Control of Breakdown of the Polyadenylate Sequence in Mammalian Polyribosomes: Role of Poly(adenylic acid)–Protein Interactions†

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ABSTRACT: The poly(adenylic acid) [poly(A)] segment in mouse sarcoma polysomes is not hydrolyzed by snake venom exonuclease under conditions which cause extensive degradation of the poly(A) in deproteinized polysomal RNA. The protecting effect of polysomes is presumably caused by the interaction between the poly(A) sequence and the protein known to be associated with it. This protection is reduced at low KCl concentration, but addition of exogenous RNA restores the protecting effect. The poly(A) segment also becomes susceptible to exonuclease after fragmentation of the polysomes by mild ribonuclease treatment. The latter treatment releases the poly(A) in association with protein. The poly(A) sequence in polysomes is readily degraded by a cytoplasmic extract of S-180 cells. Partial purification leads to a preparation active against the poly(A) in polysomes under conditions where no fragmentation of the messenger RNA is observed. Snake venom exonuclease increases the activity of the cytoplasmic preparation against poly(A) in polysomes. The active cytoplasmic factor appears to interfere with the poly(A)–protein interaction, thus rendering the polynucleotide susceptible to degradation by exonuclease. The poly(A) sequences in polysomes and in free cytoplasmic nucleoprotein particles are hydrolyzed to the same extent. The results suggest that the poly(A) sequence is normally protected from nucleases by virtue of its association with protein. The slow reduction in poly(A) size in cytoplasmic mRNA can be accounted for by a factor capable of interfering with the poly(A)–protein interaction. The latter interaction seems also dependent on the structural integrity of the polysomes or messenger ribonucleoproteins. It is suggested that a polynucleotide segment adjacent to the poly(A) can modulate the affinity of the protein for the latter sequence, thus permitting control of poly(A) stability in individual messenger RNAs.

The poly(A)[1] segment of mammalian messenger RNA has been shown to undergo a process of gradual size decrease after appearance of the RNA in the cytoplasm (Mendecki et al., 1972; Sheiness and Darnell, 1973; Brawerman, 1973a). The significance of this process is not understood. Its object does not appear to be the eradication of the poly(A) sequence since a segment of substantial size remains in the steady-state mRNA (Greenberg and Perry, 1972; Jeffery and Brawerman, 1974; Brawerman and Diez, 1975; Sheiness and Darnell, 1973). This behavior suggests that the poly(A) segment in functioning mRNA is the target of a specific hydrolytic agent, and that the cleavage process is controlled so as to avoid complete removal of this sequence. The protein or proteins that have been shown to be associated with poly(A) in polysomes (Kwan and Brawerman, 1972; Blobel, 1973) may represent a control element in the cleavage process. It has been shown in this laboratory that the protein component protects the poly(A) from digestion by RNase T_2 (Kwan and Brawerman, 1972). Resistance to polynucleotide phosphorylase has also been reported (Soreq et al., 1974). In the present report we examine the characteristics of the poly(A)–protein interaction. We have used protection of the poly(A) segment from snake venom phosphodiesterase, a 3′-exonuclease, as a measure of this interaction. We observed that incubation of polysomes with this enzyme fails to affect the poly(A) sequence. The extent of protection was reduced at low ionic strength and increased in the presence of exogenous RNA. Protection was also dependent on the structural integrity of the polysomes since mild ribonuclease treatment resulted in poly(A) degradation by the exonuclease. These findings led to the conclusion that susceptibility of the poly(A) to nucleolytic enzymes is determined by the extent of interaction between this sequence and a protein component, and that this poly(A)–protein interaction may be influenced by an adjacent RNA sequence in the polysomes. This model provides a basis for the control of poly(A) stability in individual mRNA molecules.

In order to obtain information on the nature of the cellular poly(A) cleavage process, we have also examined the cytoplasm of S-180 cells for the presence of agents capable of cleaving this sequence in the "protected state" in polysomes. We have identified a factor that renders the poly(A) susceptible to exonuclease, presumably by interfering with the poly(A)–protein interaction.

Materials and Methods

Cell Incubation, Polysome Preparation, and RNA Extraction. Procedures for the maintenance, labeling, and disruption of mouse S-180 ascites cells have been described previously (Lee et al., 1971a; Mendecki et al., 1972). Cells were labeled by incubation for 1 h with 10 μCi/ml of [2,8-^{3}H]-adenosine (32 Ci/mmol, New England Nuclear, Boston, Mass.). Cell suspensions were supplemented with 0.04 μg/ml of actinomycin D, in order to prevent the labeling of rRNA (Penman et al., 1968). Procedures for the preparation of S-180 polysomes by Mg^{2+} precipitation from cell lysates (Mendecki et al., 1972) and extraction of polysomal RNA in the presence of pH 9.0 Tris-HCl (Brawerman et al., 1972) have been previously described.

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[1] Abbreviations used: Na_2EDTA, disodium ethylenediaminetetraacetate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; mRNP, messenger ribonucleoprotein; poly(A), poly(adenylic acid); S-180, Sarcoma 180; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; rRNA and mRNA, ribosomal and messenger ribonucleic acid, respectively; UV, ultraviolet; TKM 50-50-1, 50 mM Tris-HCl (pH 7.6)-50 mM KCl-1 mM $MgCl_2$.

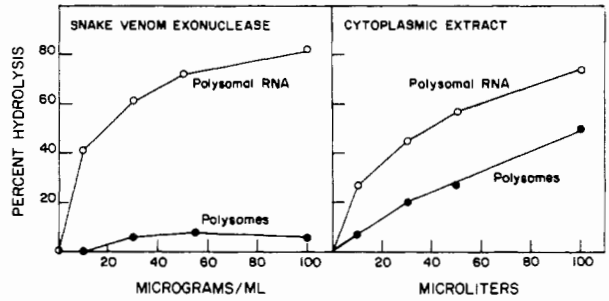

FIGURE 1: Effect of snake venom exonuclease and of cytoplasmic extract on poly(A) degradation in polysomes and in polysomal RNA. Mixtures of adenosine-labeled polysomes and unlabeled RNA, or of adenosine-labeled RNA and unlabeled polysomes, were incubated at 37 °C for 30 min, in the presence of either exonuclease (Worthington Co, N.J.) or crude cytoplasmic extract. The concentration of both labeled and unlabeled components was 0.9 A_{260} unit per ml of incubation mixture. Values expressed as percent of radioactivity in poly(A) in controls incubated under identical conditions without extract or snake venom enzyme.

Preparation of Cytoplasmic Factor. The S-180 ascites cells were collected from the peritoneal cavity and washed with 0.9% NaCl as described previously (Lee et al., 1971a). They were next washed with ice-cold 10 mM Tris-HCl (pH 7.6)–2 mM $MgCl_2$–2 mM dithiothreitol. The packed cells were resuspended in an equal volume of the same solution, left standing for 10 min in the cold to allow for hypotonic swelling, and disrupted by three passages through a hypodermic syringe (26G1/2 needle). This treatment caused no observable nuclear breakage. The homogenate was first centrifuged at 800*g* for 10 min and the supernatant subjected to centrifugation at 40 000 rpm for 2 h in a T-40 Spinco rotor. The high-speed supernatant fraction was used as crude cytoplasmic extract.

For further fractionation, solid ammonium sulfate was added to the crude cytoplasmic extract (1.14 g per 10 ml of extract) and the suspension stirred gently for 30 min in the cold. The resulting precipitate was removed by centrifugation at 35 000*g* for 10 min and additional ammonium sulfate was added to the supernatant (1.52 g per 10 ml of original crude extract). After stirring for 30 min in the cold, the precipitate was collected by centrifugation at 35 000*g* for 10 min. The pellet was dissolved in a small volume of 50 mM Tris-HCl (pH 7.6)–50 mM KCl–1 mM $MgCl_2$ (TKM 50–50–1) and any insoluble matter removed by centrifugation. The clear supernatant was subjected to gel filtration through Sephadex G-25 coarse, equilibrated with TKM 50–50–1. The latter step was carried out at room temperature. The material recovered in the void volume was used as the partially purified preparation. This preparation was stored in 50% glycerol at −20 °C. The recovery of UV-absorbing material at 280 nm was about 11%. The ammonium sulfate fractionation was not effective when cell extracts were prepared by Triton X-100 lysis (Lee et al., 1971a). Dialysis, instead of gel filtration, led to inactive preparations.

Assay for Poly(A) Hydrolysis. The poly(A) digestions were carried out at 37 °C in 0.4 ml of 50 mM Tris-HCl (pH 7.6)–100 mM KCl–5 mM $MgCl_2$ (TKM 50–100–5). Adenosine-labeled polysomes or polysomal RNA were used at a concentration of 0.9 A_{260} unit per ml of incubation mixture. This was followed by dilution with an equal volume of 50 mM Tris-HCl (pH 7.6)–100 mM KCl–30 mM EDTA and incubation with 3.5 μg/ml pancreatic ribonuclease A (Worthington Biochemical Corp., N.J.) at 37 °C for 10 min. The incubation mixtures were chilled and 100 μg of carrier yeast RNA was added before precipitation with 10% trichloroacetic acid.

TABLE I: Ammonium Sulfate Fractionation of Cytoplasmic Extract. Enrichment in Activities for Poly(A) and RNA Digestion.[a]

	Poly(A) Digestion			
	In polysomes[b]	in RNA[b]	Ratio[c] RNA/Polysomes	Nuclease Act.[b]
Crude extract	1	1	2.2	1
$(NH_4)_2SO_4$ fraction				
I	7	5.5	1.6	2.5
II	10.5	6.5	1.4	

[a] Samples of cytoplasmic extract of S-180 cells and of preparation obtained after ammonium sulfate fractionation were incubated with mixtures of labeled polysomes and unlabeled polysomal RNA, or labeled polysomal RNA and unlabeled polysomes, as in Figure 1. Poly(A) assays were done after deproteinization of the reaction mixtures. Separate samples were incubated with labeled polysomal RNA for assay of total nuclease activity (I and II represent two separate preparations). [b] Values represent specific activities relative to those in crude extract, determined by comparing amounts of material absorbing at 280 nm in samples used for hydrolysis. [c] Values represent relative rates of hydrolysis of poly(A) in deproteinized polysomal RNA and in polysomes, measured under identical conditions.

Precipitates were collected on glass fiber filters and counted as described previously (Lee et al., 1971a).

When crude extract was used for poly(A) digestion, the incubation medium was deproteinized prior to the EDTA–RNase treatment by adding 0.1 volume of 5% $NaDodSO_4$ and shaking at room temperature with an equal volume of 1:1 phenol–chloroform containing 0.1% of amyl alcohol (Perry et al., 1972). The phenol was cleared with ether and the ether was removed by evaporation.

Results

Assay for Poly(A) Hydrolysis. In order to determine the extent of poly(A) degradation after exposure of polysomes or polysomal RNA to exonuclease or to cytoplasmic preparations, the incubation mixtures were next treated with pancreatic RNase in the presence of EDTA and sufficient KCl to avoid poly(A) hydrolysis by the RNase. The EDTA was required to permit complete hydrolysis of labeled RNA bound to ribosomes. This agent also prevented further poly(A) degradation by the snake venom exonuclease or the cytoplasmic preparation. All acid-precipitable material at this stage could be adsorbed on Millipore filters after deproteinization, a characteristic of poly(A) (Lee et al., 1971b). In the initial experiments, the digests were deproteinized with phenol–chloroform prior to the RNase treatment. The latter procedure was necessary when crude cytoplasmic extracts were used for poly(A) degradation, because some proteins in the extracts apparently were interfering with the EDTA–RNase treatment. The deproteinization step was no longer necessary when purified preparations were used.

The polysomal preparations used in these studies also contained mRNA–protein particles not associated with polysomes. These preparations were obtained by Mg^{2+} precipitation from cytoplasmic extracts (Mendecki et al., 1972). We observed that the poly(A) sequences in separated polysomes and free mRNP particles are hydrolyzed to the same extent under the various conditions used in the present study (data not shown).

Enzymatic Degradation of the Poly(A) Segment in Polysomes. The poly(A) segment in polysomes is well protected

TABLE II: Activation of Cytoplasmic Factor at 45 °C.[a]

Cytoplasmic Factor	Snake Venom Exonuclease	
	Absent	Included
None		2
Preincubated, 0 °C	9	18
Preincubated, 45 °C	13	29

[a] Samples of cytoplasmic factor preparation were diluted with incubation buffer and kept either at 0 or at 45 °C for 30 min before incubation with labeled polysomes. Reactions were carried out in presence or absence of 10 μg/ml snake venom exonuclease. Values expressed as percent poly(A) hydrolysis.

TABLE III: Effect of Cytoplasmic Factor on Poly(A) Digestion by Exonuclease.[a]

Exonuclease	Cytoplasmic Factor		
	None	Included	
		No Preincubation	10-Min Preincubation
None		1	3
200 μg/ml	0	17	18

[a] Labeled polysomes were incubated for 10 min with cytoplasmic factor preparation, then snake venom exonuclease was added to 200 μg/ml, and incubation was continued for 5 min. Controls not subjected to preincubation were treated for 5 min with exonuclease in presence or absence of cytoplasmic factor. Values expressed as percent poly(A) hydrolysis.

from the action of snake venom exonuclease. Large amounts of the enzyme caused only minimal breakdown of this sequence while poly(A) from deproteinized RNA was hydrolyzed extensively (Figure 1). Exposure of the polysomes to a cytoplasmic extract of S-180 cells, on the other hand, led to extensive poly(A) degradation. In this case the rate of hydrolysis of poly(A) in deproteinized RNA was only twice as high as that of poly(A) in polysomes (Figure 1). In order to have strictly comparable conditions for poly(A) hydrolysis, equal amounts of unlabeled polysomal RNA were included in the polysome incubation mixtures, and unlabeled polysomes were similarly added to the polysomal RNA incubations.

The above results indicated the occurrence of a cytoplasmic factor capable of causing the hydrolysis of poly(A) in the "protected state in polysomes". In order to determine whether this factor might be specific for the poly(A)–protein complex rather than being a nonspecific nuclease, a partial purification was attempted. Ammonium sulfate fractionation led to a seven- to tenfold enrichment in the activity against protected poly(A) (Table I). The activity against poly(A) in free RNA was enriched to a lesser extent, and the relative amount of overall nuclease activity was substantially reduced. Thus it appears that the active agent might be specifically concerned with the degradation of the poly(A) segment in functioning mRNA. Further attempts at purification led to loss of activity, apparently because of irreversible binding of the factor to various adsorbents. The factor appeared in the void volume when subjected to gel filtration through Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.), but gel filtration through P-200 led to its adsorption on the gel matrix. No activity could be recovered from DEAE-Sephadex columns. The factor was quite stable at room temperature, and preincubation at 45–50 °C led to substantial increases in activity (Table II).

The time course of poly(A) hydrolysis with the partially purified preparation is shown in Figure 2. Two slopes were obtained consistently. The first 20% of the poly(A) was hydrolyzed relatively rapidly, and the rest appeared to be more resistant. Similar results were obtained when varying amounts of factor were used for a constant incubation time (Figure 2).

Synergistic Effects of Snake Venom Exonuclease and Cytoplasmic Factor. While the snake venom exonuclease caused no significant poly(A) breakdown in polysomes, this enzyme enhanced considerably the hydrolytic activity of the cytoplasmic preparation (Tables II and III). This effect was most evident with the use of low levels of cytoplasmic factor, which caused little poly(A) hydrolysis. Addition of excess exonuclease led to substantial hydrolysis, while the exonuclease alone had essentially no effect.

Preincubation of the cytoplasmic preparation at 45°C increased both its hydrolytic activity and the enhanced activity in the presence of exonuclease (Table II). These results indicated that the active component in the cytoplasm might be an agent that renders the poly(A) segment susceptible to hydrolysis by exonucleases. Thus the action of the cytoplasmic preparation alone would be the combined result of labilization of poly(A) segments by the active factor and hydrolysis of some of the sensitized segments by residual exonuclease in the preparation. Additional sensitized segments would be hydrolyzed by the added snake venom exonuclease. In order to determine whether the labilizing cytoplasmic agent might be a protease that destroys the proteins associated with the poly(A) segment, the following experiment was designed. Polysomes were incubated with small amounts of factor for 10 min prior to the addition of a large excess of exonuclease. If proteolytic digestion were responsible for the sensitization of the poly(A) to exonuclease, then the preincubation should have resulted in an increase in the amount of poly(A) susceptible to hydrolysis by exonuclease. As can be seen in Table III, this was not the case. Thus it appears that the exonuclease and the presumptive labilizing factor must act in concert.

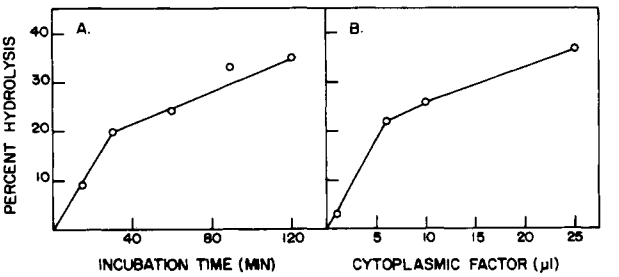

FIGURE 2: Degradation of polysomal poly(A) by cytoplasmic factor. Labeled polysomes were incubated with 10 μl of cytoplasmic factor preparation for indicated periods of time (panel A) and with indicated amounts of the same preparation for 50 min (panel B). Values expressed as percent poly(A) hydrolysis as in Figure 1.

Effect of KCl and RNA on Poly(A) Protection. The ionic environment had a strong influence on the interaction responsible for poly(A) protection in polysomes. While relatively little hydrolysis by exonuclease alone took place at 100 mM KCl in the presence of Tris buffer and $MgCl_2$, more extensive hydrolysis was observed at 30 mM KCl in Hepes buffer (Table IV). The added $MgCl_2$ had relatively little effect (data not shown) and was used primarily to maintain the integrity of the polysomes. The choice of buffer also did not appear to be critical (data not shown). The activity of the snake venom

TABLE IV: Effect of Ionic Conditions on Poly(A) Hydrolysis.[a]

		100 mM KCl–50 mM Tris–5 mM $MgCl_2$	30 mM KCl–20 mM Hepes–3 mM $MgCl_2$	
	Substrate	% Hydrolysis	% Hydrolysis	Corr Value[b]
Exonuclease	Polysomes	7	13	34
	RNA	21	8	
Cytoplasmic factor	Polysomes	8	31	
	RNA	29	31	

[a] Labeled polysomes or polysomal RNA were incubated for 20 min with snake venom exonuclease or with aliquots of cytoplasmic factor preparation. Amounts of venom exonuclease used were 50 μg/ml for polysomes and 5 μg/ml for polysomal RNA. Incubations were either with standard incubation buffer or with 30 mM KCl–20 mM Hepes (pH 7.6)–3 mM $MgCl_2$. [b] Value corrected to compensate for lower activity of snake venom exonuclease against deproteinized RNA at low KCl concentration.

TABLE V: Effect of Added RNA on Polysomal Poly(A) Hydrolysis.[a]

	100 mM KCl		30 mM KCl	
Enzyme Source	No Addition	RNA Added	No Addition	RNA Added
Cytoplasmic extract	18	25	48	18
Snake venom	8	4	44	21

[a] Labeled polysomes were incubated either with cytoplasmic factor preparation or with snake venom exonuclease (100 μg/ml) for 20 min. Incubations were either in standard incubation buffer (100 mM KCl) or in low KCl buffer (see Table IV), in presence or absence of 27 μg/ml deproteinized polysomal RNA. Values expressed as percent poly(A) hydrolysis. Data for snake venom enzyme at 30 mM KCl are corrected to account for lower activity at this salt concentration (see Table IV).

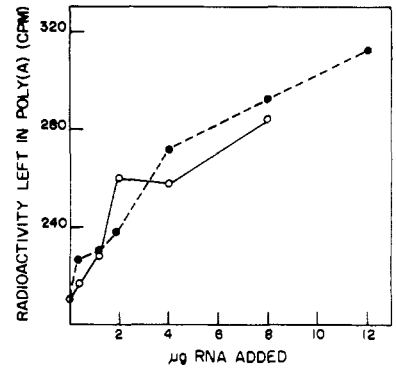

FIGURE 3: Effect of exogenous RNA on the extent of hydrolysis of polysomal poly(A) at low salt. Labeled polysomes were incubated at 37 °C for 30 min with 10 μl of cytoplasmic factor preparation in buffer containing 20 mM Hepes (pH 7.6), 30 mM KCl, and 3 mM $MgCl_2$, in the presence of indicated amounts of polysomal RNA (● - - - ●) or *Escherichia coli* transfer RNA (○ — ○). Values represent radioactivity left in poly(A) after incubation.

TABLE VI: Effect of Pancreatic Ribonuclease on Poly(A) Digestion by Exonuclease.[a]

	Poly(A) Digestion		
	No Addition	Exonuclease	RNA Digestion
No addition		1	
RNase A			
0.1 μg	1	13	46
0.5 μg	2	19	48
Cytoplasmic factor	16	28	0

[a] Labeled polysomes incubated for 10 min with 5 μg/ml snake venom exonuclease in presence or absence of indicated amounts of pancreatic RNase A or cytoplasmic factor preparation. Extent of RNA hydrolysis in polysomes measured by precipitation with cold trichloroacetic acid. Values expressed as percent hydrolysis.

exonuclease on deproteinized RNA proved to be dependent on the KCl concentration. It was considerably lower at 30 mM (Table IV). Thus the data on poly(A) hydrolysis in polysomes had to be corrected to compensate for the lower activity against RNA in low salt. This indicated a still greater susceptibility of the poly(A) segment to exonuclease at the lower KCl concentration (Table IV). Hydrolysis of poly(A) by the factor in the cytoplasmic preparation was also influenced by the KCl concentration. In this case the hydrolytic activity against deproteinized RNA was not KCl dependent (Table IV).

We also observed that the KCl effect on polysomes was itself influenced by exogenous RNA added to the incubation mixtures. The added RNA enhanced considerably the stability of the complex presumably responsible for protection (Table V). The RNA effect was pronounced at the low KCl concentration and only minimal or nonexistent at 100 mM KCl. Similar degrees of protection were observed with snake venom exonuclease and with the cytoplasmic factor. The RNA effect was dependent on concentration (Figure 3). It appears to be nonspecific since S-180 polysomal RNA and *Escherichia coli* transfer RNA had similar effects.

Labilization of the Poly(A) Sequence by RNase Treatment of Polysomes. Previous studies had indicated that the poly(A)-protein complex released from polysomes by mild ribonuclease digestion is unstable (Kwan and Brawerman, 1972). We have verified that the structural integrity of the polysomes is necessary for the poly(A) protection effect. Inclusion of small amounts of pancreatic RNase in the incubations with exonuclease led to substantial poly(A) degradation, under conditions that leave the poly(A) unaffected by either exonuclease or RNase alone (Table VI). The RNase treatment caused the release of large amounts of the poly(A) as a slowly sedimenting particle (Figure 5), as previously reported (Kwan and Brawerman, 1972). Larger amounts of RNase led to increased poly(A) digestion by exonuclease, even though little additional labeled polysomal RNA was degraded (Table VI). Under the same conditions, the cytoplasmic factor caused considerable sensitization of poly(A) without any noticeable RNA hydrolysis.

The effect of pancreatic RNase suggested that some other RNA sequence in the polysomes might participate in the poly(A) protection effect. It was important, therefore, to verify that the effect of the cytoplasmic factor was not due simply to cleavage of the poly(A)-protein complex from the rest of the polysome followed by exonuclease digestion of the labilized poly(A). As seen in Figure 4, incubation of polysomes with the cytoplasmic preparation under conditions that lead to substantial poly(A) breakdown did not alter the polysome profile. Thus no obvious nicking by endonuclease was taking place. There was also no obvious shift of poly(A)-containing material

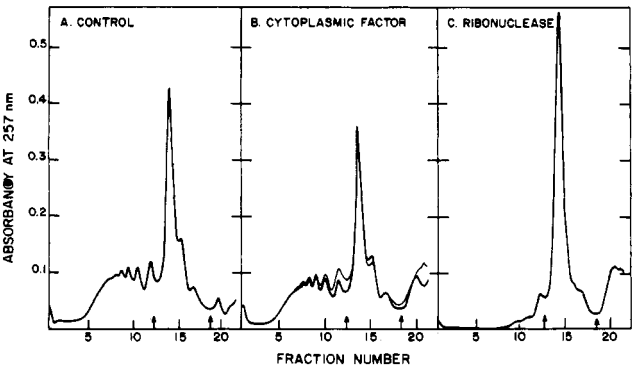

FIGURE 4: Sedimentation profiles of polysomes after treatment with cytoplasmic factor or pancreatic RNase. Labeled polysomes were incubated for 15 min with 20 μl of cytoplasmic factor preparation or with 0.1 μg/ml pancreatic RNase in presence or absence of snake venom exonuclease (10 μg/ml). Control was incubated under the same conditions without enzyme. Incubation mixtures were immediately chilled and subjected to zone centrifugation for 110 min at 41 000 rpm through 12-ml linear 10-40% sucrose gradients in 50 mM Tris-HCl (pH 7.6)-100 mM KCl-5 mM $MgCl_2$. Absorbancy was monitored at 257 nm and fractions were collected as indicated. Thin line in panel B represents profile of polysomes treated with both cytoplasmic factor and snake venom exonuclease. Arrows indicate pooled fractions for experiment described in Figure 5.

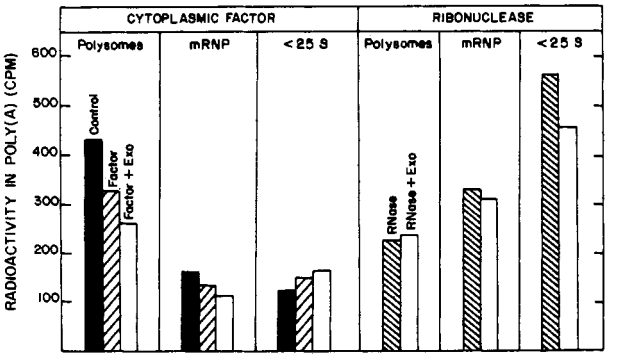

FIGURE 5: Distribution of poly(A) in centrifugal fractions after treatment of polysomes with cytoplasmic factor or pancreatic ribonuclease in the presence and absence of snake venom exonuclease. Labeled polysomes were incubated and subjected to zone centrifugation as indicated in Figure 4. Fractions were pooled as shown in Figure 4 to yield polysomes (>100 S), mRNPs (25-100 S), and small particles (<25 S). Pooled materials were digested with EDTA-RNase as described in Materials and Methods for poly(A) assay. Values represent radioactivity in poly(A).

from polysomes to lighter components (Figure 5). Most of the poly(A) loss as a result of this incubation occurred in the polysome fraction. Combined treatment with cytoplasmic factor and exonuclease caused a further loss of poly(A) from the polysome fraction, without any significant effect on the polysome profile (Figures 4 and 5). Thus the poly(A) sensitized by the factor was not in material released as small nucleoprotein complex. The RNase treatment, on the other hand, caused the release of large amounts of poly(A) protein complex sedimenting at 20 S or less (Figure 5). It is the latter material that was sensitized to exonuclease, in contrast to the situation after incubation with the cytoplasmic factor. Thus the pancreatic RNase was functioning by cleavage of the poly(A)-protein complex from the rest of the polysome, while the cytoplasmic factor was sensitizing the poly(A) segment in the polysome structure.

Since polysome fragmentation led to sensitization of the poly(A) to enzymatic hydrolysis, it was also important to verify that the incubations at low ionic strength (Table IV) were not causing polysome breakdown, perhaps through activation of an endonuclease. Such an effect could have accounted for the increased susceptibility of the poly(A) to nuclease digestion at low ionic strength. It was verified, however, that the polysome profiles were not altered after incubation at the low ionic strength either with cytoplasmic factor alone or with the factor in combination with the snake venom enzyme (data not shown).

Discussion

The results in the present study indicate that the poly(A) segment at the 3′ end of mRNA is protected from the action of snake venom exonuclease when the RNA is either in polysomes or in free ribonucleoprotein particles. Association of the poly(A) with protein appears to be the primary cause of this protection. This is indicated by the observation that the poly(A)-protein complex released from polysomes by mild ribonuclease treatment is relatively resistant to RNase T_2, an enzyme that readily degrades free poly(A) (Kwan and Brawerman, 1972). Moreover, the slow hydrolysis of poly(A) in the complex could be accounted for by its gradual dissociation under the incubation conditions (Brawerman, 1973b). The protecting effect of protein can also be inferred from the fact that deproteinization of polysomes makes the poly(A) highly vulnerable to the exonuclease. Deproteinization, however, could conceivably lead to changes in the configuration of the mRNA, with possible exposure of otherwise masked RNA segments.

Some of the present findings tend to indicate a participation of other RNA sequences in the interaction responsible for poly(A) protection. Mild RNase treatment of polysomes causes sensitization of this segment to exonuclease. Since this kind of treatment leads to labilization of the poly(A)-protein complex (Kwan and Brawerman, 1972), it is likely that the sensitization to exonuclease is due to "loosening" of the association between protein and poly(A). One possible explanation for this behavior is that the affinity of the protein component(s) for poly(A) may be influenced by another RNA segment. The latter could be another sequence in the mRNA. This arrangement offers the possibility of variations in the poly(A)-protein interaction in different mRNAs, based on differences in some polynucleotide sequences in the RNA. Our results also indicate that the poly(A)-protein interaction is "tightened" by the presence of exogenous RNA. The latter effect, however, does not appear to be specific with respect to polynucleotide sequence since polysomal RNA and *E. coli* transfer RNA are equally effective. It is possible, therefore, that the poly(A)-protein interaction in polysomes might be enhanced not by a specific polynucleotide sequence, but by any mRNA segment that may be in close proximity. In this case, variations in mRNA secondary structure, such as looping near the poly(A) segment, could lead to different susceptibilities of this segment to nucleases.

The observation that the poly(A) segment becomes susceptible to hydrolysis when the ionic strength is reduced (Table IV) can also be interpreted in terms of an mRNA secondary structure effect. A lower ionic strength could lead to alterations in RNA folding, thus removing a critical portion of the mRNA from the vicinity of the poly(A)-protein complex. It is also possible, however, that the poly(A)-protein interaction is itself dependent on KCl.

While the studies of the poly(A)-protein interaction could be carried out with a nonmammalian exonuclease, the use of S-180 extracts led to information on the nature of the agent possibly responsible for poly(A) degradation in the cytoplasm

of mammalian cells. The active factor appears to be a protein that interferes with the poly(A)-protein interaction in the complex. We provide evidence that this factor renders the poly(A) susceptible to snake venom exonuclease. The hydrolytic agent in the cytoplasm may be an exonuclease as well, but further work with more purified preparations will be required to clarify its mode of action. The inhibitory effect of exogenous RNA on poly(A) hydrolysis was observed with the cytoplasmic preparation as well as with the snake venom enzyme.

Our results indicate the possibility of a control mechanism based on differential susceptibility of the poly(A) segment to degradation in the cytoplasm. Some suggestion of differential poly(A) stability is provided by the data on the time course of hydrolysis in Figure 2. More precise data will be required, preferably with unique mRNA species, in order to verify this possibility.

Acknowledgments

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Kinetics of Dithionite Ion Utilization and ATP Hydrolysis for Reactions Catalyzed by the Nitrogenase Complex from *Azotobacter vinelandii*†

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ABSTRACT: The kinetics of $S_2O_4^{2-}$ utilization and ATP hydrolysis during the nitrogenase-catalyzed H_2 evolution and acetylene and nitrogen-reducing reactions were studied using a polarographic technique to monitor $S_2O_4^{2-}$ concentration. Rate constants for both $S_2O_4^{2-}$ utilization and ATP hydrolysis were determined as a function of temperature and corresponding activation energies determined. The activation energy for ATP hydrolysis differs from that for product formation or $S_2O_4^{2-}$ utilization by 5 kcal/mol above 20 °C and by 25 kcal/mol below 20 °C. The rate law for $S_2O_4^{2-}$ utilization was determined and describes the enzyme catalyzed rate over a 1000-fold variation in $S_2O_4^{2-}$ concentration and at least a 100-fold change in ATP concentration. The rate law for $S_2O_4^{2-}$ uilization under N_2-reducing conditions at 25 °C is given by $-d([S_2O_4^{2-}])/dt = (2.3 \times 10^{-3}E_T[S_2O_4^{2-}]^{1/2}[ATP]^2)/([ATP]^2 + K_1[ATP] + K_2)$, where E_T is total enzyme concentration in mg/ml and K_1 and K_2 are equilibrium constants for ATP binding to nitrogenase. The half-order dependence of the rate on $S_2O_4^{2-}$ concentration is interpreted in terms of the equilibrium $S_2O_4^{2-} = 2SO_2^-$, in which SO_2^- is the actual electron donor to nitrogenase. A partial mechanism incorporating these results is presented.

Nitrogenase is capable of reducing a wide range of substrates possessing unique and diverse properties. These include neutral gaseous molecules (N_2, N_2O, and acetylene), relatively large organic molecules (isonitriles, monosubstituted acetylenes, and nitriles), and anions (CN^-, N_3^-). In the absence of any of the above, $H_2O(H^+)$ serves as a substrate. In addition to these reducible substrates, nitrogenase also requires: (1) a source of low potential electrons, with $E°$ more negative than −400 mV (Watt and Bulen, 1974; Evans and Albrecht, 1974); and (2) $MgATP^{2-}$ which is hydrolyzed to $MgADP^{2-}$ and P_i.[1]

Zumft and Mortenson (1975) recently reviewed the properties of nitrogenases from bacterial sources and discussed the meager kinetic results so far reported and the problems associated with studying this aspect of nitrogenase catalysis. The rate dependence on ATP for nitrogenase-catalyzed reactions

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[1] Abbreviations used: P_i, inorganic phosphate produced from ATP hydrolysis; ATP, adenosine 5′-triphosphate; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance.