it was discretely retarded by an 18mer oligo complementary to the stem (not shown). We expect from our results that the retardation of RNA is correlated to the ability of an oligo to disrupt secondary structure. We are now investigating whether the RNA band retardation assay can be used to identify RNA sequences which are involved in secondary structure.
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