

Translational Properties of Rabbit Globin mRNA after Specific Removal of Poly(A) with Ribonuclease H

[poly(dT)/nitrocellulose column/cell-free protein synthesis systems/mRNA competition/mRNA stability]

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Communicated by Jacob Furth, July 23, 1974

ABSTRACT Highly purified RNase H (RNA·DNA hybrid ribonucleotidohydrolase, EC 3.1.4.34) from calf thymus was used to specifically remove the poly(A) sequences of purified rabbit globin mRNA after its hybridization with poly(dT). The deadenylylated globin mRNA was repurified by a one-step procedure including a nitrocellulose column. The poly(A) size and the content of unmodified mRNA were determined by hybridization with [³H]-poly(U), and it could be shown that the RNase H digestion method effectively removes this terminal poly(A) sequence. No difference in activity was found between mRNAs with and without poly(A) to initiate, elongate, terminate, and release newly synthesized globin chains in exogenous-mRNA-dependent, cell-free, protein-synthesizing systems from wheat embryo, ascites Krebs II cells, and rat liver. Furthermore, poly(A)-free globin mRNA competed with the same efficiency as authentic globin mRNA against chick ovalbumin mRNA when translated under total mRNA saturation conditions. It is apparent that the 3'-terminal poly(A) sequence is not necessary to maintain the translationally active secondary and tertiary configuration of the globin mRNA molecule. Preincubation of intact and deadenylylated globin mRNA in the Krebs II ascites translational system indicates that the presence of the poly(A) sequence may stabilize the translationally active mRNA molecule.

Ever since poly(adenylic acid) sequences were discovered as part of eukaryotic mRNAs (1-5), with the exception of histone mRNA (6), their functional role(s) in the translation and metabolism of messenger RNA remain(s) open. An early suggestion was its involvement in the transport of mRNA from nucleus to cytoplasm (7). However, mRNAs of non-nuclear origin, i.e., mitochondrial mRNA (8), and the RNA of cytoplasmic viruses (9, 10), were also found to contain 3'-terminal poly(A) sequences, their function necessarily unrelated to nuclear processing and transport. Maternal cytoplasmic mRNAs of sea urchin eggs undergo additional polyadenylation following fertilization (11), which can be observed even in enucleated activated merogons (12). The finding of a cytoplasmic poly(A) polymerase (13) supports the possibility of supplemental extranuclear poly(A) synthesis and adds to the arguments for a necessary cytoplasmic role of the messenger poly(A) sequences. The progressive shortening of the poly(A) sequences of polysomal mRNA with its age (14, 15), and the presence of at least one specific poly(A) binding protein in cytoplasmic messenger ribonucleoprotein particles (16, 17), gave rise to speculations that the poly(A) sequences have a regulatory function for messenger translational activity or messenger stability.

During the preparation of this work, two publications appeared dealing with the translational behavior of exonucleo-

lytically deadenylylated mRNA in cell-free protein synthesis (18, 19). It was shown that the poly(A) sequences were not essential for the successful translation of the messenger, but that translation of deadenylylated mouse globin mRNA resulted in a reduced efficiency to produce the specific protein product (19). This result could be due to the methods used for the removal of poly(A), which may not have been limited to the digestion of the poly(A) sequence only. We report here a very specific method for the removal of poly(A) sequences. The poly(A) of rabbit globin mRNA was digested with RNase H (hybridase, EC 3.1.4.34) after its hybridization with poly(dT). This deadenylylated mRNA shows no decrease in globin synthesis activity in various cell-free systems and in its ability to compete with another specific mRNA in an *in vitro* protein synthesizing system.

MATERIALS AND METHODS

Rabbit globin mRNA was isolated from polysomes of reticulocytes (20) and further purified by 15-30% sucrose gradient centrifugation. Chick oviduct mRNA was prepared from the polysomes of oviducts of laying hens (20), and chromatographed on an oligo(dT) cellulose column (21), which was treated with poly(A) to remove any exchangeable poly(dT). [³H]Poly(U) was synthesized with commercial polynucleotide phosphorylase from labeled UDP (22). ³H-Labeled and unlabeled poly(dT) were prepared with terminal deoxynucleotidyl transferase in the presence of 1 mM CoCl₂ and cacodylate buffer (23, 24). Homogeneous calf thymus ribonuclease H (hybridase), free of any nuclease activity of the RNase A type, was used (25).

Digestion of messenger poly(A): Rabbit globin mRNA (125 μg) was preincubated in a reaction volume of 150 μl containing 25 mM MgCl₂, 20 mM Tris·HCl, pH 8.0, and 10 μg of poly(dT) for 15 min at room temperature, enabling hybridization to occur between the poly(A) moiety of the mRNA and the poly(dT). Twenty-five micrograms of calf thymus RNase H was added and the mixture was reincubated for 20 min at the same temperature to allow hydrolysis of the poly(A) hybridized with poly(dT). For control mRNA the same procedure was followed with the omission of poly(dT).

Reisolation of mRNA after RNase H treatment: After treatment with RNase H, the reaction mixture was chromatographed on a combination column consisting of 7 ml of Sephadex G-50 layered over 0.4 ml of nitrocellulose in a plastic pipette (0.8-cm diameter), equilibrated with 0.1 M KCl. The nitrocellulose in 0.1 M KCl binds poly(dT), possible undigested hybrids, and the enzyme protein. Low-molecular-weight digestion products were retained by the upper Sephadex G-50 part of the column. By collecting only the early

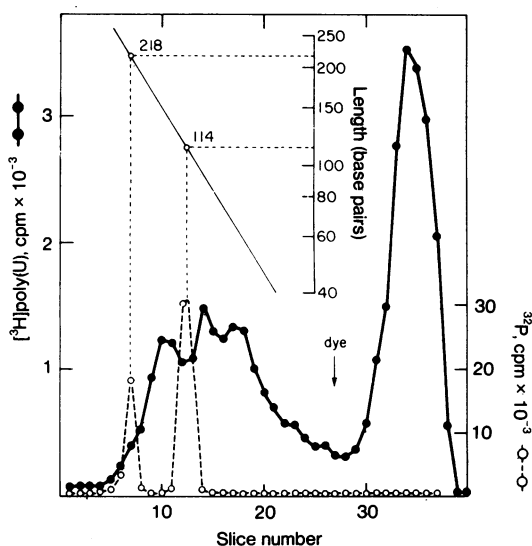


FIG. 1. Length determination of poly(A) in globin mRNA by polyacrylamide gel electrophoresis. Hybridization was carried out in a 50- μ l formamide-0.15 M NaCl-0.015 M sodium citrate, pH 7, system as described (22), using 4 μ g of globin mRNA and 0.4 μ g of [³H]poly(U) (0.5 Ci/mmmole). After incubation for 1 hr at 36°, unhybridized polyribonucleotides were hydrolyzed with 5 μ g of pancreas RNase A for 1 hr at 30°. Sodium dodecyl sulfate was added to a concentration of 0.5%, 15 μ g of *Escherichia coli* tRNA was added as carrier, and the RNA was extracted with chloroform-isoamyl alcohol (10:1). The aqueous phase was precipitated with alcohol at -20° and the RNA precipitate was collected by centrifugation and dissolved in electrophoresis buffer with bromophenol blue as marker dye. Gel electrophoresis was carried out in gels containing 7.5% polyacrylamide and 0.375% bisacrylamide according to ref. 39, except that the electrophoresis buffer contained 0.2% sodium dodecyl sulfate and 0.2 M NaCl instead of 0.02 M sodium acetate. After electrophoresis, the gels were frozen at -20°, sliced in 2-mm slices and eluted with 0.1% sodium dodecyl sulfate-, 0.02 M Tris-HCl, pH 8.0, at room temperature overnight and their radioactivity was measured in Aquasol. Parallel gels were run with well-defined ³²P-labeled double-stranded RNA markers 218 (midi variant, MDV-1) and 114 (micro variant, MCV-1) nucleotide base pairs in length (40). The high radioactivity at the end of the gel is due to residual contaminating nucleotides.

flow-through of the incubation solution, this combination column separated in a single step the poly(A)-containing or the deadenylylated mRNA from all other substances in the reaction mixture.

Cell-free protein synthesizing systems: Four different systems were used. The wheat embryo system (26), and the Krebs II ascites S30 (30,000 \times g supernatant) system (27), used without or supplemented with rabbit reticulocyte initiation factors (20), were employed. The purified rat ribosomal subunit system (28) contained 2.9 μ g of 40S, 7.2 μ g of 60S subunits, 0.045 A₂₆₀ units Krebs II ascites cell "pH 5-enzyme" and 15 μ l of an unfractionated rabbit reticulocyte initiation factor fraction (20) in 75 μ l of assay mixture. The incubation conditions for all systems were as described (20), except for the salt conditions, which were optimized to be 3.0 mM magnesium acetate and 75 mM KCl for the wheat embryo system, 3.5 mM and 60 mM for the ascites S30 system without initiation factors, 3.5 mM and 95 mM for the ascites S30 system supplemented with initiation factors and 4.0 mM and 10 mM for the purified rat liver ribosomal subunit system.

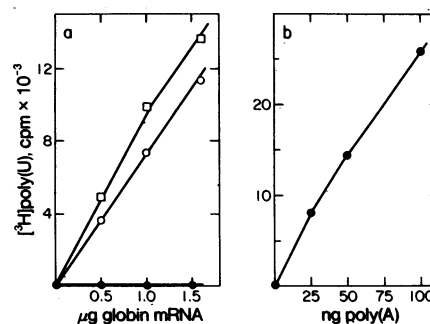


FIG. 2. Determination of poly(A) content of globin mRNA prior to and following RNase H treatment, by hybridization with [³H]poly(U). Hybridization was carried out in 100- μ l assays as described in legend to Fig. 1. After RNase H treatment, 125 μ g of carrier *E. coli* tRNA was added, followed by trichloroacetic acid precipitation. The acid-insoluble material was collected on Millipore filters and its radioactivity was measured. Indicated amounts of RNA were used for hybridization: (a) original globin mRNA (\square) globin mRNA treated with RNase H in the absence (\circ) and presence (\bullet) of poly(dT); (b) synthetic poly(A) with 40 times the amount of *E. coli* tRNA as poly(A)-free carrier RNA.

After incubation, the mRNA dependent incorporation of L-[³H]leucine (43-59 Ci/mmmole) into total protein, into released chains of newly synthesized rabbit globin, and into immunologically isolated chick ovalbumin was determined according to procedures detailed earlier (20).

RESULTS

Digestion of Messenger Poly(A) Sequences. In order to show the content and size distribution of poly(A) in rabbit globin mRNA, we employed hybridization with [³H]poly(U) (22). Fig. 1 shows the heterogeneous length of poly(A) in the hybrid form with radioactive poly(U) after polyacrylamide gel electrophoresis in high salt conditions. On a molar basis, most of the chains are between 50 and 90 nucleotides in length. This result differs slightly from that recently reported by Hunt (29), who estimated an average poly(A) chain length in rabbit globin mRNA of 30-40 bases with a method also including [³H]poly(U) hybridization but not direct length determination with double-stranded marker RNAs. By comparison with [³H]poly(U) hybridization to authentic poly(A), our original messenger preparation has a total poly(A) content of about 4% (Fig. 2), possibly reflecting some contamination of the mRNA with poly(A)-free rRNA, its presence being of no relevance in the following studies. This characterized poly(A)-containing globin mRNA fraction was treated with RNase H in the presence and absence of poly(dT) as detailed in *Materials and Methods*. In order to determine the poly(A) content of the mRNA after RNase H treatment, we quantitatively removed the poly(dT), employing the nitrocellulose column described in *Materials and Methods*. To assure completeness of poly(dT) removal after poly(A) digestion, we used highly labeled [³H]poly(dT) (56,500 cpm/ μ g). The absence of radioactivity in the reisolated mRNA fraction was direct evidence for the absence of poly(dT) from the mRNA. Reisolated mRNA after hybridization with poly(dT) and treatment with RNase H shows no detectable poly(A) when tested with [³H]poly(U). Control mRNA, reisolated following treatment with RNase H in the absence of poly(dT), shows only a slightly reduced content of poly(A) compared with the original mRNA preparation (Fig. 2a).

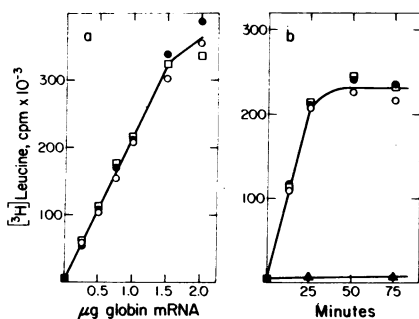


FIG. 3. Stimulation of protein synthesis in the wheat embryo cell-free S30 system upon addition of adenylated and adenylylated globin mRNA. Incorporation of [³H]leucine for 25 min at 30° into total protein was measured in a 75- μ l reaction mixture as described under *Materials and Methods*: (a) original globin mRNA (\square) or globin mRNA treated with RNase H in the absence (\circ) or presence (\bullet) of poly(dT); (b) rate of incorporation with 1 μ g of globin mRNA (symbols as above) or no exogenous RNA (\blacktriangle) added.

Translational Activity. In a preincubated wheat embryo S30 system incorporation of [³H]leucine is greatly enhanced upon addition of rabbit globin mRNA. Up to 1.5 μ g of mRNA per 75- μ l assay there is a linear dependency of the rate of incorporation of [³H]leucine into total protein (Fig. 3a). The addition of deadenylylated mRNA or, as a control, mRNA which had been treated with RNase H in the absence of poly(dT), shows no decrease or increase in translational activity as compared with the original mRNA (Fig. 3a). At no incorporation time or at any level of added mRNA could a difference be detected, whether the messenger contained its 3'-terminal poly(A) sequence or not (Fig. 3a and b). For the analysis of the messenger-dependent *in vitro* synthesized product the incubation mixture was centrifuged at 180,000 $\times g$ for 50 min and the released protein chains in the post-ribosomal supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). All three mRNA preparations caused the synthesis and release of the same amount of protein with the electrophoretic mobility of authentic rabbit hemoglobin subunits. It can be concluded that the removal of the poly(A) sequences does not interfere with the translation and termination process and that the treatment with RNase H, independent of the presence or absence of poly(dT), had not caused any breakdown within the coding sequence of globin mRNA.

In order to determine any possible effect of deadenylylation of the mRNA upon the initiation reaction it was necessary to ensure that initiation was the rate-limiting step for the synthesis of the measured product. Translation in the ascites Krebs II cell-free system is greatly increased upon addition of initiation factors derived from a ribosomal salt wash fraction of rabbit reticulocytes (20). The linear relationship between increase of protein synthesis and amount of factors added (data not shown) makes it very likely that in this system initiation is a rate limiting step under conditions of limited or no supplementary initiation factors. Deadenylylated or normal globin mRNA exhibit comparable translational activity in the ascites cell-free system without exogenous initiation factors, as they do when factors are supplied in saturating amounts (Table 1). We conclude that the poly(A) sequences are not required for maintenance of the structure of mRNA necessary for protein synthesis initiation. In addition, Table 1 shows that in several cell-free protein synthesizing systems

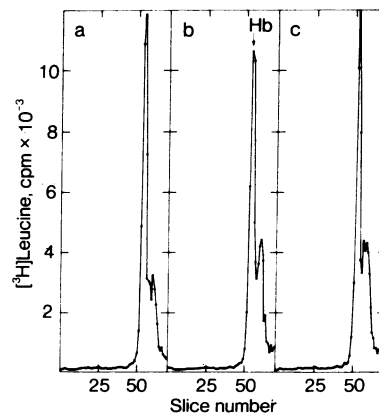


FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of globin chains synthesized and released *in vitro*. [³H]Leucine-labeled released globin chains were prepared from the post-ribosomal supernatant after 50-min incubation at 30° in the wheat embryo system (45 μ l) as described (20). They were subjected to sodium dodecyl sulfate-10% acrylamide gel electrophoresis (41). A parallel gel contained authentic rabbit globin as marker (Hb). Synthesis and release directed by 0.9 μ g of: (a) original globin mRNA; globin mRNA treated with RNase H in the absence (b) and in the presence (c) of poly(dT).

of different degree of purification, and from such diverse sources as wheat embryo, rat liver, and Krebs II ascites cells the translational activity of rabbit globin mRNA is not altered after specific removal of its 3'-terminal poly(A) sequence.

Competitive Translational Efficiencies of Normal and Deadenylylated Globin mRNA. It was reported that rabbit globin mRNA was very efficient in competitively suppressing translation of endogenous duck globin mRNA in a duck reticulocyte lysate system, whereas duck globin mRNA was almost inactive in competing against the endogenous mRNA in rabbit reticulocyte lysates (31). Furthermore, it was ob-

TABLE 1. Comparative translational activities of deadenylylated and poly(A)-containing globin mRNA in four different cell-free translational systems

Added globin mRNA*	Cell-free protein synthesizing system (pmol of [³ H]leucine incorporated†)			
	Wheat embryo S30	Ascites Krebs II S30	Ascites Krebs II S30 + initiation factors	Rat liver purified ribosomal subunits
None	1.9	0.68	26.5	9.65
Original‡	59.6	Not done	158	123
+poly(A)‡	57.5	3.62	169	119
-poly(A)	58.7	4.33	159	124

* In each experiment one μ g of mRNA was added per 75- μ l assay mixture.

† Incorporation into total protein after incubation: wheat embryo system 25 min, 30°; ascites S30 60 min, 37°; ascites S30 with initiation factors and purified rat liver ribosomal subunit system 50 min, 37°.

‡ The original globin mRNA inherently contains poly(A) and differs from the "globin mRNA + poly(A)" only operationally in that the latter is a formal control that was treated with RNase H in the absence of poly(dT).

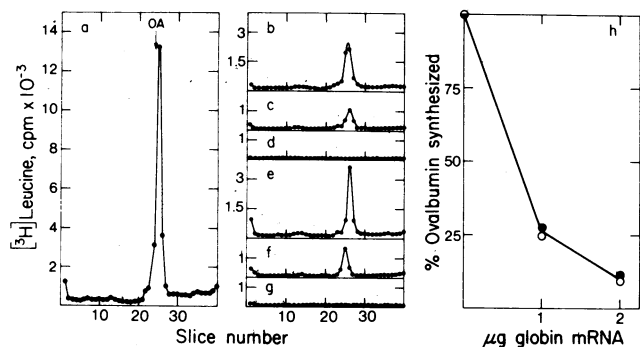


FIG. 5. Gel electrophoretic determination of ovalbumin synthesized in competition with globin mRNA with and without its poly(A) sequence. ^3H Leucine-labeled released polypeptide chains were prepared from the post-ribosomal supernatant after 60-min incubation at 37° in the ascites Krebs II cell-free system ($75\ \mu\text{l}$) supplemented with rabbit reticulocyte initiation factors. Newly synthesized ovalbumin was immunologically isolated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). A parallel gel contained authentic chick ovalbumin as marker (OA). Ovalbumin synthesis directed by $2\ \mu\text{g}$ of chick oviduct mRNA (a), in the presence of additional $1\ \mu\text{g}$ (b) and $2\ \mu\text{g}$ (c) of native globin mRNA containing poly(A), or in the presence of $1\ \mu\text{g}$ (e) and $2\ \mu\text{g}$ (f) of deadenylylated mRNA. Controls were made, in the absence of oviduct mRNA, containing only $2\ \mu\text{g}$ of native globin mRNA (d) or with $2\ \mu\text{g}$ of deadenylylated globin RNA (g). Panel (h) shows the depression in the amount of ovalbumin synthesized in competition with globin mRNA with (O) and without (●) its poly(A) sequence.

served that in a purified mouse liver ribosomal subunit system (28), in which rabbit or duck globin mRNA alone was translated equally well, only rabbit globin mRNA was expressed when both were added together in saturating amounts (32). It was concluded from these competition experiments that differences at or near the initiation sites of the mRNAs determined their respective abilities to be recognized and translated and are thus responsible for their differential efficiencies under competitive circumstances. With this in mind, we performed experiments to explore whether the removal of the poly(A) sequences would evoke subtle differences in initiation efficiency which could only be detected when magnified in a competitive situation. A near-saturating amount of poly(A)-containing total chick oviduct mRNA from laying hens, which contains more than 50% specific ovalbumin mRNA, was incubated in the Krebs II ascites cell system, supplemented with initiation factors, alone and together with two concentrations of normal and deadenylylated rabbit globin mRNA. The amount of ^3H leucine ovalbumin synthesized was determined by precipitation with monospecific antibodies and subsequent electrophoretic characterization of sodium dodecyl sulfate polyacrylamide gels. Fig. 5 shows that globin mRNA with or without its poly(A) sequence indistinguishably competes with the ovalbumin mRNA, yielding comparable decreases of ovalbumin synthesis.

Messenger Stability under In Vitro Conditions. Very little is known about the regulation of messenger stability in eukaryotes. Since it is possible that the enzymes involved in mRNA degradation *in vivo* are still active in our crude cell-free protein synthesizing system, we compared the stabilities of globin mRNA with and without its poly(A) moiety. In order to draw any conclusions it was necessary to know the time dependent decrease in the efficiency of the protein synthesizing

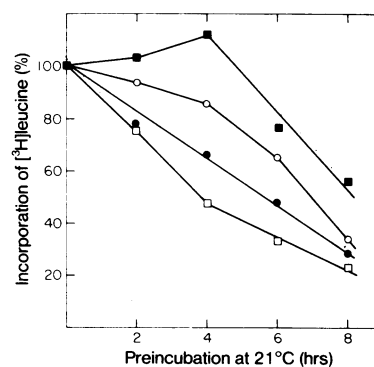


FIG. 6. Comparative stabilities of normal and deadenylylated globin mRNA activity *in vitro*. In the absence of amino acids the system was preincubated at 21° for the times indicated in the presence of $1\ \mu\text{g}$ of globin mRNA with (O) and without (●) its poly(A) sequence. The ^3H leucine incorporation into total protein was then determined upon addition of all 20 amino acids after 30-min incubation at 37° of the Krebs II ascites system ($50\ \mu\text{l}$) supplemented with reticulocyte initiation factors and the values were normalized as percent of the activity of the system without preincubation. For determination of the efficiency of the cell-free system itself after preincubation, the system was preincubated in the absence of amino acids and any exogenous mRNA at 21° for the times indicated and the translational activity was then measured upon addition of amino acids either with $1\ \mu\text{g}$ of native globin mRNA (■) or without exogenous mRNA (□).

system *per se*. We found that the ability of the Krebs II ascites system, fortified with initiation factors, to translate globin mRNA after preincubation without any exogenous mRNA is at least constant, if not slowly rising, up to 4 hr at 21° (Fig. 6). Thereafter, it decreases with a half life of about 4 hr. The endogenous activity of the system declines directly without any lag period. This property of the system made it possible to look for the decay of messenger activity. We preincubated the Krebs II ascites system, supplemented with initiation factors, in the absence of amino acids with deadenylylated or poly(A)-containing globin mRNA and at different time intervals thereafter measured the residual activity of the added mRNA. We found that the activity of globin mRNA lacking its 3'-terminal poly(A) sequence decreases linearly without any delay, whereas the activity of authentic globin mRNA bearing its poly(A) shows somewhat higher stability, with a delayed convex decay curve (Fig. 6).

DISCUSSION

Cellular RNase H (hybridase) hydrolyzes in a well-characterized endonucleolytic manner (33) phosphodiester bonds of polyribonucleotides hybridized with DNA, leaving 3'-OH termini at the polyribonucleotide strands. This enzyme can be considered a general tool for the preparative removal of terminal RNA sequences whose complementary DNAs are available or for specific cleavage of long polyribonucleotide strands if the known sequence is located internally. For this reason, highly purified calf thymus RNase H, free of RNase activity of the pancreatic RNase A type, appears to be the optimal tool for the removal of the 3'-terminal poly(A) sequences of mRNAs. After hybridization of mRNA with poly(dT), the enzyme removes specifically the 3'-terminal poly(A) moiety of the mRNA without altering any other proximal sequences, termination sequences or coding sequences. Such specificity cannot be obtained by the use of exonucleolytically

acting enzymes like polynucleotide phosphorylase (19) or other single strand 3'-OH exonucleases (18). The fact that all poly(A) can be removed after hybridization with poly(dT) and digestion in non-denaturing conditions suggests that the messenger poly(A) sequences are readily available and not buried within the structure of the mRNA molecule.

Our studies show clearly that the 3'-terminal poly(A) sequence is not directly necessary for mRNA to function as translational template. Moreover, it is evident that 3'-terminal poly(A) is not required to maintain the active conformation of the mRNA molecule, since its efficiency in initiation, elongation, termination, and release is not changed upon removal of the poly(A) sequence. We can calculate from the [³H]leucine incorporation data (Table 1), the known molecular weight of rabbit globin mRNA (30), the average leucine content of the rabbit hemoglobin subunits (34), and the determined purity of the globin mRNA preparation via its poly(A) size and content (Figs. 1 and 2) that in our Krebs II ascites S30 system, supplemented with a reticulocyte initiation factor fraction, each of the two globin mRNA strands in the average is translated at least 2.5 times. Although the level of reinitiation in even the best exogenous mRNA dependent cell-free systems is low, one can conclude that messenger poly(A) removal does not obstruct the efficiency to reinitiate. The best evidence for the absence of even subtle changes in the initiation activity after poly(A) digestion seems to us to be the unchanged translational activity of the mRNA in competition with another mRNA (Fig. 5).

Recently, cell-free protein synthesizing systems were used to determine specific mRNA tissue levels, especially after hormone induction or deinduction (35-37), by their translational activity *in vitro*. We can now infer that the *in vitro* observed changes in mRNA activity are not due to changes of mRNA activity via its poly(A) moiety.

Cytoplasmic messenger ribonucleoprotein particles contain at least one protein combined with the poly(A) moiety of the mRNA (16, 17). This protein cannot be of direct influence for the translational activity of mRNA and must be involved in other aspects of mRNA function. With decreasing evidence for the involvement of poly(A) sequences in translation *per se*, arguments for their role in the regulation of mRNA lifetime become more cogent. The progressive shortening of poly(A) with the age of mRNA (14) independent of whether protein synthesis was ongoing or not (15) led to the suggestion that gradual removal of the poly(A), its rate determined by poly(A)-protein interaction, would eventually leave the mRNA exposed to exonucleolytic attack (38). Preincubation in a cell-free protein synthesizing system suggests differential stabilities of globin mRNA with and without its poly(A) (Fig. 6).

We thank Drs. D. Mills and F. Cramer for the gift of the ³²P-labeled, double-stranded RNA markers MDV-1 and MCV-1 and Dr. J. D. Karkas for the preparation of [³H]poly(dT). The technical assistance by Richmond Ansah-Yiadom is gratefully acknowledged. This study was supported in part by research grants from the National Institutes of Health, CA-02332 and CA-12210. P.F. is a Career Investigator of the Health Research Council of the City of New York (I-104).

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