Poly(A): Possible Role in Translation of Poly(A)-Rich RNA[1] (37559)

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(Introduced by J. Holland)

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A covalently linked region, 100–200 nucleotides long, rich in polyriboadenylic acid [poly(A)], has been identified in rapidly labeled polyribosome-associated RNA and heterogeneous nuclear RNA in a number of eukaryotic cells (1–3), in several specific eukaryotic mRNAs (4–6), and in viral mRNAs (7). It has been suggested that most if not all mRNAs in eukaryotic cells, with the exception of histone mRNA, contain a poly(A) tract (8) at the 3′-OH terminal end (9).

The function of the poly(A) region is not known although a role in mRNA transport from nucleus to cytoplasm has been suggested on the basis of drug inhibitor studies (10). The presence of the poly(A) region in the mRNA of viruses whose life cycle occurs entirely in the cytoplasm (11, 12) implies additional roles. It is possible that the poly(A) tract has a regulatory function related to translation or stability of mRNA. Histone mRNA does not appear to contain a poly(A) tract (13) but can be translated in a cell-free, protein-synthesizing system (14); however, histone mRNA differs from other mRNAs by the number of genomic copies and by the rapidity of its translation and degradation (15–18) perhaps implying a unique mode of regulation.

Rapidly labeled polyribosome-associated RNA containing poly(A) tracts (poly(A)-rich RNA) from cultured human peripheral lymphocytes has been shown to be digested by a putative ribonuclease which is associated with this RNA and which retains activity despite exposure to SDS and phenol (19). This study defines the conditions of incubation for selective cleavage of the poly(A) tract of lymphocyte poly(A)-rich RNA by a putative-associated ribonuclease. The effect of this cleavage on translation has been evaluated in a cell-free, protein-synthesizing system.

Methods. Preparation and labeling of poly (A)-rich RNA. Rapidly labeled, polyribosome-associated poly(A)-rich RNA was isolated from cultures of highly purified human peripheral lymphocytes by the nitrocellulose-binding method described by Lee *et al.* (1). Lymphocyte cultures were prepared and incubated with phytohemagglutinin for 36 hr as previously described (20). At 36 hr, the media were changed to phosphate-free MEM with 10% dialyzed autologous serum, and following incubations for 30 min with actinomycin D (0.05 μg/ml) to inhibit rRNA synthesis, carrier-free [^{32}P]orthophosphate (New England Nuclear) was added and incubation continued for an additional 90 min. Polyribosomal poly(A)-rich RNA was isolated using serial extractions as previously described (20). In all procedures, acid and alkaline washed, autoclaved glassware was used. [^{3}H]Uridylate radiolabeled poly(A)-rich RNA was prepared in a similar manner, as previously described (20).

Conditions for incubation and isolation of digestion products. In these experiments, digestion of poly(A)-rich RNA was produced by incubation at 37° in 30 m*M* Tris-HCl (pH 8.3), 0.8 m*M* $MnCl_2$, and 20 m*M* $(NH_4)_2SO_4$ (Buffer 1) for varying time periods. Separation and analysis of digestion

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products was accomplished by application of RNA to linear 5-20% sucrose density gradients containing 10 m*M* Tris-HCl (pH 7.6), 10 m*M* KCl, 0.5% SDS, and centrifugation in an SW56 rotor at 56,000 rpm for 150 or 360 min.

Preparation of poly(A) tracts. Poly(A) tracts were prepared by digesting poly(A)-rich RNA for 60 min at 37° with pancreatic RNase (10 μg/ml) + T_1 RNase (500 units/ml) in 50 m*M* Tris-HCl (pH 7.6), 500 m*M* KCl, 1 m*M* $MgCl_2$. A 20-fold excess of 10 m*M* Tris-HCl (pH 7.6), 500 m*M* KCl, 1 m*M* $MgCl_2$ was added and the solution passed through a nitrocellulose (Millipore, 45 μm) filter. The RNA which was resistant to digestion, the poly(A) tract, was retained on the filter and was eluted with 0.5% SDS, 100 m*M* Tris-HCl (pH 9.0).

Base analysis. [^{32}P]Poly(A)-rich RNA was incubated at 37° for 0, 5, or 30 min in Buffer 1 and adjusted to 0.5% SDS. Samples were then subjected to rate-zonal sedimentation for 360 min as described above. The 4–6 S regions were collected, 20 μg carrier tRNA added, and RNA precipitated by addition of 4 volumes of cold ethanol. Other aliquots of [^{32}P]poly(A)-rich RNA were directly analyzed or used to prepare isolated poly(A) tracts for analysis. [^{32}P]RNA to be analyzed was dissolved in 0.3 *N* KOH and digested at 37° for 28 hr. The samples were applied to Whatman No. 1 paper and subjected to high voltage electrophoresis in pyridine acetate buffer, pH 3.5 (21). [^{32}P]tRNA was similarly digested and the position of Cp, Ap, Gp, and Up determined by autoradiography. Appropriate regions of the papers were removed and counted in Liquifluortoluene, and base composition was expressed as percentage of the total counts.

Assay for ability to direct binding of [^{3}H]met-tRNA to ribosomes. Bovine adrenal cortical ribosomes were prepared as previously described (20) following incubation of the 30,000*g* supernatant at 37° for 40 min in 10^{-3} *M* ATP, 2×10^{-4} *M* GTP, 5×10^{-5} *M* amino acid mix, 2 mg/ml phosphoenolpyruvate and 20 IU pyruvate kinase. A 0.5 *M* KCl wash of ribosomes was used as the source of initiation factors. Acylation of rabbit liver tRNA (General Biochemicals) was done as previously reported (22). Polyribosomal poly(A)-rich RNA (0.025 A_{260}) from purified human peripheral lymphocytes was preincubated in Buffer 1 at 37° for the indicated time. The ability of the poly(A)-rich RNA to direct binding of [^{3}H]met-tRNA to ribosomes (0.4 A_{260}) was then measured by a modified method of Kerwar *et al.* (23), as previously described (20). The assay mix was incubated for 4 min at 37° and the reaction was stopped by addition of 2.5 ml of buffer containing 50 m*M* Tris-HCl (pH 7.5) and 160 m*M* NH_4Cl_2. Samples were applied to Millipore filters and the filters were washed with 7.5 ml of the above buffer and counted at 27% efficiency in 10 ml of Bray's solution (24).

Millipore assay to determine the cleavage of the poly(A) tract. [^{3}H]Uridylate poly (A)-rich RNA was incubated at 37° in Buffer 1 for the time indicated and applied to Millipore filters in 10 m*M* Tris-HCl (pH 7.5), 500 m*M* KCl, and 1 m*M* $MgCl_2$. Since RNA from which the poly(A) tract has been cleaved will no longer be retained on the filter, the percentage of the cleavage of the poly(A) tracts was estimated by determining the decrease in radioactivity retained on the filters.

Results. Poly(A)-rich RNA isolated from cultured human lymphocytes was heterogeneous, the majority sedimenting at 9–22 S in a SDS-sucrose density gradient (Fig. 1A). The isolated poly(A) tracts of the poly(A)-rich RNA sedimented at 4–6 S (Fig. 1A). Incubation of [^{32}P]poly(A)-rich RNA in Buffer 1 produced a radiolabeled digestion product which sedimented at 4–6 S in sucrose density gradients (Fig. 1).

Analysis of the 4–6 S digestion product was undertaken to determine whether its base composition resembled that of the poly(A) tract of poly(A)-rich RNA. Cultured lymphocytes were incubated with [^{32}P]orthophosphate and polyribosomal poly(A)-rich RNA was isolated as described in Methods. The poly(A)-rich RNA was incubated for various times in Buffer 1, adjusted to 0.5% SDS, and subjected to rate-zonal centrifugation (Fig. 1B). The material sedimenting at

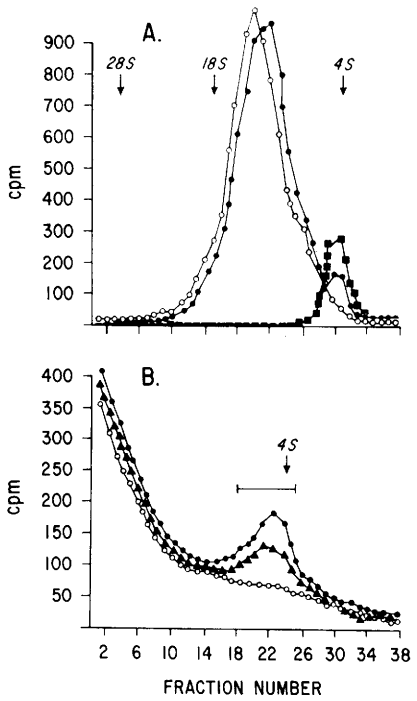

FIG. 1. Sedimentation profiles of lymphocyte poly(A)-rich RNA, lymphocyte [^{32}P]poly(A)-rich RNA, non-incubated or incubated in Buffer 1, or poly(A) tracts were sedimented through SDS, sucrose density gradients as described in Methods. (A.) Sedimentation for 150 min: (○—○) poly(A)-rich RNA non-incubated control; (●—●) poly(A)-rich RNA, incubated for 30 min at 37° in Buffer 1; (■—■) isolated poly(A) tracts. (B.) Sedimentation for 360 min: (○—○) poly(A)-rich RNA, non-incubated control; (▲—▲) poly(A)-rich RNA, incubated for 15 min in Buffer 1; (●—●) poly(A)-rich RNA, incubated for 30 min in Buffer 1. Samples of poly(A)-rich RNA containing 20-fold more radioactivity were similarly digested and sedimented, and the area indicated under the bracket was analyzed for base composition (see Table I).

4–6 S was pooled and its base composition was determined and compared to that of the poly(A) tracts (see Methods). As shown in Table I, the base composition of the 4–6 S material cleaved from poly(A)-rich RNA was indistinguishable from that of the poly (A) tracts. Similar data were obtained using poly(A)-rich RNA isolated from several other lymphocyte culture preparations.

The fact that incubation of poly(A)-rich RNA in Buffer 1 produced cleavage of the poly(A) tract permitted some functional assessment of this portion of poly(A)-rich RNA. Cultures of human peripheral lymphocytes were divided and incubated with or without [^{3}H]uridine, and polyribosome-associated poly(A)-rich RNA was prepared from each (see Methods). Incubations were performed for 5, 30 or 120 min in Buffer 1, and the percentage of the poly(A) tracts cleaved was estimated by determining the amount of radiolabeled RNA retained by a Millipore filter (Table II). The unlabeled poly(A)-rich RNA was assayed for its ability to direct binding of [^{3}H]met-tRNA to ribosomes in a cell-free, protein-synthesizing system. Incubation of the poly(A)-rich RNA under conditions which produced cleavage of the poly (A) tracts was associated with a concomitant quantitatively similar decrease in ability to direct binding of [^{3}H]met-tRNA to ribosomes (Table II).

Discussion. It is possible that mRNA functionally may consist of two portions: one serving the information function, the other, the poly(A) portion, serving a regulatory function. Conditions of incubation have been defined which produce selective cleavage of the poly(A) tracts of human lymphocyte poly(A)-rich RNA. Incubation of poly(A)-rich RNA under these conditions is associated with a decreased ability to direct binding of [^{3}H]met-tRNA to ribosomes in a cell-free, protein-synthesizing system suggesting that the poly(A) tract may be required for translation of poly(A)-rich mRNA.

There are several alternative explanations, however: (*i*) Although radiolabeled poly(A)-rich RNA is readily digested by various ribonucleases, poly(A) tracts might mediate resistance of mRNA to ribonuclease digestion. Thus, cleavage of poly(A) could render mRNA more susceptible to digestion by ribonuclease activities associated with the ribosomes and the ribosomal wash used in the initiation assay. This possibility appears unlikely since pre-incubation of [^{3}H]uridylate poly(A)-rich RNA in Buffer 1 for 30 min did not result in increased ribonuclease digestion when it was then incubated under the conditions of the initiation assay (data not shown). (*ii*) The poly(A) tract of mRNA

TABLE I. Nucleotide Composition of Poly(A)-rich RNA, the Poly(A) Tract, and the 4-6S Digestion Product of Poly(A)-rich RNA.[a]

Nucleotide	Poly(A)-rich RNA (% of total cpm)	Poly(A) tract (% of total cpm)	4–6 S Digestion product, Incubation time 5 min	4–6 S Digestion product, Incubation time 30 min
Cp	21.4	1.7	2.1	2.0
Ap	31.4	97.1	96.7	96.6
Gp	22.3	0.6	0.6	0.6
Up	25.2	0.6	0.6	0.7

[a] [^{32}P]Poly(A)-rich RNA prepared from cultured human lymphocytes was divided into aliquots. Some were incubated at 37° in Buffer 1 for 5 or 30 min, subjected to rate-zonal sedimentation, and the 4–6 S region pooled (see Fig. 1B); others were used to prepare isolated poly(A) tracts (see Methods). Results of base analysis of total poly(A)-rich RNA, isolated poly(A) tracts, and the 4–6 S digestion products are averages of duplicates differing by less than 3%.

appears to be associated with specific proteins (25). It is conceivable that loss of a protein which remains associated with the poly(A) tract and functional despite the SDS phenol extractions, rather than the poly(A) tract itself, is responsible for the decrease in ability to direct binding of met-tRNA to ribosomes. (*iii*) The poly(A) tracts released could themselves inhibit translation of mRNA (26); however, addition of synthetic poly(A) at concentrations comparable to that cleaved from lymphocyte poly(A)-rich RNA in the initiation assay did not affect translation of poly(A)-rich RNA. And (*iv*) lability of poly(A) tracts during phenol extraction dependent on RNA-associated polyribosomal proteins was demonstrated by Perry *et al.* (27). Evidence has been presented that an endoribonuclease which retains some activity despite exposure to SDS and phenol and cleaves the poly(A) tract, co-purifies with lymphocyte poly(A)-rich RNA (19). These experiments demonstrated that the associated ribonuclease cleaves the poly(A) tract, but

TABLE II. Effect of Digestion of Poly(A)-rich RNA by the Associated Ribonuclease on Ability to Direct Binding of [^{3}H]met-tRNA to Ribosomes.[a]

Preincubation of poly (A)-rich RNA in buffer 1 (min)	[^{3}H]Poly(A)-rich RNA retained on Millipore filter (%)[b]	Assay mixture	[^{3}H]met-tRNA bound to ribosomes (pmoles/A_{260} poly(A)-rich RNA)
0	100	Complete	0.778
		—A-rich RNA	0.025
		—ribosomes	0.015
		—ribosomal wash	0.025
		—ribosomes, -A-rich RNA	0.018
5	92	Complete	0.700
30	80	Complete	0.633
120	55	Complete	0.436

[a] Unlabeled poly(A)-rich RNA or [^{3}H]uridylate poly(A)-rich RNA prepared from aliquots of the same preparation of cultured lymphocytes were incubated for the indicated time in Buffer 1 and assayed for ability to be retained by a Millipore filter to direct binding of [^{3}H]met-tRNA to ribosomes (see Methods).

[b] 100% = 8500 cpm.

have not absolutely excluded the possibility that very small oligonucleotides in the non-poly(A)-rich region also are cleaved by the ribonuclease during incubation. However, analysis by the DE81 filter paper technique of Blatti *et al.* (28) has failed to demonstrate the production of small oligonucleotides following incubation of poly(A)-rich RNA in Buffer 1 (data not shown).

Assessment of the functional effect of cleavage of the poly(A) tract of a single defined deproteinized mRNA whose product is assayable *in vitro* will be necessary to unequivocally establish the mechanism by which poly (A) affects translation of poly(A)-rich mRNA.

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