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A Polynucleotide Segment Rich in Adenylic Acid in the Rapidly-Labeled Polyribosomal RNA Component of Mouse Sarcoma 180 Ascites Cells

(poly(A)/Millipore)

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ABSTRACT **The rapidly-labeled polyribosomal RNA component from mouse sarcoma 180 cells is retained on nitrocellulose (Millipore) membrane-filters at high ionic strength. This property is due to the presence of a polynucleotide sequence rich in adenylic acid that resists both T_1 and pancreatic RNase digestion. The resistant material shows sedimentation characteristics close to those of transfer RNA. The RNA molecules that contain this material can be separated from the rest of the polysomal RNA by differential phenol extraction with neutral and alkaline Tris buffers. Synthetic poly(A) exhibits the same behavior as the rapidly-labeled polysomal RNA with respect to Millipore binding and phenol fractionation. The characteristics of the rapidly-labeled polysomal RNA component permit its isolation free of ribosmal RNA.**

There is evidence for the occurrence in mRNA of polynucleotide sequences other than those required for the coding of amino acids in proteins. Large segments have been shown to serve as specific ribosomal binding sites at the beginning of each cistron in bacteriophage RNA (1). The occurrence of another type of binding site in mammalian mRNA has been suggested. This RNA has been found to occur as nucleoprotein complexes, which are found free in the cytoplasm (2–4) and are also released from polysomes when the latter are disaggregated either *in vitro* (5–7) or *in vivo* (8). Thus, substantial amounts of proteins appear to be associated with the mRNA even while it is being translated. Are there segments in the mRNA specifically concerned with the binding of these proteins? Such a possibility is supported by the recent findings that the 9S hemoglobin mRNA possesses a number of nucleotides considerably larger than that required for coding of the polypeptide chains (9, 10).

We present evidence in this report for the occurrence in the presumed mRNA of mouse sarcoma 180 ascites cells of large segments consisting primarily, if not exclusively, of adenylate residues. These sequences impart to the RNA unusual properties which make its isolation free of ribosomal RNA possible. Poly(A) has been previously demonstrated in rat liver cytoplasm (11). Evidence for the occurrence of adenylate-rich sequences in reticulocyte polysomes has been presented (12).§ The significance of this material in mRNA is unknown at the present, but a role as a binding site is possible.

MATERIALS AND METHODS

Cell labeling and polysome preparation

Mouse sarcoma 180 ascites cells were maintained by weekly transfers into the peritoneal cavity of albino mice and were incubated *in vitro* as described previously (8). The labeling procedure was modified as follows. After incubation of the washed cells in Krebs bicarbonate solution in the presence of 0.04 μg/ml actinomycin D for 30 min, the suspensions were supplemented with glucose, bovine serum, a complete mixture of amino acids, and either 5 μCi/ml [5-^{3}H]uridine (20 Ci/mmol) or 10 μCi/ml [5,8-^{3}H]adenosine (5–15 Ci/mmol), and the incubations were continued for 1 hr. The low level of actinomycin served to prevent labeling of ribosomal RNA (13). The cells, harvested after rapid chilling, and swollen in hypotonic medium, were lysed in 0.1% Triton X-100 and the polysomes were isolated as described previously (8).

RNA preparations

All operations were carried out at 0–4°C. The ribosomal samples in 50 mM Tris·HCl (pH 7.6)–50 mM KCl–1 mM $MgCl_2$ were diluted into 0.5% sodium dodecyl sulfate and 0.1 M Tris·HCl buffers of pH 7.6 or 9.0 and shaken for 10 min with an equal volume of redistilled 80% aqueous phenol. The phases were separated by centrifugation at 17,000 × *g* for 10 min. The residue after removal of the aqueous phase was re-extracted with an equal volume of 0.1 M Tris buffer. The combined aqueous phases were re-extracted twice with phenol, and the RNA was precipitated overnight with 2.5 volumes of ethanol and 0.1 volume of 1% NaCl. The precipitate was washed three times with 66% aqueous ethanol in 0.03% NaCl, dissolved in water, and stored at −20°C. When precipitation of the RNA was not required, the aqueous phase was extracted with ether and the excess ether was removed by blowing air over the solution. When sequential extractions at different pH values were performed, the

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§ For evidence of its occurrence in mRNA of HeLa cells, see refs. 19 and 20. [Ed.]

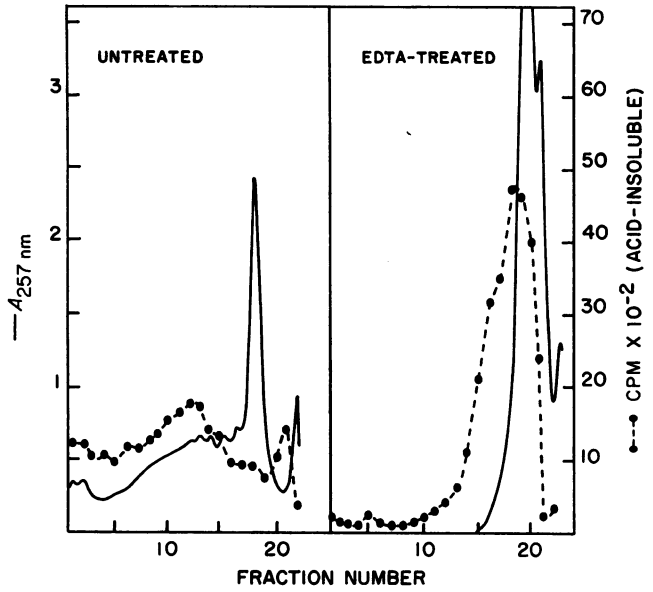

FIG. 1. Sedimentation characteristics of labeled polyribosomes. Preparation from [^{3}H]uridine-labeled cells were subjected to zone sedimentation with and without prior treatment in 15 mM EDTA at 0°C for 10 min to dissociate the ribosomes. Centrifugation was at 45,000 rpm for 25 min in an SW50 Spinco rotor through a 4.5-ml 10–40% linear sucrose gradient in 50 mM Tris(pH 7.6)–50 mM KCl–1 mM $MgCl_2$, with a 0.5-ml layer of 2 M sucrose at bottom of tubes. Sedimentation from right to left.

first phenol treatment took place in Tris buffer of pH 7.6 and the residue after removal of the aqueous phase was re-extracted successively with 0.1 M Tris (pH 7.6), with 0.5% sodium dodecyl sulfate–0.1 M Tris (pH 9.0), and with 0.1 M Tris pH 9.0. The first two and last two aqueous phases were combined.

The cation concentration was found to be critical for the RNA fractionation. K^+, and perhaps Mg^{2+}, in concentrations of at least 10 mM and 0.2 mM respectively, had to be present in addition to 0.1 M Tris(pH 7.6) during the initial phenol extraction. Re-extraction of the residue with water instead of pH 9.0 Tris led to the recovery of material with the characteristics of pH 9 RNA. The extraction with pH 9.0 Tris, however, appeared to be more effective and reproducible. For the sake of convenience, the terms pH 7.6 RNA and pH 9 RNA will be used to designate the preparations obtained by phenol extraction with the two different buffers.

TABLE 1. *Characteristics of adenosine-labeled polysomal RNA extracted with sodium dodecyl sulfate–phenol with neutral and alkaline Tris buffers*

Conditions for RNA extractions	Untreated		Ribonuclease-treated	
	Acid-insoluble	Bound to Millipore	Acid-insoluble	Bound to Millipore
(*a*) pH 7.6	179	12	28	4
(*b*) pH 9.0	346	245	111	82
(*c*) pH 7.6, then 9.0	188	217	78	98

Samples of adenosine-labeled polysomes were extracted sequentially at pH 7.6 and 9.0 (*a*, *c*), or directly at pH 9.0 (*b*). Equal volumes of buffers were used for each extraction. Aliquots of purified aqueous phases were used for measurements. Incubations with pancreatic RNase (1 μg/ml) were in 50 mM Tris (pH 7.6)–50 mM KCl–1 mM $MgCl_2$ at 30°C for 20 min. Values are expressed in counts per minute.

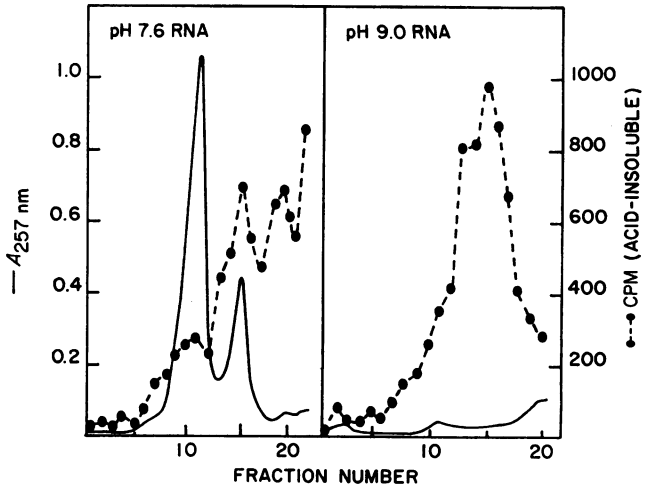

FIG. 2. Sedimentation characteristics of polysomal RNA fractions obtained by sequential pH 7.6–pH 9.0 extractions. Polysomes from uridine-labeled cells were subjected to phenol fractionation. Aliquots from purified aqueous phases were centrifuged through a 5-ml 5–20% linear sucrose gradient in 50 mM Tris(pH 7.6)–50 mM KCl–1 mM $MgCl_2$ at 45,000 rpm for 90 min. The labeled pH 9 RNA was completely retained on Millipore filters. For other details, see Fig. 1.

Counting procedures

Measurements of acid-insoluble radioactivity were as described previously (8). Total radioactivity was determined after addition of 0.5- to 0.8-ml samples to 10 ml of Triton X-100 scintillation mixture (5.5 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 333 ml of Triton X-100, 667 ml of toluene). For the Millipore binding measurements, samples were diluted into 10 volumes of ice-cold 10 mM Tris (pH 7.6)–500 mM KCl–1 mM $MgCl_2$. After 10 min in the cold, they were filtered through Millipore filters (HA 0.45 μm, Millipore Filter Corp., Bedford, Mass.) previously soaked in the same salt solution, and the filters were washed twice with 10 ml of this salt solution. After drying, they were counted in toluene scintillation mixture.

RESULTS

Characteristics of labeled polysome preparations

The bulk of the radioactivity in the preparations appears to be associated with the polyribosomes, as indicated by the rather close parallelism between the sedimentation profiles of radioactivity and UV-absorbing material and by its sensitivity to EDTA (Fig. 1). Ethidium bromide, an inhibitor of mitochondrial RNA synthesis (14), did not affect the extent of labeling of the polysome preparations. Cordycepin caused a 50% reduction in polysomal labeling, without affecting that of the nuclei, in agreement with the results of Penman *et al.* (15).

Binding of polysomal RNA fractions to Millipore filters

A large portion of the labeled RNA from preparations obtained by phenol extraction in Tris·HCl pH 9.0 was

retained on Millipore filters when the filtration is performed in the presence of 0.5 M KCl; ribosomal RNA was not adsorbed. No radioactivity was retained from RNA preparations obtained in Tris of pH 7.6. It can be seen in Table 1 that about twice as much labeled RNA is recovered from the polysomes at pH 9.0 than at pH 7.6, and that it is the additional material extracted at pH 9.0 that carries the property to be retained on Millipore. Since the ribosomal RNA is extracted at pH 7.6, the subsequent extraction at pH 9.0 yields labeled RNA of far greater specific radioactivity (see Fig. 2).

The binding of the labeled RNA could not be attributed to proteins contaminating the pH 9.0 preparations. Repeated deproteinizations with phenol and with chloroform–amyl alcohol failed to reduce the extent of binding of the RNA preparations. Moreover, addition of unlabeled pH 9 RNA to a labeled pH 7.6 preparation did not cause any binding of the latter material. Aggregation of the RNA in high KCl was also eliminated as a possible cause for retention on the filters. Zonal sedimentation in 0.5 M KCl, the concentration used for the binding experiments, indicated little tendency for aggregation in either RNA fraction. Moreover, the binding to Millipore was nearly as effective at 22°C as at 0–4°C, and exposure to 0.5 M KCl at 0°C for 15 min did not increase the extent of binding.

The labeled RNA fractions obtained by sequential extractions at pH 7.6 and 9.0 showed very similar sedimentation characteristics, except for the presence of some slowly-sedimenting material in the pH 7.6 fraction (Fig. 2). In a preparation with an overall retention capacity of 70% of the total radioactivity, the material that binds to Millipore exhibited the same range of sedimentation values as the total labeled RNA (Fig. 3).

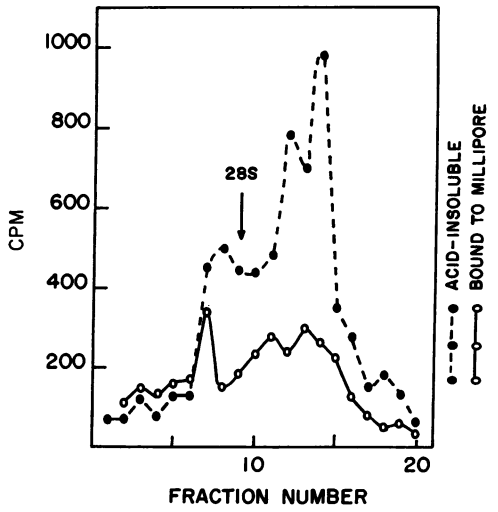

FIG. 3. Sedimentation chracteristics of acid-insoluble and Millipore-binding components in pH 9 polysomal RNA. RNA from U-labeled polysomes was sedimented as in Fig. 2. Samples from each fraction were used for measurements of Millipore binding and acid-insoluble radioactivity. 70% of the radioactivity in the RNA preparation was retained on Millipore prior to sedimentation.

Nature of the material responsible for the Millipore-binding capacity

Treatment of polysomal RNA from adenosine-labeled cells with either pancreatic or T_1 RNase left a large portion of the radioactive material still capable of binding to Millipore (Tables 1, 2). The same treatments applied to uridine-labeled RNA completely eliminated the binding capacity (Table 2). This indicated that relatively large polypurine segments in the pH 9 RNA were capable of binding to the filters, and that no polypyrimidine sequences left after T_1 RNase digestion shared in the binding capacity. The adenosine-labeled RNase-resistant fragments appeared to be free of internal guanylic acid, since simultaneous treatments with the two enzymes caused only a small additional reduction in the amount of radioactive binding material.

TABLE 2. *Effect of RNase treatments on adenosine-labeled and uridine-labeled polysomal RNA*

Treatment	Adenosine-labeled		Uridine-labeled	
	Acid-insoluble	Bound to Millipore	Acid-insoluble	Bound to Millipore
None	1,490	827	1,323	672
Pancreatic RNase	379	397	214	3
T_1 RNase	910	382	379	15

Labeled polysomes were extracted directly at pH 9.0. For other details see Table 1.

Treatment with DNase (5 μg/ml) in addition to the two RNases caused no further reduction.

More than 90% of the radioactivity in the adenosine-labeled polysomal RNA was present in the adenylic acid residues. This was determined by alkaline hydrolysis followed by paper chromatography to separate the nucleotides (16). No labeled DNA was present, as indicated by the fact that all the radioactivity was rendered acid-soluble after KOH treatment (20 min at 100°C in 0.1 N KOH).

It appeared from the above results that the RNase-resistant material that binds to Millipore might represent relatively large poly(A) sequences. It was important to determine whether the binding property was due to the polynucleotide itself or to some other material firmly attached to it. Synthetic poly(A) was found to bind to Millipore filters under conditions similar to those used for the polysomal RNA (Table 3). In both cases the binding was highly dependent on the KCl concentration. Thus the capacity of polysomal RNA to bind to Millipore filters could be attributed solely to the RNase-resistant sequences in the RNA molecules.

TABLE 3. *Effect of KCl concentration on the binding of poly(A) and polysomal RNA to Millipore filters*

KCl concentration (mM)	Millipore binding	
	Poly(A)	Polysomal RNA
500	187	101
200	137	56
50	53	11

[^{14}C]Poly(A) (1 Ci/g-atom P, Miles Laboratories, Elkart Ind.), and uridine-labeled pH 9 RNA, were mixed in 10 mM Tris·HCl (pH 7.6)–1 mM $MgCl_2$ and the appropriate concentration of KCl, and filtered as described in *Methods*. Poly(A) was quantitatively adsorbed in 500 mM KCl.

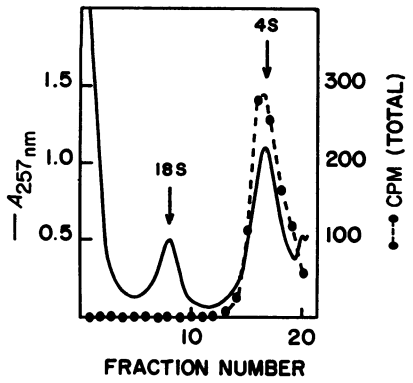

FIG. 4. Sedimentation characteristics of RNase-resistant fragment from adenosine-labeled polysomal RNA. pH 9 polysomal RNA was subjected to treatment with both T_1 and pancreatic RNases as in Table 2. Digested material was subjected to phenol extraction at pH 9.0. Purified aqueous phase was adjusted to pH 7.6, diluted with 10 volumes of 10 mM Tris (pH 7.6)–500 mM KCl–1 mM $MgCl_2$, and filtered through Millipore. Material bound to filter was eluted with 0.5% sodium dodecyl sulfate in 0.1 M Tris pH 9.0 by shaking at 0°C for 1 hr. Eluate was mixed with ribosomal RNA and *E. coli* tRNA, and subjected to zonal sedimentation at 45,000 rpm through a 5-ml 5–20% linear sucrose gradient in 20 mM Tris(pH 7.6)–10 mM KCl.

Characteristics of the adenylate-rich fragments from polysomal RNA

The labeled material that binds to filters after pancreatic RNase treatment represents polyribonucleotides, since virtually all the radioactivity migrates with 2′- and 3′-adenylic acid after alkaline hydrolysis. The sedimentation characteristics of the RNase-resistant material after elution from Millipore are shown in Fig. 4. The material appears to be quite homogeneous, and cosediments with tRNA. All the radioactivity was acid-insoluble. Treatment with pancreatic RNase alone yielded fragments with the same sedimentation characteristics as those of the fragments obtained by treatment with the two enzymes.

DISCUSSION

The evidence presented indicates that the rapidly-labeled polysomal RNA component of mouse sarcoma 180 cells contains a large sequence, or sequences, rich in adenylic acid. It also shows that a major portion of the labeled RNA molecules can bind to nitrocellulose membrane filters, and that the adenylate-rich sequence present in these molecules can fully account for this unexpected property. Neither ribosomal RNA, nor rapidly-labeled RNA apparently free of this sequence, shows the binding capacity. The adenylate-rich sequence can be tentatively identified as poly(A) on the basis of its resistance to pancreatic and T_1 RNases, as well as its Millipore-binding capacity and its behavior during the phenol fractionation. These last two characteristics are shared by synthetic poly(A) (see below).

The RNA species lacking the capacity to bind to Millipore filters can be separated from those with this capacity by sequential phenol extraction of the polysomes with neutral and alkaline Tris buffers. The labeled RNA of the pH 9 fraction binds quantitatively to Millipore. This indicates that each RNA molecule in this fraction is associated with the presumed poly(A). The proportion of the total polysomal RNA that could bind to Millipore ranged from 50 to 70%. This proportion did not vary significantly with different periods of labeling, nor after interruption of RNA synthesis by a high concentration of actinomycin D. It is possible that all the rapidly-labeled polysomal RNA molecules normally contain poly(A) segments, and that the pH 7.6 RNA arises from fragmentation of the polysomal RNA during the manipulations, with loss of the sequence responsible for the binding capacity.

The behavior of the polysomal RNA in the phenol fractionation procedure is caused by the special property of the adenylate-rich segments. Synthetic poly(A), as well as deproteinized pH 9 RNA, were found to shift to the nonaqueous phase when subjected to the phenol treatment at pH 7.6 in the presence of unlabeled polysomes. This appeared to be determined primarily by the cation concentration rather than by the pH. Other types of polynucleotides have been shown to respond in a similar fashion to salt during phenol treatment (17). This finding points to the necessity of avoiding even moderate salt concentrations in any polysomal RNA extraction procedure. It may also be noted that the behavior of the adenylate-rich segments in the rapidly-labeled RNA molecules, both with respect to Millipore binding and differential phenol extraction, provides a means for the isolation of these molecules free of ribosomal RNA.

The occurrence of poly(A) sequences in polysomal RNA appears to be a common feature of mammalian cells. A rapidly-labeled RNA fraction rich in adenylic acid has been isolated previously from rat liver cytoplasm by means of a pH 7.6–pH 8.3 phenol fractionation procedure (16), and poly(A) itself has been isolated from the same material (11). The 9S reticulocyte RNA has been reported to contain adenylate-rich material (12). Preliminary experiments in this laboratory indicate that this 9S RNA can be selectively adsorbed on Millipore filters. The 9S RNA molecule could accommodate a large poly(A) segment, since it appears to contain a number of nucleotides considerably greater than that required to code for the globin polypeptide chains (9, 10).

The function of the adenylate-rich sequence in polysomal RNA remains to be determined. Preliminary experiments indicate that the bulk of the rapidly-labeled nuclear RNA lacks RNase-resistant material that binds to Millipore. The absence of adenylate-rich material from rat liver nuclear RNA has already been reported from this laboratory (16). It is possible, however, that a small proportion of poly(A)-containing RNA occurs in nuclei (18).¶ We suggest that the presence of this sequence in newly-transcribed RNA may be a prerequisite for its processing as mRNA and its transport to the cytoplasm. The adenylate-rich segments could be involved in the binding of the mRNA to cell components involved in the transport process.

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¶ See also ref.19. [Ed.]

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