THE ISOLATION AND PROPERTIES OF RETICULOCYTE SOLUBLE 5 S RNA

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1. Introduction

In recent years 5 S RNA was detected on the ribosomes of many organisms, including bacteria, fungi, echinoderms and vertebrates [1–5]. It was, therefore, considered to be a universal component of both prokaryotic and eukaryotic ribosomes. While further analysis of the cellular location of 5 S RNA has shown that the molecule attached to each ribosome is always associated with the large ribosomal subunit [6–9], it was also found on the more mature precursor particles of the 50 S subunit [10–12]. No soluble 5 S RNA was detected in the cytoplasm of either prokaryotic or eukaryotic cells, although the existence of minor quantities of such molecules was not excluded [13, 14]. Recently the existence of free 5 S RNA was described in the soluble fraction of previtellogenic oocytes of *Xenopus laevis* [15]. In the present study, we found that soluble RNA prepared from the cytoplasmic fractions (S_{100}) of reticulocytes, when subjected to gel filtration, displayed in addition to the tRNA peak another peak of low molecular weight RNA. Studies of the physical and chemical properties of this unidentified RNA, together with hybridization competition experiments between this molecule and ribosomal 5 S RNA, provided ample evidence that this low molecular weight RNA is soluble 5 S RNA. The amounts of the soluble 5 S RNA found in the S_{100} fraction of reticulocytes reached 75% of that attached to the ribosomes. Labeling kinetics of soluble and ribosomal 5 S RNA strongly suggest that the soluble 5 S RNA originates from the ribosomal 5 S RNA that was released from the degrading ribosome during the maturation process of the reticulocyte.

2. Materials and methods

Reticulocyte-rich blood was obtained by bleeding common stock rabbits [16] or injecting mice daily with phenylhydrazine [17]. ^{32}P-labeled reticulocytes were prepared by injecting intravenously anemic rabbits with 5–8 mCi [^{32}P]phosphate (Radiochemical Centre, England). The methyl groups of RNA were labeled by injecting mice (~ 50 g) intraperitoneal with 50 μCi of L-([^{14}C]methyl) methionine (Sigma).

The fractionation of reticulocytes into ribosomes and S_{100} fractions was essentially as described previously [16]. For ribosomal 5 S RNA isolation, the RNA extracted from the ribosomes was brought to a final concentration of 1 M NaCl and left overnight at 0°. The precipitated RNA (mainly rRNA) was removed by centrifugation and the 1 M NaCl supernatant was chromatographed on a Sephadex G-100 column [16]. tRNA and soluble 5 S RNA were isolated from the S_{100} fraction that prior to phenol extraction was concentrated by pH 5 precipitation (see fig. 1).

RNA–DNA hybridization: DNA was isolated from rabbit bone-marrow cells by the method of Marmur [18] with minor alterations. Since the rate of labeling of reticulocyte RNA *in vivo* is not sufficient for hybridization experiments, chemical methylation of RNA with [^{3}H]dimethylsulphonate was carried out [19]. The RNA methylated was soluble 5 S RNA and its specific radioactivity reached 8,000 cpm per μg RNA. The hybridization experiments were carried out essentially by the procedure of Hatlen and Attardi [20].

The standard incubation mixture for determining the methyl accepting activity of various RNA species of reticulocytes was essentially as described by Comb [21]. 1 mg protein from fresh prepared *E. coli* S_{100} fraction was added per incubation mixture as a source

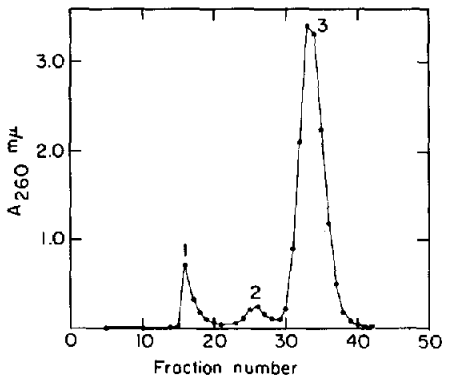

Fig. 1. The purification of soluble 5 S RNA on a Sephadex G-100 column. pH 5 Precipitate prepared from S_{100} fraction of reticulocytes was phenol extracted and alcohol precipitated. 7.5 mg of 5 S RNA were applied to a Sephadex G-100 column (2 X 150 cm) and eluted with 1 M NaCl. 8 ml fractions were collected.

for methylating enzymes. The determinations of ribosomal and soluble 5 S RNA sedimentation coefficients ($S_{20,w}$) were carried out in a Spinco Model E ultracentrifuge using a split beam photoelectric scanner set at 280 nm. The melting profiles of RNA were performed according to the method of Marmur and Doty [22]. Base analysis and gel-electrophoresis of tRNA and soluble and ribosomal 5 S RNA was done as described elsewhere [16].

3. Results and discussion

3.1. *Isolation and properties of a low molecular weight RNA (soluble 5 S RNA) from the S_{100} fraction of reticulocytes*

Soluble RNA prepared by phenol extraction of the pH 5 precipitate from the S_{100} fraction of reticulocytes displayed considerable size heterogeneity when subjected to gel filtration (fig. 1). The major part of the S-RNA extracted from the cytoplasm of reticulocytes consisted of tRNA (fig. 1, elution region 3). There was a small peak of RNA (elution region 1) that corresponded to a mixture of rRNA and mRNA, which most probably originated from residual subunits that were not removed during centrifugation. Between the two peaks of rRNA and tRNA there was an unidentified peak (elution region 2) evidently composed of RNA in that it was labeled by [^{32}P]phosphate, resistant to degradation by pronase and DNAase and degraded by pancreatic RNAase and alkali. The sedimentation coefficient of this RNA (5.1 $S_{20,w}$) appeared to be identical to that of 5 S RNA of ribosomal origin. The low quantities of rRNA found in the S_{100} fraction (fig. 1) exclude the possibility that 5 S RNA found in this fraction was derived from ribosomes not removed during centrifugation. To establish the relationship between soluble and ribosomal 5 S RNA, their different physical and biological properties were compared. The

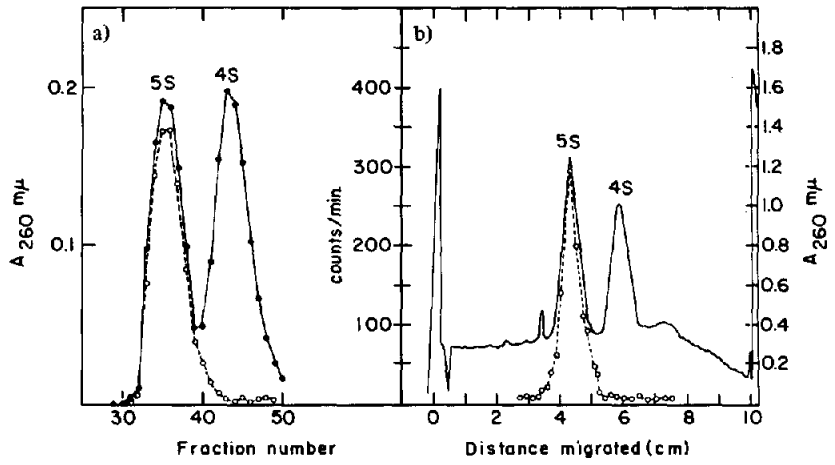

Fig. 2. Chromatographic and electrophoretic analysis of soluble 5 S RNA. a) A tracer amount of ^{32}P-labeled soluble 5 S RNA (20 μg, 5300 cpm) was added to a mixture of unlabeled tRNA and ribosomal 5 S RNA (180 μg each) and filtered through a Sephadex G-100 column (1 X 160 cm). 2.4 ml fractions were collected. b) 20 μg of the labelled material together with cold tRNA and ribosomal 5 S RNA (50 μg each) was applied to a 7.5% polyacrylamide gel. Scanning and slicing of gels as described elsewhere. (●—●—●) A_{260}; (○- -○- -○) radioactivity.

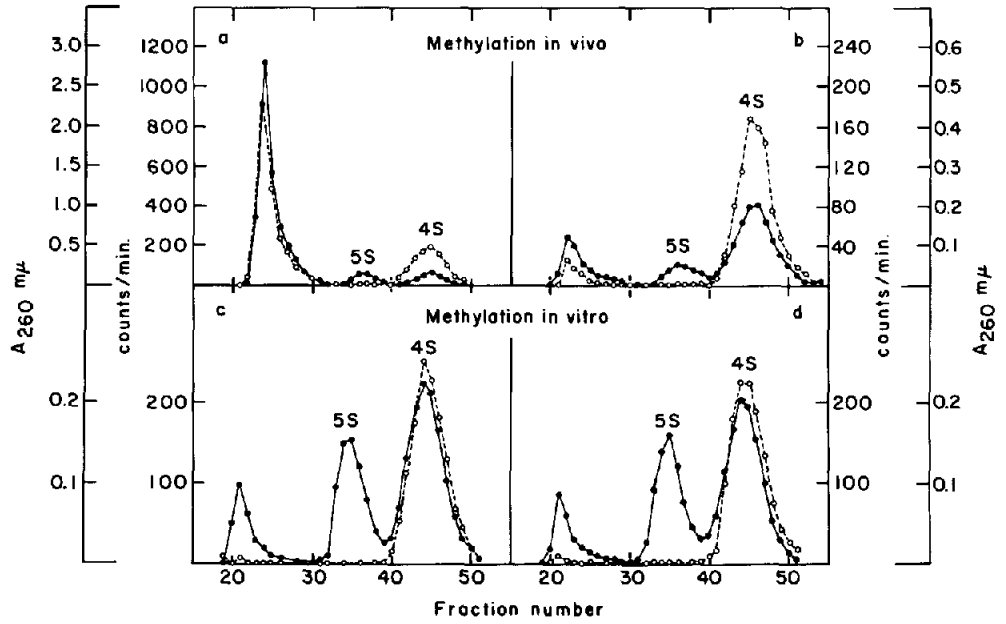

Fig. 3. Distribution of methylated bases in different RNA species following *in vivo* and *in vitro* methylation. a and b) The radioactivity profile is due to *in vivo* base methylation following 40 hr ([^{3}H]methyl) methionine administration to reticulocytic mice. a) 885 μg of [^{3}H]methyl RNA extracted from ribosomes was filtered through a Sephadex G-100 column (1 × 160 cm). b) 115 μg RNA extracted from the S_{100} fraction and applied to gel filtration. c) *In vitro* methylation of ribosomal 5 S RNA and tRNA (each 150 μg). d) *In vitro* methylation of soluble 5 S RNA and tRNA (each 150 μg). For details of *in vitro* methylation see Materials and methods. (•–•–•) A_{260}; (○–○–○) radioactivity.

chromatographic properties of soluble 5 S RNA were further explored by subjecting the ^{32}P-labeled RNA together with cold ribosomal 5 S RNA and tRNA as markers to polyacrylamide gel electrophoresis and rechromatography on Sephadex G-100. It appears that the chromatographic properties of the soluble 5 S RNA as established by both techniques were identical to those of ribosomal 5 S RNA (fig. 2). When soluble and ribosomal 5 S RNA were exposed to thermal denaturation, the melting profiles of both were fully comparable with a midpoint absorbancy rise of 62°. The amounts of soluble 5 S RNA found in the S_{100} fraction of reticulocytes of different preparations ranged from 4–7% of total tRNA of the cells.

The major nucleotide composition of soluble and ribosomal 5 S RNA was identical (Up, 23.4; Gp, 31.6; Cp, 26.8; Ap, 18.2; by per cent). One of the characterizing features of ribosomal 5 S RNA molecule is the absence of any detectable methylated base in it [4, 6, 7], and its lack of methyl accepting activity as demonstrated *in vitro* (Tova Zehavi-Willner, unpublished results). Therefore it was interesting to test whether the soluble 5 S RNA also lacked methylated bases. In fig. 3a, b is compared the radioactivity due to methylated bases in soluble and ribosomal 5 S RNA isolated from mice reticulocytes after *in vivo* exposure to [^{14}C]methyl-methionine. It is clear from these results that radioactivity due to methylated bases is neither associated with the ribosomal 5 S RNA peak nor with the soluble 5 S RNA peak, as expected. For further comparison between the two 5 S RNA molecules, their methyl-accepting activity was assayed using as control tRNA which is an efficient methyl acceptor. Neither ribosomal 5 S RNA nor soluble 5 S RNA were methylated *in vitro* by heterologous methylases from *E. coli*, whereas tRNA was easily methylated (fig. 3c, d).

3.2. *Hybridization competition experiments*

Chromatographic properties as well as other physical and chemical properties strongly suggest that both soluble and ribosomal 5 S RNA belong to a unique species of RNA, the so-called 5 S RNA. To confirm this suggestion, hybridization competition experiments between ([^{3}H]methyl) soluble 5 S RNA and cold

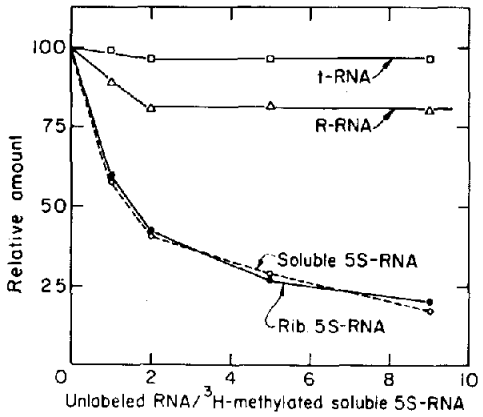

Fig. 4. Competition for sites in bone-marrow DNA between [^{3}H]methylated soluble 5 S RNA and unlabeled tRNA, ribosomal 5 S RNA and rRNA of reticulocytes. Each incubation mixture contained 100 μg DNA, 1 μg [^{3}H]methylated soluble 5 S RNA and varying amounts of unlabeled RNA. Conditions of incubation and isolation of RNA–DNA hybrids are described in Materials and methods.

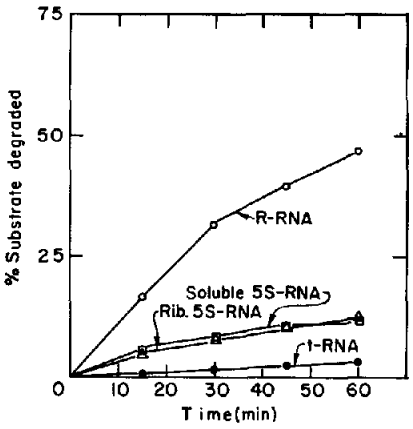

Fig. 5. Comparison of degradation rates of various RNA species by S_{100} fraction. Approx. 50 μg RNA (~ 15,000 cpm) of each species of RNA was separately added to 1 ml S_{100} fraction of reticulocytes. The various mixtures were incubated at 37° and every 15 min 0.1 ml aliquots were removed and their TCA-precipitable radioactivity determined.

ribosomal 5 S RNA, tRNA and rRNA were performed. Optimal conditions were established for hybrid formation between labeled soluble 5 S RNA and bone marrow DNA at saturation. The saturation value obtained for soluble 5 S RNA was 0.014 ± 0.002% of DNA, close to the value obtained by Williamson and Morrison for rabbit reticulocyte ribosomal 5 S RNA. When formation of ([^{3}H]methyl) soluble 5 S RNA–DNA hybrids was followed in the presence of increasing amounts of different unlabeled RNA species, the competition curve between ([^{3}H]methyl) soluble 5 S RNA and ribosomal 5 S RNA substantially coincided with the homologous competition curve. Only a slight competition was observed between labeled soluble 5 S RNA and unlabeled tRNA or rRNA (fig. 4). Thus, these experiments demonstrate that soluble and ribosomal 5 S RNA compete for complementary sites on the DNA molecule, namely the two RNA's belong to the same species of RNA.

3.3. *Relative resistance of 5 S RNA from ribosomal and cytoplasmic origin to reticulocyte RNAase degradation*

The existence in *E. coli* of 5 S RNA regions resistant to nucleolitic attack provided evidence for the secondary structure of this molecule [23]. For further comparison of the conformational state of the ribosomal and soluble 5 S RNA, their resistance to reticulocyte RNAase digestion was tested. Purified ^{32}P-labeled RNA fractions were incubated with unlabeled S_{100} fractions that served as the RNAase source [24], and the extent of RNA degradation was measured. From fig. 5 it can be seen that during 1 hr of incubation almost 50% of the rRNA was degraded. The degradation rate of both ribosomal and soluble 5 S RNA was identical and much lower (~ 15% per hr) than that of rRNA. The lowest degradation rate was that of tRNA (~ 5% per hr), a result already observed by others [24].

3.4. *Metabolic relationship between soluble and ribosomal 5 S RNA*

In order to investigate the nature of the association between the two 5 S RNA molecules from the different fractions of the reticulocyte, the kinetics of their labeling by [^{32}P]phosphate was measured. Reticulocytic rabbits were administered with 7 mCi [^{32}P]phosphate each and blood was collected from them after 16, 40, 63 and 86 hr. From each blood sample the various RNA species were isolated on Sephadex G-100 columns as described in Materials and methods and the specific activity of the RNA's determined. The kinetics of ribosomal 5 S RNA labeling by [^{32}P]phosphate correlated well with that of rRNA (fig. 6), indicating that the synthesis of the two molecules is coordinative, a

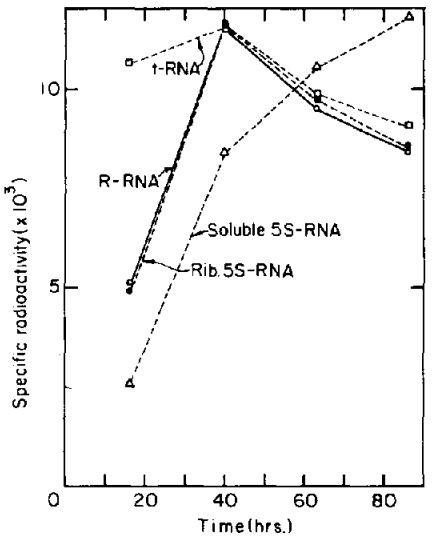

Fig. 6. Kinetics of labeling of soluble 5 S RNA as compared to ribosomal 5 S RNA, tRNA and rRNA. Blood samples were removed at varying times from a reticulocytic rabbit following [^{32}P]phosphate administration. RNA was extracted from ribosomes and S_{100} fraction of the various blood samples and subjected to further fractionation on Sephadex G-100 columns. The specific radioactivity of each RNA species isolated from Sephadex G-100 was determined.

result observed also by others [3, 8]. For brief exposures to [^{32}P]phosphate, when young reticulocytes are preferentially labeled [25, 26], the rate of labeling of soluble 5 S RNA was much lower than that of ribosomal 5 S RNA and rRNA. Forty hours following [^{32}P]phosphate administration, when practically all reticulocytes were labeled, the specific activities of all RNA species were equal. When the time of labeling was prolonged to over 60 hr, a period during which the specific activity of young reticulocytes decreases and that of mature reticulocytes increases, the specific activity of soluble 5 S RNA exceeded that of ribosomal 5 S RNA and rRNA (fig. 6). The mode of labeling of ribosomal 5 S RNA and rRNA as opposed to soluble 5 S RNA suggests that the newly formed 5 S RNA is bound to ribosomes being assembled, and only during the maturation process of reticulocytes, when their ribosomes are degraded, is the ribosomal 5 S RNA released into the cytoplasm as a soluble molecule. From the previous data it can be seen that transfer of the 5 S RNA from the bound state to the free state did not cause changes in either the physical or chemical properties of the molecule. Accumulation of the soluble 5 S RNA in the cytoplasm of the maturing reticulocyte (maturation time, 24 hr [16]) can take place most probably because of the relative resistance of the molecule to the endogenous RNAase (see previous section).

We looked also for soluble 5 S RNA in the S_{100} fraction of rabbit liver and chicken embryo cells. In the S_{100} fraction of liver cells with a ribosomal half life of 5 days [27] we were able to demonstrate the existence of a small pool of soluble 5 S RNA (1–2% of tRNA, as compared to a reticulocyte pool that corresponded to 4–7% of tRNA). In the S_{100} fraction of the fast dividing cells of chicken embryo only traces of soluble 5 S RNA could be detected.

It seems therefore that the content of soluble 5 S RNA in the cytoplasm of cells of various origin may depend greatly on the turnover or degradation rate of their ribosomes. Another factor that may also largely affect the pool of soluble 5 S RNA is its relative resistance to degradation by endogenous RNAase. As far as reticulocytes are concerned, their cytoplasmic RNAase is quite inefficient in degrading the 5 S RNA. The capacity of RNAase from cells of different origin to degrade 5 S RNA should be tested. Another possibility, somewhat less likely for the control of the pool of soluble 5 S RNA in cells, is that 5 S RNA released from degrading ribosomes is transferred to the nucleus and reincorporated into ribosomes being assembled. The existence of such a process might be in accord with an alternative explanation to the phenomenon described by Knight and Darnell [9] and later by Weinberg and Penman [14], that the labeling kinetics of 28 S RNA and ribosomal 5 S RNA in HeLa cells differ.

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