PRESENCE OF POLYADENYLIC ACID SEQUENCES IN RNA OF MEMBRANE-BOUND POLYRIBOSOMES

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Received 8 August 1972

1. Introduction

Polynucleotide sequences rich in adenylic acid have been shown to be covalently bound to mRNA extracted from free polyribosomes of animal cells (for refs. see the review by Darnell et al. [1]). Poly-A sequences have also been found in mRNA of both DNA viruses [2, 3] and RNA viruses [4–6]. The poly-A sequences are approx. 150–200 nucleotides long [1]. We have recently shown that duck globin mRNA isolated from avian erythroid cells incubated *in vitro* with labeled adenosine also contains a poly-A sequence of similar size [7]. We report here that newly synthesized mRNA isolated from polyribosomes bound to the endoplasmic reticulum of mouse myeloma cells contains a poly-A sequence similar to that isolated from free polyribosomes.

2. Methods

Mouse myeloma cells of the P-3 line were grown as previously indicated [8]. RNA was labeled by incubating the cells at a concentration of 3 to 5×10^5 cells/ml for 30 min with 40 ng/ml of actinomycin D and then for 3 hr with 1 μCi/ml of either [^{3}H]adenosine or [^{3}H]uridine. The cells were collected and homogenized as previously described [8]. Nuclei were pelleted during centrifugation at 500 *g* for 5 min. Membrane-bound polyribosomes were separated from the post-nuclear supernatant by centrifugation at 27,000 *g* for 5 min. This membrane fraction was washed once with 10 ml of 10 mM NaCl, 10 mM Tris-HCl pH 7.5 and 1.5 mM $MgCl_2$ (RSB) and resedimented as above. It was then resuspended in 0.5 ml of RSB and the ribosomes were released from the membrane by addition of sodium deoxycholate and Brij-58 each to a final concentration of 1%. Supernatant and membrane fraction were applied to 15–50% sucrose gradients in RSB and polyribosomes fractionated by an 18 hr centrifugation at 16,000 rpm in a SW 25.3 rotor. The gradients were analyzed in a recording spectrophotometer for A_{260} and fractions corresponding to polyribosomes pooled and precipitated by the addition of NaCl to 0.1 M and sodium dodecyl sulphate to 0.5% followed by 2 vol of ethanol.

The precipitate formed overnight at –20° was dissolved in 0.5 ml of 10 mM Tris-HCl pH 7.4, 0.1 M NaCl, 1 mM EDTA and 0.5% Na dodecyl sulphate (SDS buffer) and applied to 15–30% sucrose gradients in SDS-buffer. The gradients were centrifuged 19 hr at 26,000 rpm in the SW 27 rotor. The A_{260} was monitored and the fractions containing RNA sedimenting slower than 4 S were discarded; the remaining portion of the gradient was precipitated by the addition of 2 vol of ethanol. The RNA was dissolved in 0.2 M NaCl, 0.01 M EDTA, 10 mM Tris pH 7.4 and incubated 30 min at 37° with 2 μg/ml of RNA-ase A (Sigma Chemical Co.) and 10 units/ml of T1 RNAase (Worthington Biochemical). Carrier tRNA (0.1 mg) was then added to each sample and the RNA precipitated with 3 vol of ethanol.

After enzyme digestion, the RNA was analyzed on 6 cm 10% acrylamide gels by electrophoresis at 5 mA/gel for 2.5 hr according to Weinberg et al. [9]; the gels were sliced, hydrolyzed with NH_4OH and counted as previously described [9].

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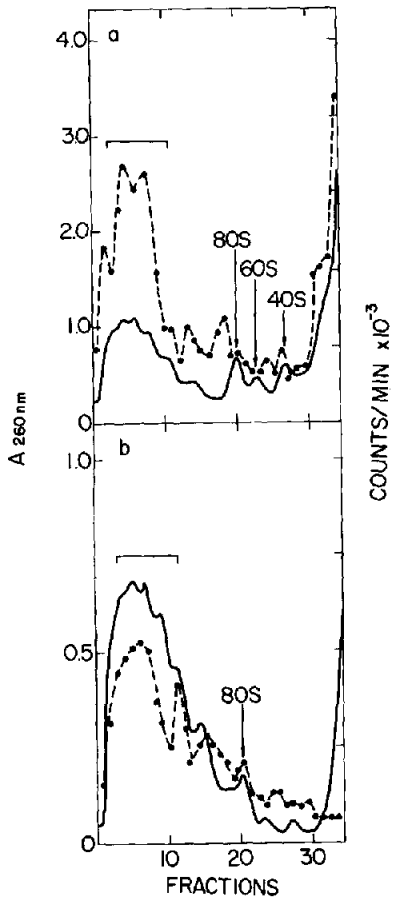

Fig. 1. Isolation of free and membrane-bound polyribosomes. A culture of 300 ml of P-3 cells was incubated for 30 min with 40 ng/ml of actinomycin D and subsequently for 3 hr with 0.3 mCi of [^{3}H]uridine. The free and membrane-bound ribosomes were fractionated by sucrose density gradient centrifugation as described in Methods. The 15–50% sucrose gradients were spun for 18 hr at 16,000 rpm in a SW 25.3 rotor. The gradients were analyzed for A_{260} (——); 0.5 ml fractions were collected and an aliquot counted. The cpm/fraction are indicated (- - - -). Free polyribosomes, (a); membrane-bound polyribosomes, (b). The fractions of gradients *a* and *b* that were pooled for further analysis are indicated.

3. Results

In order to label RNA, myeloma cells were incubated with either [^{3}H]uridine or [^{3}H]adenosine in the presence of 40 ng/ml of actinomycin D. All RNA species, with the exception of ribosomal RNA, were labeled during this incubation [10]. The cells were then homogenized and fractionated to isolate free and membrane-bound polyribosomes (fig. 1). RNA was recovered from polyribosomes by precipitation with ethanol (see Methods). The ^{3}H-labeled RNA is predominantly mRNA since after treatment of polyribosomes with EDTA the labeled RNA sediments at the top of a sucrose gradient, while short treatment with a low concentration of RNAase causes polyribosomes to breakdown to 80 S ribosomes and hydrolysis of the labeled RNA (C. Baglioni and I. Bleiberg, unpublished results). These criteria have been used to iden-

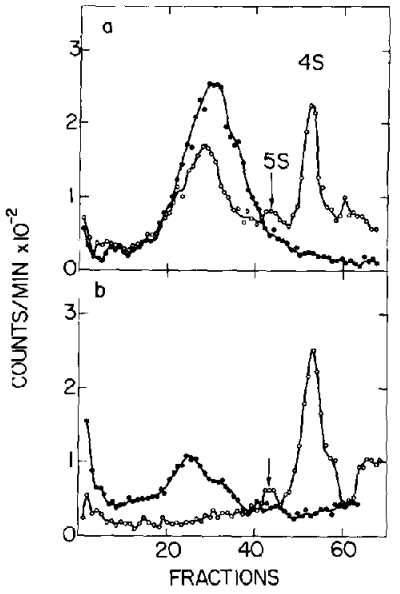

Fig. 2. Acrylamide gel electrophoresis of [^{3}H]adenosin-labeled RNA extracted from free and membrane-bound polyribosomes. The RNA of P-3 cells was labeled with [^{3}H]adenosine in an experiment identical to that described in fig. 1. The ribosomes were separated by sucrose gradient centrifugation and the fractions corresponding to those of gradient *a* and *b* of fig. 1 isolated. The fractions from each gradient were combined and centrifuged 3 hr at 160,000 g_{av}. The pellets obtained were resuspended in 1 ml of SDS-buffer with the aid of a small magnetic stirrer; 0.2 ml were applied to 15–30% sucrose gradients in 0.01% SDS buffer. The gradients were centrifuged 19 hr at 26,000 rpm in the SW 27 rotor. The top portion of each gradient was removed and the RNA precipitated from the remaining part of the gradient with ethanol as described under Methods. The RNA was dissolved in 2.5 ml of RNAase digestion buffer; four fifths of the sample was digested as described under Methods and analyzed by acrylamide gel electrophoresis after addition of marker 5 S [^{14}C] RNA. This marker (arrows) allowed us to superimpose the patterns obtained with undigested RNA (o–o–o) on those obtained with the RNA digested by RNAase (•–•–•). The positions of migration of 4 S and 5 S RNA are indicated. a) RNA extracted from free polyribosomes; b) RNA of membrane-bound polyribosomes.

tify mRNA associated with polyribosomes [11].

The RNA was subsequently fractionated on sucrose gradients; this procedure allowed us to deproteinize RNA by avoiding phenol extractions, which may lead to a selective loss of mRNA or of A-rich sequences of mRNA [12, 13]. The RNA precipitated from sucrose gradients was digested with pancreatic and T1 RNAase. The RNAase resistant portion was precipitated with ethanol and characterized by electrophoresis on 10% acrylamide gels. As a control, an aliquot of undigested RNA was analyzed in parallel.

Fig. 2 shows the superimposed electrophoretic patterns of polyribosome-associated [^{3}H]adenosine labeled RNA from free and membrane-bound ribosomes and of the respective RNAase digests. A peak with the electrophoretic mobility described for poly-A [1] was observed in both the RNAase digests of free and membrane-bound polyribosomal RNA. By contrast, no RNAase resistant material appeared in the digests of [^{3}H]uridine labeled RNA analyzed in the same way.

The electrophoretic pattern of the labeled RNA obtained from free polyribosomes shows a peak migrating slightly faster than the poly-A peak and two peaks corresponding to 5 S and 4 S RNA (fig. 2). The RNA species larger than 10 S do not enter into this 10% gel and so are not displayed in this analysis [4]. By comparison, the electrophoretic pattern of RNA of membrane-bound polyribosomes reveals the 5 S and 4 S RNA peaks, but the peak migrating slightly faster than poly-A is absent. This result suggests that a species of RNA is specifically associated with free but not with membrane-bound polyribosomes; it seems possible that this is histone mRNA, since treatment of the cells with the inhibitor of DNA synthesis cytosine arabinoside leads to the disappearance of this peak (M. Zauderer and P. Liberti, personal communication).

We have calculated that 13–15% of the untreated RNA applied to the gels is recovered with 5 S and 4 S RNA. About 7% to 10% of the RNAase treated RNA is recovered in the poly-A peak; RNAase digestion is quite effective since no trace of 5 S or 4 S RNA is found in the electropherogram (fig. 2).

We have confirmed that an RNAase-resistant fraction is present in membrane polyribosome-associated RNA labeled with [^{3}H]adenosine in the presence of 40 ng/ml of actinomycin D by analyzing RNA fractions separated by sucrose density gradient sedimentation as previously described [7]. RNAase resistant material has been observed throughout the gradient with a peak sedimenting between 4 S and 18 S. The RNAase resistant fraction represents as much as 25% of the radioactive RNA in this peak.

4. Conclusions

RNAase resistant [^{3}H]adenosine labeled polynucleotide sequences have been shown in RNA obtained from both free and membrane-bound polyribosomes. The results obtained indicate that the proportion of poly-A in RNA associated with free and membrane-bound polyribosomes is not substantially different. This suggests that poly-A sequences are not involved in directing mRNA into one or the other type of polyribosomes.

Acknowledgements

This work has been supported by grants of the National Science Foundation and of the National Institute of Health.

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