CLEAVAGE OF THE POLYADENYLATE-RICH REGION OF POLYADENYLATE-RICH RNA

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Summary: The polyadenylate-rich portion of rapidly-labeled polyribosomal RNA isolated from lymphocytes or adrenal cortex was cleaved by a ribonuclease co-purifying with this RNA and retaining activity despite exposure to SDS and phenol. A ribosomal factor which inhibited the digestion of polyriboadenylate-rich RNA by the associated ribonuclease was partially purified. Cleavage of the polyadenylic acid-rich region was associated with the loss of RNA-directed binding of ^{3}H-met-tRNA to ribosomes, suggesting a possible role for the polyadenylic acid-rich region in mRNA translation.

A covalently bound region rich in polyriboadenylic acid (poly A) has been identified in eukaryotic mRNA (1), in rapidly labeled polyribosome-associated RNA and heterogeneous nuclear RNA of eukaryotic cells in culture (2,3), in the mRNA of vaccinia virus (4) and in the genomes of RNA tumor viruses and single-stranded non-oncogenic RNA viruses functioning as mRNA (5,6). The function of the poly A region is at present unknown. A role in the transport of mRNA from nucleus to cytoplasm has been suggested (7), but the presence of poly A in mRNA of viruses replicating in the cytoplasm (5,6) suggests additional roles. Possible cytoplasmic functions of the poly A region include secondary or tertiary structural alterations which might regulate mRNA stability, attachment to ribosomes, or other translational events. The present studies characterize a cytoplasmic ribonuclease co-purified with polysomal A-rich RNA which cleaves the poly A portion of RNA and identify a specific ribosome-associated inhibitor of this nuclease digestion.

Methods: Rapidly labeled polyribosomal RNA containing poly A regions (A-rich RNA) was isolated from cultures of phytohemagglutinin-stimulated pure human peripheral lymphocytes and adrenocorticotropin (ACTH)-stimulated guinea pig adrenal cortices by the method of Lee et al. (2) using serial pH 7.6 and pH 9 extractions as previously described (8).

Assays for digestion of A-rich RNA were done in 65 µl of buffer containing 30 mM Tris-

HCl (pH 8.3), 0.8 mM $MnCl_2$, and 20 mM $(NH_4)_2SO_4$ (Buffer 1) at 37° C. Reactions were terminated by addition of 4 ml. of a buffer containing 10 mM Tris-HCl (pH 7.6), 500 mM KCl, and 1 mM $MgCl_2$ (Buffer 2), and filtered through nitrocellulose filters (Millipore, 0.45μ) which were washed with Buffer 2 and counted in Bray's solution (9).

Ribosomal eluate was prepared from guinea pig adrenal cortical ribosomes by addition of 0.5M KCl. The 30-40% ammonium sulphate precipitate of this eluate was dialyzed against 0.02 M Tris-HCl (pH 7.6), 0.5 M KCl, and 0.0001 M 2-mercaptoethanol (Buffer 3).

Binding of ^{3}H-met-tRNA to ribosomes was measured by a modification of the method of Kerwar et al. (10), as previously described (8).

Results and Discussion: Characterization of A-rich RNA. The A-rich RNA gave a heterogeneous velocity gradient pattern, most of the RNA sedimenting at 11-18S (Figure 1).

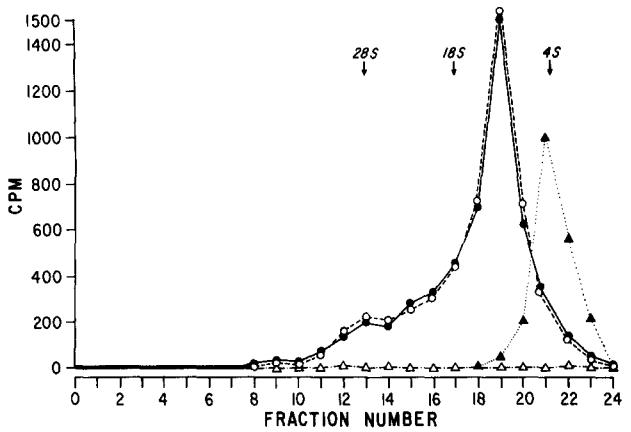

Figure 1. Sedimentation characteristics of A-rich RNA. ^{3}H-uridine- or ^{3}H-adenosine-labeled lymphocyte A-rich RNA was used directly or equal amounts were incubated with pancreatic RNase (8 μg/ml.) for 30 minutes at 37° C in 50 mM Tris-HCl (pH 7.6), 50 mM KCl and 1 mM $MgCl_2$. Samples were applied to nitrocellulose filters, eluted, mixed with marker beef adrenal ribosomal RNA and yeast tRNA, and subjected to zonal sedimentation in an SW 50.1 rotor at 45,000 r.p.m. for 120 minutes at 4° C through a 5 ml. 5-20% linear sucrose gradient in 20 mM Tris-HCl (pH 7.6) and 10 mM KCl, and fractions counted in 10 ml. of Bray's solution. Sedimentation from right to left. (●——●) ^{3}H-adenosine-labeled A-rich RNA; (○——○) ^{3}H-uridine-labeled A-rich RNA; (▲.....▲) ^{3}H-adenosine-labeled A-rich RNA retained on a nitrocellulose filter following pancreatic RNase; (△-·-·-·△) ^{3}H-uridine-labeled A-rich RNA retained on a nitrocellulose filter following pancreatic RNase.

Pancreatic RNase digestion of A-rich RNA labeled with ^{3}H-uridine left no radioactivity retainable by nitrocellulose filters and rendered 96% of radioactivity CCl_3COOH-soluble. Pancreatic RNase digestion of A-rich RNA labeled with ^{3}H-adenosine to label, in addition, the poly A portion, left 30-40% of the radioactivity retainable by nitrocellulose filter and CCl_3COOH-precipitable. RNA retained on the filters following pancreatic RNase digestion sedimented at 4-6S (Figure 1).

Cleavage of the Poly A region of A-rich RNA by an Associated Ribonuclease. When radiolabeled A-rich RNA was incubated in Buffer 1, there was a marked time- and temperature-dependent digestion of the RNA as reflected by a decrease in the amount of radioactivity retained on a nitrocellulose filter (Table 1). Optimal conditions for digestion were 20 mM

Table 1. Digestion of A-rich RNA

^{3}H-adenylate-labeled A-rich RNA	Incubation at (°C)	Counts per minute retained on a nitrocellulose filter
+	none	1,070
+	4°	746
+	37°	342
⊕	37°	1,020

^{3}H-adenosine-labeled lymphocyte A-rich RNA was incubated for 60 minutes in Buffer 1 and assays performed as described in Methods. ⊕ indicates that the A-rich RNA was heated at 90° C for 15 minutes prior to incubation in Buffer 1. Results are the average of triplicate determinations differing by less than 2%.

$(NH_4)_2SO_4$, 0.8 mM $MnCl_2$, 30 mM Tris-HCl (pH 8.3). $MgCl_2$ could not be substituted for $MnCl_2$. The portion of the RNA which was initially digested appeared to be only the poly A region. ^{3}H-adenosine-or ^{3}H-uridine-labeled A-rich RNA was incubated in Buffer 1 for 30 minutes at 37° C and subjected to rate-zonal centrifugation through sucrose density gradients such that species migrating at 11-12S were sedimented to the bottom of the gradient. A 4-6S peak of radioactivity appeared after incubation of ^{3}H-adenosine-labeled A-rich

RNA; no such digestion product was observed after incubation of ^{3}H-uridine-labeled A-rich RNA (Figure 2). The ^{3}H-adenosine-labeled material migrating at 4-6S resisted digestion with

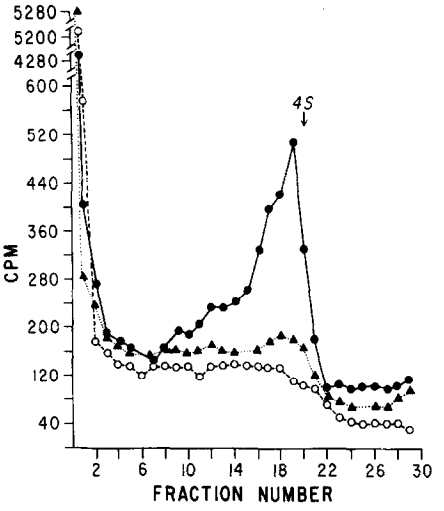

Figure 2. Digestion of A-rich RNA by associated ribonuclease and inhibition by ribosomal eluate. Equal amounts of radioactivity of ^{3}H-adenosine- or ^{3}H-uridine- labeled A-rich RNA were incubated in Buffer 1 with either ribosomal eluate (50 µg/ml.) or equivalent volume of Buffer 3 for 30 minutes at 37° C. The reaction mixture was made 1% SDS, applied to a linear 5-20% sucrose gradient containing 10 mM Tris-HCl (pH 7.6), 20 mM KCl, 1% SDS, and centrifuged for 5 hours at 56,000 r.p.m. in an SW 56 rotor at 20° C. Values on the ordinate represent radioactivity in the pellet; 90-95% of the counts applied to the sucrose gradients were recovered. Sedimentation right to left. (●——●) ^{3}H-adenosine-labeled A-rich RNA; (O-----O) ^{3}H-uridine-labeled A-rich RNA; (▲• • • • • ▲) ^{3}H-adenosine-labeled A-rich RNA plus ribosomal eluate.

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pancreatic RNase and was quantitatively retained on a nitrocellulose filter, further supporting its identity as poly A. Incubation of ^{3}H-adenosine-labeled A-rich RNA for 2 hours in Buffer 1 showed further accumulation of radioactivity in the 4-6S region with some digestion of this peak to smaller fragments; there were still no radiolabeled 4-6S digestion products of ^{3}H-uridine-labeled A-rich RNA in 2 hours.

Cleavage of the poly A region appeared to be produced by a ribonuclease which copurified with the A-rich RNA. Incubation of ^{3}H-adenosine-labeled A-rich RNA for 15 minutes at 90° C abolished its degradation when subsequently incubated in Buffer 1 at 37°

C for one hour (Table 1). There was also no degradation of radiolabeled A-rich RNA sedimented at 20° C in a 1% SDS, sucrose density gradient prior to the usual phenol, ether extractions to remove SDS, suggesting that an associated ribonuclease was either separated from A-rich RNA or inactivated during the centrifugation. The enzymatic nature of the degradation of the poly A region was further confirmed by using A-rich RNA as a source of ribonuclease. Unlabeled A-rich RNA prepared from guinea pig adrenal glands, when incubated with synthetic ^{3}H-polyriboadenylic acid, degraded this labeled homopolymer as determined by a decrease in CCl_3COOH-precipitable counts. Digestion of synthetic polyriboadenylic acid was confirmed by alterations of rate-zonal centrifugation patterns (Figure 3).

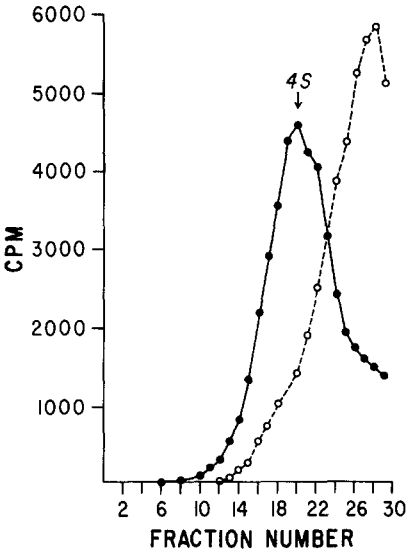

Figure 3. Digestion of synthetic polyriboadenylic acid by A-rich RNA. Synthetic ^{3}H-polyriboadenylic acid (Miles Laboratories, Inc., 35.6 mCi/mM) was incubated with A-rich RNA (0.3 µg) isolated from guinea pig adrenal cortex or buffer blank carried through the same extraction procedure in 65 µl. of Buffer 1 for 8 hours at 37° C. Reaction mixtures were adjusted to 1% SDS and sedimented as described in the legend to Figure 2 (●——●). Synthetic ^{3}H-polyriboadenylic acid plus buffer; (O- - -O) synthetic ^{3}H-polyriboadenylic acid plus A-rich RNA.

Synthetic ^{3}H-polyriboadenylic acid incubated with buffer alone sedimented at 4S. Addition of the A-rich RNA resulted in digestion and the appearance of radioactivity near the top of

the gradient. Heating the A-rich RNA at 90° C for 5 minutes or incubating with pronase at 37° C for 1 hour abolished its ability to digest synthetic ^{3}H-polyriboadenylic acid. As assessed by CCl_3COOH-precipitable radioactivity, A-rich RNA produced no digestion in 8 hours of radiolabeled ribosomal RNA, double- or single-stranded DNA, double-stranded RNA, synthetic polyriboadenylic acid, polydeoxythymidylic acid hybrids, or synthetic polyriboguanylic acid.

In data which will be presented elsewhere (11), a cytoplasmic ribonuclease, referred to as ribonuclease poly Aase, has been purified 3,000-fold from beef adrenal cortex. Ribonuclease poly Aase digested synthetic ^{3}H-polyriboadenylic acid and cleaved the poly A region of A-rich RNA and had salt, divalent cation, and pH optima, and substrate specificity identical to those of the ribonuclease co-purifying with the A-rich RNA.

Ribosomal Eluate Prevented A-rich RNA Digestion. Addition of ribosomal eluate inhibited completely the digestion of A-rich RNA by the associated ribonuclease (Table 2 and Figure 2). This inhibitor was heat-labile, affording no protection following incubation for 10 minutes at 60° C, and was destroyed by incubation with trypsin. Ribosomal eluate also inhibited digestion of the poly A region of A-rich RNA by purified ribonuclease poly Aase (Table 2), but did not inhibit digestion of synthetic polyriboadenylic acid by this ribonuclease. This factor did not protect A-rich RNA from digestion with pancreatic (2 μg/ml.), T_1 (1 μg/ml.), or T_2 (2 μg/ml.) RNase. Ribosomal eluate contained ribonuclease activity which was apparent only following inactivation of the inhibitor by heat (Table 2).

A-rich RNA Directed Binding of ^{3}H-met-tRNA to Ribosomes. Messenger RNA function for A-rich RNA was suggested by its ability to direct binding of ^{3}H-met-tRNA to preincubated ribosomes which bound no met-tRNA in the absence of A-rich RNA (Table 3), and incorporation of ^{14}C-phenylalanine into TCA-precipitable polypeptides in an *in vitro* protein synthesis assay (8). Incubation of A-rich RNA under conditions which appeared to cleave only the poly A region (Figure 2) caused a progressive decrease in the ability of A-rich RNA to direct binding of ^{3}H-met-tRNA to ribosomes (Table 3).

Table 2. Protection of A-rich RNA from Digestion by an Associated Ribonuclease and by Ribonuclease Poly Aase by Ribosomal Eluate

Complete Assay Mixture	Counts per minute retained on nitrocellulose filter
Not incubated	1,650
Incubated	707
+ Ribosomal eluate	1,624
+ Ribosomal eluate added post incubation	703
+ Heated ribosomal eluate *	590
+ Ribonuclease poly Aase	385
+ Ribonuclease poly Aase + ribosomal eluate	1,641

^{3}H-adenosine-labeled lymphocyte A-rich RNA was incubated at 37° C for 30 minutes in Buffer 1. Where indicated, ribosomal eluate (75 µg protein/ml.) and purified ribonuclease poly Aase (0.8µg/ml were added. Results are the average of duplicates differing by less than 2%.

These experiments demonstrated a ribonuclease activity in lymphocytes and adrenal cortex which co-purified with polyribosomal A-rich RNA, retained activity despite exposure to SDS and phenol, and cleaved initially only the poly A-rich region of A-rich RNA. The method used to purify A-rich RNA has been shown to depend on its association with proteins (12). The demonstration that a heat-labile, non-dialyzable ribosome-associated protein prevented digestion of A-rich RNA by both the associated ribonuclease and ribonuclease poly Aase, but not by other ribonucleases, yet failed to protect synthetic polyriboadenylic acid from degradation by ribonuclease poly Aase, suggests a specific interaction of the inhibitor and A-rich RNA which may regulate the associated ribonuclease activity.

Enzymatic digestion of A-rich RNA under conditions which appeared to cleave only the poly A portion was associated with a concomitant loss of ability to direct binding of ^{3}H-met-tRNA to ribosomes. These data suggest that the poly A-rich region may be involved in translation of these mRNAs. Rigorous proof of the function of the poly A region requires demon-

Table 3. A-rich RNA Directed Binding of ^{3}H-met-tRNA to Ribosomes

Assay Mixture	Preincubation of A-rich RNA (minutes)	^{3}H-met-tRNA bound to ribosomes (c.p.m./0.4 OD_{254})
Complete	0	1,098
- Ribosomal wash, A-rich RNA	0	33
- A-rich RNA	0	36
- Ribosomes	0	20
- Ribosomal wash	0	33
Complete	4	1,006
Complete	30	897
Complete	120	588

A-rich RNA was prepared from ACTH-stimulated guinea pig adrenal cortices as described in Methods, and preincubated at 37° C for the time indicated in Buffer 1 before addition to the assay. Results are the average of duplicate determinations differing by less than 1%. ^{3}H-adenosine-labeled A-rich RNA from ACTH-stimulated guinea pig adrenal glands incubated in Buffer 1 under the conditions used in this experiment showed a 10-15% digestion at 30 minutes and a 45-50% digestion at 120 minutes of the poly A regions as assessed by retention of the A-rich RNA on nitrocellulose filters. Similar results were obtained using lymphocyte A-rich RNA.

stration that cleavage of the poly A region of a specific mRNA prevents translation of this mRNA and that no other alterations of the mRNA occur under these experimental conditions.

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