On the Structure of Rapidly Labeled Rat Liver RNA

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Rat liver total RNA, extracted by three phenol methods relying upon 4-aminosalicylate, naphthalene-1,5-disulfonate, and heat, respectively, gave different migration patterns, most pronounced for rapid label. The difference could not be attributed to differential extraction.

By the 4-aminosalicylate method, large amounts of rapidly labeled structures sedimenting up to 20 000 S were isolated. These structures, consisting of aggregates of heterogeneous RNA, were examined in the present report. They complied with most of the criteria used to classify giant heterogeneous nucleoplasmic RNA as a separate entity of covalent molecules, but they were not resistant to treatment with dimethyl sulfoxide or to heating in low-ionic-strength acetate buffers or in formaldehyde.

When such disaggregating treatment was performed on the RNAs extracted by the different methods, the rapidly labeled RNA > 45 S disappeared, and several additional UV-absorbing constituents appeared in the cold-extracted RNAs. This caused a resemblance in the migration and sedimentation patterns of the different RNAs. Radioactive markers added to the extraction buffers prior to homogenization of the livers did not reveal any degradation during extraction or analysis. The results suggest that giant heterogeneous nucleoplasmic RNA from rat liver could essentially be an artifact of extraction.

The presence of giant heterogeneous nucleoplasmic RNA (giant hnRNA) (10–200 S) in eucaryotic cells has been established by many authors (review by Darnell¹). It was identified in rat liver by Schütz *et al.*² A precursor relationship to cytoplasmic messenger RNA (mRNA) (6–30 S)^{3,4} is widely accepted. However, conclusive evidence for specific cleavage has not been found. A remarkably reproducible base

composition has been reported, with uridine content in excess of the corresponding DNA, and in excess of that of the cytoplasmic mRNA of the same cells.^{3,5–8} Its biological role is thus still uncertain.

The existence of covalent molecules of corresponding size was questioned by Mayo and de Kloet⁹ who showed that formaldehyde treatment converted rapidly sedimenting rapidly labeled RNA (rlRNA) to structures sedimenting as mRNA. Recent reports, using different disaggregating treatments, and different methods for analysis of the treated RNA, have been contradictory.^{10–15} Even if such a conversion could be found to be accompanied by a corresponding change in the electrophoretic pattern, the possibility of giant size transcription with later conversion to smaller molecules could not be excluded.

In an examination of different methods for extraction of rat liver total RNA¹⁶ large variations were found in the rapidly sedimenting rlRNA. These could not be attributed to differential extraction of the rapidly labeled molecules. Preliminary experiments showed that the amount and size of such molecules, when extracted by a 4-aminosalicylate method, were out of proportion to what could reasonably be expected to represent an *in vivo* state. The present study was undertaken to see whether these structures were consistent with the criteria of giant hnRNA. If so, such molecules could be mere artifacts of extraction, and not necessarily expression of giant size transcription. Besides, the criteria used to classify giant hnRNA as a separate entity of covalent molecules could be evaluated.

The results showed that the extracted aggregates met many of the criteria used for giant hnRNA, and that several of the disag-

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gregating treatments previously used to establish the covalency of this class of molecules in fact did not disaggregate them. However, they could be disaggregated by heating in low-ionic-strength acetate buffer at 50 °C, or by treatment with dimethyl sulfoxide (DMSO). When such treatment was applied to materials extracted by different methods, evidence was obtained that emphasized the tendency of newly synthesized RNA to aggregate during extraction. To exclude the possibility of RNA degradation, controls using both homogeneous and heterogeneous markers were included.

MATERIALS AND METHODS

Animals. Male 250 g CD*F inbred rats (Charles River Breeding Laboratories, Wilmington, Mass.) were used.

Cell fractionation procedures. Liver nuclei were prepared by homogenizing the liver in 13 ml 1.5 M sucrose containing 2 mg/ml bentonite (treated according to Fraenkel-Conrat *et al.*¹⁷). The homogenate was filtered through gauze and layered on a 35 ml linear 1.5–2.25 M sucrose gradient, using a 10 ml 2.3 M sucrose cushion. All sucrose solutions were made up in buffer (Tris 0.05 M, KCl 0.025 M, CaCl₂ 3.3 mM; pH 7.1) containing 0.1 mg/ml dextran sulfate. Centrifugation was performed in a Spinco SW 25.2 rotor for 45 min at 25 000 rpm and 5 °C. The pellet was collected.

Polysomes were prepared after homogenization of the liver in two volumes chilled 0.25 M sucrose in TKM-buffer (Tris 0.05 M, KCl 0.025 M, MgCl₂ 2.5 mM; pH 7.0), containing 2 mg/ml bentonite. The homogenate was then centrifuged at 15 000 *g* for 10 min at 2 °C, and the supernatant made 0.4 % w/v with respect to deoxycholate. 15 ml was applied on top of two layers of sucrose in TKM-buffer, 10 ml 0.5 M sucrose and 5 ml 1.0 M sucrose. Centrifugation was performed in a Spinco SW 25.1 rotor for 3 h at 25 000 rpm and 2 °C. The pellet was used as polysome source.

Extraction procedures. Method 1 was a cold-phenol method based on the 4-aminosalicylate method of Parish and Kirby.¹⁸ Method 2 was modified from the cold-phenol naphthalene-1,5-disulfonate method of Attardi *et al.*⁵ Method 3 was modified from the hot-phenol method of Warner *et al.*¹⁹ Details have been given previously.¹⁸

Preparation of E. coli 16 S (10 S) rRNA marker. *Escherichia coli* K12 cells were grown logarithmically for 2 h in 500 ml of a minimal salt medium containing 0.4 % w/v glucose and 200 μ Ci ³H-uridine. Ribosomal RNA (rRNA) was extracted by a modified Method 3, extracting at 4 °C, and the 16 S rRNA purified by repeated ultracentrifugations. After DMSO-

treatment, the peak was recovered as a 10 S peak.

Chemical analysis. Protein was determined by the method of Lowry *et al.*²⁰ DNA was determined by a diphenylamine reaction.²¹

Ultracentrifugation. Sucrose gradient analysis and sedimentation coefficient determinations have been described previously.¹⁶ The very high *s* values (≥ 2000 S) were determined by examining pellet material after short runs at reduced speed (10 000 rpm), taking the sedimentation during the acceleration and deceleration of the rotor into consideration. The possibility that the pelleting could be due to irregular sedimentation, was excluded by running in a sector-formed partition cell in a Spinco model E analytical ultracentrifuge.

Before density equilibrium centrifugation, RNA was fixed at 4 °C in 0.05 M sodium phosphate-citrate pH 7.0, containing 5–8 % w/v formaldehyde. Cesium chloride gradients were run in a Spinco SW 65 rotor for 66 h at 25 °C. Mixed cesium chloride: cesium sulfate 4:1 v/v (equal densities) gradients were run in a Spinco 65 fixed angle rotor at 30 °C and 61 000 rpm. All solutions contained 1.5 % w/v formaldehyde and 0.05 M sodium phosphate-citrate pH 7.0.

Polyacrylamide gel electrophoresis was performed as described previously.¹⁶

³²P Base composition analysis. Sucrose gradient fractions were pooled according to the UV-pattern, precipitated after addition of carrier RNA, and hydrolyzed in 0.3 M potassium hydroxide at 37 °C for 17 h. The nucleotides were separated by high voltage electrophoresis, using Whatman No. 1 paper as support.

Treatment with disaggregating agents. Formaldehyde treatment was performed by heating RNA in 0.01 M sodium acetate pH 6.0, containing 7.5 % w/v formaldehyde, at 63 °C for 15 min before chilling. RNA samples to be treated with DMSO were dissolved in DMSO containing 0.01 M LiCl and 1 mM EDTA.²²

RESULTS AND DISCUSSION

Aggregate characteristics

Physical state. rRNA₁^{*} isolated 20 min after administration of radioactive orotic acid, sedimented heterogeneously with 3 % of the radioactivity at 2000 S or faster, and 1 % at 20 000 S or faster.

No DNA was found in material ≥ 140 S. The protein content was 5–30 μ g per mg total RNA₁, all sedimenting at *s* values ≥ 140 S. A reduction in protein content upon re-extraction with phenol with no change in RNA-pattern,

* Subscript ₁, ₂, or ₃: That which pertains to Extraction Method 1, 2, or 3.

as well as cesium salt density gradient centrifugations, indicated that this protein was merely co-sedimenting. The sedimentation patterns were also unaffected by treatment of the isolated RNA with the lipid solvents ethanol, ethyl-ether, or methanol-chloroform 1:2 v/v, or exposure to pronase, DNAase, or amylase. RNAase treatment caused both rapid label and optical density to be confined exclusively to the top fractions of the gradients. The rapidly sedimenting substances therefore appear to consist of RNA without other macromolecules. Their conversion to slower sedimenting, faster migrating structures on moderate heating in a low-ionic-strength acetate buffer or by DMSO-treatment (see below), indicates that they consist of hydrogen-bonded aggregates.

Origin. By separate extraction of RNA from nuclei and polysomes, rRNA₁ sedimenting at *s* values comparable to those obtained by extracting whole liver tissue could be demonstrated only in the nuclear fraction. Polysomal rRNA sedimented in the region 5–35 S.

Kinetics. All RNA sedimenting faster than 65 S had identical labeling kinetics. Except for the growing polynucleotide chains,¹⁶ it was labeled faster than any other fraction. The absolute amount of radioactivity ≥ 140 S was maximal about 20 min after administration of radioactive orotic acid (Fig. 1a). Its relative specific activity

showed a steady decline with time (Fig. 1b), demonstrating the high turnover of the fast sedimenting RNA₁, and indicating that newly synthesized or even incomplete RNA strands are especially liable to aggregation.

Apparent base composition. Base composition analysis of RNA₁ isolated 1 h after intraperitoneal injection of ³²P_i showed varying composition of the RNA in the heavy parts of the gradient. Even runs of the same material on parallel gradients showed considerable differences in base composition at the same gradient level. (Repeated analysis of material from the same hydrolysis tube showed identical results). However, the RNA in the regions > 65 S had adenine + uracil/guanine + cytosine ratios ≥ 1 . With references to the polyadenylic sequences in nuclear and cytoplasmic mRNA, a relatively high adenine content, ranging from 25 to 31 mol per cent, was noteworthy. No substantial participation of rRNA/pre-rRNA could be detected.

Susceptibility to disaggregating conditions. Exposure to low ionic strength at room temperature²³ did not affect the rapidly sedimenting rRNA₁, but retarded the 10–45 S rRNA (Fig. 2a, b). This retardation was reversible (Fig. 2c), indicating a mere conformational change.

Heating at 50 °C for 3 min in the presence of 0.1 M sodium chloride left the rapid label pattern almost unaffected (Fig. 2d). When such heating was performed in 0.1 M sodium acetate, a substantial amount of rapidly sedimenting radioactivity was converted to material sedimenting in the 5–28 S region, and after heating in low-ionic-strength acetate buffer, nearly all radioactive material sedimented in the 5–35 S region (Fig. 2e). A parallel conversion was seen by gel electrophoresis (Fig. 5a, d). No specific release of material from the aggregates was found corresponding to rRNA or tRNA (Fig. 2f). Molecules without a firm secondary structure therefore seem to aggregate most readily. Local concentrations in the cell might also be of importance.

Five mM magnesium prevented any change during heating at 50 °C for 5 min. Concentrations of RNA above 1 mg/ml stabilized the original structures somewhat, increasing with greater concentrations. Treatment at room temperature with 1 % w/v SDS, 3–8 M urea or ethylurea and/or 1–10 mM EDTA did not

Fig. 1. Kinetics of labeling of apparent giant hnRNA₁. RNA₁ was isolated at different intervals after injection of 50 μ Ci ¹⁴C-orotic acid. 1 mg RNA was subjected to ultracentrifugation (10–40 % w/w sucrose in 0.1 M sodium acetate pH 6.0, Spinco SW 25.1, 24 000 rpm, 5 °C, 14 h). The pellet (≥ 140 S) was eluted, and the amount of RNA and of radioactivity determined.

Fig. 2. The effects of low ionic strength and of elevated temperatures on the sedimentation pattern of RNA₁. (a–e) 1 mg RNA, extracted 10 min after administration of 300 μ Ci 3 H-orotic acid, was examined after incubation under the following conditions: (a) Control. No incubation. (b) In 5 mM EDTA-tris pH 7.0, at 25 °C for 1 h. (c) As under (b), with sodium acetate added to 0.1 M before chilling. (d) In 0.01 M sodium acetate containing 0.1 M sodium chloride and 1 mM EDTA at 50 °C for 3 min. (e) In 0.01 M sodium acetate pH 6.0 containing 1 mM EDTA, at 50 °C for 3 min before addition of acetate to 0.1 M. (f) rRNA ≥ 140 S from an animal labeled for 30 min with 50 μ Ci 14 C-orotic acid was heated as under (e), and recentrifuged.

10–40 % w/w sucrose, Spinco SW 25.1, 24 000 rpm, 5 °C, 14 h, gradient buffer: (a,c–f) 0.1 M sodium acetate pH 6.0, (b) 5 mM EDTA-tris pH 7.0. —, OD_{260 nm}; (a–e) - - -, 3 H-dpm $\times 10^{-3}$; (f) - - -, 14 C-dpm $\times 5 \times 10^{-4}$.

affect the gradient patterns, but abolished the adsorption of rRNA to the centrifuge tubes.

After treatment with formaldehyde, the changes were more pronounced than after heating in low-ionic-strength buffer, but some fast-sedimenting, rapidly labeled structures persisted (Fig. 3a). The 28 S rRNA was then partly excluded from the gels, indicating a severe derangement of the molecular conformation, possibly also formation of intermolecular methylene bridges.²⁴ DMSO-treatment caused essentially the same changes as heating in buffer but some rRNA, less than after formaldehyde-treatment, still sedimented at 2000 S or more (Fig. 3b).

Aggregate formation. When 20 min labeled RNA₁ > 80 S was collected, dissociated by heating, and added to the aqueous phase before unlabeled rat liver total RNA was extracted, aggregation could directly be demonstrated, although to a less extent than observed after *in vivo* labeling (Fig. 4a, b). After re-dissociation, the radioactivity maximum sedimented as after the first dissociation (Fig. 4c).

Relationship to giant hnRNA. The rapidly sedimenting rRNA₁ possesses several of the characteristics reported for giant hnRNA. It is the most rapidly labeled fraction found, except for the growing polynucleotide chains. It consists of pure RNA, and can be demonstrated

Fig. 3. Dissociation of rat liver RNA. RNA labeled for 10 min was examined by sucrose gradient centrifugation after treatment with (a) formaldehyde, (b) DMSO. The material had been stored for four years between the experiments reported in Fig. 2a–e and the experiments reported in this figure. Legend otherwise as in Fig. 2a.

only in the nuclear fraction. Its sedimentation pattern is fairly reproducible with 25–35 % of the total radioactivity sedimenting ≥ 140 S after a 20 min pulse. It resists treatment with 3–8 M urea or ethylurea, 1–2 % SDS, 1–10 mM EDTA, or low ionic strength at room temperature. It is also unaffected by heating at 50–60 °C in 0.1 M sodium chloride for 3–5 min. In spite of this, it is obviously an artifact of extraction, because it sediments at s values up to 20 000 S, and aggregation can directly be demonstrated.

To be designated as covalent, RNA molecules should be completely resistant to treatment with DMSO or to heating in low-ionic-strength acetate buffer containing 1 mM EDTA for removal of divalent ions. The difference between sodium chloride and sodium acetate is not understood, but Tal²⁵ noticed that potassium acetate depressed the melting temperature of rRNA. Parallel examination by ultracentrifugation and gel electrophoresis should be performed to determine whether changes in charge or in conformation *versus* aggregation/disaggregation have taken place. Because formaldehyde-treated RNA is partly excluded from acrylamide gels, the use of this denaturing agent is not advocated.

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Fig. 4. Aggregation/disaggregation of rRNA₁. (a) 20 min labeled RNA, originally sedimenting ≥ 80 S, was collected and dissociated by heating at 57 °C for 7 min in 0.01 M sodium acetate pH 6.0. The dissociated material was added to aqueous solution₁ before extraction of unlabeled rat liver total RNA. The extracted material was then dissociated by heating. (a) Dissociated rRNA before addition to aqueous solution₁. (b) Total RNA after extraction. (c) Total RNA after dissociation.

10–40 % w/w sucrose in 0.1 M sodium acetate pH 6.0, Spinco SW 25.1, 24 000 rpm, 5 °C, 14 h. —, OD_{265 nm}; ----, ¹⁴C-dpm $\times 10^{-2}$.

Although DNA-like with respect to a high adenine+uracil content, our apparent giant hnRNA₁ did not have the high uracil content reported for giant hnRNAs. A specific base composition would suggest a separate molecular species, but determinations made by phosphorus-32 nearest neighbor technique must be regarded with caution.

Fig. 5. Disaggregation of rat liver total RNA extracted according to three methods. RNA, extracted 20 min after administration of 50 μ Ci 14 C-orotic acid, heated in 0.01 M sodium acetate pH 6.0 at 50 $^{\circ}$ C for 3 min prior to electrophoresis. (a) RNA₁, (b) RNA₂, and (c) RNA₃: Unheated, run in magnesium-buffer. (d) RNA₁, (e) RNA₂, and (f) RNA₃: After heating, run in EDTA-buffer.

2.6 % w/v acrylamide, 12 mA/tube, 20 $^{\circ}$ C, 2 3/4 h. —, densitometer tracing_{265 nm}; ●, 14 C-dpm/mg gel.

Disaggregation patterns

When materials extracted by the 3 different methods were examined after heating in low-ionic-strength acetate buffer, it was found that the aggregates of rRNA₃ at 48, 37, and 33 S were harder to dissociate than those obtained by the other methods (Fig. 5a–f). The 28 S peak of RNA₁ and RNA₂ became reduced, while the 18 S peak remained unaffected. Additional peaks between the ribosomal peaks and in the

4–18 S region made the UV-patterns of RNA₁ and RNA₂ approach that of RNA₃, demonstrating which components of RNA₃ that could be attributed to heating during extraction. A comparison of the UV-patterns of unheated RNA with those obtained after heating (Fig. 5) indicates that the 25 S, 21.5 S, and 20 S peaks as well as the 12.8 S, 9.5 S, and 7.5 S peaks may represent *in vivo* existing separate molecules.

Heating at 50 °C for 3 min in 0.01 M sodium acetate usually made all rRNA₁ enter the gels, with only traces of radioactivity migrating slower than the 45 S component, and with a main peak at 32 S (Fig. 5d). The amount of rRNA₃ on top of the gels was reduced, but some radioactivity usually persisted (Fig. 5f), unless all aggregates of rRNA were dissociated. Such heating made these radioactivity patterns approach that of unheated rRNA₂. However, parallel heating of rRNA₃ also altered the migration patterns of this rRNA species, with a decrease in the amount of radioactivity in the region > 18 S, and an increase in the 4–18 S region. Some label still migrated as a shoulder at 45 S (Fig. 5e). Heating of rRNA₁ and rRNA₃ at higher temperatures (> 60 °C) caused a further conversion in their patterns towards that of heated rRNA₂.

After heating at 50 °C, similarity in the patterns of the different rRNAs was in general found when materials with labeling times ≤ 10 min were investigated, and the UV-absorbing rRNA aggregates were dissociated. In these materials the amount of radioactivity in pre-rRNA/rRNA was negligible. Increase in the incubation times from 3–5 min to 1 h did not affect the patterns significantly. A further conversion was seen on increased heating, occurring in a parallel fashion for the different materials.

The conversion of the isolated giant hnRNAs to slower sedimenting, faster migrating structures demonstrates their aggregate nature, and supports the findings of Mayo and de Kloet.⁹ All phenol methods examined caused aggregation of RNA. The preferential aggregation of newly synthesized RNA probably reflects a less firm secondary and tertiary structure of these molecules. The tendency towards aggregate formation varies according to the extraction method,¹⁶ as does the dissociation temperature of the formed aggregates. One cannot exclude a transcription giving giant molecules which are immediately cleaved without dissociation of the fragments. However, hard evidence for the *in vivo* existence of rRNA molecules larger than corresponding to 45–50 S cannot be found.

The rapid label in the 45 S and 36 S regions became reduced on heating, and the radioactivity accumulated as a 32 S peak. Convincing evidence has been obtained by electron microscopy (review by Bush and Smetana²⁶) and by

disaggregating experiments^{9,27} to demonstrate the transcription of 45 S pre-rRNA as a single polynucleotide chain. The possibility exists, however, that the molecules do not immediately dissociate after scission. Possible co-migration of other rRNA species in our experiments necessitates the use of subcellular fractionation to settle the question.

After heat treatment, rRNA peaks were observed at 28–32 S, 26 S, 20 S and 4.5 S. Long label (24–48 h) followed the UV-pattern closely.

The possibility of RNA degradation

The size of the rat liver rRNA molecules seemed smaller than could be expected from most previous reports. It was therefore imperative to exclude the possibility of degradation of the molecules during their extraction, storage or analysis.

RNAase activity during the extraction seemed unlikely: The ice-cold homogenized livers were mixed with phenol within 1 min after decapitation of the animals, and RNAase inhibitors were used throughout. Removal of DNA and glycogen by chemical¹⁸ instead of enzymatic methods did not influence the dissociation. The sedimentation pattern of dissociated heterogeneous rRNA seemed unaffected by passage of the rRNA through the extraction procedure (Fig. 4a, c). Also unaffected was the migration pattern of a homogeneous marker, 16 S (10 S) rRNA from *E. coli* (Fig. 6a–c). As these molecules were partly unfolded by the DMSO-treatment during their purification, their sensitivity towards degradation should be increased. These results, therefore, strongly argue against any degradation during the extraction.

Degradation during storage could be excluded, as the dissociation patterns were unchanged, even after 4 years of storage.

The changes found on dissociation by heating at 50 °C could not be attributed to degradation during the incubation: Parallel changes were observed after treatment with DMSO at room temperature, which obviates both RNAase activity and thermal scissions,²⁸ and no further changes were found when the incubation was increased from 5 min to 1 h. At temperatures ≥ 60 °C, the patterns were not as stable,

Fig. 6. Disaggregation of rat liver total RNA₂, extracted with *E. coli* 16 S (10 S) rRNA marker. The marker was prepared as described under Materials and Methods. Traces of 23 S rRNA were present. The purified ³H-labeled marker was added to aqueous solution₂, before the chopped liver from a rat labeled for 45 min with 50 μ Ci ¹⁴C-orotic acid was added, homogenized, and extracted. Disaggregation was

indicating a possible contribution from hydrolytic/thermal scissions.

As degradation could not be detected during extraction or analysis of the RNA, only trace amounts — if any — of the rat liver RNA molecules can be larger than corresponding to about 45 S. The strong tendency to aggregation of the rapidly labeled molecules should be considered whenever their structure, or factors affecting their turnover, are discussed.

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performed by heating at 50 °C in 0.01 M sodium acetate pH 6.0 for 3 min. (a) Purified disaggregated marker material (25 μ g). (b) Extracted total RNA (300 μ g). (c) Extracted total RNA after disaggregation.

2.6 % w/v acrylamide, EDTA-buffer, 12 mA/tube, 20 °C, 3 h. —, densitometer tracing_{265 nm}; ····, ³H-dpm $\times 10^{-2}$ /mg gel; ●, ¹⁴C-dpm/mg gel.

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